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Pressure and Temperature Effects on Human Red Cell Cation Transport

A.C. Hall*, J.C. Ellory* and R.A. Klein**

* Physiological Laboratory, University of Cambridge and
** Medical Research Council, Molteno Institute, Cambridge, United Kingdom

Summary. The effects of hydrostatic pressure and temperature on the three components of K^+ uptake in human red cells have been investigated, using ouabain and bumetanide to distinguish between the pump, passive diffusion and cotransport. The pressure sensitivity for passive diffusion has been shown to depend on the counter-ion present. The order of this effect, $Cl^- > Br^- > NO_3^- > I^-$, is the same as for the ionic partial molal volumes and the Hofmeister series. We have analyzed our experimental results thermodynamically, and propose a model for the activated transition-state complex of the potassium ion which involves the loss of water molecules from the secondary hydration shell, cosphere II.

Key words hydrostatic pressure · potassium flux · erythrocyte membrane · water of hydration · anion effect · thermodynamic analysis

Introduction

Na^+ and K^+ transport across the erythrocyte membrane can be divided into 3 major components: the flux mediated by the sodium pump (Glynn, 1957), the Cl-dependent NaK cotransport system (Wiley & Cooper, 1974; Dunham, Stewart & Ellory, 1980), and the residual passive "leak" component which shows a characteristically linear concentration dependence (Glynn, 1956). These components can be conveniently separated pharmacologically using ouabain to selectively inhibit the Na pump (Schatzmann, 1953) and furosemide or bumetanide to inhibit the cotransport system (Wiley & Cooper, 1974; Stewart, Ellory & Klein, 1980).

We became interested in the use of both pressure and temperature as complementary physical tools for distinguishing transport processes in biological membranes, and comparing them with similar phenomena in model systems. It is possible to use the pressure and temperature variance of transport parameters as a means of deriving a quantitative thermodynamic description of the systems being compared (Johnson, Eyring & Stover, 1974; Macdonald, 1975).

Changes in pressure or temperature beyond the normal physiological range may demonstrate interesting physico-chemical features of membrane function. For example, a reduction in temperature causes a paradoxical increase in the passive cation permeability of human red cells, which is associated with a temperature-dependent minimum (Stewart et al., 1980).

This paper demonstrates that pressure, as well as temperature, can profoundly alter cation transport in a selective manner in human erythrocytes. Some of these effects are also anion-dependent. Possible mechanisms for the action of pressure on the various cation transport processes are discussed.

Materials and Methods

Blood

Fresh human blood was collected by venepuncture into heparinized syringes, washed three times by centrifugation and resuspension in (mM): NaCl 145; D-glucose 10; HEPES (or Tris, 10) 10; adjusted to pH 7.4; and the buffy layer removed by aspiration.

Technique for Flux Measurements Under Pressure

The unpressurized control samples were placed in 1.5 ml Eppendorf micro-reaction tubes. The pressurized samples were contained in 1-ml disposable plastic syringes, mixed by stainless steel slugs, and sealed with plastic caps after careful exclusion of air. Any change in fluid volume in the syringe due to pressure was thus accommodated by the movement of the piston. On decompression all the samples were placed on ice. Exactly 1 ml of the cell suspension was decanted rapidly into Eppendorf micro-reaction tubes and the cells prepared for the estimation of radioisotope.

The pressure vessel consisted of a stainless steel cylinder of 60 mm bore, which could be compressed hydraulically with distilled water driven by a Haskel MCP 188 water pump. Pressure was measured to $\pm 2\%$ using a Bourdon-type gauge. Temperature could be maintained to ± 0.2 °C (including during compression and decompression) using a thermostatically controlled water jacket. The vessel could hold up to 24 1-ml syringes.

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Compression and decompression rates were found to be unimportant. Fluxes measured in unpressurized cells were found to be the same as those of erythrocytes subjected to five rapid compression (400 ATA)-decompression (1 ATA) cycles, to within experimental error. No red cell lysis was detected in pressurized samples. Values for the three flux components at various temperatures were obtained on separate occasions but using the same donor, and hence represent true replicates. For technical reasons it was not possible to cover the entire temperature range with the same sample of red blood cells in one experiment because of the large thermal capacity of the pressure vessel.

Influx Studies

Washed red cells were suspended in the appropriate incubation medium to give a hematocrit of 5%, determined by measuring the hemoglobin released with Drabkin's reagent (*see* Catton, 1957) spectrophotometrically at 540 nm with an appropriate dilution of the cell suspension.

The normal influx incubation medium contained (mM): NaCl 145; D-glucose 10; $CaCl_2$ 2.5; HEPES 10; adjusted to pH 7.4. When required ouabain (Sigma Chemical Company) and bumetanide (Leo Pharmaceuticals, Princes Risborough, Bucks) were added to give final concentrations of 1 mM and 5×10^{-5} M, respectively. In the K^+ influx experiments, either ^{42}KCl or $^{86}RbCl$ were added with a final KCl concentration of 7.5 mM. Control experiments have established the identity of ^{42}K and ^{86}Rb for measuring K fluxes. For the studies of ^{24}Na transport, isotope in isotonic NaCl was added to the cell suspension. Isotopes were obtained from the Radiochemical Centre, Amersham, Bucks. Red cell fluxes were performed as described previously (Dunham & Ellory, 1980).

