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Effects of High Hydrostatic Pressure on 'Passive' Monovalent Cation Transport in Human Red Cells

A.C. Hall* and J.C. Ellory†
Physiological Laboratory, Cambridge CB2 3EG, United Kingdom, and †University Laboratory of Physiology, Oxford OX1 3PT, United Kingdom

Summary. The effects of high hydrostatic pressure (up to 400 ATA) on the 'passive' (defined as ouabain + bumetanide + EGTA-insensitive) influx and efflux of radiotracer cations (K^+ Rb^+, Na^+, Cs^+) has been studied in human red cells suspended at different medium tonicities giving altered cell volumes. Under all conditions studied, cation permeability was raised at pressure, and at least two distinct components were found to comprise this flux. Thus, increasing pressure (1) caused a generalized increase in cation permeability which was unaffected by the anion present, demonstrated linear concentration dependence, and was *reduced* with cell swelling, and (2) stimulated a specific KCl pathway which was Cl^- dependent, demonstrated saturation kinetics with raised $[K]_o$ and was *increased* with cell swelling. High hydrostatic pressure caused a significant alteration to red cell morphology from the normal biconcave disc to cup-shaped forms and it is proposed that this is associated with the unmasking of the volume-sensitive KCl system (2).

Key Words human red cell · hydrostatic pressure · 'passive' cation transport · volume-sensitive KCl transport · activation volume · erythrocyte morphology

Introduction

The effects of high hydrostatic pressure on membrane cation transport processes are difficult to predict. From its effects on enzymes and in other biochemical systems (Macdonald, 1975) one might expect that increasing pressure would inhibit transport; however, pressure has also been shown to increase the cation permeability mediated by some systems (Hall et al., 1982; Ellory et al., 1985). We have demonstrated previously (Hall et al., 1982) that in human red blood cells, high pressure (up to 400 ATA) inhibits ouabain-sensitive (i.e., Na^+/K^+ pump) and bumetanide-sensitive (i.e., $Na^+ + K^+$ cotransport) components of K^+ transport. In marked contrast, however, the residual flux remaining after ouabain and bumetanide treatment was markedly stimulated under pressure and was Cl^- dependent.

Red cells of many species demonstrate a specific volume-sensitive KCl transport pathway which is activated by cell swelling produced by varying medium tonicity (Dunham & Ellory, 1981; Kregenow, 1981; Ellory et al., 1982, 1985; Lauf, 1985). Erythrocytes from normal human donors are, however, unusual as 'passive' K^+ transport is generally unresponsive to changes in cell volume (Ellory et al., 1985). Treatment with the thiol-reactive agent NEM does, however, reveal a volume-sensitive KCl flux suggesting that the pathway (or one very similar to it) is present in the untreated human erythrocyte but is latent under normal conditions (Lauf et al., 1984, 1985; Ellory et al., 1985). The Cl^- dependence of the pressure-induced K^+ flux reported in our earlier study raises the possibility that a substantial part of the K^+ permeability observed under pressure may be due to the activation of an otherwise latent volume-sensitive KCl transport system.

In the present paper we have examined further the action of pressure on 'passive' (defined as ouabain + bumetanide + EGTA-insensitive) monovalent cation transport in human red cells. Our data support the hypothesis that high hydrostatic pressure activates a volume-sensitive KCl transport pathway present in the human red cell membrane. In addition, high pressure was also found to increase cation permeability in a more general fashion, with Na^+ and Cs^+ also showing enhanced permeability. Thus, when the volume-sensitive KCl flux was inhibited by cell shrinking or Cl^- replacement, K^+ transport through this additional pathway(s) was clearly observed, and was kinetically distinct from the Cl^--dependent system. On the basis of scanning electron microscope studies, it is proposed that the effect of pressure on volume-sen-

*Present address: University Laboratory of Physiology, University of Oxford, Parks Road, Oxford OX1 3PT, England.

sitive KCl transport may be related to the fact that pressure alters the morphology of a large fraction of red cells from biconcave discs to a cup-shaped appearance, i.e. this pressure-induced morphological change is associated with the activation of the KCl flux. A preliminary account of some of this work has already been published (Ellory & Hall, 1985).

Materials and Methods

1. Blood

Fresh blood was taken from normal healthy human donors (male and female, aged 20 to 40) by venepuncture into heparinized syringes. The red cells were washed three times by centrifugation (2,000 × *g*, 5 min) and aspiration in a medium of the following composition (mM); NaCl 150; MOPS 15; glucose 10 (pH 7.4 with NaOH) and the buffy coat removed.

2. Tracer Fluxes

The techniques employed for radioisotope influx and efflux at atmospheric and high hydrostatic pressure were as previously described (Hall et al., 1982; Young & Ellory, 1982). Briefly, flux experiments were performed in an incubation medium containing the appropriate cations (Na^+, K^+, Cs^+, Rb^+) to give the required final concentrations (*see below*), with (mM): MOPS (15), glucose (10), ouabain (0.5), bumetanide (0.1) and EGTA (0.1), (pH 7.4 with Tris base). The uptake of radiotracer cations (^{86}Rb, ^{43}K, ^{22}Na, ^{134}Cs) was determined in cell suspensions with radioactivity at about 1 μCi/ml. Intracellular isotope accumulation was measured at the end of the flux period (usually 30 min) by washing cells free of extracellular isotope at 5°C using (mM): $MgCl_2$ (106), MOPS (15), (pH 7.4 at 5°C with Tris base), by centrifugation (10,000 × *g*, 10 sec) and aspiration.

For efflux experiments, fresh, washed red cells were suspended at 10% hematocrit in (mM): NaCl (150), KCl (7.5), MOPS (15), glucose (10), EGTA (0.1), (pH 7.4 with NaOH) and incubated at 37°C for about 3 hr in the presence of the appropriate isotope at about 10 μCi/ml. At the end of the loading procedure the cells were quickly washed in ice-cold, isotope-free flux medium and resuspended in the appropriate experimental solution. The cell suspensions were then transferred to Eppendorf centrifuge tubes for the control (i.e., unpressurized flux), and to disposable syringes containing mixing bars for the flux at high pressure. Care was taken to remove air bubbles to ensure hydrostatic compression, and control experiments established that there was no measurable change in the temperature of the pressure vessel with rapid compression to 400 ATA (*see* Hall et al., 1982 for further methodological details). The rate constants for K^+ or Na^+ efflux were calculated as previously described (Hall & Willis, 1984). The time spent by pressurized samples at 1 ATA during analysis and prior to repressurization was small (about 2 min) and a correction was found to be unnecessary.

Red cell volume was varied and monitored as previously described (Dunham & Ellory, 1981), by adding sucrose or distilled water for shrunken or swollen cells, respectively, thereby maintaining ionic strength constant. Relative cell volume (r.c.v.) was determined by measuring the percent packed cell volume (% PCV) of a concentrated sample of the cell suspension (usually 40% hematocrit to increase the accuracy of the measurement) using microhematocrit centrifuge tubes (Hawksley Ltd., Lancing, Sussex, U.K.). The hemoglobin concentration of these cell suspensions was then measured spectrophotometrically (*see below*), and taking the r.c.v. of the cells in isotonic saline (i.e. normal value) to be 1.00, corrections to the r.c.v. due to differences in hematocrit between the cell suspensions were then made (Dunham & Ellory, 1981). Anion substitution was performed as previously reported (Dunham et al., 1980). Briefly, this involved washing cells in a Cl^--free medium, with $CH_3SO_4^-$ (or for some experiments, Br^-, NO_3^- or I^-) acting as the anion replacement, and incubating at 37°C. When external concentrations of cations or anions were varied, ionic and osmotic strength were maintained constant, and further details of the experimental solutions are given in the relevant Figure legends.

The experimental temperature used throughout this study was 37 ± 0.1°C. The hematocrit for flux experiments was about 5%, the exact value being determined by measuring the hemoglobin released with Drabkin's reagent at A_{540} in an appropriate dilution of the cell suspension (Dacie & Lewis, 1975).

3. Volume and Morphology of Red Cells Fixed at Pressure

Red cells were fixed at atmospheric and high hydrostatic pressure by the following method. Washed erythrocytes were suspended at high hematocrit (30 to 50%) for the volume determinations within sealed syringes containing mixing bars. Cell suspensions of lower hematocrit (about 5%) were used for the morphological studies. The saline used was identical in composition to that used for the flux studies including inhibitors (*see above*, Tracer Fluxes). The cells were separated from the fixative (usually 0.5% vol/vol glutaraldehyde) by a layer of the inert oil di-*n*-butylpthalate. The cell suspensions were equilibrated at 37°C in the water bath or pressure vessel for 5 min. The samples to be pressurized were then compressed and allowed to equilibrate for 30 min, i.e., a length of time comparable to that used for the flux studies. Next, the pressure vessel was inverted several times causing the mixing bars to fall through the layer of oil and thoroughly mix the cells with the fixative. After 30 min the cells were decompressed and allowed to stand for a few minutes as the oil layer separated out. For volume measurements, samples of the fixed cell suspension were taken into microhematocrit centrifuge tubes, centrifuged and the cell volume estimated as described above with corrections made as appropriate for differences in hematocrit. Control experiments (*see* Results section 5) confirmed that fixed red cells were unresponsive to medium tonicity supporting the notion that the volume of cells remained unchanged during decompression.

For scanning electron microscopy, following the separation of cells from the di-*n*-butylpthalate, erythrocytes were washed free of unreacted glutaraldehyde by low-speed centrifugation (50 × *g*, 2 to 3 min) and aspiration using a Ca^{2+}-free saline, and allowed to settle for 30 min on cover slips coated with poly-*l*-lysine (Sanders et al., 1975). The cover slips were then placed in 100 mM sodium cacodylate buffer (pH 7.2 with HCl) containing 2% glutaraldehyde for 30 min, washed briefly in buffered saline, and post-fixed with 1% osmium tetroxide in cacodylate buffer for 30 min. Next, the cells were dehydrated in a graded series of ethyl alcohol solutions. The red cells were then critical-point dried in CO_2, coated with 20 nm of gold, and examined in a J.E.O.L. J.S.M. 35 CF scanning electron microscope.

4. Reagents and Isotopes

Analytical grade reagents were obtained from BDH Ltd., Poole, Dorset, and solutions prepared with glass-distilled water. EGTA, EDTA, MOPS, Tris, di-*n*-butylpthalate, poly-*l*-lysine and ouabain were purchased from Sigma Ltd. Osmium tetroxide and glutaraldehyde (E.M. grade) were obtained from Emscope Laboratories Ltd., Ashford, Kent. Sodium and potassium methylsulfate were obtained from ICN Pharmaceuticals Inc., Plainview, N.Y. Bumetanide was a gift from Leo Laboratories, Princes Risborough, Bucks. RbCl and CsCl were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The radioisotopes ^{86}Rb, ^{22}Na, and ^{134}Cs were obtained from Amersham International. ^{43}K was purchased from the MRC Cyclotron Unit at the Hammersmith Hospital, London.

