

Effects of Bicarbonate on Lithium Transport in Human Red Cells

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ABSTRACT Lithium influx into human erythrocytes increased 12-fold, when chloride was replaced with bicarbonate in a 150 mM lithium medium (38°C, pH 7.4). The increase was linearly related to both lithium- and bicarbonate concentration, and was completely eliminated by the amino reagent 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). DIDS binds to an integral membrane protein (mol wt $\sim 10^5$ dalton) involved in anion exchange. Inhibition of both anion exchange and of bicarbonate-stimulated lithium influx was linearly related to DIDS binding. 1.1×10^6 DIDS molecules per cell caused complete inhibition of both processes. Both Cl^- and Li^+ can apparently be transported by the anion transport mechanism. The results support our previous proposal that bicarbonate-induced lithium permeability is due to transport of lithium-carbonate ion pairs (LiCO_3^-). DIDS-sensitive lithium influx had a high activation energy (24 kcal/mol), compatible with transport by the anion exchange mechanism. We have examined how variations of passive lithium permeability, induced by bicarbonate, affect the sodium-driven lithium counter-transport in human erythrocytes. The ability of the counter-transport system to establish a lithium gradient across the membrane decreased linearly with bicarbonate concentration in the medium. The counter-transport system was unaffected by DIDS treatment. At a plasma bicarbonate concentration of 24 mM, two-thirds of the lithium influx is mediated by the bicarbonate-stimulated pathway, and the fraction will increase significantly in metabolic alkalosis.

INTRODUCTION

One purpose of this work is to investigate the effect of bicarbonate on the passive lithium permeability of human red cells. Bicarbonate causes selective increases of passive lithium and sodium permeabilities (Funder and Wieth, 1967*b*; Wieth, 1970). In contrast, the passive potassium permeability is not increased, and the cesium permeability is even reduced by 30%, when 150 mM chloride is substituted with bicarbonate. We have previously proposed that the increased permeabilities are due to the ability of carbonate to form ion pairs with sodium and lithium, but not with potassium and cesium. According to this ion pair hypothesis, negatively charged ion pairs, NaCO_3^- and LiCO_3^- , are transported through the cation-tight membrane by the specific anion exchange system.

The ion pair hypothesis has received some support by the observation that bicarbonate-stimulated cation fluxes are inhibited by compounds that are considered inhibitors of the anion exchange system (Callahan and Goldstein, 1972; Duhm and Becker, 1977*b, c*, 1978). We have pursued this line of investigation further by comparing membrane binding and inhibitory effects of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). DIDS was developed as a specific inhibitor of anion transport (Cabantchik and Rothstein, 1974). It reacts only with a limited number of amino groups in the membrane, and our results support the concept that anion exchange and bicarbonate-stimulated lithium fluxes are mediated by the same transport mechanism.

A second purpose of the present article is to evaluate the role of passive lithium permeability in the maintenance of a concentration gradient for lithium between red cells and the extracellular environment. Lithium concentrations in red cells from psychiatric patients are considerably lower than they would be if lithium ions were distributed passively between cells and plasma (Mendels and Frazer, 1974). In vitro studies have revealed that lithium is extruded against an electrochemical gradient by a counter-transport mechanism, which depends on the presence of an oppositely directed gradient for sodium ions (Haas et al., 1975; Duhm et al., 1976). A large capacity for lithium-sodium counter-transport has been found in bovine red cells (Funder and Wieth, 1978) in contrast to the much smaller capacity of human red cells (Duhm and Becker, 1977*c*; Pandey et al., 1977*a*; Pandey et al., 1977*b*; Sarkadi et al., 1977). The lithium gradient across the human red cell membrane can, therefore, only be sustained if the passive lithium permeability remains low. We have examined the extent to which variations of passive lithium permeability affect net movements of lithium by varying the bicarbonate concentration between 0 and 150 mM under conditions where the counter-transport is fully operative. The outward net flux of lithium from cells containing 1.6 mM lithium, suspended in a medium containing 2 mM lithium, was completely eliminated by the increased lithium influx in a 100 mM bicarbonate medium.

METHODS

Freshly drawn, heparinized human blood was centrifuged and washed three times in 150 mM NaCl at room temperature. Plasma and buffy coat were removed after the first centrifugation. In the different classes of experiments the cells were prepared differently as indicated below, but in each, the cells were finally washed and resuspended at a hematocrit of 40% in a medium, in which the cells were at steady state with respect to chloride, bicarbonate, and hydrogen ions at 38°C. Isolation of cells and media by centrifugation at 0°C and determination of extracellular trapped medium was performed as described by Funder and Wieth (1967*a*). All media contained ouabain 10^{-4} M. Lithium and sodium in media and red cells were determined by flame photometry, and the cellular concentrations were corrected for the trapping of extracellular ions. The water content of cells was determined by drying cells to constant weight at 105°C for 24 h and was corrected for the trapping of 2% (wt/wt) of extracellular medium in the packed cell column.

Bicarbonate-Stimulated Lithium Influx

The media were made up from 150 mM solutions of the following salts: LiCl, LiHCO₃,

NaCl, NaHCO₃, KCl, and KHCO₃. The bicarbonate media were prepared by dissolving the respective carbonates, which were converted into bicarbonate by titration with CO₂ to pH 7.4 (38°C) before use. CO₂/bicarbonate was the only extracellular buffer system present in bicarbonate-containing media, and the pH was kept constant at pH 7.4 (38°C) in cell suspension by use of the pH-stat described previously (Funder and Wieth, 1967*a*). In the same work we have shown that ouabain-treated red cells produce 2.6 mmol lactate (liter cells × h)⁻¹ at 38°C, pH 7.4. Therefore, the decrease of bicarbonate concentration due to the formation of lactic acid at a hematocrit of 40% is 1.7 mmol (liter medium × h)⁻¹. This decrease is insignificant at high bicarbonate concentrations, and even at a concentration of 25 mM bicarbonate the decrease during a 1-h incubation period is too small to affect the precision of the flux measurements to any measurable degree. The bicarbonate-free chloride media were buffered with 20 mM glycylglycine. The pH decreased slightly (<0.05 U/h) during 1-h incubation periods in chloride media at 38°C (Funder and Wieth, 1967*a*).

Media with varying lithium and bicarbonate concentrations (Figs. 1 and 2) were prepared by appropriate mixing of the above-mentioned solutions as indicated in the legends of the figures. Influx of lithium was determined over periods, during which cellular lithium content increased linearly with time. It was found that lithium influx remained constant with time during incubation of cells up to 1 h in a 150 mM LiHCO₃ medium, the condition which caused the maximal lithium influx seen in this study. The DIDS-sensitive lithium influx was calculated by subtracting the lithium influx determined in DIDS-treated cells from the total lithium influx.

DIDS Treatment of Erythrocytes

4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) was synthesized from 4,4'-diaminostilbene-2,2'-disulfonic acid (DADS) by Dr. M. P. Hancock, Chemical Laboratory 2, University of Copenhagen. The method was modified from that developed by Maddy (1964) for the synthesis of an analogue stilbene derivative. The purity was checked by elementary analysis for C, H, N, and S, and by infrared and ultraviolet spectroscopy. The molar extinction coefficients at the absorption maximum at 343 nm measured in phosphate buffer were 5.36×10^4 liter mol⁻¹ (pH 8.8) and 4.99×10^4 liter mol⁻¹ (pH 1). Thin layer chromatography in three solvent systems (pyridine/acetic acid/water [10:1:40 vol/vol], n-propanol/ammonium hydroxide/water [6:3:2 vol/vol], ethanol/water [3:2 vol/vol]) showed that the final preparation did not contain demonstrable amounts of DADS. The compound was found to be stable, when stored in the dark as a dry powder at -20°C. Fresh stock solutions and dilutions were prepared for each experiment, because control experiments showed that the inhibitory effect of DIDS on anion exchange decreased by 5%/24 h, when DIDS was stored in concentrated aqueous solution at neutral pH.

In experiments where complete (i.e., >99%) inhibition of the anion exchange system was wanted, red cells were incubated for 45 min at 38°C at a DIDS concentration sufficient to give a ratio of DIDS molecules to cells of above 10⁷ (about 10 times the number of DIDS molecules sufficient to cause maximum inhibition, cf. Fig. 3). To produce graded inhibition of anion exchange and of bicarbonate-stimulated lithium influx (between 10 and 100%), the ratio was varied between 10⁵ and 1.2×10^6 DIDS molecules per cell (Figs. 3 and 5). All DIDS molecules were taken up by the cells when the ratio was kept within this range (cf. Results). The number of cells per milliliter of the incubates was determined by counting (Coulter Counter model DN, Coulter Electronics Inc., Hialeah, Fla.), and the number of DIDS molecules in the suspension was calculated by multiplying the amount of DIDS (moles per milliliter suspension) with Avogadro's number (6.023×10^{23} mol⁻¹).