Efflux Studies

Freshly washed erythrocytes were incubated for 3 hr at 37 °C in (mM): NaCl 145; KCl 7.5; $CaCl_2$ 2.5; D-glucose 10; HEPES 10; adjusted to pH 7.4, in the presence of the appropriate radioisotope. At the end of the incubation period the cells were washed rapidly three times in ice-cold, isotope-free medium and resuspended to a final hematocrit of about 5%. The efflux measurements were started by transferring the Eppendorf reaction tubes to the water bath, and syringes to the pressure vessel, both being maintained at 37 °C. After 5 min one set of samples was placed on ice (5 min) and centrifuged (12,000 × g, 30 sec), and then an aliquot (0.8 ml) of the supernate taken for counting of the radioisotope present by the Čerenkov effect in a liquid scintillation spectrometer. As these samples were taken, the remainder inside the vessel were pressurized. At fixed intervals the vessel was decompressed, samples of each supernatant taken for counting, and the vessel quickly repressurized. The rate constant for Na^+ efflux was calculated according to the method of Willis, Ellory and Becker (1978).

Statistical Treatment

Three or four replicate samples were taken for each experimental condition. Data are presented as mean ± standard error of the mean.

Abbreviations

HEPES. N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; ATA, atmospheres absolute; (1 ATA = 0.101 MPa); SITS, 3-acetamido-4'-iso-thiocyanato-stilbene-2,2'-disulfonic acid

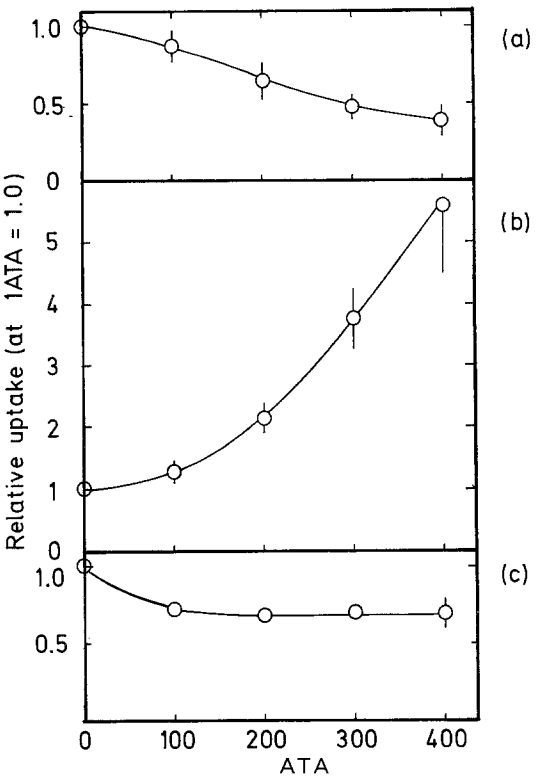


Fig. 1. Effect of hydrostatic pressure, at 37 °C, on the three components of K^+ influx; (*a*) pump; (*b*) passive diffusion; (*c*) cotransport

Results and Discussion

At 37 °C, 1 ATA and K_o 7.5 mM, the 3 components of K influx in one of 3 normal donors used for these experiments were sodium pump 1.63 ± 0.05 (14), cotransport 0.57 ± 0.03 (14) and leak 0.167 ± 0.010 (14) mmol/liter cells hr^{-1}. Figure 1 shows that as the pressure is raised, with the temperature held constant at 37 °C, there is a gradual reduction in potassium uptake by both the pump and cotransport mechanisms, but a very marked increase in the K^+ passive leak particularly above 100 ATA. In Fig. 2*a* it is apparent, on the one hand, that K^+ uptake via the pump is reduced by an increase in pressure from 1 ATA to 400 ATA at all temperatures (i.e. +3 °C to +40 °C). On the other hand, the passive K^+ leak is increased markedly by raising the pressure also from 1 ATA to 400 ATA over the same temperature range (Fig. 2*b*). Increased pressure was also found to depress cotransport over this temperature range.

The effect of pressure on the pump component of the K^+ flux was studied in more detail. Ouabain-sensitive K^+ influx was measured at various external K^+ concentrations (1 − 15 mM) using NaCl to maintain isotonicity. The data were analyzed by plotting