5. Abbreviations

MOPS: 4-morpholinepropanesulfonic acid; Tris: tris-(hydroxymethyl) aminomethane; EGTA: ethyleneglycol-bis(B-aminoethylether)-N,N,N',N',-tetraacetic acid; EDTA: ethylenediamine-tetraacetic acid; ATA: atmospheres absolute (1 ATA = 0.101 MPa); ΔV^*: apparent activation volume; K_m is the substrate concentration at half-maximal flux (apparent Michaelis constant); V_{max} the apparent maximal velocity of the flux (*see below*) and α is the proportionality constant (in units of [(mmol/liter cells · hr)/mM] and represents the slope of the nonsaturable component of cation uptake as a function of the external cation concentration.

K^+ uptake experiments which demonstrated saturation kinetics (example given in Fig. 1) were fitted to a two-component kinetic model representing saturable and nonsaturable components (Dunham et al., 1980). The apparent kinetic constants were computed using the Hanes-Woolf plot as described by Cornish-Bowden (1976). Other data were fitted to a least-squares linear regression, or curves drawn by eye as indicated. Significant differences were determined using an unpaired Students *t*-test.

6. Calculation of ΔV^*

At constant temperature, the rate of a chemical reaction at high pressure is influenced by the volume change which occurs in the rate-determining step. Thus the rate constant k is related to pressure in the following way;

$$\frac{d \log_{10} k}{dP} = \frac{-\Delta V^*}{2.303\, RT}. \quad (1)$$

Data are usually plotted as the logarithm of the reaction velocity as a function of pressure, and from the slope of the relationship, the activation volume may be calculated using the following equation (*see* Hochachka & Somero, 1973):

$$\Delta V^* = 2.303\, RT \frac{(\log_{10} k_1 - \log_{10} k_2)}{(P_2 - P_1)}. \quad (2)$$

The symbols are defined as follows: k_1 and k_2 represent the flux rate at pressures P_1 and P_2, respectively; R is the gas constant (82.07 ml - atmosphere/C°/mole), and T the absolute temperature (310°K) yielding the activation volume in units of ml/mole. When the reaction proceeds with an increase in volume (i.e. + ΔV^*) then increasing pressure inhibits the process, and if values of ΔV^* are negative then raising pressure stimulates the reaction. If a graph of the relationship between the logarithm of the flux and hydrostatic pressure is a straight line, then the activation volume is unaffected by high pressure. Graphs of the data describing 'passive' (i.e., ouabain, bumetanide and EGTA-insensitive) K^+ or Na^+ uptake in swollen or shrunken cells suspended in either Cl^- or $CH_3SO_4^-$ media were found to be linear (examples are given in Fig. 2). This indicates that a single activation volume is involved and therefore values for ΔV^* may also be determined from data obtained at a single pressure (usually 400 ATA).

Results

1. Action of Hydrostatic Pressure on 'Passive' K^+ Influx

(a) *Effect of Varying Red Cell Volume*

Figure 1 shows the effects of 400 ATA on K^+ uptake as a function of $[K]_o$ in swollen and shrunken red cells suspended in a Cl^- medium. At 1 ATA K^+ uptake was a linear function of $[K]_o$ over the range studied, and for the data pooled from several donors there was a small (but not significant) increase in K^+ uptake with cell swelling (Table 1 and *see below*). In both swollen and shrunken cells increasing pressure to 400 ATA elevated K^+ uptake; however, the kinetic characteristics of K^+ influx were markedly different in each case.

K^+ uptake in swollen cells at pressure (Fig. 1) was well described by a two-component system consisting of a saturable flux obeying 'simple' Michaelis-Menten type kinetics, and a linear component (*see* Dunham et al., 1980). In contrast, the data for shrunken cells under pressure were consistent with an increased linear (nonsaturable) component of K^+ influx, there being no evidence of a saturable transport system under these conditions. The saturable flux observed in swollen cells under pressure exhibited a relatively high apparent K_m and V_{max} (Table 1).

The effect of pressure on the 'residual' nonsaturable component in swollen cells is difficult to determine because the saturable flux exhibits a low apparent affinity (Table 1). Thus, even at high values of $[K]_o$, the saturable flux will still give a significant contribution to total K^+ uptake, and will therefore tend to exaggerate the magnitude of the nonsaturable component. An estimate of the involvement of the nonsaturable flux may, however, be obtained at high values of $[K^+]_o$ provided that an appropriate correction is made for the K^+ influx mediated by the saturable component. Over the range 90 to 130 mM $[K]_o$, the slope of total K^+ uptake was 0.0417 ± 0.0090 [(mmol/liter cells · hr)/

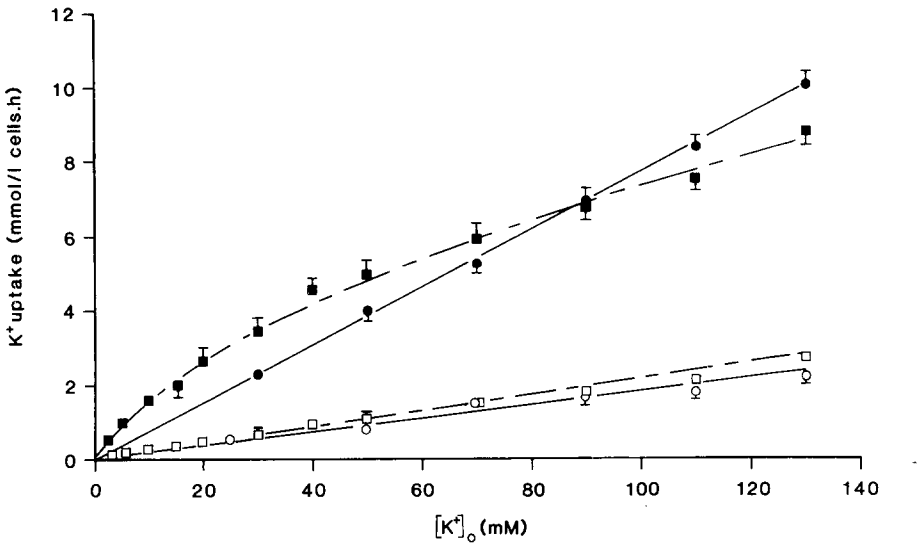

Fig. 1. Effect of pressure on 'passive' K^+ uptake as a function of $[K]_o$ in red cells of different volume in a Cl-containing incubation medium. K^+ influx was measured at 1 (open symbols) and 400 ATA (closed symbols) in shrunken (○, ●, respectively) and swollen (□, ■, respectively) cells as a function of the external K^+ concentration with Na^+ as replacement cation, in the incubation medium including inhibitors as described in Materials and Methods. In this experiment the relative cell volumes of shrunken and swollen cells (normal cells = 1.00) were 0.88 and 1.13, respectively. At 1 ATA the values for α in swollen and shrunken cells were 0.0215 ± 0.0012 and 0.0186 ± 0.0011 [(mmol/liter cells · hr)/mM], respectively. In swollen cells at 400 ATA the K_m was 33.5 mM, the V_{max} 5.1 mmol/liter cells · hr. The slope of the nonsaturable component over the range 90 to 130 mM corrected for the contribution of the saturable K^+ flux (*see* text) was 0.0350 ± 0.0009 [(mmol/liter cells · hr)/mM]. In shrunken cells at 400 ATA, α was 0.0769 ± 0.0062. The curve describing K^+ uptake in swollen cells at 400 ATA was drawn using the two-component kinetic model (Dunham et al., 1980) incorporating the values for the K_m, V_{max} and α given above. In this and in subsequent figures, results are means (± SD); for pooled data *see* Table 1

Table 1. Effects of hydrostatic pressure on the apparent kinetic properties of K^+ uptake as a function of $[K^+]_o$ in swollen and shrunken cells.[a]

Cell volume	Pressure (ATA)	V_{max} (mmol/liter cells · hr)	K_m (mM)	α [(mmol/liter cells · hr)/mM]
Shrunken	1	—	—	0.0118 ± 0.0012
				(0.0119 ± 0.0008)
	400	—	—	0.0680 ± 0.0080
				(0.0591 ± 0.0065)
Swollen	1	—	—	0.0133 ± 0.0013 NS
				(0.0132 ± 0.0013)
	400	6.38 ± 0.63	45.2 ± 7.0	0.0342 ± 0.0050*
		(5.67 ± 0.55)	(39.4 ± 3.3)	(0.0350 ± 0.0032)

[a] 'Passive' K^+ uptake was determined using the radiotracer ^{86}Rb, with the apparent Michaelis Menten constants computed as described (Materials and Methods, and *see* Fig. 1). Also given in parentheses are results obtained from experiments using ^{43}K as the tracer for K^+. Results are means (± SEM) from blood obtained from at least five donors for the Rb^+ data, and three donors for the ^{43}K data. An asterisk (*) indicates a significant difference ($P < 0.001$) between shrunken and swollen cells; NS indicates no significant difference ($P > 0.05$).

mM], and over the same range the saturable component contributed 22 ± 3% to total K^+ influx. Thus the corrected value for the nonsaturable flux was 0.0342 ± 0.0050 (results are means ± SEM from five experiments on different donors). Therefore at 400 ATA, the nonsaturable component of K^+ influx was increased by approximately 2.6× compared to the unpressurized control.

The K^+ flux in shrunken cells was more pressure-sensitive with uptake being stimulated approx-

imately 6× (Table 1), yielding a higher value for the apparent activation volume (Table 5). Close agreement was also found between saturable and nonsaturable fluxes determined using the radiotracers ^{86}Rb or ^{43}K (Table 1, *see also* Table 5), confirming the validity of the former as a tracer for K^+.

The effects of intermediate pressures on the kinetic properties of the saturable K^+ flux in swollen cells were also determined over the range 1 to 50 mM $[K]_o$ with the Na^+ as replacement cation. In results from three separate experiments, the V_{max} and K_m were (means ± SEM), 2.76 ± 0.21 mmol/liter cells · hr, and 58.00 ± 0.8 mM, respectively, at 200 ATA, this changing to 7.81 ± 0.42 and 38.8 ± 0.5, respectively, at 400 ATA. Thus increasing pressure raised both the magnitude and the affinity of K^+ uptake in swollen cells.