Chloride Self-Exchange

Determination of chloride self-exchange was carried out with ^{36}Cl -loaded cells in a 150 mM NaCl medium (0°C, pH 7.4) by a millipore filtering technique (Dalmark and Wieth, 1972). It may be noted that determinations of bicarbonate-stimulated lithium influx in DIDS-treated red cells were carried out at 38°C, but Brahm (1977) has shown that the fractional inhibition of facilitated anion transport by DIDS is constant in the whole temperature interval between 0 and 38°C. The fractional inhibition was calculated from determinations of the rate of chloride exchange in DIDS-treated and in untreated control cells.

Lithium Sodium Counter-Transport Experiments

Preloading of cells with lithium was performed by incubating red cells at a hematocrit of 30% in a medium containing 100 mM LiCl and 50 mM NaCl for 30 min at 38°C. Next, the cells were washed and incubated in a medium containing 2 mM LiCl, 150 mM NaCl, and 20 mM glycylglycine or, in the experiments performed in the presence of bicarbonate (Fig. 7-10), in mixtures of 150 mM NaCl and 150 mM NaHCO_3 containing 2 mM LiCl. The capacity of the counter-transport system shows a pronounced interindividual variability (Duhm and Becker, 1977c), whereas the intraindividual variability is remarkably small (Duhm and Becker, 1977b). Apart from the experiments reported in Table V, all counter-transport experiments were, therefore, performed with red cells from a single donor (J.F.), whose cells showed a counter-transport near the average of the values found in experiments with cells from 16 donors (Table V). All counter-transport experiments were performed at 38°C, pH 7.4. The hematocrit was 30-40%. In experiments performed in the presence of bicarbonate, pH and thereby P_{CO_2} was kept constant as described above.

Reference Values for Human Erythrocytes

As first pointed out by Keitel et al. (1955), concentrations expressed per liter of cells or cell water do not refer to a constant number of cells, or to a constant surface area, if the water content of the cells varies under the experimental conditions employed. In the present study the cells swelled in the bicarbonate media, because cells incubated in NaHCO_3 or LiHCO_3 media take up Na^+ or Li^+ without a concomitant loss of potassium (Funder and Wieth, 1967a; Wieth, 1970). Keitel et al. (1955) suggested the use of cell solids as a reference, when information about changes of red cell contents is sought, rather than information about changes of concentrations. We have followed that convention of our studies of lithium influx, but Table I states values which can be used, if conversion of one reference unit to another is to be made. For instance, the water content of cells in all experiments reported in this study vary maximally 20% from the normal water content of ~2 kg water per kg solids, so that units expressed, e.g., in mmol/kg solids can be converted into units of mmol/kg water by dividing with 2.

Calculation of Ion Pair Concentrations

The possible concentration of LiCO_3^- ion pairs in a bicarbonate solution at pH 7.4 was evaluated from the mass equilibrium:

$$K_{\text{LiCO}_3^-} = \frac{\alpha_{\text{Li}^+} \times \alpha_{\text{CO}_3^{2-}}}{\alpha_{\text{LiCO}_3^-}}$$

The ion activities (denoted by α) were calculated by means of activity coefficients for lithium and carbonate at an ionic strength of 0.15: $\gamma_{\text{Li}^+} = 0.75$, $\gamma_{\text{CO}_3^{2-}} = 0.31$ (Garrels et al., 1961). The value of the dissociation constant $K_{\text{LiCO}_3^-}$ is not known, but its order of magnitude can be evaluated: the corresponding dissociation constant for sodium-carbon-

ate ion pairs (K_{NaCO_3}) has in two studies been found to be 0.05 (Garrels et al., 1961; Siggaard-Andersen, 1962). The tendency of lithium to form ion pairs with oxy-anions is stronger than that of sodium (Robinson and Harned, 1941). Thus, the dissociation constant for the ion pair LiOH (1.2) is four times smaller than that for NaOH (5.0; Davies, 1962). We have assumed that this ratio may also hold for the carbonate ion pairs. There is some basis for this assumption, because the bicarbonate stimulation of lithium influx in fact is 4–5 times higher than bicarbonate stimulation of sodium influx (Wieth, 1970; Duhm and Becker, 1978). Using a value of 0.012 for the dissociation constant K_{LiCO_3} , an activity coefficient for the ion pair (LiCO_3^- of 0.75 [Garrels et al., 1961] and a pK_2 for carbonic acid of 9.8 (Siggaard-Andersen, 1962), the concentration of LiCO_3^- is found to be:

$$\text{LiCO}_3^- = \frac{\alpha\text{Li}^+ \times \alpha\text{CO}_3^{2-}}{\gamma_{\text{LiCO}_3^-} \times K_{\text{LiCO}_3^-}} = \frac{(0.75 \times 0.15)(0.31 \times 5.9 \times 10^{-4})}{0.75 \times 0.012} = 0.002.$$

The concentration of LiCO_3^- in a 150 mM lithium bicarbonate solution at pH 7.4 is thus estimated to be 0.002 M. This value is only an "order of magnitude estimate", but it is

TABLE I
REFERENCE VALUES FOR NORMAL HUMAN RED BLOOD
CELLS

No. of cells per kg cell solids	3.1×10^{13}
Membrane area per kg cell solids, $\text{cm}^2 (\text{kg solids})^{-1}$	4.4×10^7
* Volume of cells per kg cell solids, $\text{cm}^3 (\text{kg solids})^{-1}$	2.7×10^3
* Water content per kg cell solids, $\text{g cell water} (\text{kg solids})^{-1}$	2.0×10^3

Membrane area $1.42 \times 10^{-6} \text{ cm}^2$; cell volume $8.7 \times 10^{-11} \text{ cm}^3$.

* Varies with water content of cells.

clear that it is worth considering, whether the presence of 1–2% of the lithium in the form of anions may cause the increased lithium permeability of red cells incubated in bicarbonate media.

RESULTS

Bicarbonate-Stimulated Lithium Influx

Fig. 1 shows that lithium influx was a linear function of lithium concentration both in a 150 mM chloride medium and in a 150 mM bicarbonate medium. Lithium influx increased 12-fold when 150 mM chloride was substituted with bicarbonate. The effect of bicarbonate on lithium influx was completely eliminated in cells treated with sufficient doses of DIDS to inhibit anion exchange >99%.

Similarly, lithium influx from a medium containing 150 mM lithium was found to increase linearly with bicarbonate concentration, when chloride in the medium was gradually replaced with bicarbonate (Fig. 2). Also in these experiments, it was observed that the effect of bicarbonate could be completely eliminated by treating the cells with DIDS.

Lithium influx is thus a linear function of both extracellular lithium concentration and of extracellular bicarbonate concentration following the relation

$$M_{\text{net}} = 0.036 \times \text{Li}_o + (0.003 \times \text{HCO}_3^-) \times \text{Li}_o \text{ mmol (kg cell solids} \times \text{h)}^{-1}. \quad (1)$$

The coefficients of Eq. 1 were determined by linear regression analysis of the results shown in Figs. 1 and 2. The first term of Eq. 1 describes the linear increase of lithium influx with extracellular lithium concentration in a bicarbonate free medium ($5.4 \text{ mmol [kg solids} \times \text{h]}^{-1}$ in a 150 mM LiCl medium), and the second term describes the additional increase found in the presence of bicarbonate ($67.5 \text{ mmol [kg cell solids} \times \text{h]}^{-1}$ in a 150 mM LiHCO_3 medium at

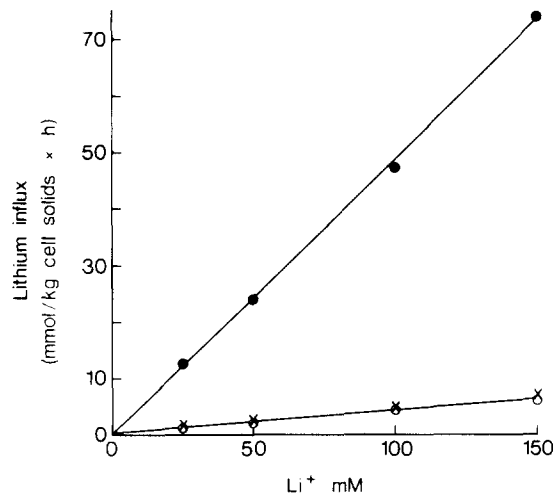


FIGURE 1. Lithium influx into human red cells as a function of extracellular lithium concentration (38°C , pH 7.4, ouabain 10^{-4} M). Lithium influx was a linear function of extracellular lithium concentration in the 150 mM bicarbonate medium both in normal (●) and in DIDS-treated cells (○). Note that the DIDS treatment of cells reduce the lithium influx to a level identical with that found, when normal erythrocytes were suspended in a medium containing 150 mM chloride (×). Potassium was used as substituting cation keeping the sum of ($\text{K}^+ + \text{Li}^+$) constant at 150 mM in all experiments. The saturable component of lithium influx which is due to lithium-sodium exchange (Duhm and Becker, 1977a, Fig. 3) is too small to be seen in the figure.

pH 7.4, 38°C). The bicarbonate-sensitive influx is completely eliminated in DIDS-treated cells, and this component of lithium influx can, therefore, also be denoted "the DIDS-sensitive lithium influx." The water content of the cells varied considerably in these experiments (range 1.9–2.37 kg water/kg solids). The net flux of water accompanying lithium net flux was found to be 5.8 g/mmole (SE 0.3, $n = 5$). Flux values in mmol/cm^2 can be obtained by multiplying with the factor: $2.27 \times 10^{-8} \text{ kg cell solids} \times \text{cm}^{-2}$, cf. Table I.

