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THE DEPENDENCE ON EXTERNAL CATION OF SODIUM AND POTASSIUM FLUXES ACROSS THE HUMAN RED CELL MEMBRANE AT LOW TEMPERATURES

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SUMMARY

1. The fluxes of Na and K across the human red cell membrane have been studied as functions of temperature and external cation composition.

2. In media containing any of a variety of organic compounds as the principal cation (choline, *N*-methyl-D-glucamine (NMDG), arginine, L-lysine and trimethylphenylammonium), the ouabain plus bumetanide-insensitive influxes and effluxes of Na and K displayed marked paradoxical temperature dependence such that the flux minimum, which normally occurs at about 8–10 °C in a NaCl medium, was shifted up to about 20 °C.

3. Inhibitor and anion replacement studies excluded contributions by the major carrier-mediated systems evident at 37 °C.

4. At 0 °C in NMDG, about 1 mM-external Na and 10 mM-external K were required half-maximally to inhibit the K and Na influxes respectively.

5. When the K(^{86}Rb) efflux in NMDG media at 0 °C was measured in the presence of low concentrations of a series of external inorganic ions and guanidine, the order of potency for reduction of the efflux was Li > Mg = Ca > Ba > Sr > Na > Rb = Cs = guanidine > K.

6. The influxes of the neutral amino acid L-leucine and the cationic species L-lysine both showed simple monotonic temperature dependence in Na and NMDG media.

7. These effects show that the permeability of the human red cell membrane to inorganic univalent cations at low temperatures is markedly dependent on the external ionic conditions. Low permeability is favoured by the presence of cations with a high charge density.

INTRODUCTION

Wieth (1970) observed that, in the presence of the anions salicylate and thiocyanate, the ouabain-insensitive fluxes of Na and K across the human red cell membrane showed 'paradoxical' temperature dependence such that a minimum occurred at about 20 °C. We extended this observation (Stewart, Ellory & Klein, 1980) to show that a similar effect could be observed in chloride media with a minimum at 8 °C if

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a loop diuretic (bumetanide or frusemide (furosemide)) was present to inhibit the Na–K co-transport system (Wiley & Cooper, 1974).

The experiments to be described here arose from studies of the organic cation *N*-methyl-D-glucamine (NMDG) as a potential inert replacement for Na and K. This base, whose p*K* is 9·39 at 30 °C and 9·62 at 0 °C (Perrin, 1965), is available as a pure powder and can easily be made into either the chloride or the nitrate salt. In K influx experiments conducted in NMDG or choline media, we found that 'cold-start' experimental techniques, in which cells and isotope were mixed at 0 °C prior to transfer to the 37 °C water bath, gave spuriously high results compared to those in which the cells were washed at 20 °C and to which labelled cation was added at 37 °C. The suspicion that a temperature effect was involved was confirmed by the studies reported here.

We have measured K and Na influxes and effluxes and Cs and amino acid influxes using tracer techniques. The results show that K, Na and Cs (but not amino acid) fluxes are considerably increased at 0 °C in the presence of a number of organic cations including NMDG and choline, and that the flux increase can be prevented by the addition of either further univalent or divalent cations and by some organic cations including guanidine. Although most experiments have been conducted in NMDG, we have not found any major differences between this cation and choline. These results have methodological implications for workers wishing to use organic cations as replacements for Na and K in red cell transport experiments, and highlight a marked and unsuspected dependence of cation permeability on external cationic conditions at low temperatures.

Accounts of this work have been given previously (Blackstock, Ellory & Stewart, 1985; Blackstock & Stewart, 1985).

METHODS

Materials

NMDG and most other chemicals were obtained from Sigma Ltd., Poole, Dorset. Choline was obtained from BDH, Poole, and was recrystallized from hot ethanol before use. NMDG was obtained as the base in powder form, titrated with HCl or HNO_3 and used without further purification. Bumetanide was a gift from Leo Laboratories, Princes Risborough, Bucks. ^{86}Rb, ^{22}Na and [^{14}C]L-leucine and L-lysine were obtained from Amersham International, Amersham, and ^{43}K and ^{129}Cs were obtained from the M.R.C. Cyclotron Unit, Hammersmith Hospital, London.

Flux measurements

Fresh human red cells from normal donors were used throughout. All cell handling solutions routinely contained: cations, 150 mM; Cl, 150 mM; morpholinopropanesulphonic acid (MOPS), 15 mM (pH 7·4 at temperature of experiment) and glucose, 5 mM. Prior to experiments, the cells were thrice washed in a 150 mM-solution of the cation of choice. When required, anion replacement with nitrate was conducted according to Dunham, Stewart & Ellory (1980).