It was important also to investigate the effects of intermediate pressures on the nonsaturable components of 'passive' cation uptake. Figure 2 shows two examples of these experiments where Na^+ or K^+ (^{86}Rb) uptake was studied in shrunken cells suspended in a Cl^- medium. When plotted semilogarithmically, there was a linear relationship ($r > 0.985$) between cation influx and pressure. Similar linear relationships were also obtained for Na^+ uptake in swollen cells in Cl^- or $CH_3SO_4^-$ media, and for the nonsaturable component of K^+ uptake in swollen cells in a Cl^- medium (*data not shown*). These results indicate that within experimental error, the apparent activation volume (ΔV^*) is unaltered by high pressure and therefore values of ΔV^* may also be estimated by taking data at a single pressure (*see* Materials and Methods, and Table 5 for pooled data).

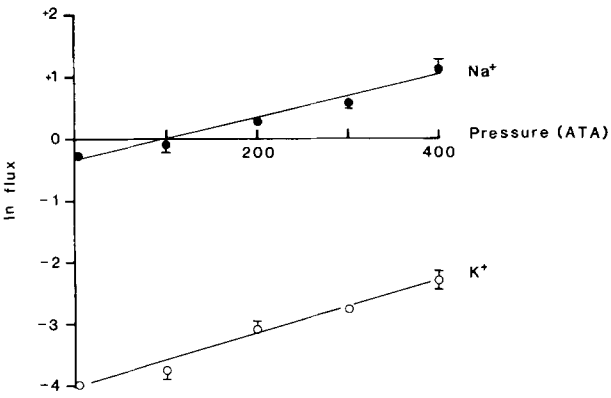

Fig. 2. Effect of increasing hydrostatic pressure on 'passive' Na^+ and K^+ uptake in shrunken red cells. For Na^+ influx (●; r.c.v. = 0.88) the external Na^+ concentration was 65 mM with K^+ as replacement, and for K^+ uptake (○; r.c.v. = 0.90) the slope of the relationship K^+ uptake *vs.* $[K]_o$ over the range 90 to 130 mM (Na^+ replacement) was determined as described in a Cl^- incubation medium. The natural logarithm of the flux is plotted as a function of hydrostatic pressure. The slopes of the linear regression describing the data for Na^+ and K^+ uptake were 0.003031 ± 0.000016 atm^{-1} and 0.004076 ± 0.000015 atm^{-1} respectively, yielding values for the ΔV^* of −77.11 ± 0.3 and −103.3 ± 0.3 ml/mole for Na^+ and K^+, respectively (*see* Table 5 for pooled data)

(b) Effect of Anion Substitution on 'Passive' K^+ Influx

(i) K^+ uptake vs. $[K]_o$. As the volume-sensitive K^+ flux in other species has been shown previously to be dependent on Cl^- (Dunham & Ellory, 1981; Ellory et al., 1982; Lauf et al., 1985), the replacement of Cl^- by $CH_3SO_4^-$ should inhibit the saturable component of K^+ uptake in swollen cells observed at pressure. Figure 3 shows that in a $CH_3SO_4^-$ medium this is indeed the case and there was no evidence of a saturable flux either in swollen or shrunken cells at pressure in a Cl^--free medium, with linear concentration dependence being observed under both conditions. Furthermore, at 400 ATA and in the absence of Cl^-, swelling the cells *reduced* 'passive' K^+ uptake. In pooled results from experiments on four different donors, the slope of the nonsaturable component α was not significantly different ($P >$ 0.05) between shrunken and swollen cells at 1 ATA (0.014 ± 0.004 and 0.015 ± 0.002 [(mmol/liter cells · hr)/mM], respectively; means ± SEM). At pressure, α was significantly ($P < 0.01$) greater in shrunken cells compared to swollen cells (0.066 ± 0.010 and 0.044 ± 0.008 [(mmol/liter cells · hr/mM], respectively). This effect of cell swelling in a $CH_3SO_4^-$ medium is in marked contrast to the effects of pressure on K^+ uptake in swollen cells suspended in a Cl^- medium (Fig. 1), but similar to the effect of swelling on the calculated nonsaturable linear component of K^+ uptake, and for the other cations (i.e., Na^+, Cs^+) studied (Tables 1 and 5).

(ii) Cl^- dependence of pressure-induced K^+ flux. In a further series of experiments, the Cl^- dependence of volume-sensitive K^+ transport was investigated at pressure (Fig. 4). At atmospheric pressure in a Cl^--free incubation medium there was no difference between K^+ influx in swollen and shrunken cells in agreement with previous studies (Ellory & Hall, 1985; Ellory et al., 1985). As the $[Cl]_o$ was progressively increased however, 'passive' K^+ uptake showed an increase in swollen cells, whereas in shrunken cells there was no significant change (< ± 3%) in flux. This suggests that at least in some donors there is a small Cl^--dependent K^+ flux present in swollen cells at 1 ATA (i.e., in 'normal' conditions) and the significance of this is considered in detail in the Discussion. At 400 ATA

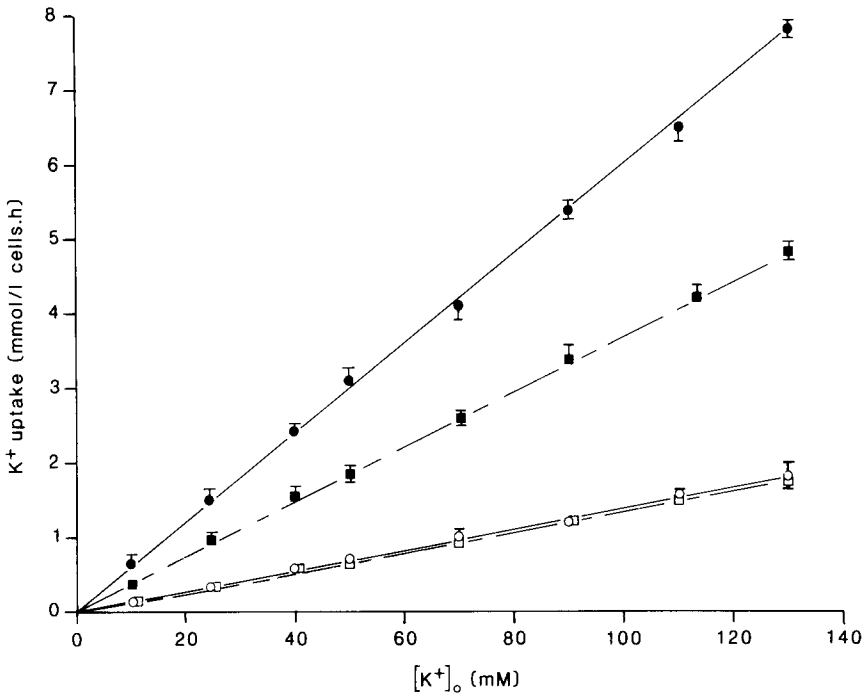

Fig. 3. Effect of pressure on 'passive' K^+ uptake as a function of external $[K]_o$ in red cells of different volume in a $CH_3SO_4^-$-containing incubation medium. K^+ influx was measured at 1 (open symbols) and 400 ATA (closed symbols) in shrunken (○, ● r.c.v. = 0.88) and swollen (□, ■ r.c.v. = 1.12) red cells, respectively. Ionic and osmotic strength were maintained constant using Na^+ as replacement cation, with the other constituents of the flux medium as described in Materials and Methods. In this experiment, values of α at 1 ATA in shrunken and swollen cells were 0.0138 ± 0.0003 and 0.0131 ± 0.0006 [(mmol/liter cells · hr)/mM], respectively, and at 400 ATA 0.0601 ± 0.0010 and 0.0369 ± 0.0010, respectively. For pooled data *see* text

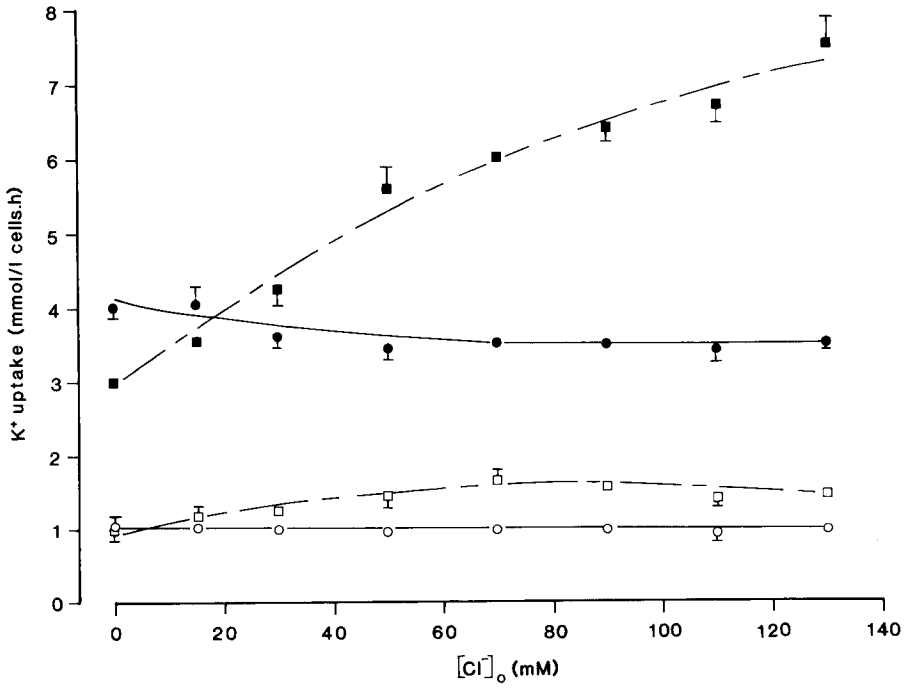

Fig. 4. Effect of hydrostatic pressure on the $[Cl]_o$ dependence of 'passive' K^+ uptake in red cells of different volume. K^+ influx was determined at 1 and 400 ATA in shrunken (○, ●, respectively; r.c.v. = 0.88) and swollen (□, ■, respectively; r.c.v. = 1.13) red cells. $[Cl^-]_o$ was replaced by $CH_3SO_4^-$ with $[Na]_o$ and $[K]_o$ maintained constant at 65 mM with the other constituents present as described in Materials and Methods. The curves were drawn by eye

Table 2. Effect of hydrostatic pressure on the anion dependence of 'passive' (a) K^+ and (b) Na^+ uptake[a]

	n	Anion	Cation uptake (mmol/liter cells · hr)		ΔV^* (ml/mole)
			1 ATA	400 ATA	
(a) K^+ Uptake	8	Cl^-	0.160 ± 0.009	0.761 ± 0.064	−99.5 ± 5.2
	8	$CH_3SO_4^-$	0.140 ± 0.013 NS	0.653 ± 0.056 NS	−98.2 ± 5.8
(b) Na^+ Uptake	3	Cl^-	1.812 ± 0.036	5.225 ± 0.116	−67.5 ± 2.1
	3	$CH_3SO_4^-$	2.116 ± 0.100*	6.017 ± 0.215*	−66.7 ± 2.5

[a] Cation influx was measured at $[Na^+]_o$ = 145 mM and $[K^+]_o$ = 7.5 mM with the other constituents as described (*see* Materials and Methods), in (a) cells shrunken to an r.c.v. of approximately 0.86 (range 0.84 to 0.88) or (b) cells of 'normal' volume. NS indicates no significant difference ($P > 0.05$), and an asterisk (*) a significant difference at the level $0.05 > P > 0.02$ between fluxes measured in either medium at 1 ATA or 400 ATA. Results are means (± SEM) from *n* independent experiments on blood from various donors.