In the experiments presented in Figs. 1 and 2, lithium influx was examined in an open system, i.e., pH is kept constant by continuous titration with CO_2 . We have checked that a free access to CO_2 was of no importance for the lithium accumulation by comparing (a) the uptake in the open system with (b) the uptake in the cells incubated anaerobically at 38°C in a syringe. Virtually the

same uptake (69.5 and 68.6 $\text{mmol} [\text{kg cell solids} \times \text{h}]^{-1}$) was found in both samples. We have also examined the effect of 1 mM acetazolamide on bicarbonate-stimulated lithium influx, because the hydration and dehydration of CO_2 , as shown in the Discussion section, might play a role for the rate of transfer of LiHCO_3 from medium to cells. In two experiments lithium uptake was 65 $\text{mmol} (\text{kg cell solids} \times \text{h})^{-1}$, showing that the complete inhibition of the carbonic anhydrase decreased the rate of lithium influx at the most by 10% .

As mentioned before, it is possible that the DIDS-sensitive lithium influx is caused by the formation of ion pairs, LiCO_3^- , which can be transported by the

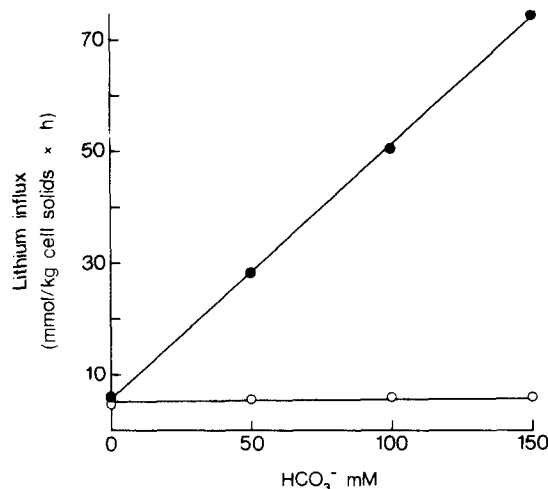


FIGURE 2. The effect of bicarbonate on lithium influx into normal (●) and into DIDS-treated (○) human erythrocytes (38°C , pH 7.4 , ouabain 10^{-4} M). The media contained 150 mM lithium, and chloride was used as substituting anion, keeping the sum of $(\text{Cl}^- + \text{HCO}_3^-)$ constant at 150 mM in all experiments. Note that lithium influx into the DIDS-treated cells was unaffected by the bicarbonate concentration, whereas lithium influx into the untreated cells was a linear function of bicarbonate concentration: $M_{\text{in}}^{\text{Li}} = 0.453 \times \text{HCO}_3^- + 5.6$ (coefficient of correlation 0.999), where $M_{\text{in}}^{\text{Li}}$ is the lithium influx in $\text{mmol} (\text{kg solids} \times \text{h})^{-1}$ and HCO_3^- is the extracellular bicarbonate concentration. The mean value of lithium influx into the DIDS-treated cells was 5.5 $\text{mmol} (\text{kg cell solids} \times \text{h})^{-1}$.

anion exchange system of the red cell membrane. DIDS is thought to be a very specific inhibitor of anion exchange and to block chloride transport by binding to a limited number of binding sites in the membrane. We therefore, decided to carry out a quantitative comparison of the effect of DIDS binding on the anion exchange and on the bicarbonate-stimulated (DIDS-sensitive) lithium influx.

Inhibition of the Anion Exchange Mechanism by DIDS

Anion exchange is irreversibly inhibited when erythrocytes are pretreated with DIDS as described in the Methods section. Fig. 3 shows that we found a linear relation between the number of DIDS molecules bound per cell and the fractional inhibition of chloride exchange. 50% inhibition was found when 5.5×10^5 DIDS molecules were bound per cell, and maximal inhibition (99.5 – 99.7%)

was found at a DIDS binding of 1.1×10^6 molecules per cell. These figures for binding are maximum numbers. The determination of binding was based on our observation that all DIDS molecules added to the suspension were bound by the cells, as long as the hematocrit and the DIDS concentration were matched not to exceed 1.1×10^6 molecules per cell. The results of Table II demonstrate

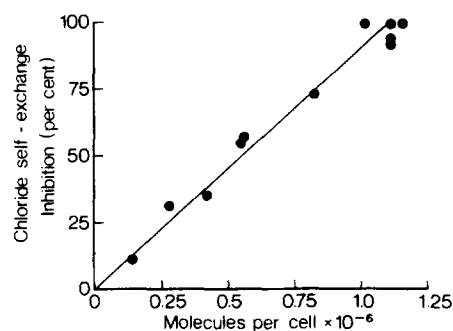


FIGURE 3. Inhibition of chloride self-exchange in DIDS-treated human red cells as a function of the number of DIDS molecules bound per cell. The results indicated that inhibition varies linearly with DIDS binding, and from the present data and from the data presented in Tables II and III, it was calculated that complete inhibition corresponds to a binding of 1.08×10^6 molecules per cell (SE 0.03×10^6 , $n = 15$).

TABLE I I
INDEPENDENCE OF DIDS BINDING ON DIDS CONCENTRATION

DIDS		Cells	Binding	Inhibition of chloride exchange
<i>mol/liter suspension</i>	<i>molecules/liter suspension</i> $\times 10^{-18}$	<i>no./liter suspension</i> $\times 10^{-12}$	<i>molecules/cell</i> $\times 10^{-6}$	%
10^{-7}	0.060	0.098	0.612	62.6
5×10^{-7}	0.301	0.49	0.614	64.3
10^{-6}	0.602	0.98	0.614	66.3
2×10^{-7}	0.120	0.11	1.12	99.1
4×10^{-7}	0.241	0.21	1.15	99.4
10^{-6}	0.602	0.66	0.91	99.5
2×10^{-6}	1.120	1.12	1.01	99.6

The irreversible DIDS binding and the inhibition of chloride self-exchange were independent of the DIDS concentration in the medium, and depended only on the number of DIDS molecules bound per cell. It was checked that all DIDS molecules were removed by the cells. With the sensitivity of the biological assay (cf. Fig. 4), a concentration of DIDS remaining in the supernate of 2×10^{-8} M would have been detected. The total removal of DIDS from a medium containing 2×10^{-6} M DIDS initially, therefore, means that at least 99% of the molecules had been bound by the cells during the DIDS treatment.

that the same degree of inhibition was obtained in different cell samples, in spite of large variations of DIDS concentration and of hematocrit, when the number of DIDS molecules per cell was not varied during pretreatment. Evidence that virtually all DIDS molecules were removed by the cells was obtained by measuring that there was no DIDS left in the supernates of the DIDS-treated cells. These supernates were isolated by centrifugation and used

for a biological determination of DIDS concentration. The assay was based on the observation by Lepke et al. (1976) that anion exchange is inhibited immediately when red cells are exposed to DIDS, although the irreversible binding to membrane components requires prolonged incubation at higher temperatures. The DIDS content in the supernates could, therefore, be determined by using them as flux media for chloride self-exchange using ^{36}Cl -loaded erythrocytes at a low hematocrit ($\sim 0.5\%$). A standard graph was determined by running separate control experiments in media with known DIDS concentrations (Fig. 4). The DIDS concentration in the supernates could then be determined from the standard graph. It can be seen that the biological assay is sensitive to DIDS concentration between 20 and 200 nM. By this method we