Fluxes were measured as described previously (Young & Ellory, 1982; Ellory, Flatman & Stewart, 1983) in the media described above with ouabain and bumetanide, each 0·1 mM, if required. The precise composition will be stated in the Figure or Table legends. The inhibitors, which made no difference to the results at 0 °C (Table 1), were usually used to inhibit the Na–K pump and co-transport system at temperatures above 0 °C. ^{86}Rb and ^{43}K were used as tracers for K and ^{22}Na for Na. The cells were exposed to isotope for up to 1 h prior to washing in cold $MgCl_2$ (106 mM) with Tris, 10 mM. Although the uptakes of Na and K were not strictly linear in time (Fig. 6), influx experiments were generally performed using a single time point, while care was taken to ensure

that exposure times to isotope were kept constant in any one experiment. The quantity of cells was estimated spectrophotometrically in Drabkin reagent at 540 nm. After washing, the cells were lysed in distilled water with Triton X-100, 0·1 % (v/v), and treated with trichloroacetic acid, 2·5 % (w/v), followed by centrifugation (10 000 *g*, 2 min). The activity of ^{43}K and ^{86}Rb in the supernatants were determined by the Cerenkov effect in a β-scintillation counter. Although the ^{43}K was contaminated by about 10 % ^{42}K, errors due to complex decay characteristics were avoided by counting standards soon after the samples. ^{22}Na activity was measured by gamma counting. Measurements were conducted in at least triplicate and the results are expressed as the mean ± S.E. of mean. In spite of the non-linearity of the time course of cation uptake, all results have been expressed, for convenience, in units of mmol l cells^{-2} h^{-1}. The flux rates in these conditions are in any case subject to variation as a result of pre-flux incubation conditions, and quantitative comparisons of the fluxes between experiments are not possible. Effluxes were conducted as described previously (Ellory *et al.* 1983).

Amino acid fluxes were measured according to Young, Jones & Ellory (1980). The ^{14}C-labelled amino acids were used. The external L-leucine and L-lysine concentrations were 0·2 mM each. The cells were exposed to isotope for 10 min at 3 °C and for up to 80 min at lower temperatures before washing in ice-cold $MgCl_2$ as described for the cation influxes above. The ^{14}C activity was measured by liquid scintillation counting in Pico-Fluor 30 scintillant (Packard).

RESULTS

K influx at 0 and 37 °C in Na and NMDG media

The data in Table 1 shows K influx in NMDG and Na media at 0 and 37 °C. The effects of the inhibitors ouabain and bumetanide and of chloride substitution with nitrate are also shown. At 0 °C, the flux in NMDG was threefold greater than that in Na and neither inhibitor reduced it, while anion substitution at this temperature increased the flux, consistent with the results of Wieth (1970). At 37 °C, the fluxes in NMDG were comparable to or less than those in Na and the inhibitors and anion substitution had marked effects. The ouabain-sensitive component was reduced in NMDG, consistent with our previous comparison of this flux in 75 mM-choline plus 75 mM-Na with 150 mM-Na (Ellory *et al.* 1983, Table 1). The bumetanide-sensitive component was reduced in NMDG consistent with that pathway's partial Na dependence (Wiley & Cooper, 1974). The residual fluxes were slightly greater in Na than in NMDG.

Since this study indicated that the increased fluxes in NMDG were not attributable to any of the major carrier-mediated systems evident at 37 °C, further experiments involving temperatures above 0 °C were usually conducted in the presence of both ouabain and bumetanide in order to suppress the Na–K pump and Na–K co-transport systems respectively.

K and Na fluxes vs. temperature in NMDG and inorganic cation

We investigated the temperature dependence of the K and Na fluxes in more detail. Fig. 1*A* shows the ouabain plus bumetanide-insensitive K influx (7·5 mM-external K; ^{86}Rb tracer) over the range 0–37 °C in Na and NMDG media, while Fig. 1*B* shows a parallel Na influx experiment (^{22}Na tracer) in K and NMDG media. In the inorganic cation media, both K and Na influxes showed minima at about 5–8 °C. In NMDG, the minima occurred at 20 °C for K influx and at about 15 °C for the Na flux, and in fact, the fluxes of both ions at 0 °C were greater than those at 37 °C. This effect occurred irrespective of whether the pH was kept constant with temperature (as in

TABLE 1. K influx at 0 °C and 37 °C in NMDG and Na. Effect of ouabain and bumetanide and of chloride substitution

Temp. (°C)	External cation	Anion	Inhibitor (0·1 mM)	K influx (mmol l cells^{-1} h^{-1})	Ouabain sensitive	Bumetanide sensitive	Chloride dependent
0	Na	Cl	None	0·038 ± 0·001			
			Ouabain	0·035 ± 0·002	N.s.		
			O+B	0·028 ± 0·001		0·008 ± 0·002	
		NO_3	Ouabain	0·059 ± 0·002			< 0
0	NMDG	Cl	None	0·102 ± 0·001			
			Ouabain	0·097 ± 0·008	N.s.		
			O+B	0·100 ± 0·001		N.s.	
		NO_3	Ouabain	0·129 ± 0·001		—	< 0
37	Na	Cl	None	1·809 ± 0·136			
			Ouabain	0·940 ± 0·066	0·870 ± 0·151		
			O+B	0·391 ± 0·007		0·548 ± 0·066	
		NO_3	Ouabain	0·144 ± 0·003			0·796 ± 0·067
37	NMDG	Cl	None	1·078 ± 0·019			
			Ouabain	0·379 + 0·014	0·699 ± 0·022		
			O+B	0·222 ± 0·003		0·157 ± 0·015	
		NO_3	Ouabain	0·124 ± 0·002			0·254 ± 0·014