Table 3. Effect of divalent cations and their chelators on the pressure-induced K^- flux in swollen red cells[a]

Condition (mM)	K^+ Influx (mmol/liter cells · hr)		ΔV^* (ml/mole)
	1 ATA	400 ATA	
0.1 EGTA	0.565 ± 0.055	2.318 ± 0.195	−90.0 ± 5.4
0.1 EDTA	0.552 ± 0.012	2.519 ± 0.112	−96.8 ± 2.8
'0' Ca^{2+}	0.534 ± 0.042 NS	2.240 ± 0.186 NS	−91.4 ± 5.3
5 Ca^{2+}	0.436 ± 0.036 NS	1.709 ± 0.182*	−87.1 ± 6.6
10 Ca^{2+}	0.424 ± 0.032*	1.562 ± 0.230*	−83.1 ± 6.9
20 Ca^{2+}	0.416 ± 0.020*	1.556 ± 0.230*	−84.1 ± 8.9
20 Mg^{2+}	0.442 ± 0.012**	1.514 ± 0.061**	−78.5 ± 2.6
10 Ca^{2+} : 10 Mg^{2+}	0.418 ± 0.023**	1.364 ± 0.150*	−75.4 ± 6.8

[a] 'Passive' K^+ uptake was measured as described (*see* Materials and Methods) with $[K^+]_o$ = 65 mM, and sucrose and NaCl added as appropriate to maintain osmolarity and ionic strength constant. Red cells were swollen by about 14%, and for the condition '0' Ca^{2+}, no Ca^{2+} was added, although contaminant Ca^{2+} was estimated to be 10 μM by atomic absorption spectrophotometry. NS indicates no significant difference ($P > 0.05$); a single asterisk (*) indicates a significant difference at the level $0.05 > P > 0.02$, and a double asterisk (**) a difference at the level $P < 0.01$ between the divalent cations and their chelators either at 1 or 400 ATA. Results are means (± SEM) from four independent experiments on blood from different donors.

in the absence of Cl^-, K^+ influx was *higher* in shrunken cells compared to swollen cells (*cf.* Fig. 3). Increasing $[Cl]_o$ had no significant effect on K^+ uptake in shrunken cells (Fig. 4) and in pooled experiments on shrunken cells, K^+ influx was unaffected when Cl^- was replaced by $CH_3SO_4^-$ (Table 2a). In swollen cells there was a large progressive *increase* in K^+ flux (Fig. 4). These data, taken with those presented in Figs. 1 and 3 and Tables 1 and 2 suggest that high hydrostatic pressure activates a saturable K^+ flux sensitive to changes in cell volume and dependent on Cl^-. In addition, high pressure also stimulates a nonsaturable K^+ flux which is *reduced* by cell swelling (*see below*), and in shrunken cells is unaffected by the replacement of $CH_3SO_4^-$ for Cl^-.

(iii) Anion dependence of volume-insensitive K^+ flux. One would expect that with the volume-sensitive K^+ flux inactivated by cell shrinking, anion substitution should have little effect on the pressure-induced volume-insensitive cation flux. Table 2a shows that in shrunken cells 'passive' K^+ uptake was elevated at pressure; however, as expected, there was no significant difference between the pressure-induced K^+ flux in the Cl^- or $CH_3SO_4^-$ media. In two separate experiments, the replacement of Cl^- by other anions (Br^-, NO_3^-, or I^-) also failed to influence the K^+ flux observed at pressure, and values for the ΔV^* in these media were comparable to those obtained for Cl^- and $CH_3SO_4^-$ (−87, −80 and −82 ml/mole, respectively). Therefore in shrunken cells, the anion present did not markedly

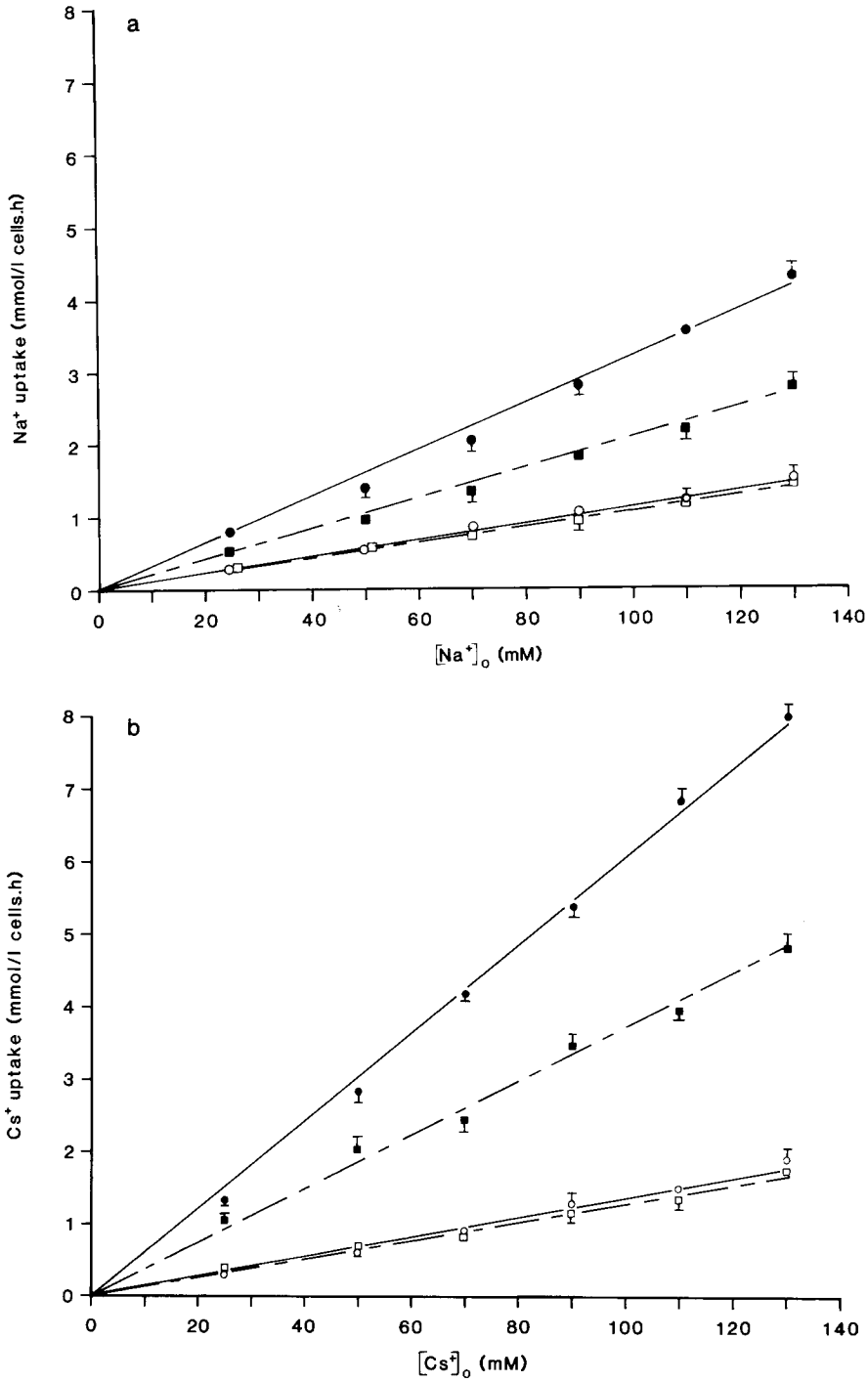

Fig. 5. Effect of hydrostatic pressure on the 'passive' uptake of (*a*) Na^+ as a function of $[Na]_o$, and (*b*) Cs^+ as a function of $[Cs]_o$ in red cells of different volume. Na^+ or Cs^+ influx were measured at 1 and 400 ATA in shrunken (○, ●) and swollen (□, ■) cells, respectively. K^+ was used as the replacement cation for both experiments with the other constituents of the incubation medium as described in Materials and Methods. The r.c.v. of shrunken cells for the Na^+ and the Cs^+ experiment were 0.86 and 0.88, respectively, and for swollen cells were 1.13 and 1.14, respectively. For the Na^+ experiment, values of α in shrunken cells at 1 and 400 ATA were 0.0111 ± 0.0010 and 0.0319 ± 0.0014 [(mmol/liter cells · hr)/mM], respectively, and in swollen cells 0.0103 ± 0.0011 and 0.0213 ± 0.0015, respectively. For the Cs^+ experiment the comparable values were 0.0136 ± 0.0008, 0.0610 ± 0.0018 and 0.0127 ± 0.0007, 0.0351 ± 0.0014, respectively. For pooled data, *see* Table 5

influence the pressure-induced K^+ flux (*see also* Table 5), and this is in marked contrast to the anion dependence of the K^+ flux in swollen cells (Fig. 4).