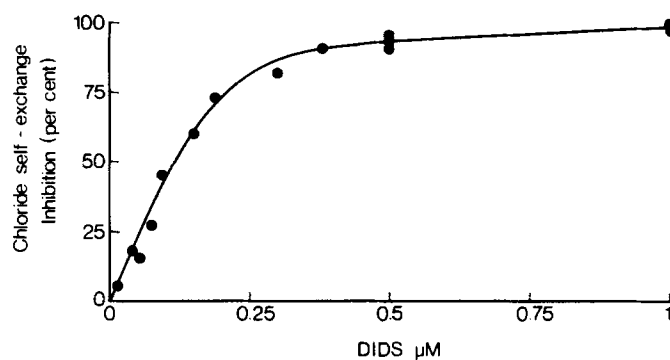


FIGURE 4. Inhibition of chloride self-exchange in normal human red cells, which were injected acutely into a well-stirred 150 mM NaCl medium containing DIDS in the concentrations shown on the abscissa (0°C , pH 7.4, hematocrit 0.5%, corresponding to 5×10^{10} cells per liter). Because the irreversible binding of DIDS is slow at 0°C , the cells bind DIDS in a reversible reaction, and the concentration of "free" DIDS molecules in the suspension is lower than the "total" DIDS concentrations shown on the abscissa (viz. Discussion). The graph was fitted by eye. The results show that the biological assay for DIDS is sensitive in the range of concentration between 20 and 200×10^{-9} M.

found that virtually all ($>99\%$) of the DIDS molecules were bound to the cells. Free DIDS in the supernates was only found when the number of DIDS molecules exceeded 1.1×10^6 molecules per cell. Table III shows the results of an experiment where inhibition of chloride self-exchange was determined in cells that had bound 1.1×10^6 and 7.7×10^6 DIDS molecules per cell. It is apparent that the inhibition of chloride exchange was similar in the two sets of cells, so that the additional binding of 6.6×10^6 DIDS molecules per cell caused no additional inhibition of anion exchange.

Quantitative Comparison of the DIDS Effect on Anion Exchange and on DIDS-Sensitive Lithium Influx

The next step was to compare the degree of inhibition of anion exchange and of bicarbonate-stimulated lithium influx in red cells treated with DIDS to cause graded degrees of inhibition. The results are shown in Fig. 5. It is apparent that there was a simple relation between the inhibition of the two transport processes:

DIDS-sensitive lithium influx was inhibited to exactly the same degree as the chloride exchange. From comparison with the results of the DIDS binding study (Fig. 3), it is apparent that both chloride exchange and DIDS-sensitive lithium influx were reduced by 50% by the binding of $\sim 5 \times 10^5$ molecules per cell. In view of the limited number of DIDS binding sites, this finding makes it probable that the DIDS-inhibitable lithium transport is dependent on the integrity of that integral membrane protein, which appears to be involved in anion transport through the erythrocyte membrane.

Temperature Dependence of DIDS-Sensitive Lithium Transport

The temperature dependence of the DIDS-sensitive lithium transport was found to be more pronounced than that of other cation transport processes (Whittam, 1964). The results of Table IV show that the DIDS-sensitive influx increased almost 200-fold in the interval 0–38°C (apparent activation energy 24

TABLE III
LACK OF EFFECT OF EXCESS DIDS BINDING ON THE RATE OF CHLORIDE EXCHANGE

DIDS binding			Cells	DIDS binding	Rate coefficient of chloride exchange	Inhibition
Total number	No. bound to cells	No. free				
	molecules/liter suspension $\times 10^{-10}$		no./liter suspension $\times 10^{-12}$	molecules/cell $\times 10^{-6}$	(min) ⁻¹	%
1.20	1.20	0	1.12	1.1	0.0101	99.6
3.01	1.48	1.53	0.19	7.8	0.0098	99.6

The rate coefficient of uninhibited chloride self-exchange was 2.66 min^{-1} (SE 0.04, $n = 22$). The concentrations of DIDS in the two suspensions was 2 and 5 μM .

kcal/mol), whereas the residual (DIDS-resistant) lithium influx only increased by a factor of 10 in the same temperature interval (apparent activation energy 11 kcal/mol). The activation energy of 24 kcal/mol is somewhat lower than the value found for iodide (Dalmark and Wieth, 1972), but similar to that found for chloride and bromide between 0 and 38°C by Brahm (1977).

Lithium/Sodium Counter-Transport in Chloride Media

As reported by Haas et al. (1975), lithium is extruded against an electrochemical gradient, when red cells with an intracellular lithium concentration of 1–2 mmol/kg cell water are suspended in an isotonic sodium chloride medium containing 2 mM lithium. In the experiment shown in Fig. 6 the medium contained 10^{-4} M ouabain, so it is clear that the lithium net flux is not mediated by the classic sodium/postassium transport system.

Table V shows the average net movement found in 16 experiments. It may be noted that the mean value of the ratio between lithium concentrations in extra- and intracellular water phases increased from 1.26 to 2.53 over the 4 h period. Assuming the equilibrium potential for lithium to be equal to that of chloride, the ratio $\text{Li}_o^+/\text{Li}_i^+$ would have been ~ 0.7 , if lithium ions were distributed at equilibrium at a membrane potential of -10 mV .

The Effect of Bicarbonate on the Lithium/Sodium Counter-Transport

Substitution of chloride with bicarbonate increases the passive lithium permeability as shown in the previous sections. We have, therefore, investigated how substitution of chloride with bicarbonate at constant pH (7.4) modified net movements of lithium in counter-transport experiments. Fig. 7 shows that the

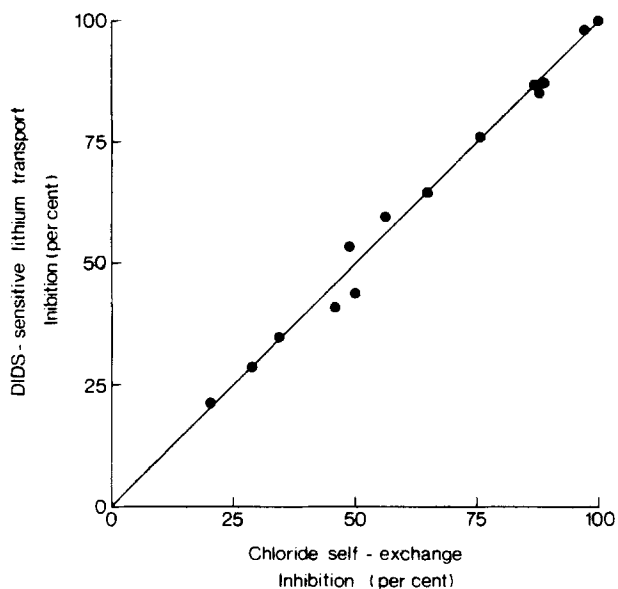


FIGURE 5. Correlation between inhibition of chloride self-exchange (abscissa) and inhibition of bicarbonate-induced (DIDS-sensitive) lithium influx into human erythrocytes. Red cells were treated with varying amounts of DIDS to cover the range of binding between 2.5×10^5 to 1.1×10^6 molecules per cell. After the DIDS treatment the cells were divided into two portions: one portion was used for the determination of chloride self-exchange, and the other for the determination of DIDS-sensitive lithium transport. The latter cells were washed repeatedly with 150 mM NaHCO_3 (to ensure that all cellular chloride was exchanged with bicarbonate), and thereafter suspended in a 150 mM LiHCO_3 medium to determine the lithium influx as in the experiments shown in Fig. 2. 100% inhibition of DIDS-sensitive lithium influx in a 150 mM LiHCO_3 medium corresponds to a reduction of lithium influx from 70 to 5.6 $\text{mmol (kg cell solids} \times \text{h)}^{-1}$.

lithium net extrusion decreased gradually with increasing bicarbonate concentration. A net flux of zero was obtained at a bicarbonate concentration of 100 mM, and a slight uptake of lithium was observed in an isotonic bicarbonate medium (150 mM).

It was demonstrated in the following section that bicarbonate ions do not interfere with that transport mechanism which moves lithium ions against a gradient at the expense of energy from the dissipation of the electrochemical gradient of sodium ions. The effect of bicarbonate on lithium net flux appears to be indirect (a) by inducing an increased passive lithium permeability, which will cause an increased influx of lithium, in a direction opposite to the lithium

transport by the counter-transport system, and (b) by reducing the electrochemical gradient for sodium, which is necessary to fuel the lithium counter transport. The sodium gradient is reduced because sodium influx is also increased in the presence of bicarbonate (Wieth and Funder, 1965; Funder and Wieth, 1967b).

Lithium/Sodium Counter-Transport in DIDS-Treated Cells

DIDS had no effect on the lithium/sodium transport system. Fig. 8 shows that the net efflux of lithium into a sodium chloride medium had the same time-course in DIDS-treated cells and in control cells.