K influx (^{86}Rb tracer; 7·5 mM-external K) was measured in media containing (mM): Na or NMDG, 142·5; Cl or NO_3, 150; MOPS, 15 (pH 7·4 at 0 °C or 37 °C); glucose, 5; and ouabain (O) and bumetanide (B), if required, 0·1. Anion replacement was conducted according to 'Methods'. The measurements denote the mean ± S.E. of mean of six observations. The terms 'ouabain sensitive' and 'bumetanide sensitive' refer to fluxes in Cl media only; 'chloride dependent' represents the difference in ouabain-insensitive flux between Cl and NO_3 media. N.s. denotes no significant difference.

these experiments) or if it was allowed to vary as the solution, set at pH 7·4 at 37 °C, was cooled. Similar results were also observed for ^{43}K-labelled K and ^{129}Cs-labelled Cs (not shown).

The ouabain plus bumetanide-insensitive effluxes of Na and K showed a similar effect. Fig. 2 shows the K efflux (^{86}Rb tracer) in NMDG and Na media and Table 2 shows the ^{43}K and ^{22}Na effluxes at 37 °C and 0 °C in Na and NMDG media. At 37 °C, the Na efflux was greater in Na than in NMDG, presumably reflecting the activity of the Na–Na exchange system (Dunn, 1970; Beaugé & Lew, 1977; Duhm & Becker, 1979). At 0 °C, both the Na and K effluxes were at least eightfold greater in NMDG than in Na, consistent with the above experiments and demonstrating the symmetry of the effect both in terms of cations under study and direction of transport.

Specificity of this effect amongst the organic cations

High fluxes at 0 °C were not confined to NMDG media. The specificity of this effect was tested by measurement of the ouabain plus bumetanide-insensitive K influx in

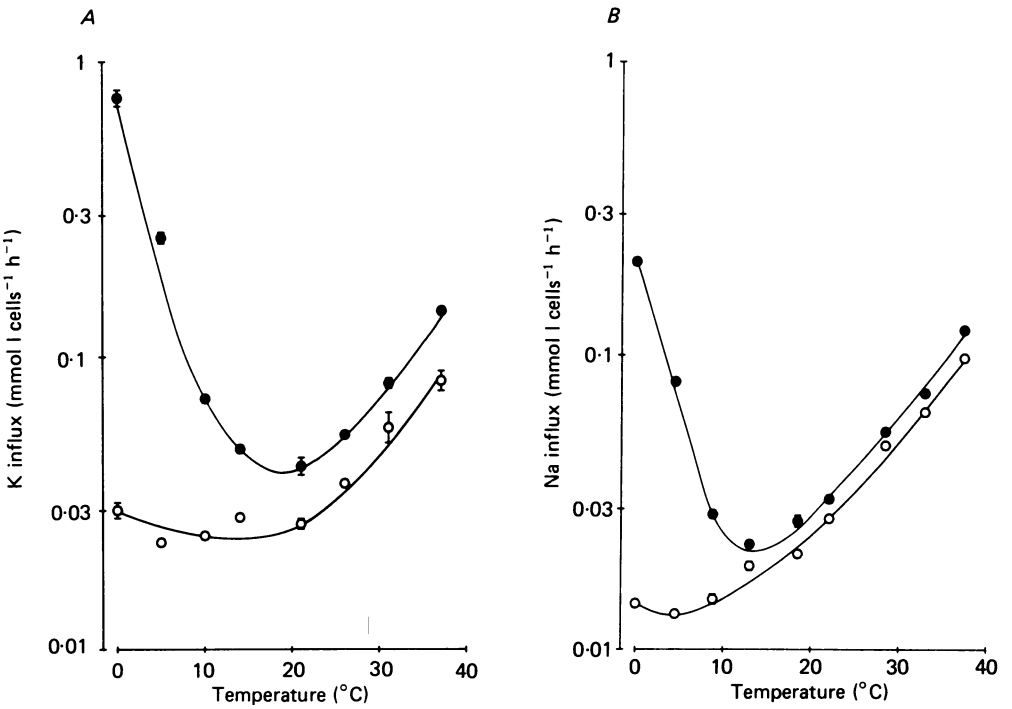

Fig. 1. *A*, ouabain plus bumetanide-resistant K influx *vs.* temperature in NMDG (●) and Na (○) media. K influx (^{86}Rb tracer) was measured in media containing (mM): K, 7·5; NMDG or Na, 142·5; Cl, 150; MOPS, 15 (pH 7·4 at whatever temperature); glucose, 5; and ouabain and bumetanide, both 0·1. After washing in the medium of choice at 20 °C, the cells were suspended and incubated at the temperatures shown for 30 min prior to the addition of labelled K. The points denote the mean of six determinations in a single experiment. The lines were drawn by eye. The error bars denote 1 S.E. of mean and were omitted if the error was less than the diameter of the point. *B*, Na influx *vs.* temperature in NMDG (●) and K (○) media. The experiment was conducted in an identical fashion to that in *A* except that Na (^{22}Na tracer) was substituted for K(^{86}Rb) and K was substituted for Na.