(c) Effects of Divalent Cations

It is conceivable that high pressure may, in addition to activating volume-sensitive KCl transport, cause a rise in $[Ca]_i$, for example by elevating Ca^{2+} influx, and thereby activate the Ca^{2+}-sensitive K^+ pathway, the so-called Gárdos channel (Gárdos, 1956; Lew & Ferreira, 1978). In order to exclude the involvement of this transport pathway in our experimental conditions, most experiments have been performed in the presence of the Ca^{2+}-chelating agent EGTA. Nevertheless, Lauf (1985) has recently proposed a role for divalent cations in regulating inducible KCl transport in sheep red cells, and therefore it was important to determine whether external Ca^{2+} and Mg^{2+} had any effect on

the pressure-induced fluxes. Table 3 shows the results from these experiments performed on swollen cells to maximize the pressure-induced K^+ flux. At 1 ATA the addition of divalent cations significantly reduced 'passive' K^+ uptake, in agreement with previous studies (Ellory et al., 1983). At pressure, K^+ influx was elevated under all conditions tested. However, as observed at 1 ATA, increasing the divalent cation concentration reduced the pressure-induced flux and there was no difference in efficacy between Ca^{2+} or Mg^{2+}. The pressure effect on K^+ uptake was the same in the presence or absence of the chelators showing that 'contaminant' levels of these divalent cations were without significant effect. Notice also that because the addition of divalent cations reduces permeability at 1 ATA as well as at pressure, the reduction in ΔV^* is quite small (about 10% for Ca^{2+} and Mg^{2+} at 20 mM) compared to the absolute fluxes at pressure (about 60%). Thus, Ca^{2+} or Mg^{2+} decrease the pressure-induced K^+ flux, and therefore the pressure effects on K^+ permeability reported in this paper are not the result of the activation of Ca^{2+}-sensitive K^+ transport.

2. Effects of Pressure on Na^+ and Cs^+ Influx

(a) Influence of Cell Volume

The volume-sensitive KCl flux in red cells has been shown to be specific for K^+ (and Rb^+) transport over Na^+ (Ellory et al., 1982) and therefore one would expect high pressure to exert a different effect on 'passive' Na^+ or Cs^+ permeability in the presence of Cl^-. A series of kinetic experiments similar to those for K^+ uptake was performed, by measuring Na^+ or Cs^+ uptake over a range of external concentrations (25 to 130 mM) in cells of different volume (Fig. 5*a* and *b*, respectively). At 1 ATA there was no evidence of saturation, and no significant effect ($P > 0.05$) of cell swelling on 'passive' Na^+ influx (values for α in shrunken and swollen cells were 0.012 ± 0.002 and 0.010 ± 0.001 [(mmol/liter cells · hr)/mM], respectively; means ± SEM from four experiments). Similarly at 400 ATA, linear concentration dependence was observed both in swollen and shrunken cells, but in contrast, cell swelling significantly ($P < 0.01$) *reduced* Na^+ influx (Fig. 5*a*). In pooled experiments on four different donors, in shrunken and swollen cells α was 0.035 ± 0.005 and 0.020 ± 0.003 [(mmol/liter cells · hr)/mM], respectively. A similar picture emerged for Cs^+ uptake (Fig. 5*b*). There was no significant difference ($P > 0.05$) between shrunken and swollen cells at 1 ATA but at 400 ATA cell swelling significantly ($P < 0.01$) decreased 'passive' Cs^+ influx (*see* Table 5).

These pressure effects on 'passive' Na^+ and Cs^+ permeability are similar to the action of pressure on the nonsaturable component of K^+ uptake (Fig. 1 and Table 1). These data are therefore consistent with the idea that, in the absence of the specific Cl^--dependent volume-sensitive K^+ flux, there is a general increase in monovalent cation permeability at high hydrostatic pressure, which is *reduced* by cell swelling.

(b) Effect of Anion Substitution

The anion-dependence of the pressure-induced Na^+ flux was also studied at a single value of $[Na]_o$ in cells of 'normal' volume (Table 2b). At atmospheric pressure, Na^+ uptake was slightly elevated when $CH_3SO_4^-$ replaced Cl^-. At 400 ATA the Na^+ flux was increased; however, there was no significant difference between the Na^+ influx in these media. In two separate experiments, replacement of Cl^- by other anions (Br^-, NO_3^-, I^-) did not alter the pressure-induced Na^+ flux (*data not shown*) and values for the ΔV^* were similar to those observed for Cl^- and $CH_3SO_4^-$ (−68, −73 and −62 ml/mole, respectively). Thus the anion dependence of the pressure-induced Na^+ flux is markedly different from the anion dependence of K^+ uptake at pressure at low values of $[K]_o$ (*see* Hall et al., 1982), confirming the notion that at high pressure Na^+ transport via the specific KCl pathway is negligible.

3. Action of Hydrostatic Pressure on K^+ and Na^+ Efflux

The effects of high pressure on 'passive' K^+ and Na^+ efflux in red cells of different cell volume in the presence of Cl^- or $CH_3SO_4^-$ were also investigated (Table 4). At 1 ATA cell swelling had no significant effect on cation efflux either in Cl^{-1} or $CH_3SO_4^-$ incubation media. The replacement of Cl^- by $CH_3SO_4^-$ did, however, reduce cation efflux both in swollen cells and those of 'normal' volume. Pressure increased K^+ efflux from cells of 'normal' volume, the effect being significantly less in swollen cells as shown by the decrease in ΔV^* (Table 4). This is similar to the effect of pressure on K^+ uptake when $[K]_o$ is high (*see* Fig. 1), consistent with the present experimental conditions of a high $[K]_i$ (approximately 90 mmol/liter cells) for the efflux experiments. At 400 ATA, Cl^- replacement by $CH_3SO_4^-$ revealed that a significant fraction (approximately 15%) of the K^+ efflux was Cl^- dependent; however, as observed earlier, the generalized pressure-induced permeability increase dominates the pressure-activated, volume-sensitive KCl flux at high K^+ concentrations (*see* Fig. 1). Thus, the ΔV^* for K^+ efflux in swollen cells is slightly reduced by Cl^--replacement indicating a small contribution from the volume-sensitive KCl flux which demonstrates

Table 4. Effects of hydrostatic pressure and anions on (a) K^+ and (b) Na^+ efflux from red cells of different volume[a]

Cation	Anion	Volume	Efflux rate constant (h^{-1})		ΔV^* (ml/mole)
			1 ATA	400 ATA	
(a) K^+	Cl	N	0.0271 ± 0.0012	0.1151 ± 0.0030	−92.6 ± 2.5
		SW	0.0268 ± 0.0010 NS	0.0834 ± 0.0021	−72.4 ± 2.1
	$CH_3SO_4^-$	N	0.0205 ± 0.0011	0.0811 ± 0.0053*	−87.7 ± 4.2
		SW	0.0185 ± 0.0009 NS	0.0501 ± 0.0060*	−63.5 ± 4.6
(b) Na^+	Cl^-	N	0.0482 ± 0.0022	0.1625 ± 0.0068	−77.5 ± 6.2
		SW	0.0441 ± 0.0016 NS	0.0873 ± 0.0062	−43.4 ± 5.4
	$CH_3SO_4^-$	N	0.0410 ± 0.0025	0.1450 ± 0.0060 NS	−80.6 ± 5.8
		SW	0.0395 ± 0.0018 NS	0.0862 ± 0.0055 NS	−49.8 ± 4.8

[a] 'Passive' cation efflux was determined in cells of normal (*N*) volume and swollen by approximately 13% into incubation media containing Na^+ (Cl^- or $CH_3SO_4^-$) at 120 mM and K^+ (Cl^- or $CH_3SO_4^-$) at 7.5 mM with the other constituents as described (*see* Materials and Methods). An asterisk (*) indicates a significant difference ($P < 0.05$) at 400 ATA between fluxes measured in cells of a given volume suspended in a Cl^- or $CH_3SO_4^-$ incubation medium. NS indicates no significant difference ($P > 0.05$). Results are means ± SEM from at least three experiments on blood from different donors.

a large activation volume (>120 ml/mole; Hall et al., 1982). For normal high intracellular K^+ values, the K^+ efflux rate constant data translates into a Cl^--dependent K^+ efflux in swollen cells at 400 ATA of about 3 mmol/liter cells · hr, in reasonable agreement with the K^+ influx computed from the apparent kinetic constants given in Table 1 at $[K]_o$ = 90 mM of 4.3 mmol/liter cells · hr.

Na^+ efflux was also raised under pressure, and as observed for K^+ efflux was reduced with cell swelling. In contrast to K^+ efflux, there was no significant effect on Na^+ efflux when Cl^- was replaced by $CH_3SO_4^-$ (Table 4). Notice also that the values for the ΔV^* of Na^+ efflux are lower than for K^+ efflux as reported for the influx experiments (*see below* and Table 5).

4. Comparison of ΔV^* Values for 'Passive' Cation Permeability

Table 5 shows a summary of the apparent activation volumes for 'passive' (i.e., ouabain + bumetanide + EGTA-insensitive) cation fluxes obtained from experiments on many donors. For all cations studied (including the 'diffusive,' i.e. linear component of K^+ uptake in swollen cells; *see* Fig. 1 and Table 1) the apparent activation volumes were large and negative. Raising cell volume using a hypotonic saline significantly ($P < 0.01$) *decreased* the ΔV^* for all cations studied, although the magnitude of this effect varied over the range 1.2 to 1.8× [K^+ efflux (Cl^- medium) to Rb^+ influx (Cl^- medium)] depending on the cation studied. The smallest effect of cell swelling was probably the result of two opposing effects on K^+ permeability which accompany an increase in red cell volume. Thus, cell swelling will *increase* K^+ efflux mediated by the specific KCl pathway, but will also *decrease* K^+ efflux via the generalized nonspecific flux through which the other cations are also transported.

Excluding the data for K^+ efflux in a Cl^- medium, which are a special case because two components are involved, at the same cell volume there was no significant effect ($P > 0.05$) on the apparent activation volume of Cl^- replacement by $CH_3SO_4^-$. Pooling the data for K^+ (^{86}Rb, and ^{43}K), Rb^+ and ^{134}Cs influx and efflux, demonstrated good agreement for the ΔV^* both in shrunken (−94.6 ± 2.8 ml/mole, mean ± SEM, n = 29 experiments) and swollen cells (−62.1 ± 1.8 ml/mole; n = 24). The values for Na^+ fluxes were significantly ($P < 0.001$) lower compared to the other cations investigated at both cell volumes (shrunken cells −73.1 ± 1.5 ml/mole, n = 18; swollen cells −47.7 ± 2.1 ml/mole, n = 17). For a given cell volume, there was good agreement between values of ΔV^* influx or efflux.