TABLE IV
TEMPERATURE DEPENDENCE OF LITHIUM INFLUX INTO NORMAL AND DIDS-TREATED HUMAN RED CELLS SUSPENDED IN 150 mM LiHCO₃ MEDIA

Temperature °C	Lithium influx		
	(A) Untreated red cells	(B) DIDS-treated red cells	(A-B) DIDS-sensitive lithium influx
		<i>mmol (kg cell solids × h)⁻¹</i>	
38	71.8	5.9	65.9
28	26.4	2.1	24.3
18	7.4	1.1	6.3
10	2.45	0.54	1.91
0	0.87	0.58	0.29
Activation energy, <i>kcal (mol)⁻¹</i>	20.1	10.7	24.1
SD	0.7	2.2	1.1
Correlation coefficient	0.998	0.942	0.997

The influx values are mean values of 2-4 determinations at each temperature. The Arrhenius activation energies were calculated by linear regression analysis of the relation between ln (influx) and the reciprocal absolute temperature. The standard deviation of the slope was used for calculating the SD of the activation energies, which are presented together with the correlation coefficients of the regression analyses.

As shown in the previous sections bicarbonate does not induce any increased lithium permeability in DIDS-treated erythrocytes. One might, therefore, anticipate that the effect of bicarbonate on the time-course of lithium counter-transport would be eliminated after DIDS treatment, if the sodium/lithium counter-transport system operates normally in the presence of 150 mM bicarbonate and at a Pco₂ of 250 mm Hg. Fig. 9 shows that this was the case. The time-course and the degree of lithium net transport was indistinguishable from that found in a chloride medium, when the cells had been treated with DIDS before running the experiment in a bicarbonate medium. We can, therefore, conclude that the effect of bicarbonate on lithium net movements is due to the increased passive permeabilities to lithium and sodium: the increased lithium influx swamps the capacity of the counter-transport system for lithium extrusion. The increase of sodium permeability decreases the sodium gradient, which drives the counter-transport mechanism. This is shown in the following section.

The Effect of Bicarbonate on the Sodium Gradient

Fig. 10 illustrates that sodium accumulation in ouabain-treated red cells is also a function of extracellular bicarbonate concentration, although the effect of

bicarbonate on sodium permeability is about five times smaller than the effect on lithium permeability (cf. legend of Fig. 10). In the presence of ouabain the intracellular sodium concentration increased from 8 to 16 mmol/kg cell water over a 4-h period in the all-chloride medium, whereas the corresponding increase in the presence of 150 mM bicarbonate was from 8 to 38 mmol/kg cell water. The extracellular sodium concentration remained almost constant under

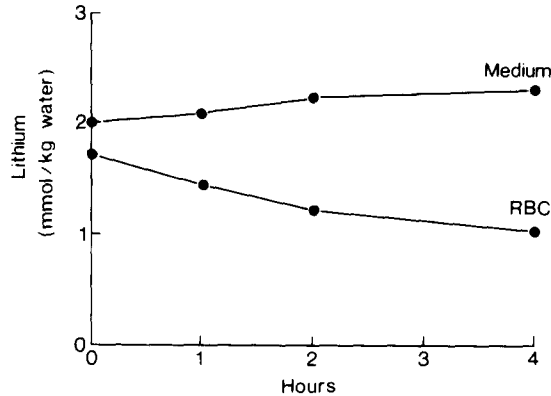


FIGURE 6. Sodium-lithium counter-transport in human red cells. Lithium-loaded cells with an intracellular lithium concentration of 1.7 mmol/kg cell water were suspended in a medium containing 2 mM LiCl and 150 mM NaCl buffered with glycylglycine to pH 7.4 (38°C, ouabain 10^{-4} M, hematocrit 32%). The water content of the cells was constant during the experiment (1.84 kg water/kg cell solids). The 4-h lithium net flux was 0.7 mmol/kg cell water, corresponding to 1.3 mmol/kg cell solids.

TABLE V
CHANGES OF INTRA- AND EXTRACELLULAR LITHIUM
CONCENTRATIONS AND OF RED CELL LITHIUM CONTENT DURING 4-h
COUNTER-TRANSPORT EXPERIMENTS

	Time = 0 h			Time = 4 h		
	Medium	Red cells	Red cells	Medium	Red cells	Red cells
	mmol/kg water		mmol/kg solids	mmol/kg water		mmol/kg solids
Mean	2.01	1.60	2.87	2.30	0.91	1.71
* SE	0.01	0.05	0.09	0.03	0.04	0.08
Li_o^+/Li_i^+	1.26			2.53		

* $n = 16$.

both sets of conditions. The sodium gradient (here expressed by the ratio of extracellular to intracellular concentrations) in both cases decreased from an initial value of ~ 20 . After 4 h the ratio had decreased to 10 in the chloride medium, and to 4 in the bicarbonate medium. It is, therefore, obvious that the sodium gradient across the membrane at the end of an experiment in the all-chloride medium was steeper than the sodium gradient from the bicarbonate medium to the intracellular water phase. It should be noted that the bicarbonate-induced increase of sodium permeability was also completely eliminated in DIDS-treated cells (Fig. 10).

DISCUSSION

The discussion deals with three topics: (a) the role of a low lithium permeability for the maintenance of a lithium gradient by the counter-transport system; (b) the relation between DIDS binding and inhibition of chloride and lithium

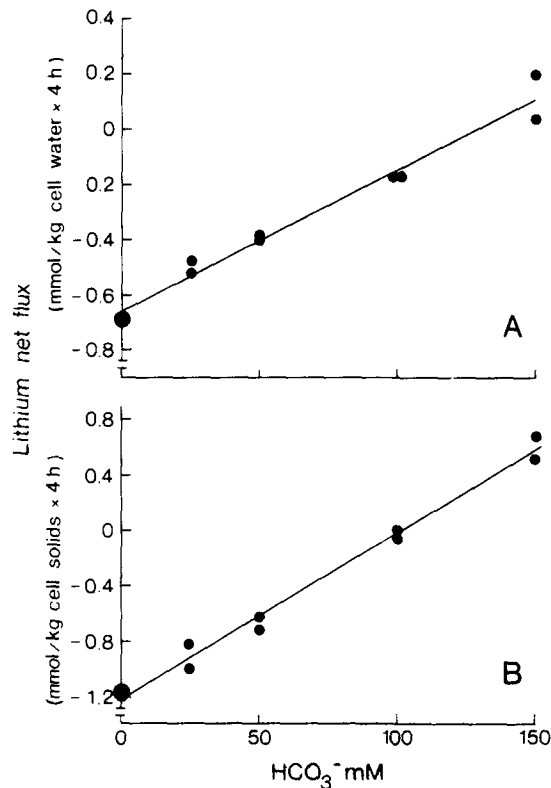


FIGURE 7. 4-h changes of (A) lithium concentration and (B) lithium content in human red cells as a function of the bicarbonate concentration in the medium. The cells were preloaded with lithium to a concentration of 1.6 mmol/kg cell water and suspended in media containing 2 mM LiCl (the media are described in Methods). The values indicated for a bicarbonate concentration of zero (150 mM NaCl medium) are the mean values from 16 experiments (of. Table V). The cells swell moderately during incubation in high bicarbonate media because of the uptake of NaHCO_3 and water. The data of (A) are, therefore, not an exact measure of the lithium net flux. The net flux can be evaluated from (B), because values expressed in mmol/kg cell solids refer to a constant number of cells (of. Table I). The bicarbonate concentration at which net movements of lithium was zero was 100 mM as read off from (B).

fluxes; and finally (c) the mechanism of the DIDS-sensitive lithium transport, especially its possible relation to the anion transport system.

Leak Pathways to Lithium

Operationally defined, there appear to be three pathways for lithium transport under the experimental conditions employed by us, in which lithium transport

by the active Na-K transport system (Duhm and Becker, 1977*a*; Pandey et al., 1977*a*) has been completely inhibited with ouabain. One pathway is the leak to lithium found when red cells are suspended in a bicarbonate-free chloride medium. It amounted to 5–6 mmol (kg red cell solids \times h)⁻¹ in a 150 mM LiCl

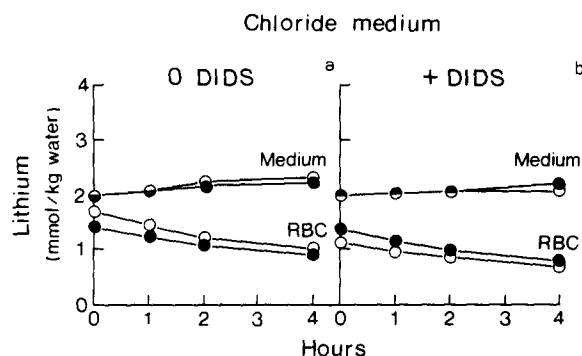


FIGURE 8. Sodium-lithium counter-transport in (a) normal and (b) DIDS-treated human erythrocytes. Duplicate experiments with cells from one donor incubated in 150 mM NaCl medium containing 2 mM Li⁺. Except for the DIDS treatment of the cells used in the experiments shown in (b), the experimental conditions were similar to the experiment shown in Fig. 6.