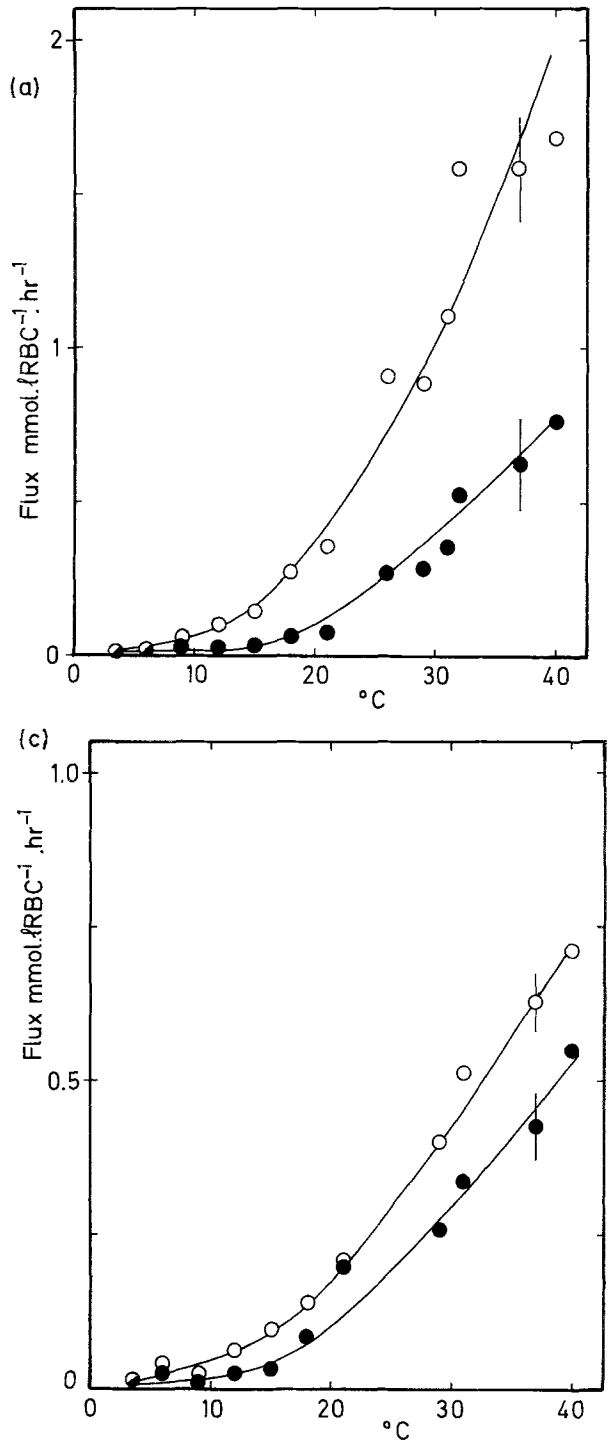
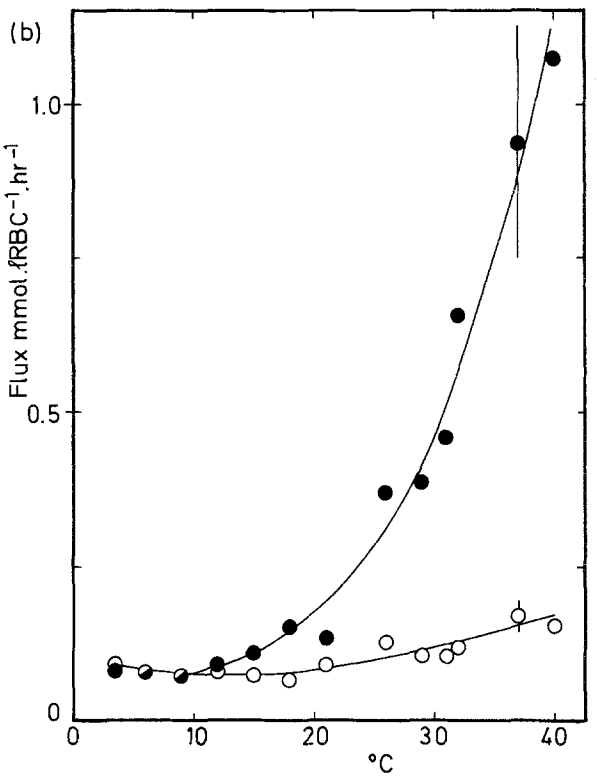


Fig. 2. The effect of temperature at 1 ATA (○) and 400 ATA (●) on the three components of K^+ influx: (*a*) pump; (*b*) passive diffusion; (*c*) cotransport

$\frac{[S]}{v}$ against $[S]$ (Dixon & Webb, 1965) and straight lines obtained as shown in Fig. 3.

$$\frac{[S]}{v}=\frac{[S]}{V_{max}}+\frac{K_m}{V_{max}}. \quad (1)$$



Values of the slope and x intercept give the reciprocal of V_{max} and K_m, respectively. At the two pressures examined K_m and V_{max} were found to be 2.31 $\pm$0.06 mM and 2.00$\pm$0.02 mmol liter RBC^{-1} hr^{-1} (1 ATA), and 1.67$\pm$0.13 mM and 0.76$\pm$0.02 mmol liter RBC^{-1} hr^{-1} (400 ATA), suggesting a mixed kinetic inhibition effect.

The effect of increased pressure on Na^+ and K^+ leak fluxes (defined as the ouabain and bumetanide insensitive components) was examined further (Table 1). Increasing the pressure results in large increases in the fluxes, demonstrating that both Na^+ and K^+ leak influx and efflux are elevated. The ratio of the flux at 300 ATA to that at 1 ATA is given, together with the estimated value of ΔV^*, the volume of the activated transition state complex (*see below*).

The effect of anion substitution on the magnitude of the pressure coefficient for K^+ uptake was also investigated. Experiments were conducted at 1 ATA and 400 ATA at 37 °C in an incubation medium containing (mM); KX 145 (X is the anion of interest, i.e. Cl^-, Br^-, NO_3^- or I^-), D-glucose 10, $Ca(OH)_2$ 2.5, ouabain 1, bumetanide 0.05, HEPES 10, adjusted to pH 7.4. The results for ouabain and bumetanide-insensitive K^+ uptake are shown in Fig. 4, demonstrating that considerable differences occur on changing the counter-ion. It is useful to compare the pressure coefficient for K^+ uptake in the presence of different anions, calculated as the apparent ΔV^* for the process. Table 2 shows the values ob-