a number of cations at 0 and 37 °C (Fig. 3). At 37 °C, all of the ions indicated gave a comparable flux to that in Na. At 0 °C, low fluxes (i.e. comparable to those in Na at about 0·03 mmol l cells^{-1} h^{-1}) were found in Li, Mg, guanidine and methylguanidine, but high fluxes at around 0·5–1·0 mmol l cells^{-1} h^{-1} were found in NMDG, choline, arginine, L-lysine and trimethylphenylammonium. Because it was so convenient to use, further experiments were usually conducted with NMDG.

Dependence of K and Na influxes on external Na and K

The following experiments were designed to investigate the manner in which the fluxes at 0 °C changed as NMDG was replaced by an inorganic cation. The cells were exposed to the non-labelled ion at 20 °C prior to cooling and addition of labelled cation. Fig. 4*A* shows that the high K influx in NMDG (7·5 mM-external K) was sharply reduced by progressive replacement by Na, such that about 1 mM-external Na caused half-maximal inhibition. Addition of further Na beyond 10 mM had little

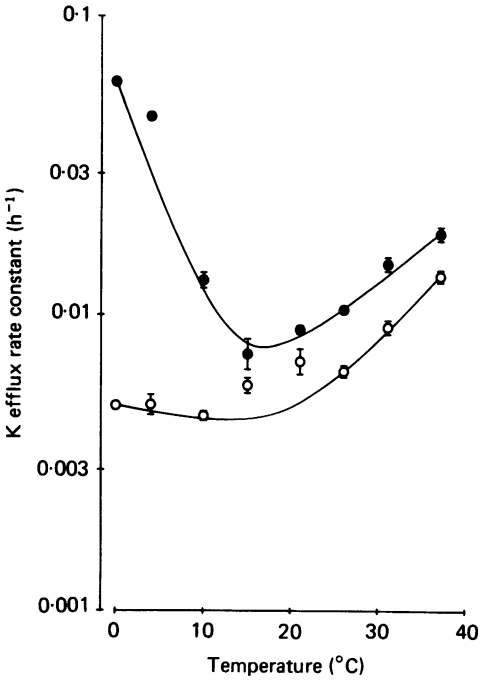

Fig. 2. Ouabain plus bumetanide-insensitive K efflux (^{86}Rb tracer) *vs.* temperature in Na (○) and NMDG (●) media. After ^{86}Rb loading and washing, the cells were suspended in media containing either 150 mM-NaCl or NMDG Cl with buffer, glucose and inhibitors as described for Fig. 1. Quadruplicate 1 ml aliquots were taken and centrifuged at five time points over an 80 min period. The lines were drawn by eye. The error bars denote 1 S.E. of mean.

TABLE 2. Cation effluxes at 0 and 37 °C in Na and NMDG

Temp (°C)	External cation	Na efflux (rate constant: h^{-1})	K efflux (rate constant: h^{-1})
37	Na	0·0614 ± 0·0047	0·0199 ± 0·0013
	NMDG	0·0301 ± 0·0008	0·0417 ± 0·0036
0	Na	0·0056 ± 0·0016	0·0056 ± 0·0007
	NMDG	0·0456 ± 0·0015	0·1043 ± 0·0036

Na (^{22}Na) and K (^{43}K) effluxes were measured as described in 'Methods' in media containing either 150 mM-NaCl or 150 mM-NMDG Cl with buffer, glucose and inhibitors as in Fig. 1.

effect. In a complementary Na influx experiment (Fig. 4*B*), external K produced a similar flux reduction at low concentrations, but about 8–10 mM-K was required to yield half-maximal inhibition.

A natural extension to the above studies was to look at K influx as a function of the external K concentration itself. This was of interest because it might give information on the nature of the transport process: usually, a 'leak' is expected to obey Fick's law and show linear dependence on external cation concentration (e.g. ouabain plus loop-diuretic-insensitive K influx at 37 °C: Wiley & Cooper, 1974). The experiment is shown in Fig. 5, where K influx was measured at 0 °C as a function