5. Effects of Pressure on Red Cell Volume

The flux experiments described above suggest that a major effect of pressure is the activation of volume-sensitive KCl transport. This may be related to a pressure-induced change in red cell volume and in this section we describe experiments designed to test this hypothesis by determining the effects of high

Table 5. Apparent activation volumes (ΔV^*) for 'passive' cation transport in erythrocytes of different cell volumes suspended in Cl^- or $CH_3SO_4^-$ incubation media[a]

Cation (isotope)	Flux	Anion	n	Cell volume	ΔV^* (ml/mole)
K^+(^{86}Rb)	K^+ influx	Cl^-	8	SH	−90.2 ± 3.6
			5	SW	−60.2 ± 5.4
		$CH_3SO_4^-$	5	SH	−86.6 ± 4.5
			5	SW	−63.2 ± 4.3
	K^+ efflux	Cl^-	4	SH	−89.5 ± 4.6
			4	SW	−72.4 ± 3.8
		$CH_3SO_4^-$	3	SH	−93.1 ± 6.3
			3	SW	−63.5 ± 5.3
K^+ (^{43}K)	K^+ influx	Cl^-	3	SH	−101.7 ± 3.1
			3	SW	−62.2 ± 4.3
Rb^+ (^{86}Rb)	Rb^+ influx	Cl^-	3	SH	−105.7 ± 3.2
			3	SW	−58.6 ± 4.4
Na^+ (^{22}Na)	Na^+ influx	Cl^-	6	SH	−68.3 ± 6.1
			5	SW	−44.2 ± 4.7
		$CH_3SO_4^-$	5	SH	−78.2 ± 4.5
			5	SW	−50.1 ± 3.3
	Na^+ efflux	Cl^-	4	SH	−71.9 ± 6.3
			4	SW	−46.5 ± 4.3
		$CH_3SO_4^-$	3	SH	−73.9 ± 4.5
			3	SW	−49.8 ± 6.3
Cs^+ (^{134}Cs)	Cs^+ influx	Cl^-	3	SH	−95.7 ± 3.6
			3	SW	−64.8 ± 2.2

[a] Cation influx and efflux, and the values for the ΔV^* were determined in shrunken (SH) and swollen (SW) cells as described (*see* Materials and Methods) using the radioisotopes given in parentheses. For influx experiments the ΔV^* was independent of the external cation concentration and was unaltered by increasing pressure (*see* Fig. 2 and Hall et al., 1982). The apparent activation volume for K^+ (and Rb^+) influx in swollen cells was obtained as described (*see* text). Results are means (± SEM) on n experiments on blood from different donors.

hydrostatic pressure on red cell volume. In fact, fixing cells at high pressure with glutaraldehyde revealed no significant volume increase occurred.

The effects of pressure on cell volume were determined using the microhematocrit centrifugation technique (*see* Materials and Methods). These experiments relied on the basic principle that fixed red cells did not significantly change their volume during decompression. Therefore, in order to determine the osmotic sensitivity of fixed red cells, the following control experiment was performed. Red cells were suspended in the standard incubation medium of 'normal' tonicity which was used for these experiments, and fixed with glutaraldehyde as described (*see* Materials and Methods). The tonicity of the medium was then increased or decreased by 15%, and the relative cell volume (i.e. compared with cells in 'normal' saline) determined by the microhematocrit method. In four experiments the ratio of the cell volume of control cells to those suspended in media of altered tonicity was 1.00 ± 0.02 and therefore these variations in medium osmolarity did not significantly change cell volume. Larger alterations to medium tonicity of about a third, did, however, start to reveal a change in the volume of fixed red cells. In this case increasing tonicity from 300 to 400 mOsm gave a 6% decrease in volume, while a decrease in osmolarity by the same amount gave the complementary effect. Thus a small but significant volume change did occur for large osmotic pressure changes. However, it would appear that red cells when fixed with glutaraldehyde are unresponsive to changes in medium osmolarity at least over the lower range of current interest. Thus the technique, within experimental error, accurately records the volume of red cells when fixed under given experimental conditions.

Using the technique described above, the volume of red cells suspended in a medium of identical composition to that used for the morphological and flux studies was determined at 1 and 400 ATA. In four experiments on blood from different donors, the volume of erythrocytes exposed to 400 ATA (30 min) was significantly ($P < 0.05$) reduced (i.e. cell shrinkage occurred) by 3.93 ± 0.62% compared to cells suspended in 'normal' saline. No significant

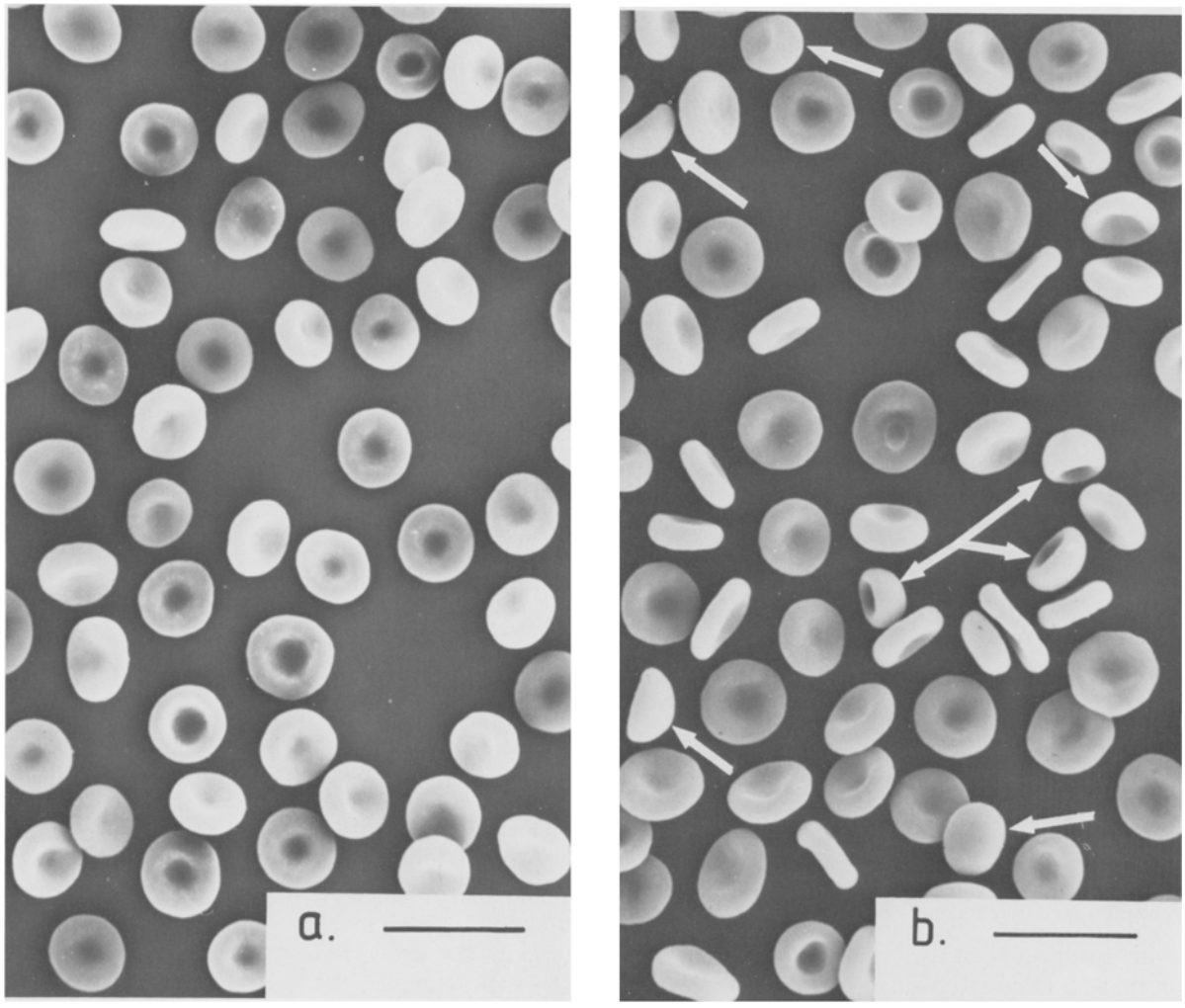

Fig. 6. Effect of high hydrostatic pressure on the morphology of human erythrocytes. Red cells from normal human donors were fixed at (*a*) 1 ATA and (*b*) 400 ATA as described in Materials and Methods. The magnification was 1600×, and the scale bar represents 10 μm. The abnormal cells are indicated by arrows. Results shown are from one experiment, with similar data being obtained from experiments performed on three more different donors

change in cell volume was detected in the 1 ATA control cells.

These data taken with the cation flux measurements at pressure suggest that cell volume is *reduced* under prolonged compression, presumably as a result of the activation of the volume-sensitive KCl flux leading to a loss of osmotically active KCl. The finding that cell *shrinkage* accompanies stimulation of the volume-sensitive KCl flux suggests that cell volume *per se* is not the major determinant of the activation of this transport system, although it may have an important modulatory role (*see* Discussion). Alterations to the morphology of normal red cells does, however, significantly alter membrane transport properties (e.g. Fortes & Ellory, 1975) and therefore it was important to determine if red cell shape was perturbed at high pressure.

6. Effects of Pressure on Red Cell Morphology

The morphology of a significant fraction of red cells fixed at high hydrostatic pressure was altered and Fig. 6 shows results obtained from one of these experiments. At 1 ATA (Fig. 6*a*), normal human red cells exhibited their characteristic biconcave disc appearance, and cup-shaped red cells were never observed. When pressure was raised to 400 ATA (Fig. 6*b*), however, an increasing number of abnormal cup-shaped or prestomatocytic cells were evident. The fraction of abnormal cells typically ranged from 5 to 35% of the total cell population studied, and although the absolute number of cup-shaped cells depended on the individual under investigation, for a given donor a reproducible frac-

tion of abnormal cells was recorded when experiments were repeated. These data taken with the measurements of red cell volume at pressure (Section 5) suggest that at 400 ATA there is a slight reduction in red cell volume and an alteration to the morphology of a significant fraction of the erythrocyte population from the normal biconcave disc to cup-shaped forms.

Discussion

The measurement of cation transport under high hydrostatic pressure offers the possibility of gaining new insights into the fundamental mechanisms of red cell membrane permeability. In particular, high pressure may be a useful 'tool' with which to separate different specific transport processes, and may reveal properties of permeability pathways not observed under 'normal' conditions. This paper represents an extension to our earlier work (Hall et al., 1982), as in the present study we have investigated 'passive' (defined as ouabain + bumetanide + EGTA-insensitive) cation transport (K^+, Rb^+, Na^+ and Cs^+) as a function of cell volume at normal and high hydrostatic pressures. In the absence of specific inhibitors of the pressure-induced fluxes, we have used substrate kinetics, anion dependence and volume sensitivity in an attempt to define and separate the various flux components.