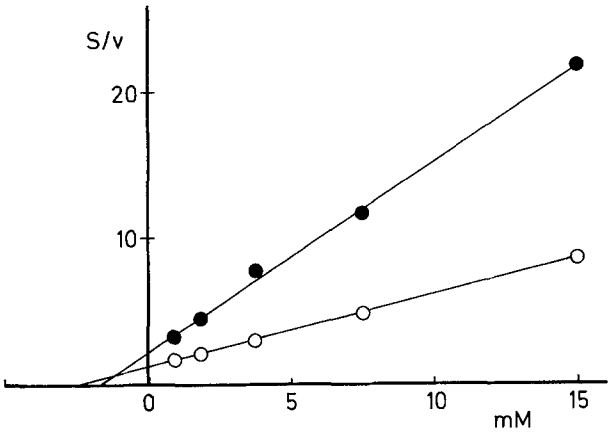


Fig. 3. Kinetic analysis of the effect of hydrostatic pressure on the ouabain-sensitive K^+ influx. Substrate concentration [S] is plotted against [S]/v, where v is the reaction velocity: (○), 1 ATA; (●), 400 ATA

Table 1. Effect of pressure on the ouabain and bumetanide-insensitive cation fluxes

	Control	Flux ratio R_{300}/R_1	ΔV^* (37 °C)
Na^+ efflux[a]	0.048 ± 0.003	2.375	− 73.6 cm³
K^+ efflux[a]	0.029 ± 0.001	4.821	−133.9 cm³
Na^+ influx[b]	0.408 ± 0.006	3.652	−110.2 cm³
K^+ influx[b]	0.967 ± 0.037	3.767	−112.9 cm³

[a] Rate constant (hr^{-1}).
[b] Rate (mmol liter RBC^{-1} hr^{-1}).

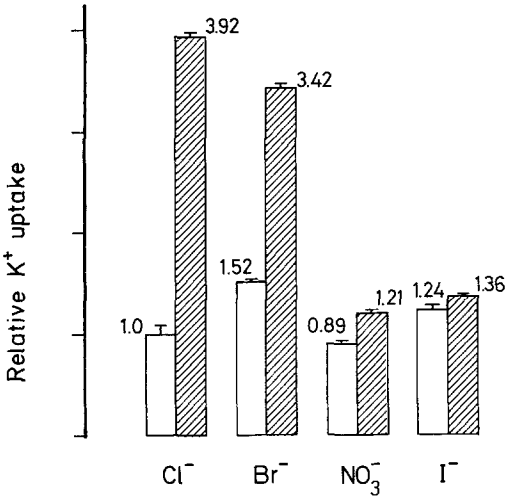


Fig. 4. The effect of different anions on the pressure sensitivity of ouabain-sensitive (passive) K^+ uptake. Uptakes at 1 and 400 ATA (crosshatched) are shown with the standard deviation for the measurements

tained for ΔV^* with different anions. It is significant that the order for the anions' effect on ΔV^* is similar to the lyotropic series (Hofmeister, 1888; *see* Davson, 1940 for early references) and to the rank order for anion permeation of cellulose acetate membranes (Lakshminarayanaiah, 1969, p. 300). Table 2 also presents estimates taken from the literature for ionic radii and partial molar volumes of the anions.

Table 2. Pressure coefficient, expressed as ΔV^*, for K^+ uptake by erythrocytes over the range 1–400 ATA

	Cl^-	Br^-	NO_3^-	I^-
ΔV^* (cm^3 $mole^{-1}$)	−87.2	−52.0	−19.4	−6.0
Ion radius[a] (Å)	1.81	1.96	–	2.19
Partial molal volume[b] (cm^3 $mole^{-1}$)	+23.2	+30.1	+34.4	+41.6

[a] Thorne & Roberts, 1954.
[b] Franks, 1973, or Friedman & Krishnan, 1973.

Table 3. Pressure coefficient for "leak" K^+ uptake, expressed as ΔV^*, against external potassium concentration $[K^+]_o$

$[K^+]_o$ (mM)	0.94	1.88	3.75	7.5	15.0	145
ΔV^* (cm^3)	−83.2	−78.6	−80.9	−82.4	−94.1	−87.2

Average value $\Delta V^* = -84.4 \pm 5.5$ cm^3 $mole^{-1}$.

There was no significant difference in K^+ uptake at 400 ATA when measured in the presence of 50 μM SITS (0.754 ± 0.139 mmol liter RBC^{-1} hr^{-1}) or its absence (0.724 ± 0.069 mmol liter RBC^{-1} hr^{-1}), suggesting that the band 3 anion exchange protein is not involved in this process (Knauf & Rothstein, 1971). Experimental determination of ΔV^* for passive K^+ uptake ("leak") over a range of $[K^+]_o$ values, indicated that ΔV^* was substantially independent of potassium concentration (Table 3) in the range 1 to 400 ATA.

The effect of pressure on the three components of K^+ uptake was very similar for the three normal subjects studied (JCE, ACH & VLL). Both pump and cotransport components showed negative coefficients, i.e., positive values of ΔV^*, and the passive leak gave a positive coefficient (Table 4). However, the donor (JG) with dehydrated stomatocytosis and her mother (AG) (Wiley, Ellory, Shuman, Shaller & Cooper, 1975; Wiley, 1977) showed marked differences compared to the normal donors. Although pressure had a similar effect on the pump, there appeared to be an opposite or zero effect on cotransport with increasing pressure. One characteristic of these cells is their high passive leak (0.387 ± 0.014 mmol liter RBC^{-1} hr^{-1} at 1 ATA). Under pressure there was a dramatic increase corresponding to $\Delta V^* = -153$ cm^3.