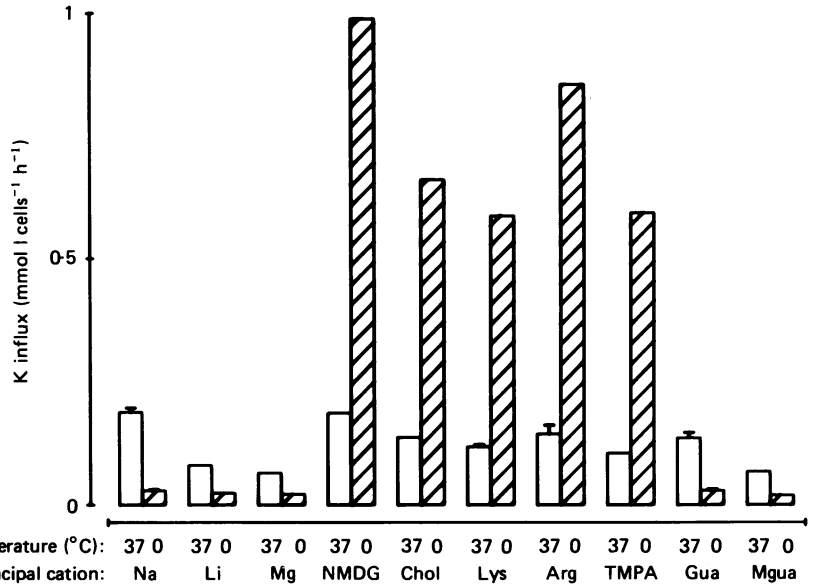

Fig. 3. K influx (^{86}Rb tracer) at 37 °C (open bars) and 0 °C (hatched bars) in cells suspended in a variety of different cations. In addition to 7·5 mM-external K and 142·5 mM-cation (75 mM in the case of Mg with 75 mM-sucrose), the media contained Cl, MOPS, glucose and inhibitors as described for Fig. 1. The abbreviations denote: chol, choline; lys, L-lysine; arg, arginine; TMPA, trimethylphenylammonium; gua, guanidine; and mgua, methylguanidine. The cells were incubated in the cation of choice for 30 min at the temperature shown prior to the addition of labelled K. The error bars denote the S.E. of mean of six observations and are omitted if the S.E. of mean was less than 2 % of the mean.

of external K over the range 2·5–150 mM, under conditions of NMDG and Na replacement for K. With NMDG replacement, the flux showed a curvilinear dependence on external K in the range 2·5–50 mM, falling off at higher concentrations to become linear. With Na replacement, the flux was a linear function of external K up to 100 mM, but deviated upwards at 150 mM to meet the NMDG replacement line at [K] = 150 mM. Although superficially this result in NMDG media suggested a saturable, carrier-mediated process, this need not necessarily be the case in this context (see Discussion).

Time course of K and Na uptake in NMDG at 0 °C

The absolute magnitudes of the fluxes at 0 °C were found to be variable, and the following experiments were designed to investigate the effect of exposure to NMDG at various temperatures prior to the flux measurement. In the experiment shown in Fig. 6*A*, it is shown that the rate of K uptake was roughly three times as great in those cells which were incubated at 0 °C for 60 min prior to the addition of labelled K when compared with those cells to which isotope was added either at 20 °C or just after transfer to the ice-bath. In a parallel experiment conducted with ^{22}Na-labelled Na (Fig. 6*B*), a similar but more striking result was seen: the rate of uptake in cells exposed to NMDG at 0 °C without Na for 60 min was enhanced sixfold. These