Although at first sight the results look complex, they can be accommodated satisfactorily by a relatively straightforward two-component model. Overall, our interpretation of the data is, therefore, that high hydrostatic pressure causes (1) a general increase in membrane permeability to monovalent cations (K^+, Rb^+, Na^+, Cs^+). This pressure-induced effect has some of the properties of a 'pore' or simple channel [no saturation kinetics, no anion dependence, and essentially equally effective for the four monovalent cations (although Na^+ permeation is slightly less pressure-sensitive (*see below*)], but is responsive to cell shape or volume, since the pressure effect is *reduced* when measured in cells suspended in hypotonic media. The other effect of pressure, (2), is the activation of a more specific, volume-sensitive K(or Rb)Cl transport pathway, which is *increased* in hypotonic media and shows Michaelis Menten-type kinetics. The results indicate that Na^+ and Cs^+ do not participate in this pathway. It is this second effect of pressure which dominates when K^+ uptake is measured at low $[K]_o$ (i.e., in the 'physiological range') as shown in our previous study (Hall et al., 1982). Thus, at high substrate concentrations, in shrunken cells, or in a Cl^--free medium, the generalized effect of pressure on cation permeability is the dominant pathway for 'passive' K^+ transport. This interpretation accounts for the results on K^+ efflux, where cell swelling or Cl^- substitution was associated with a *decrease* in the pressure-induced flux (Table 3).

Although the present data strongly implicate the unmasking of the volume-sensitive KCl pathway by high pressure, it is important to confirm that this interpretation is not complicated by pressure effects on other carrier-mediated systems. In particular it is essential to exclude the involvement of the ouabain-sensitive Na^+/K^+ pump (Schatzmann, 1953; Glynn, 1956), the bumetanide-sensitive ($Na^+ + K^+$) cotransport system (Wiley & Cooper, 1974; Chipperfield, 1980; Dunham et al., 1980) and the Ca^{2+}-activated K^+ channel (Gárdos 1956; Lew & Ferreira, 1978). The concentrations of ouabain, bumetanide and EGTA used in this study are maximal for the inhibition of these transport pathways (Schatzmann, 1953; Ellory & Stewart, 1982) and for the chelation of any contaminant Ca^{2+} present in the incubation medium (Lew & Ferreira, 1978) under standard experimental conditions. If any of these transport systems were activated at high pressure, then since they are known to be independent of cell volume (Dunham & Ellory; 1982; Ellory et al., 1985) we would expect the presence of a saturable K^+ flux in shrunken as well as in swollen cells (e.g. Fig. 1). Furthermore, Na^+ uptake, which can also be mediated by ouabain and bumetanide-sensitive transport systems, shows linear concentration dependence at pressure in the presence of these inhibitors both in swollen and shrunken cells (Fig. 5*a*). These results therefore support the notion that pressure does not significantly reverse the binding of ouabain or bumetanide. Indeed, the lack of involvement of both ouabain and bumetanide-sensitive cation transport pathways is supported by our previous experiments which show that these systems are markedly inhibited by increasing pressure (Hall et al., 1982). Finally, our experiments with increasing concentrations of Ca^{2+} and Mg^{2+} (Table 3) rule out the involvement of the Ca^{2+}-sensitive K^+ channel under our experimental conditions.

It should be remembered that the interpretation of the Na^+ flux data may be complicated by the involvement of other transport systems insensitive to ouabain, bumetanide and EGTA. Thus Na^+ transport measurements at high pressure may be mediated to a small, but nevertheless significant extent by, for example, the Na^+-Li^+ exchange system (Pandey et al., 1978) and capnophorin as $NaCO_3^-$ ion pairs (Funder, 1980). The participation of these pathways, which in common with other carrier-mediated systems are likely to have positive values for the ΔV^* (i.e. will be inhibited by increasing pressure) (Hall et al., 1982; Canfield & Macey, 1984;

Hall & Ellory, 1985), may explain the lower (i.e. less negative) values for ΔV^*.

An important question which does arise is whether normal human red cells at atmospheric pressure demonstrates volume-sensitive K^+ transport in the presence of EGTA and inhibitors for Na^+/K^+ pump and ($Na^+ + K^+$) cotransport. Our pooled obtained from different donors (Table 1) show no significant effect of a hypotonic medium, although in some donors we have detected a small, reproducible increase in K^+ uptake with cell swelling, which appears to be Cl^--dependent (e.g. Fig. 4 this paper; *see also* Ellory et al., 1985). The literature on this topic is controversial. Thus some workers find that in red cells obtained from normal human donors swelling decreases 'passive' cation transport (Poznansky & Solomon, 1972), others (Ellory et al., 1982) report that swelling has no effect, a third group describe the effects of cell volume change on net outward cation transport as 'complex' (Adragna & Tosteson, 1984). More recently there have been reports of the existence of a small basal KCl flux which is Cl^- dependent and volume sensitive (Ellory et al., 1985; Kaji, 1985; Lauf et al., 1985).

Red cells from many other species demonstrate large volume-sensitive K^+ fluxes (Dunham & Ellory, 1981; Ellory et al., 1985) and therefore human red cells appear to be exceptional in this respect. Nevertheless, it has been proposed that since a Cl^--dependent K^+ flux which is volume sensitive, can be revealed in human erythrocytes by treatment with the thiol-reactive agent NEM (Wiater & Dunham, 1983; Lauf et al., 1984, 1985), this system may well be present in the membrane, but latent under normal conditions. Recent evidence suggests that a functionally active volume-sensitive KCl transport systems is present in 'young' immature human red cells, and that with cell maturation the pathway becomes masked (Ellory et al., 1986), resembling the situation in sheep reticulocytes (Lauf, 1983). This raises the possibility that there is a significant degree of heterogeneity in a sample of red cells prepared for flux studies using conventional techniques and this may account for some of the conflict in the literature. An obvious extension to this caveat is that it is 'young' cells which are principally involved in the pressure response. Experiments with red cells from patients with hemolytic anemias and therefore high degrees of reticulocytosis would support this hypothesis (Gardiner et al., 1983; Ellory et al., 1985).

It is perhaps unexpected to report that high hydrostatic pressure activates volume-sensitive KCl transport and increases the monovalent cation permeability rather than inhibiting these systems, as we have demonstrated for other cation transport pathways (Na^+/K^+ pump, ($Na^+ + K^+$) cotransport; Hall et al., 1982), and amino acid permeation (Hall & Ellory, 1985). The stimulation of the volume-sensitive flux may, however, be explained by the morphological change (biconcave disc to cup-shaped forms) demonstrated in red cells fixed under high pressure (Fig. 6). Shape changes induced by high hydrostatic pressure have previously been reported in red cells (Ebbeke, 1936; Haubrich, 1937) although in these studies much higher pressures were used than in the present work (>1500 ATA) and therefore cell morphology may have been comprised by additional effects, for example the heat of compression. These early experiments were included by Ponder (1948) with other 'obscure phenomena' associated with the isovolumic disc-sphere transformation, and he suggested that the effect of pressure may be "the result of a reorientation of the structural components upon the arrangement of which the discoid shape of the red cell depends" (Ponder, 1948; p. 44).

These 'structural components' have, in recent years been identified as the complex cytoskeletal protein network which underlies the red cell membrane, and which plays a central role in the maintenance of red cell shape (Lux, 1979; Branton et al., 1980; Shotton, 1983). It is well known that the dissociation of many structural proteins proceeds with a decrease in volume (i.e., has a $-\Delta V^*$) and therefore high pressure causes profound changes to the morphological properties of many cell types (Zimmerman, 1970; Macdonald, 1975). It therefore seems reasonable to propose that an important effect of high pressure is to disrupt the cytoskeletal network of the erythrocyte membrane and thereby produce the morphological changes we have observed. It is interesting to note that the resultant cup-shaped, or prestomatocytic forms are morphologically similar to those present in red cells from donors with hereditary stomatocytosis (HS) (Wiley, 1977), which apparently contain a structurally weakened protein cytoskeleton (Lux, 1979). Similar cup-shaped erythrocytes are observed when red cells from normal donors are treated with NEM and it has been suggested that this morphological change plays an important role in the exposure of the volume-sensitive KCl flux (Ellory et al., 1985). At atmospheric pressure HS cells show some of the cation transport characteristics of erythrocytes from normal donors subjected to 400 ATA, and are themselves even more sensitive when subjected to high pressure (*see* Table 4 of Hall et al., 1982; Gardiner et al., 1983; Ellory, et al., 1985). Thus, the activation of volume-sensitive KCl transport by high pressure, NEM or in certain pathological states may be related to an

alteration to the structural integrity of the red cell cytoskeleton leading to a morphological change which reveals the otherwise latent KCl flux.

Our experiments suggest that under experimental conditions identical to those used for the flux studies, a significant reduction in red cell volume occurred at high hydrostatic pressure. In contrast, Macdonald and Shelton (1985) using modified Coulter Counter apparatus, have not observed any significant changes to volume of erythrocytes from the plaice (*Pleuronectes platessa*) when determined at pressures similar to those used in the present report. Such measurements may, however, be inaccurate for studying cells of abnormal morphology (Glader & Nathan, 1978; Kamentsky, 1980) which may be present at high pressure (*see* Fig. 6*b*). The present observations that, paradoxically, cell shrinkage accompanies the activation of the volume-sensitive KCl flux, provokes the suggestion that it is the morphological change to cup-shaped forms which represents the primary signal for the stimulation of the KCl flux, rather than a gross change in cell volume. Clearly, further experimentation is required to elucidate the relative roles of cell volume and morphology, and the intermediate steps which occur between the 'sensor' and the activation of this complex transporter.

Experiments described in the present study show that values of ΔV^* for cation transport are relatively unaffected by the anion present (Tables 2 and 5). This may appear to conflict with our previous report (Hall et al., 1982; *see also* Macdonald, 1984), in which it was proposed that anions played a major role in determining pressure-induced permeability by way of the water structure of the secondary hydration shell surrounding the K^+ ion. This theory was based on the anion dependence of K^+ uptake at values of $[K]_o$ up to 15 mM. As we have demonstrated in the present paper, at high pressure the volume-sensitive KCl pathway dominates under these low $[K]_o$ conditions (Fig. 1) and therefore it was the anion dependence of this specific transport pathway which was being investigated. From experiments on volume-sensitive KCl transport in sheep red cells (Dunham & Ellory, 1981) this is known to be $Cl^- > Br^- \gg NO_3^- > I^-$, i.e. the Hofmeister series, and identical to the anion dependence of the pressure-induced K^+ reported previously (Hall et al., 1982). Confirmation of this interpretation is obtained from the fact that when the volume-sensitive KCl flux is inhibited by cell shrinking, anion substitution had no significant effect on the pressure-induced K^+ flux (Table 2a). Similarly, Na^+ influx which is not apparently mediated by the KCl transporter was also unaffected by anion replacement (Table 2b).