One possible mechanism by which pressure could cause an apparent increase in leak would be via a displacement of the inhibitor, either ouabain or bumetanide, from its site of action. It was, there-

Table 4. Molar volume change of activation ΔV^* for the three components of the K^+ uptake in different subjects. The control flux at 1 ATA is also shown (mmol liter RBC^{-1} hr^{-1}) in parentheses

Subject	Pump	Cotransport (cm^3 $mole^{-1}$)[a]	Leak
JCE	+50.3 (1.767±0.07)	+24.3[c] (0.58±0.01)	−103.3 (0.17±0.004)
	+65.2 (1.631±0.182)	+29.8 (0.562±0.209)	−115.9 (0.167±0.036)
			− 84.4 (different $[K^+]$)
ACH	+53.2 (1.822±0.066)	+29.0 (0.581±0.014)	−120.6 (0.132±0.035)
VLL	+56.2 (1.541±0.016)	+21.0 (0.353±0.008)	− 82.5 (0.239±0.007)
AG	+120.0 (1.221±0.005)	+ 5.9 (0.326±0.007)	−132.5 (0.124±0.005)
JG[b]	+100.6 (1.713±0.02)	−52.4 (0.273±0.017)	−152.7 (0.387±0.014)

[a] Values estimated from data at 1 and 400 ATA unless otherwise indicated.
[b] JG has dehydrated stomatocytosis and is the daughter of AG.
[c] Note pressure dependence: apparent values for comparative purposes only (but *see text*).

fore, important to confirm that pressure did not cause such an artefactual effect. There are two compelling pieces of evidence which lead to the conclusion that pressure does not affect the binding of either ouabain or bumetanide. First, the results from cells incubated in anion-substituted media, were obtained at potassium concentrations that would have saturated the pump and cotransport systems (Dunham et al., 1980). Even if inhibitor binding were to have been altered by increased pressure, these pathways would not have transported more K^+. Thus the increase observed arose from a true increase in the passive leak. Moreover, the pressure coefficient ΔV^* for K^+ uptake does not change significantly over a large range of potassium concentration (Table 3).

Secondly, under pressure the leak for K^+ showed a linear concentration dependence. If the binding of ouabain or bumetanide were to have been altered by application of increased pressure, then a nonlinear concentration dependence or even saturation would have been observed as either the pump or the cotransport system, or both, became activated by inhibitor displacement.

Goldinger, Kang, Choo, Paganelli and Hong (1980) have reported that a pressure of 150 ATA was without effect on the ouabain-insensitive component of Na^+ efflux. This might appear to conflict with the present results (although most of the present data are for K fluxes). The discrepancy between their and our data may be resolved since these authors were probably observing both an inhibition of cotransport together with an increase in the Na^+ leak outwards. At this relatively low pressure the effect on these pathways is almost equal in magnitude, yet opposite in direction. At higher pressures, however, the effect on the passive leak begins to dominate, since little further effect on cotransport is seen at pressures above 100 ATA. At any rate cotransport appears to have a low pressure coefficient ($\Delta V^* = +20-30\ cm^3$), which varies markedly with pressure.

Detailed thermodynamic analysis of enzymatic and other multi-step reactions may be extremely complicated (Johnson et al., 1974; Macdonald, 1975; Roberts, 1977). Experimentally determined thermodynamic parameters are useful for comparative purposes, however, particularly if it can be shown that the rate-limiting step does not change over the range of experimental variables used. The linearity of plots of pressure or the reciprocal of absolute temperature against the logarithm of the reaction rate is a good indication that the rate-limiting step has not changed. On the other hand, curvature or frank discontinuities suggest a change in the limiting step or master reaction.

The rate of a reaction or magnitude of an equilibrium constant is determined by the free energy of activation or equilibrium, respectively.

$$\Delta G^* = \Delta H^* + P\Delta V^* - T\Delta S^*$$
$$\Delta G^\theta = \Delta H^\theta + P\Delta V^\theta - T\Delta S^\theta$$

and

$$\text{rate} \propto \exp(-\Delta G^*/RT)$$
$$K = \exp(-\Delta G^\theta/RT)$$

where ΔG, ΔH, ΔV and ΔS are, respectively, the free energy, enthalpy, volume change and entropy of the process in question. A study of the experimental dependence of a reaction rate against temperature and pressure, enables one to estimate the value of the enthalpy and volume change associated with formation of the activated transition-state complex.

We have analyzed our data for the three components of K^+ uptake by plotting $\frac{1}{T}{}^0K^{-1}$ and pressure against the natural logarithm of the flux as shown in Figs. 5 and 6, and Table 5. With the exception of the temperature dependence of the passive leak (Fig. 5*c*), all the other graphs are convincingly linear. Zimmermann, Pilwat, Pégueux and Gilles (1980) have recently shown that the pressure-induced net K^+ loss from human erythrocytes is