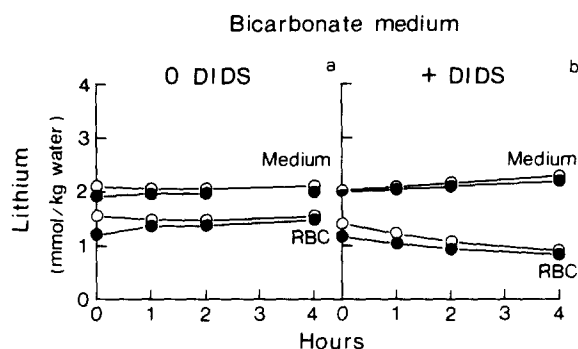


FIGURE 9. Sodium-lithium counter-transport in (a) normal and (b) DIDS-treated human erythrocytes. Duplicate experiments with cells from one donor incubated in 150 mM NaHCO₃ media containing 2 mM Li⁺. There was a small net uptake of lithium into the untreated cells, because the leak to lithium increased in the presence of bicarbonate (cf. Figs. 2 and 7). The lithium counter-transport proceeded normally, when the cells had been treated with DIDS before the experiment.

medium (Figs. 1 and 2). It was linearly related to extracellular lithium concentration (Fig. 1), and was not affected by DIDS treatment of the cells.

An additional leak to lithium was induced, when chloride was replaced with bicarbonate (at constant pH). Fig. 1 shows that this component of lithium influx was also linearly related to extracellular lithium concentration, and it increased linearly with bicarbonate concentration from 5 to 75 mmol (kg red cell solids \times h)⁻¹, as 150 mM LiCl was gradually replaced with 150 mM LiHCO₃ (Fig. 2). The

bicarbonate-induced lithium influx was completely eliminated in DIDS-treated cells, and lithium influx into the DIDS-treated cells was accordingly unaffected by the substitution of chloride with bicarbonate (Fig. 2).

The third pathway studied in the present work is the sodium/lithium counter-transport system (Haas et al., 1975; Duhm et al., 1976). The experimental conditions employed by us were similar to those used by Haas et al. (1975). Red

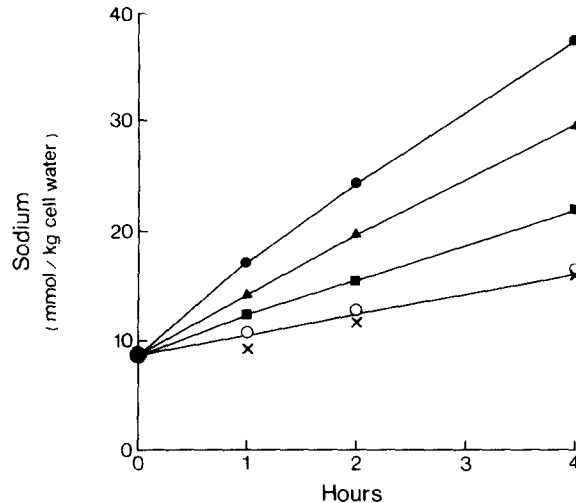


FIGURE 10. The change of intracellular sodium concentrations during incubation of red cells in 150 mM sodium media containing varying concentrations of chloride and bicarbonate (38°C, pH 7.4, ouabain 10^{-4} M). The bicarbonate concentrations are indicated as follows: 150 mM (●); 100 mM (▲); 50 mM (■); and 0 mM (×). Sodium influx into DIDS-treated cells incubated in 150 mM NaHCO₃ (○) was reduced to the level found when normal cells were incubated in an all-chloride medium. The results were obtained on the cells used for the counter-transport experiments shown in Fig. 7. The extracellular sodium concentration was constant during the experiments, and the ratio Na_o^+/Na_i^+ therefore decreased during the experiments as described in the text. It was calculated that sodium net flux during the 1st h of incubation in the presence of 150 mM sodium was a linear function of bicarbonate concentration: $M_{in}^{Na} = 0.091 HCO_3^- + 1.87$ (coefficient of correlation 0.988), where M_{in}^{Na} is the sodium influx in $mmol (kg\ solids \times h)^{-1}$ and HCO_3^- is the extracellular bicarbonate concentration.

cells were preloaded with lithium to an intracellular concentration of 1.6 mM and suspended in an isotonic NaCl medium containing 2 mM lithium (Fig. 6). The examination shown in Fig. 7 was carried out to investigate how lithium net movements are affected by variations of bicarbonate concentrations. The net flux decreased with increasing bicarbonate concentration in media where chloride was gradually replaced with bicarbonate, and no net movements of lithium took place, when bicarbonate concentration was 100 mM. DIDS has no effect on lithium movements in a bicarbonate-free medium (Fig. 8), so it can be concluded that the counter-transport system is separate from the bicarbonate stimulated lithium leak. As predicted from this conclusion, net counter-move-

ments of lithium were restored, when bicarbonate-induced increases of lithium permeability had been eliminated in DIDS-treated cells (Fig. 9).

At a bicarbonate concentration of 100 mM the DIDS-sensitive lithium influx was just sufficient to prevent a net flux of lithium by the counter-transport mechanism. Under these conditions, where lithium influx through the leak pathways was equal and opposite to lithium efflux by the counter-transport mechanism, one can evaluate the magnitude of lithium counter-transport at intra- and extracellular lithium concentrations of 1.6 and 2 mM. Influx through the leaks is according to Eq. 1:

$$0.036 \times Li_o + (0.003 \times HCO_3^- \times Li_o = 0.036 \times 2 \\ + (0.003 \times 100) \times 2 = 0.67 \text{ mmol (kg solids} \times \text{h)}^{-1}$$

at extracellular lithium and bicarbonate concentrations of 2 and 100 mM. In a bicarbonate-free NaCl medium, where the bicarbonate-induced leak is absent, the initial net flux should thus amount to 0.6 mmol (kg solids \times h)⁻¹ corresponding to 0.3 mmol (kg cell water \times h)⁻¹, of. Table I. This agrees with our finding of a net flux of 0.3 mmol/kg cell water during the 1st h of the experiment shown in Fig. 6.

The present results imply that steady-state ratios (Li_o^+/Li_i^+) of lithium in vivo will be lower than those obtained in vitro in the absence of bicarbonate. Variations of plasma bicarbonate will affect the steady-state distribution of lithium between cells and plasma of patients treated with lithium salts (Duhm and Becker, 1977*b*), just like the bicarbonate-induced variations in red cell sodium, which have been demonstrated during metabolic alkalosis (Funder and Wieth, 1974*a, b*). Eq. 1 shows the role plasma bicarbonate will play for the magnitude of the lithium influx. At a physiological bicarbonate level in plasma of 24 mM, two-thirds of the lithium influx takes place through the bicarbonate-dependent leak, and one third through the leak which is independent of bicarbonate.

DIDS Binding and Inhibition of Anion Exchange

The DIDS binding studies were performed to obtain a tool for the comparison of inhibition of anion exchange and of lithium transport. Thorough kinetic and biochemically oriented DIDS binding studies have recently been published by Lepke et al. (1976) and by Ship et al. (1977). Our studies confirm that the relation between DIDS binding and inhibition of chloride self-exchange is linear as shown in Fig. 3. Complete (i.e., >99.5%) inhibition of anion exchange was achieved by the binding of 1.1×10^6 DIDS molecules per cell (of. legend of Fig. 3). This should be compared with values of $1.2\text{--}1.3 \times 10^6$ molecules per cell found by different methods in three recent studies (Lepke et al., 1976; Halestrap, 1976; Ship et al., 1977). The values determined by Lepke et al. and by Ship et al. depend on determinations of the number of cells per milligram membrane protein, which as discussed in both articles may introduce variations of up to 50% when the binding per cell is calculated. Therefore, it is worth noting that determination of the number of DIDS molecules bound per cell in our study and in that of Halestrap (1976) was based only on known concentrations of DIDS and of cells, because all DIDS molecules present in the medium were bound to the cells.