Podolsky (1956), in one of the earliest reports of the actions of pressure on transport, suggested that a pressure-induced alteration to anion hydration diameter might explain the effects of 80 ATA on Na^+ efflux from cat erythrocytes. The results in the present study indicate that the anion present does not, however, markedly influence pressure-sensitive cation transport when the specific KCl pathway is inhibited. Thus, an alternative and perhaps more plausible explanation, is that pressure influences anion-dependent Na^+ transport, which has been shown to exist in red cells from other carnivore species (Parker, 1983).

An explanation for the generalized increase in cation permeability is, perhaps, more difficult. The values of ΔV^* for the permeation of all cations studied in the absence of the volume-sensitive KCl flux (Table 5) demonstrated four important features: (1) they were negative, (2) for a given cation the ΔV^*'s were essentially the same for influx and efflux, (3) they were unaffected by the anion present (*see also* Table 2) and (4) they were significantly reduced by cell swelling (Table 5). The ΔV^* of this pressure-induced flux is opposite in sign to comparable experiments performed on lipid membranes treated with either pore-forming or carrier-mediated ionophores. In these liposome experiments, increasing pressure has consistently been found to *reduce* ionophore-mediated cation transport or cation conductance (Johnson & Miller, 1975; Bruner & Hall, 1983). One might expect that in the absence of volume-sensitive KCl transport (i.e. in shrunken cells, or with Cl^- replacement) the cation permeability of the red cell membrane would approximate that of the artificial lipid membrane, and thus the pressure effects on cation transport would be similar. The results presented in this study clearly indicate that the 'passive' cation permeability of the red cell membrane at high pressure even with all the known carrier-mediated cation transport pathways inhibited, is quite different when compared to the liposome. At the present time we cannot explain this anomaly although it is obvious that the presence of proteins in the red cell membrane make the crucial difference. Further studies using high pressure may clarify the nature of this pathway(s), and yield valuable information on the 'ground state' permeability of biological membranes and its control.

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References

Adragna, N.C., Tosteson, D.C. 1984. Effect of volume changes on ouabain-insensitive net outward cation movements in human red cells. *J. Membrane Biol.* **78**:43–52

Branton, D., Tyler, J., Hargreaves, W. 1980. Protein interactions in the erythrocyte cytoskeleton. *In*: Membrane Transport in Erythrocytes. Alfred Benzon Symposium No. 14. U.V. Lassen, H.H. Ussing, and J.O. Wieth, editors. pp. 23–30. Munksgaard, Copenhagen

Bruner, L.J., Hall, J.E. 1983. Pressure effects on alamethicin conductance in bilayer membranes. *Biophys. J.* **44**:39–47

Canfield, V.A., Macey, R.I. 1984. Anion exchange in human erythrocytes has a large activation volume. *Biochim. Biophys. Acta* **778**:379–384

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

Cornish-Bowden, A. 1976. Principles of Enzyme Kinetics. Butterworths, London

Dacie, J.V., Lewis, S.M. 1975. Practical Haematology. 5th Ed. Churchill Livingstone, London

Dunham, P.B., Ellory, J.C. 1981. Passive potassium transport in low potassium sheep red cells: Dependence upon cell volume and chloride. *J. Physiol. (London)* **318**:511–530

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

Ebbecke, U. 1936. Uber plasmatische Kontraktionen von roten Blutkorperchen, Paramacien und Algenzellen unter der Einwirkung hoher Drucke. *Pfluegers Arch. Gesamte Physiol.* **238**:452–466

Ellory, J.C., Dunham, P.B., Logue, P.J., Stewart, G.W. 1982. Anion-dependent cation transport in erythrocytes. *Philos. Trans. R. Soc. London B* **299**:483–495

Ellory, J.C., Flatman, P.W., Stewart, G.W. 1983. Inhibition of human red cell sodium and potassium transport by divalent cations. *J. Physiol. (London)* **340**:1–17

Ellory, J.C., Hall, A.C. 1985. The effects of high hydrostatic pressure on volume-sensitive cation transport in human red cells. *J. Physiol. (London)* **362**:15P

Ellory, J.C., Hall, A.C., Stewart, G.W. 1985. Volume-sensitive cation fluxes in mammalian red cells. *Mol. Physiol.* **8**:235–246

Ellory, J.C., Hall, A.C., Stewart, G.W. 1986. Volume-sensitive KCl transport in human red cells. *J. Physiol. (London)* **374**:33P

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. Pharmacol.* **75**:183–188

Fortes, P.A.G., Ellory, J.C. 1975. Asymmetric membrane expansion and modification of active and passive cation permeability of human red cells by the fluorescent probe 1-anilino-8-naphthalene sulphonate. *Biochim. Biophys. Acta* **413**:65–78

Funder, J. 1980. Alkali metal cation transport through the human erythrocyte membrane through the anion exchange mechanism. *Acta Physiol. Scand.* **108**:31–37

Gardiner, R.M., Barnes, N.D., Ellory, J.C. 1983. Congenital haemolytic anaemia associated with abnormal inducible red cell cation permeability. *Arch. Disease Childhood* **58**:547–549

Gárdos, G. 1956. The function of calcium in the potassium permeability of human erythrocytes. *Biochim. Biophys. Acta* **30**:653–654

Glader, B.E., Nathan, D.G. 1978. Cation permeability alterations during sickling: Relationship to cation composition and cellular hydration of irreversibly sickled cells. *Blood* **51**:983–989

Glynn, I.M. 1956. Sodium and potassium movements in human red cells. *J. Physiol. (London)* **134**:278–310

Hall, A.C., Ellory, J.C. 1985. The effects of high hydrostatic pressure on amino acid transport in sheep red cells. *1st Congr. Comp. Physiol. Biochem.* B96, I.V.B.S. Liege, Belgium

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

Hall, A.C., Willis, J.S. 1984. Differential effects of temperature on three components of passive permeability to potassium in rodent red cells. *J. Physiol. (London)* **348**:629–643

Haubrich, R. 1937. Uber die Druckresistenze der Erythrocyten. *Pfluegers Arch. Gesamte Physiol.* **239**:304–313

Hochachka, P.W., Somero, G.N. 1973. Strategies of Biochemical Adaptation. W.B. Saunders Co., Philadelphia, Pa.

Johnson, S.M., Miller, K.W. 1975. The effect of pressure and the volume of activation on the monovalent cation and glucose permeabilities of liposomes of varying composition. *Biochim. Biophys. Acta* **375**:286–291

Kaji, D.M. 1985. Volume-activated K flux in human erythrocytes. *J. Gen. Physiol.* **86**:40a

Kamentsky, L.A. 1980. Objective measures of information from blood cells. *Blood Cells* **6**:121–140

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

Lauf, P.K. 1983. Thiol-dependent passive K/Cl transport in sheep red cells: II. Loss of Cl^- and N-ethylmaleimide sensitivity in maturing high K^+ cells. *J. Membrane Biol.* **73**:247–256

Lauf, P.K. 1985. K^+:Cl^- cotransport: Sulfhydryls, divalent cations, and the mechanism of volume activation in a red cell. *J. Membrane Biol.* **88**:1–13

Lauf, P.K., Adragna, N.C., Garay, R.P. 1984. Activation by N-ethylmaleimide of a latent K-Cl flux in human red blood cells. *Am. J. Physiol.* **246**:C385–C390

Lauf, P.K., Perkins, C.M., Adragna, N.C. 1985. Cell volume and metabolic dependence of NEM-activated K-Cl flux in human red blood cells. *Am. J. Physiol.* **249**:C124–C128

Lew, V.L., Ferreira, H.G. 1978. Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. *In:* Current Topics in Membranes and Transport. A. Kleinzeller and F. Bronner, editors. Vol. 10, pp. 217–277. Academic Press, New York

Lux, S.E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Haematol.* **16**:21–51

Macdonald, A.G. 1975. Physiological aspects of deep sea biology. *In:* Monographs of the Physiological Society, No. 31. Cambridge University Press, Cambridge, U.K.

Macdonald, A.G. 1984. The effects of pressure on the molecular structure and physiological functions of cell membranes. *Philos. Trans. R. Soc. London B* **304**:47–68

Macdonald, A.G., Shelton, C.J. 1985. Ionic regulation under high pressure: Some observations of comparative and theoretical interest. *In:* High Pressure Effects on Selected Biological Systems. A.J.R. Pequeux and R. Gilles, editors. pp. 51–67. Springer, Berlin

Pandey, G.N., Sarkadi, B., Haas, M., Gunn, R.B., Davis, J.M., Tosteson, D.C. 1978. Lithium transport pathways in human red cells. *J. Gen. Physiol.* **72**:233–247

Parker, J.C. 1983. Volume-sensitive sodium movements in dog red cells. *Am. J. Physiol.* **244:**C324–C330

Podolsky, R.L.J. 1956. A mechanism for the effect of hydrostatic pressure on biological systems. *J. Physiol. (London)* **132:**38P-39P

Ponder, E. 1948. Hemolysis and Related Phenomena. Grune Stratton, New York

Poznansky, M., Solomon, A.K. 1972. Regulation of human red cell volume by linked cation fluxes. *J. Membrane Biol.* **10:**259–266

Sanders, S.K., Alexander, E.L., Braylan, R.B. 1975. A high yield technique for preparing cells fixed in suspension for scanning electron microscopy. *J. Cell. Biol.* **67:**476–480

Schatzmann, H.J. 1953. Herzglykoside als Hemmstoffe fur den aktiven Kaliumundn Natriumtransport durch die Erythrocytenmembran. *Helv. Physiol. Pharmacol. Acta* **11:**346–354

Shotton, D. 1983. The proteins of the erythrocyte membrane. *In:* Electron Microscopy of Proteins. J.R. Harris, editor. pp. 205–330. Academic, London

Wiater, L.A., Dunham, P.B. 1983. Passive transport of K^+ and Na^+ in human red blood cells: Sulfhydryl binding agents and furosemide. *Am. J. Physiol.* **245:**C348–C356

Wiley, J.S. 1977. Genetic abnormalities of cation transport in the human erythrocyte. *In:* Membrane Transport in Red Cells. J.C. Ellory and V.L. Lew, editors. pp. 337–361. Academic, London

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

Young, J.D., Ellory, J.C. 1982. Flux measurements. *In:* Red Cell Membranes: A Methodological Approach. J.C. Ellory and J.D. Young, editors. pp. 119–133. Academic, London

Zimmerman, A.M. 1970. High Pressure Effects of Cellular Processes. Academic, New York

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