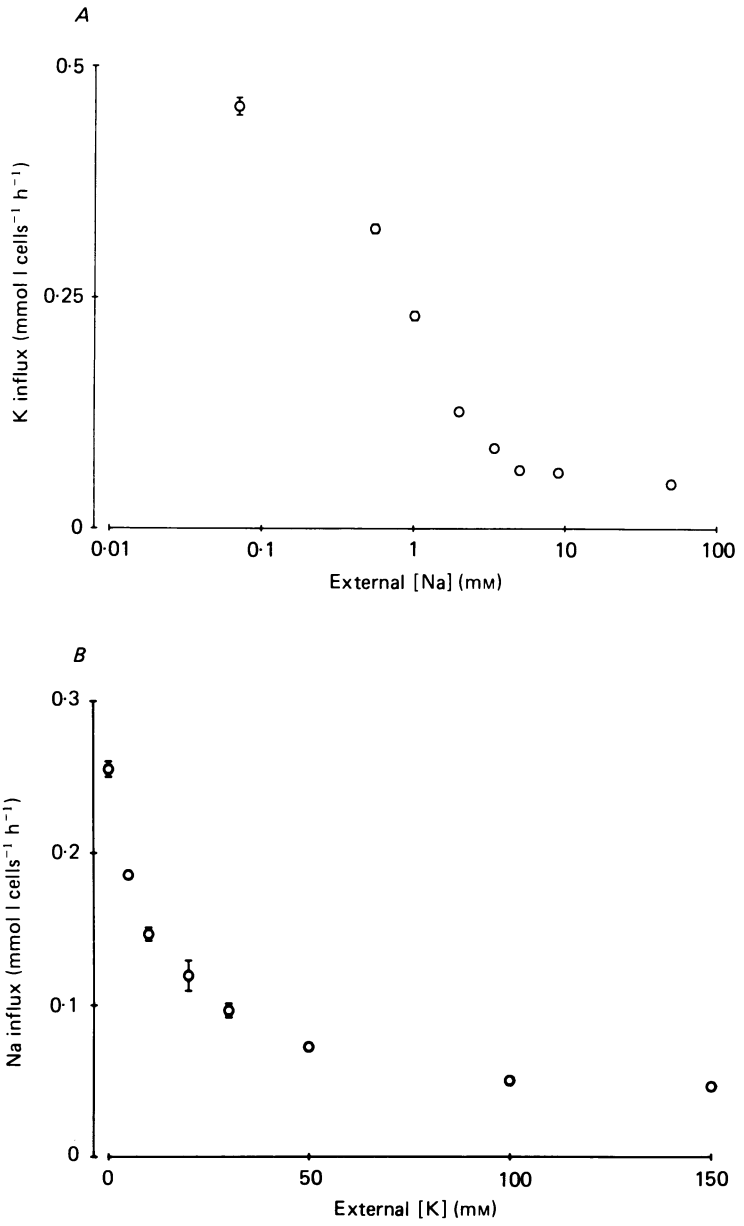

Fig. 4. *A*, dependence of K influx on external Na at 0 °C, NMDG replacement. After washing in 150 mM-NMDG with buffer and glucose (see Fig. 1) at 20 °C, the cells were suspended in media containing various concentrations of Na, with NMDG added to maintain isotonicity, and cooled to 0 °C where they were held for 30 min prior to the addition of ^{86}Rb-labelled K to a final concentration of 7·5 mM. The points denote the mean ± S.E. of mean of four observations. *B*, dependence of Na influx on external [K] at 0 °C, NMDG replacement. This experiment was conducted identically to that just described except that external K (unlabelled) was varied while the influx of ^{22}Na-labelled Na was measured.

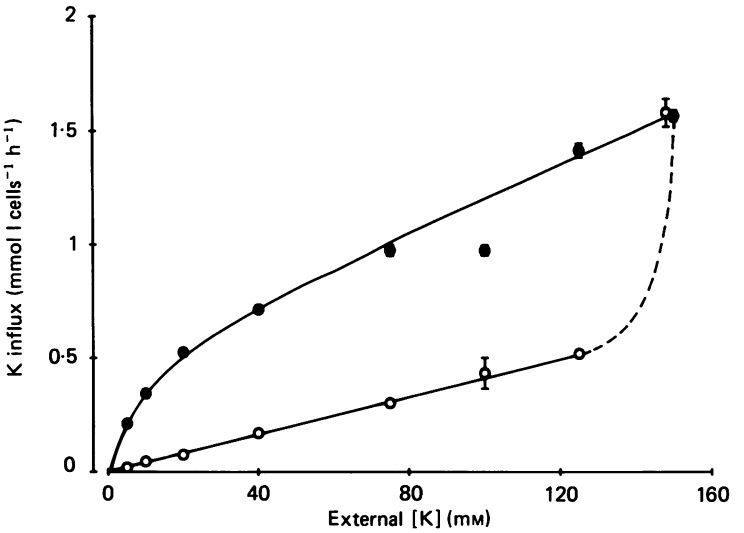

Fig. 5. Dependence of K (^{86}Rb tracer) influx on external K at 0 °C, NMDG and Na replacement. After washing in either 150 mM-NMDG or Na at 20 °C, the cells were suspended in media containing various concentrations of K, with maintenance of isotonicity by either NMDG (●) or Na (○). After incubation at 0 °C for 30 min, a small but constant volume of 150 mM-NMDG containing ^{86}Rb was added to each tube. After exposure to isotope for 60 min, the cells were centrifuged and samples of the supernatant kept for estimation of specific activity, after which the cells were washed in $MgCl_2$ as usual. The points denote the mean ± S.E. of mean of four observations. The lines were drawn by eye.

experiments suggested that high permeability developed only after exposure of the cells to NMDG at 0 °C, and that while the addition of 7·5 mM-Na or K at 20 °C prevented this increase in permeability on transfer to the ice-bath, addition of these cations at 0 °C did not quickly reverse the permeability. The rate of change in K uptake on cooling to 0 °C is shown in Fig. 7, in which only 1 mM-external K was used to minimize the effect of external K on the flux. The uptake was monitored for 60 min at 20 °C prior to cooling to 0 °C, after which the rate of uptake gradually increased to reach a new steady state after about 30 min. When Na was added to one aliquot to give a final concentration of 3 mM, the rate of K uptake slowed significantly compared to the control aliquot without Na.