The rate of the irreversible reaction between DIDS and membrane groups decreases steeply with temperature. By treating the red cells with DIDS at 38°C for 45 min we ensured a covalent, irreversible binding of DIDS to the membrane. Our studies confirm the prediction of Lepke et al. (1976) that the irreversible binding will not take place during short-lasting exposures of cells to DIDS at 0°C. DIDS binding to the anion transport system is assumed to take place in two steps: (a) electrostatic adsorption of the molecule to a membrane protein and (b) irreversible binding through the formation of a covalent thiourea-binding between a thiocyanate group in the DIDS molecule and a nitrogen of the protein. When both steps are allowed to proceed, all DIDS molecules present in the suspension are bound, as long as the number of molecules does not exceed 1.1×10^6 molecules per cell. The second irreversible step of the DIDS binding did not have time to take place, when red cells were exposed briefly to DIDS of 0°C. In the experiment shown in Fig. 4, inhibition was not a linear function of DIDS concentration (or in other words not a linear function of the number of DIDS molecules available per cell). The shape of Fig. 4 looks like an adsorption isotherm, and it is reasonable to assume that it represents primarily the first step of the DIDS binding. In that case an estimate of the affinity of the reversible first step can be made: it is assumed that a given number of DIDS molecules causes the same degree of inhibition, whether or not the binding is reversible (Lepke et al., 1976). Knowing the number of cells in the suspension and the degree of inhibition one can calculate the number of DIDS molecules taken up by the cells, and accordingly the concentration of unadsorbed DIDS molecules in the extracellular phase. The calculations showed that at low DIDS concentrations the cells in a suspension with a hematocrit of 0.5% take up ~65% of the DIDS molecules (Fig. 4). The true concentration of DIDS is, therefore, only one-third of the "total" DIDS concentration indicated on the abscissa of Fig. 4. The graph is shifted to the left if inhibition is related to "free" DIDS concentration, and the DIDS concentration causing 50% inhibition appears to be about 40 nM, indicating a very high affinity of DIDS for rapid reversible binding to the cell membrane.

DIDS-Sensitive Lithium Transport

The complete concordance between DIDS inhibition of anion exchange and of bicarbonate-stimulated lithium influx (Fig. 5) makes it worth considering whether inhibition of both transport processes is caused by the binding of DIDS to a single membrane component which is involved in the transport of both ions. It is known from the biochemical studies of Lepke et al. (1976) and of Ship et al. (1977) that some 80% of the DIDS molecules bind to the so-called "band III protein". This is an integral membrane protein with a molecular weight of $\sim 10^5$ dalton. The number of DIDS molecules bound to band III at complete inhibition of transport (Fig. 3) agrees extremely well with the estimate of 0.9×10^6 copies of band III molecules per cell (Steck, 1974), suggesting that there is one DIDS binding site per protein monomer. The present findings, therefore, make it likely that both the transport of anions and of lithium in the presence of bicarbonate depend on the integrity of a single membrane protein.

Reduction of bicarbonate-induced transport of sodium and lithium by inhibi-

tors of anion transport has previously been reported. Callahan and Goldstein (1972) observed that the bicarbonate-stimulated sodium permeability was reduced by furosemide, fluorodinitrobenzene, and by 4-acetamido, 4'-isothiocyanostilbene-2,2' disulfonic acid (SITS). All three compounds are known as inhibitors of anion transport, and all caused a reduction of bicarbonate-stimulated sodium influx, although complete inhibition was not obtained. Duhm and Becker (1977*b, c*, 1978) found inhibition of lithium influx in the presence of bicarbonate by dipyridamole, phlorizin, phenobutazone, salicylate and furosemide, all of which are inhibitors of anion transfer across the red cell membrane, although it must be noted that most of these compounds also affect other transport processes.

DIDS has been called "a specific inhibitor of anion transport" (Ship et al., 1977), and the effects on sodium and lithium transport mentioned here are the only exceptions observed so far. Two possibilities exist: (a) DIDS is not so specific an inhibitor of anion transport as has been believed; in that case, the close agreement between inhibition of chloride self-exchange and lithium transport (Fig. 5) is fortuitous, and lithium transport, e.g., might be inhibited by the binding of some of the 200,000 DIDS molecules per cell that bind to other membrane components rather than to the band III protein. (b) Another, and in our judgement more attractive, possibility is that the anion exchange and DIDS-sensitive lithium fluxes are in fact mediated by the same transport system. This would not necessarily imply that the anion transport system is also able to transfer positively charged ions: sodium and lithium form negatively charged ion pairs (NaCO_3^- and LiCO_3^-) in bicarbonate solutions. Such ion pairs could be transported by the anion exchange system, and dissociate again in the intracellular phase, where dissociation is favored because both pH and the lithium (or sodium) concentration is lower than in the extracellular phase. The overall result is that cations are smuggled in an anion disguise through a membrane, which has not lost its permselective properties (Wieth, 1970; 1971). The question whether this ion pair hypothesis is sound from a quantitative point of view is considered in the following section.

The Ion Pair Hypothesis

Is it reasonable to assume that the necessary number of lithium ions can be transported as ion pairs by the anion exchange mechanism? In order to evaluate this, it is necessary to know the extracellular LiCO_3^- concentration, and to relate the transfer of lithium by the bicarbonate-sensitive route to the turnover number of the transport system.

It is well established that alkali metal ions form ion pairs with inorganic anions in aqueous solution (Davies, 1962). The tendency of the cations to form such ion pairs varies with the nature of the anion. In the case of oxyanions, Robinson and Harned (1941) proposed a theory of "localized hydrolysis" to explain the fact that only lithium and sodium (not potassium, rubidium, and cesium) form ion pairs with proton-accepting oxyanions. This theory implies that the strongly hydrated cations can accommodate the oxygen atom of the anion in their innermost hydration shell. Predictions of this hypothesis have been verified for several anions (Davies, 1962). Thus, lithium and sodium hydroxide form electroneutral ion pairs

in aqueous solutions, whereas ion pairing of the hydroxides of potassium, rubidium, and cesium does not take place. The dissociation constant of NaOH is 4.2 times larger than that of LiOH, because the OH⁻-group binds most strongly to the cation with the smallest crystal radius. Garrels et al. (1961) determined the dissociation constant of the ion pair NaCO₃⁻ and found a value of 5.4×10^{-2} and found, moreover, that ion pairing between potassium and carbonate could not be demonstrated (Garrels and Thompson, 1962). Data for lithium are not available, but because the crystal radius of the cations determines the tendency of ion pairing, a rough estimate can be based on the assumption that the ratio between the dissociation constants of LiCO₃⁻ and NaCO₃⁻ is 1:4, as in the case of the hydroxides. In that case the concentration of LiCO₃⁻ in a lithium bicarbonate medium will be four times higher than that of NaCO₃⁻ in a sodium bicarbonate medium at the same pH. Bicarbonate-stimulated lithium permeability is 4–5 times higher than bicarbonate-stimulated sodium permeability (Fig. 10, Wieth, 1970; Duhm and Becker, 1978). This apparent difference of permeabilities may thus be due to the difference in concentrations of the ion pairs, in which case LiCO₃⁻ and NaCO₃⁻ are transported at the same rate by the transport system.

Assuming thermodynamic dissociation constants for NaCO₃⁻ and LiCO₃⁻ of 0.054 and 0.013, the ion pair concentrations in 150 mM bicarbonate solutions are 0.5 and 2.0 mM, respectively (cf. Methods). Is it reasonable to assume that the presence of these amounts of the ion pairs can cause a sufficient cation influx through the anion transport mechanism?

The turnover number for the anion exchange mechanism has been found to be 5×10^{10} ions per cell \times s in studies of chloride exchange at 38°C (Brahm, 1977). A DIDS-sensitive lithium influx of 67.5 mmol/kg cell solids \times h corresponds to an influx of 3.6×10^5 ions per cell \times s. This lithium influx is thus 1.3×10^5 times lower than the turnover number of the exchange mechanism. The calculated concentration of LiCO₃⁻ was 2 mM. If the affinity of the ion pair for the transport system is within the range found for other inorganic anions (apparent half-saturation at anion concentrations between 10 and 30 mM without any relation to the rate with which the anion is transported), LiCO₃⁻ influx could be mediated by the anion exchange mechanism, even if the rate of transfer of the ion pair was 2,000-fold slower than that of chloride (Brahm, 1977) and bicarbonate.¹ This rate of transfer is an order of magnitude faster than that of the most slowly transported inorganic anions (sulphate and phosphate), and an order of magnitude smaller than that of iodide, the most slowly transported halide. In our discussion it has been assumed that it is the aqueous concentration of ion pairs which is of importance for transport. There is evidence that the anion transport mechanism is located in a hydrophobic environment in the membrane (Brahm and Wieth, 1977). The tendency to form ion pairs will increase if the local dielectric constant is lower than that of water, and the binding between an- and cation will be stabilized if the ion pair is transferred through a hydrophobic region of the membrane.