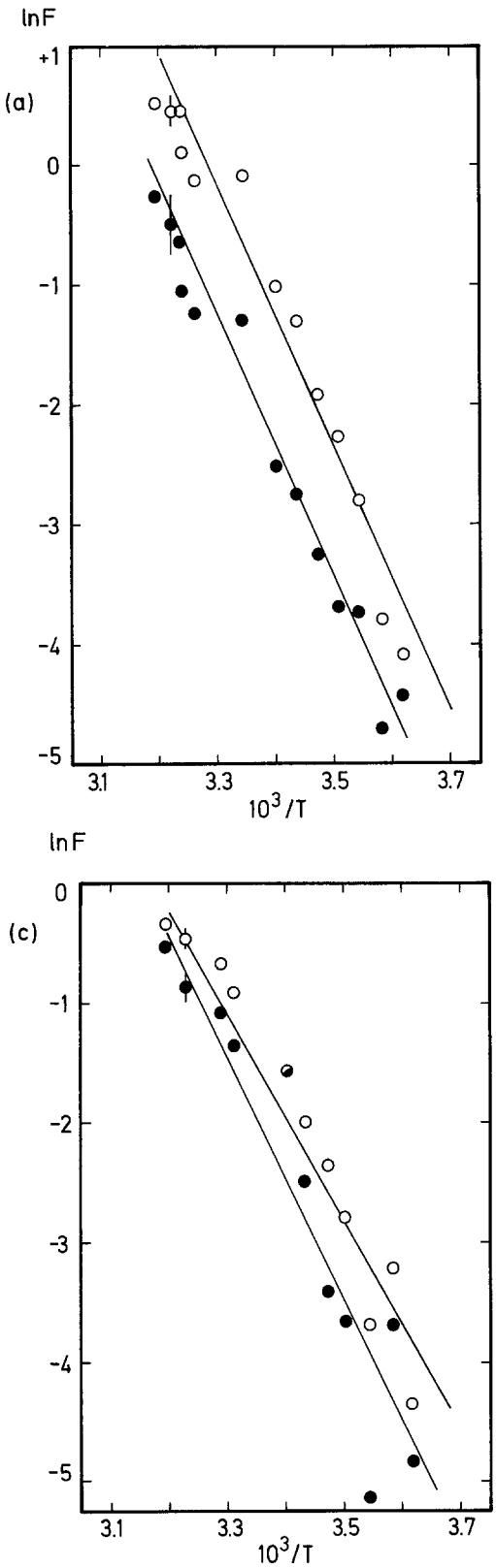
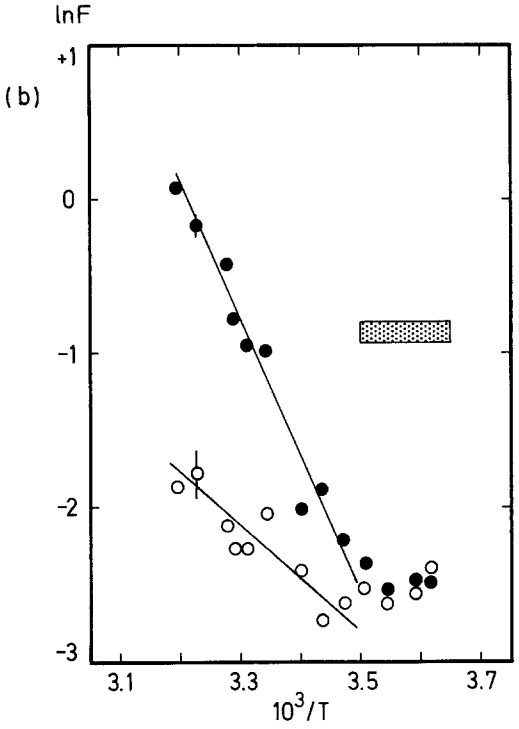


Fig. 5. Arrhenius plots of the effect of temperature on the three components of K^+ uptake. The reciprocal of the absolute temperature is plotted against the natural logarithm of uptake: (*a*) pump; (*b*) passive diffusion; stippled area shows region of known paradoxical behavior (Stewart et al., 1980); (*c*) cotransport



linear up to 600–700 ATA. These authors did not, however, distinguish experimentally between the components of K^+ permeation. Although the data presented here for the pump (Fig. 5*a*) are most reasonably represented by straight line regressions, we have observed a discontinuity in the Arrhenius plot in the region of 25 °C using more accurate and closely spaced flux measurements obtained within single experiments using a Forbes' bar (replotted from Stewart et al., 1980). We have already reported that the potassium leak shows a paradoxical increase below about 10–12 °C (Stewart et al., 1980) and have therefore restricted our analysis for the enthalpy and volume change of activation to above 12 °C.

The effect of pressure on the pump component (Fig. 3) indicates values for molar volume change, ΔV, for V_{max} and K_m of $+61.1\,cm^3$ and $-20.3\,cm^3$, respectively. The values of the molar volume change for V_{max} ($+61.1\,cm^3$) and the overall pump flux ($+65.2\,cm^3$) are close because of near saturation of the potassium binding site under our experimental conditions. Molar volume changes are only directly comparable within a particular series of experiments because of other minor effects.

The molar volume change for the passive leak is large and negative. Shrivastav and Parmentier (1980) tabulate a series of typical ΔV^* values for reactions involving hydrophobic interactions and ionization. Our value for the K^+ leak is large by comparison, particularly for a nonenzymatic process which could be expected to have a ΔV^* around 20–