It was of interest to know whether these effects of incubation at 0 °C in NMDG might affect flux measurements at 37 °C. When ouabain plus bumetanide-insensitive K influx (7·5 mM-external K) was measured in NMDG media in two batches of cells washed in NMDG at 0 and 20 °C respectively, it was confirmed that the flux at body temperature was increased by 80 % in the cold-washed cells (0·3339 ± 0·006 compared with 0·1866 ± 0·0024), indicating that the high permeability induced at 0 °C was not simply reversed by warming in that medium. However, the fluxes in a NaCl medium at 37 °C were not found to be affected by the temperature of the cell preparation steps (cells washed in NMDG at 0 °C, 0·1355 ± 0·0043; NMDG at 20 °C, 0·1350 ± 0·0021; and

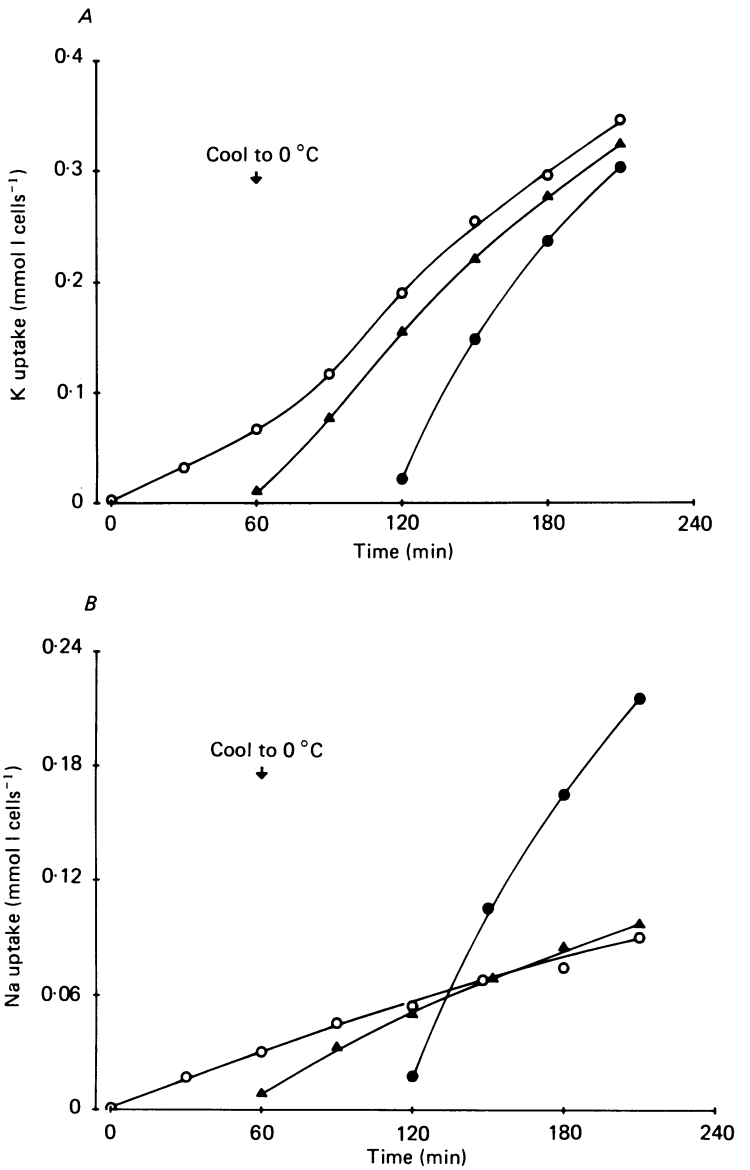

Fig. 6. *A*, time course of K uptake (^{86}Rb tracer) into human red cells in NMDG at 0 °C. After washing and suspension at 20 °C in a 150 mM-NMDG solution with buffer, glucose and inhibitors as described for Fig. 1, the cells were divided into three aliquots but all kept at 20 °C. To one aliquot (○), labelled K was added at 0 min, to a final concentration of 7·5 mM. After incubation at 20 °C for 60 min the tubes were transferred to an ice-bath, when labelled K was immediately added to the second group (▲). K was added to the third group (●) after a further 60 min at 0 °C. Samples were taken from each group for washing and counting at 30 min intervals after the addition of K. The points denote the mean of four observations. The lines were drawn by eye. *B*, time course of Na uptake in NMDG at 0 °C. This experiment was conducted identically except that ^{22}Na-labelled Na was substituted for K.

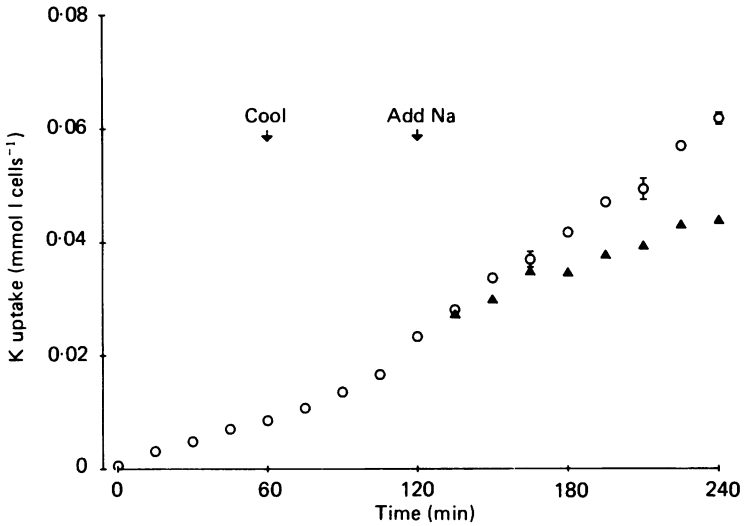

Fig. 7. The cells were first washed in NMDG at 20 °C and suspended with buffer, glucose and inhibitors as for Fig. 1 at that temperature, in a solution previously set to pH 7·4 at 0 °C. ^{86}Rb-labelled K was added to a final concentration of 1 mM and sequential quadruplicate aliquots taken for cell washing at 15 min intervals thereafter. At 60 min, the cells were transferred to the ice-bath and at 120 min, the cell suspension was divided into two and NaCl was added to one aliquot (▲) to a final concentration of 3 mM. The symbols denote the mean ± S.E. of mean of four observations.