The temperature dependence of DIDS-sensitive lithium influx is high (Table IV). The activation energy of 24 kcal/mol corresponds well to the 25 kcal/mol

¹ Wieth, J. O., and J. Brahm. Unpublished observation.

found for chloride between 0 and 38°C (Brahm, 1977). The activation energy was somewhat lower than that found for slowly transported inorganic anions (Dalmark and Wieth, 1972), but it is likely that the determination of the activation energy of the bicarbonate-induced fluxes leads to values that are too low: the experiments were performed at pH 7.4 at all temperatures, and because the dissociation constant of HCO_3^- increases with increasing temperature, and K_{LiCO_3} is likely to do the same, one must expect that the concentration of LiCO_3^- decreases with increasing temperature at the fixed pH. Bicarbonate-stimulated lithium influx, therefore would not increase as much as it would have done, if the concentration of the ion pair could have been kept constant over the whole temperature interval. Still the activation energy is substantially higher than the activation energies of other cation fluxes through the red cell membrane (Whittam, 1964), and it should be noted that activation energy of the DIDS-resistant lithium influx is only 11 kcal/mol.

As recently discussed by Knauf et al. (1977), the red cell membrane has a much lower permeability to net movements than to exchange diffusion of anions, and it is too early to decide whether the two different transport modes can be mediated by the same transport system (Vestergaard-Bogind and Lassen, 1974), or whether there exist two independent transport systems: an exchange and a conductance pathway. The slow rate of LiCO_3^- transfer makes it worth considering whether this transfer could be mediated by a pathway allowing a slow net transfer of anions. This does not appear likely. It has been observed that anion net movements are not inhibited by phlorizin and phloretin which are potent inhibitors of exchange fluxes (Kaplan and Passow, 1974; Cotterrell, 1975). Duhm and Becker (1978) have found that bicarbonate-stimulated lithium fluxes are inhibited both by phlorizin and by phloretin, and we have shown in unpublished experiments that bicarbonate-induced sodium fluxes are inhibited by phloretin. It appears, therefore, that it is not a net transport pathway, but the anion exchange mechanism in the red cell membrane that is involved in lithium and sodium transfer.

As an alternative to the ion pair hypothesis, Callahan and Goldstein (1972) have proposed that the formation of carbamino groups in the membrane by the overall reaction: $\text{RNH}_3^+ + \text{CO}_2 \rightarrow \text{RNHCOO}^- + 2 \text{H}^+$, may be responsible for the augmented permeability to sodium and lithium in bicarbonate media. Carbon dioxide partial pressure is elevated ($P_{\text{CO}_2} = 250 \text{ mm Hg}$ in a 150 mM bicarbonate medium at pH 7.4, 38°C), so there is no doubt that fixed positive charges by this reaction might be converted into fixed negative charges, thereby altering the physical nature of the permeation barrier in the membrane. In that case our results obtained with DIDS suggest that a surprisingly low number of amino groups in the membrane are involved in maintaining the cation barrier, and that most of them are located in the anion transport protein. However, although several of the compounds that inhibit both anion and bicarbonate-stimulated cation fluxes are amino reagents, others such as furosemide, dipyridamole, and phloretin are not likely to act by blocking carbamino group formation. It is also worth noting that CO_2 is a very weak inhibitor of chloride exchange (Dalmark and Wieth, 1972, Fig. 2), so there is no basis for assuming that an anion binding group is converted into a cation binding group after the binding of CO_2 .

Strongest indirect support for the ion pair hypothesis, and evidence against the possible role of carbon dioxide, has come from the studies of lithium and sodium permeabilities in media containing divalent oxyanions other than bicarbonate/carbonate (Duhm and Becker, 1978). Duhm and Becker predicted, and verified, that divalent anions like sulfite, oxalate, and phosphite, that have an interchange distance similar to that of carbonate (2.2 Å), form ion pairs with Li^+ and Na^+ , and, therefore, facilitate their influx through the red cell membrane without affecting the potassium permeability. The interpretation of such experiments is simplified because they can be run in the absence of CO_2 . The anion facilitated lithium and sodium fluxes studied by Duhm and Becker (1978) were inhibited by inhibitors of anion exchange such as SITS and dipyrindamole. They further showed that the oxalate-dependent lithium influx between pH 6 and 7 had a pH dependence similar to that of monovalent anion exchange (Gunn et al., 1973). It appears very useful that the hypothesis of ion pair transport can now be explored in systems where the number of variables is importantly reduced.

Events Leading to Net Transport of LiHCO_3 or NaHCO_3 According to the Ion Pair Hypothesis

Duhm and Becker (1978) have recently proposed that the net result, when LiCO_3^- or NaCO_3^- is exchanged with a bicarbonate ion is a Li^+/H^+ or Na^+/H^+ exchange, one hydrogen ion being transferred to the extracellular phase for every lithium or sodium ion entering. This is correct, if the events taking place through the anion exchange mechanism are considered alone, and the subsequent reactions between CO_2 , bicarbonate, and carbonate are disregarded. It has previously been shown that measurements of anion distribution between red cells and NaHCO_3 media indicated no major change in the state of dissociation of the intracellular buffers, as would have been the case if sodium ions were in fact exchanged for hydrogen ions (Wieth, 1969; Table I). Further evidence against a simple exchange comes from measurements of water net movements: cells exchanging Na^+ or Li^+ for H^+ in an isotonic salt solution would take up ~3 g water per mmol Na^+ or Li^+ , whereas the uptake of ($\text{Na}^+ + \text{HCO}_3^-$) or of ($\text{Li}^+ + \text{HCO}_3^-$) would cause a water uptake of ~6 g per mmol Na^+ or Li^+ , because both a cation and an anion move from medium to cells. We have previously presented data showing that red cells incubated in a NaHCO_3 medium take up 5.7–6.5 g water per mmol Na^+ (Funder and Wieth, 1967*b*; Wieth, 1969), and the water uptake accompanying lithium influx in the present work was 5.8 g water per mmol Li^+ . Our interpretation of the events taking place is presented in Fig. 11. The exchange of an ion pair for bicarbonate leads to an exchange of a hydrogen ion for the alkali metal cation. However, the hydrogen ion will be transported back into the cell by the mechanism, which is known as the "Jacobs-Stewart cycle" (Jacobs and Stewart, 1942). The cycle starts its run when, after the removal of carbonate from the extracellular phase by the transport of the ion pair, LiCO_3^- in the cell dissociates into Li^+ and CO_3^{2-} . The intracellular pH is lower than the extracellular pH, and the carbonate ion combines in the cell with a hydrogen ion to form bicarbonate. The hydrogen ion is derived from the dissociation of carbonic acid, and the removal of CO_2

creates that gradient which recruits carbon dioxide molecules from the extracellular phase. The small effect of acetazolamide on the rate of lithium transfer suggests that the spontaneous rate of CO_2 hydration at 38°C is sufficiently rapid to keep pace with the relatively slow cation influx.

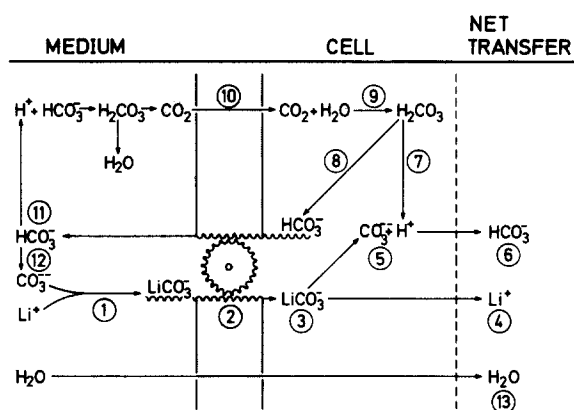


FIGURE 11. Bicarbonate-stimulated lithium influx into human red cells. The figure describes the sequence of events leading to intracellular accumulation of Li^+ , HCO_3^- , and H_2O as a combination of the ion pair hypothesis for the transfer of LiCO_3^- with the Jacobs-Stewart cycle for the transfer of H^+ through the red cell membrane (see text). The sequence is indicated by numberings on the figure: (1) formation of LiCO_3^- ; (2) exchange of extracellular LiCO_3^- for intracellular HCO_3^- via the coupled anion exchange mechanism; (3) intracellular dissociation of LiCO_3^- into (4) Li^+ and (5) CO_3^{2-} ; (6) conversion of CO_3^{2-} to HCO_3^- by the uptake of a H^+ (7), formed by dissociation of H_2CO_3 into H^+ and HCO_3^- (8); the H_2CO_3 is formed by hydration of CO_2 (9); the removal of intracellular CO_2 creates a gradient that makes CO_2 diffuse from the extra- to the intracellular phase (10); by the dissociation of extracellular bicarbonate is formed a hydrogen ion (11), which is consumed in the formation of CO_2 , and a carbonate ion (12), which completes the cycle, when it combines with a lithium ion to form LiCO_3^- (1). The net result of all reactions is shown in the right hand side of the figure. One Li^+ and one HCO_3^- is transferred from medium to cells. The accumulation of ions will induce osmotic water transport into the cells (13), so that osmotic equilibrium is maintained across the membrane.

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