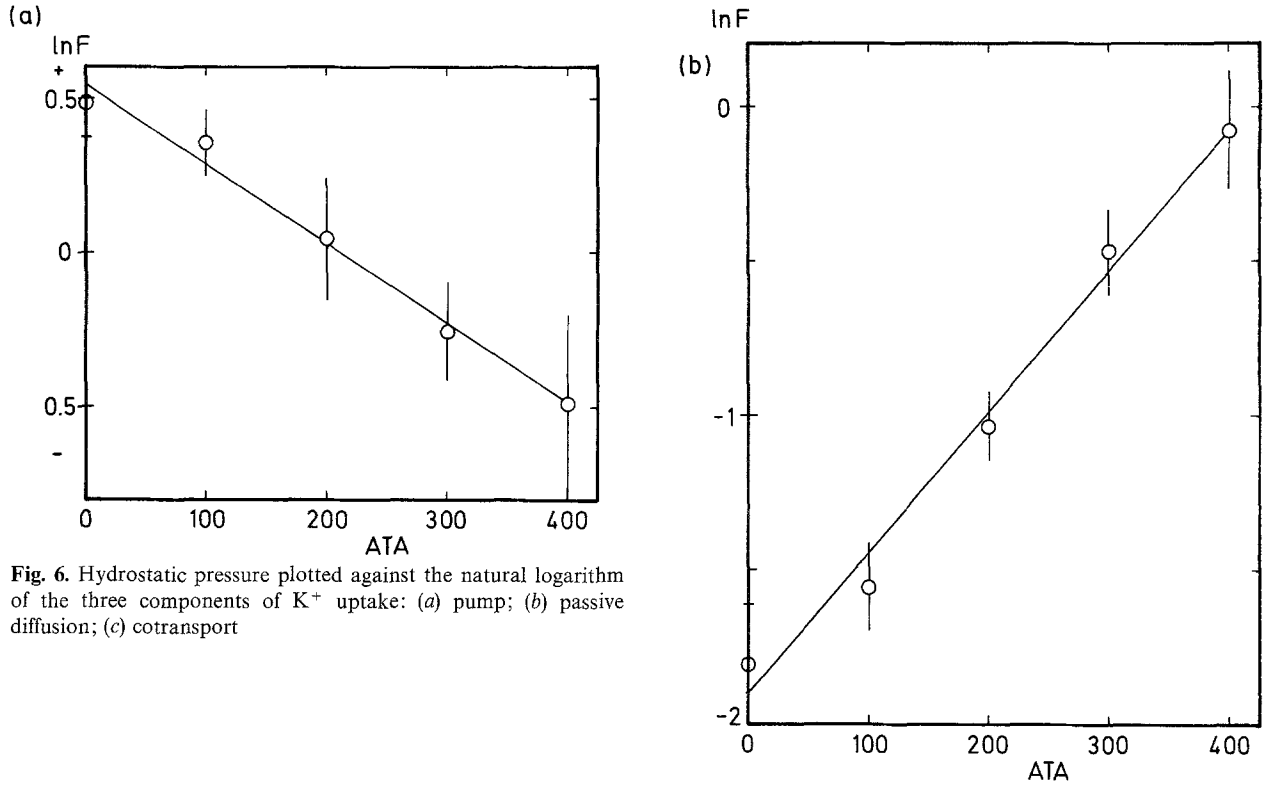


Fig. 6. Hydrostatic pressure plotted against the natural logarithm of the three components of K^+ uptake: (*a*) pump; (*b*) passive diffusion; (*c*) cotransport

30 cm^3 $mole^{-1}$. The effect of anion substitution (Table 2 on the value of the molar volume change throws some light on the possible mechanism. ΔV^* shows a progressive reduction from -87.2 to $-6.0\,cm^3\,mole^{-1}$ on passing through the series $Cl^- > Br^- > NO_3^- > I^-$. The anions follow the order of the Hofmeister or lyotropic series, and it has been realized for many years that cation permeability is susceptible to the particular counterion present (Davson, 1940; Podolsky, 1956; Funder & Wieth, 1967; Wieth, 1972). Various suggestions have been made regarding the structure-making or -breaking effects of anions on the solvent, and the effect that this might have on the membrane (*see* discussion in Funder & Wieth, 1967, and Franks, 1973). The physical basis for the concept of structure-making or -breaking by ions is well covered by Kavanau (1965). A similar order is obtained for the electrical effects of potassium salts at air-water interfaces (Aveyard & Haydon, 1973).

Thermodynamic analysis of permeability data is of practical use only in terms of the physical insight that such an approach gives into the membrane transport process. In the light of our results for the K^+ leak we should like to suggest the following interpretation. The value for $\Delta V^* = -80$ to $-90\,cm^3\,mole^{-1}$ could represent the stripping off of approximately 5 water molecules ($5 \times 18 = 90\,cm^3\,mole^{-1}$) from the hydration shell of the potassium ion. It seems likely that these solvent molecules would be removed from the secondary hydration shell (Fig. 7) rather than from the tightly bound primary shell because of the high free energy of hydration for K^+ of $-314\,kJ\,mole^{-1}$ (Bockris, 1949; Samoilov, 1957*a*, *b*, 1961; Mullins, 1959), with the primary hydration water being less mobile than secondary or bulk water. Alternatively one could consider this value to represent a large number of

Table 5. Temperature and pressure coefficients for the pump, cotransport and passive leak components of K^+ uptake in erythrocytes

	Pressure ΔV^*_{37}	Temperature	
		ΔH^*_p (kJ mole^{-1})	$\Delta H^{\theta\,a}_p$ (kJ mole^{-1})
Pump	$+65.1\pm0.4$ cm^3	90.7 ± 5.0 (1)	-
	($V_{max}+61.1$ cm^3:	90.9 ± 3.9 (400)	88.3 (400)
	$K_m-20.3$ cm^3)		
Leak	-115.9 ± 0.7 cm^3 [b]	28.7 ± 4.3 (1)	-
	-84.4 cm^3 [c]	74.0 ± 4.9 (400)	78.7 (400)
Cotransport	$+83.0$ cm^3 (1–100)	73.6 ± 4.3 (1)	-
	$+2.6\pm0.3$ cm^3 (100–400)	84.0 ± 7.2 (400)	83.9 (400)

[a] Corrected to standard state by $\Delta H^*_p=\Delta H^\theta_p+p\Delta V^*$.
[b] Obtained by regression over the pressure range 1–400 ATA.
[c] Obtained at 1 and 400 ATA over a range of $[K^+]_o$.

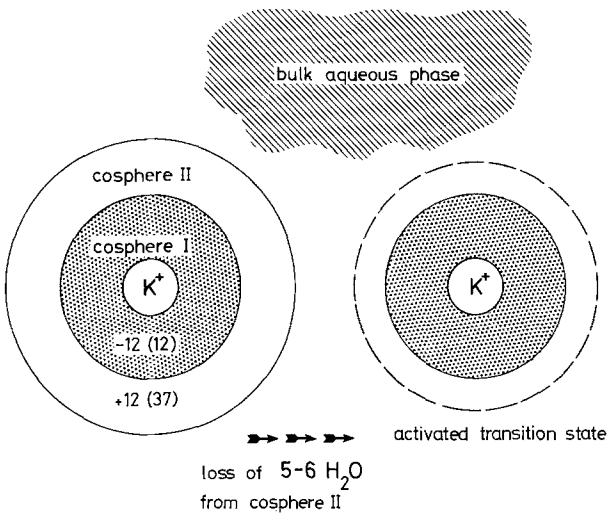


Fig. 7. Suggested activation step by loss of water in the permeation of K^+ ion in human erythrocytes. Cospheres of hydration are shown drawn to scale (after Friedman & Krishnan, 1973). The total entropy change for each cosphere is shown with the probable number of water molecules involved, in parentheses

water molecules (50–60) removed from both the potassium cosphere II and also the hydration shell of the pore, with a ΔV approximating the value for the Ice I-water transition ($\simeq -1.63$ ml (mole H_2O)$^{-1}$). The effect of the anions in the series $Cl^- > Br^- > NO_3^- > I^-$ would be increasingly to destroy the structure of the secondary hydration layer (cosphere II) thus reducing the molar volume change required for formation of the activated transition-state complex (Friedman & Krishnan, 1973; Verrall, 1973). Mullins (1959) suggested that one of the requirements for cation tunnelling in muscle membrane was that "... the ion can largely replace its hydration beyond the first shell with a solvation of similar magnitude obtained from the pore wall...". We believe that our experimental results may provide numerical support for such a mechanism. Podolsky (1956), working with frog sartorius muscle, found that the maximum isometric tension was affected by the series Cl^-, Br^-, NO_3^-, I^-. This effect he ascribed to the different hydration of the anions in this group. We feel, however, that the anion effect is more likely in our system, and perhaps in others also, to be related to the hydration state of the cations present such as sodium or potassium.

It is of interest to note that the very marked positive pressure dependence for the passive K^+ leak vanishes at temperatures below 12–15 °C (Figs. 2 and 5*b*), corresponding to the position of the flux minimum reported by us previously for chloride-containing media (Stewart et al., 1980). Moreover the enthalpy of activation *appears* to be drastically affected by pressure (Fig. 5*b* and Table 5) only in the case of the passive leak. A more satisfactory explanation, since $\Delta H^*_{app}=\Delta H^*+p\Delta V^*$, would be that ΔV^* is a function of temperature. Thus a change in the molar volume term for K^+-activation and diffusion brought about by a reduction in temperature may be related to order-disorder phenomena within the membrane, and alterations in the structure of the K^+ site for passive diffusion.

The two other components of K^+ uptake in human erythrocytes are clearly distinguished from the passive leak by the effect of increased pressure. The pump exhibits a ΔV^* of $+65$ cm^3 mole^{-1} K^+ which is fairly typical for an enzymatically mediated process (Hochachka & Somero, 1973). With two K^+ bound per (Na^+, K^+)-ATPase molecule this is equivalent to $+130$ cm^3 mole^{-1}. If there is a negative ΔV^* for the K^+ ion itself similar in magnitude to that observed for the passive leak, then the volume increase for the protein itself could be as high as 300 cm^3 mole^{-1} on binding K^+. This may be interpreted as the volume increase which occurs as a result of the conformational change brought about by potassium binding. The temperature dependence and the values for the enthalpy of activation support

such an entropic interpretation. Similar high values have been reported for the assembly of myosin polymer from monomeric subunits (Dreizen & Kim, 1971; Kim & Dreizen, 1971; Hochachka & Somero, 1973). Cotransport, on the other hand, shows a relatively low ΔV^* of $+2.6\,cm^3$ mole between 100 and 400 ATA, and an apparent value of $+83\,cm^3$ between 1 and 100 ATA. We feel unable, at this stage, to ascribe any particular physical significance to the marked pressure dependence of ΔV^* for cotransport, other than to suggest a highly pressure-sensitive 'conformational' change which is substantially complete on reaching a pressure of 100 ATA.

In summary we have shown that the thermodynamic analysis of pressure effects may provide a physical insight into the mechanism for ion permeation in biological membranes. In particular, we interpret our results to indicate that the K^+ ion loses solvated water molecules on formation of the activated transition-state complex for passive diffusion.

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Note Added in Proof

We should like to stress that our interpretation implies the removal of water molecules from the hydration shell of ionized potassium as the rate-determining step involved in the formation of an activated potassium ion-membrane pore complex, and as such does not suggest removal of water molecules from the system as a whole.

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