cells washed in Na at 20 °C, 0·1365±0·0016), indicating that the increase in flux rate which was observed in NMDG media was reversed by suspension of the cells in 150 mM-NaCl at 37 °C.

Effect of buffer

We have traditionally used MOPS buffer because of its low coefficient of pK variation with temperature (Perrin & Dempsey, 1974). The temperature effects described here were not confined to measurements in this buffer, and in fact fluxes at 0 °C in NMDG were greater in Tris(hydroxymethyl)aminomethane (Tris) and *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonate (HEPES) than in MOPS. In one experiment, the K influxes in NMDG media at 0 °C were 0·126±0·002 mmol l cells^{-1} h^{-1} in MOPS buffer, and 0·241±0·006 and 0·873±0·012 when MOPS was replaced with 15 mM-HEPES and 15 mM-Tris respectively.

Effect of pH

Since Na, the smaller cation, seemed to be considerably more effective in inhibiting the fluxes (Fig. 4), the effect of pH on K influx at 0 °C was tested in both NMDG and Na media (Fig. 8). In the Na medium, pH had little influence on the flux, whereas in the NMDG medium there was a fiftyfold increase in flux as the pH was increased from 7·0 to 8·0. This effect was repeated in choline using cells from a second donor (not shown).

Since pH can influence cell volume (Hladky & Rink, 1977), the effect of two

methods of osmotic volume change were tested: the addition of sucrose to a constant concentration of NMDG, increasing the osmolarity from 200 to 400 mosmol l^{-1}, led to a 60% increase in K influx, while the addition of NMDG Cl to give an identical change in osmolarity but with a change in ionic strength, led to a 40% decrease, showing that cell volume changes *per se* were unlikely to be important in the pH experiment above.

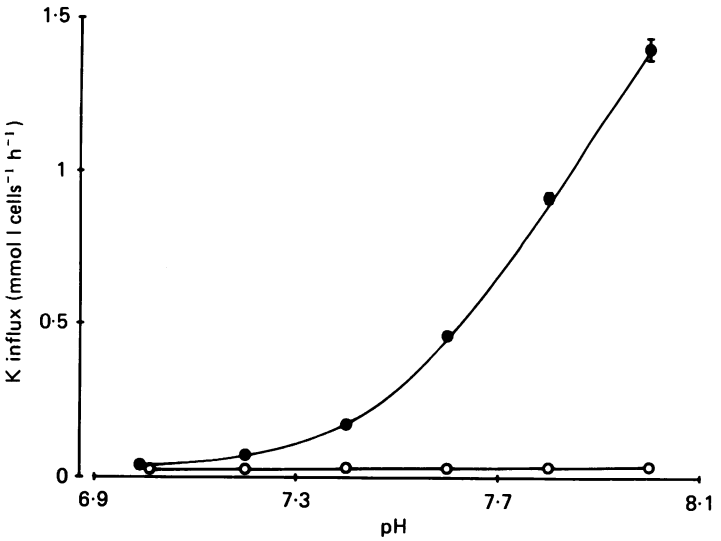

Fig. 8. The dependence of K influx on pH at 0 °C. K influx (^{86}Rb tracer) was measured in Na (○) and NMDG (●) media as described for Fig. 1, except that pH was varied as shown. The points denote the mean ± S.E. of mean of four observations.

The effect of various external cations on K efflux

The experiments to be described here were designed to compare the potency of the inorganic cations and guanidine in reducing permeability in NMDG at 0 °C. We used K efflux (^{86}Rb tracer) as a test flux to allow the maximum possible variation in external cation concentration. Two experiments are shown in Fig. 9. In the left-hand panel, the ions were present at 3 mM. At this concentration, the divalents (not shown at this concentration) all gave low fluxes comparable with those in Li. The univalents showed the ranking Li > Na > Rb = Cs = guanidine > K. Two further experiments, using fresh solutions, confirmed the order Na > Rb > K, which represents a deviation from the Hofmeister series. In the right-hand panel, the cations were present at 0·1 mM, at which concentration the univalents other than Li had little effect. Both the control and the flux in Li were slightly lower in this experiment compared with that in the left-hand panel, probably due to slight differences in cell preparation. These data showed the ranking Li > Mg = Ca > Ba > Sr. Since the divalents all gave low fluxes (i.e. comparable with those in Li) at 3 mM, the ranking Li > Mg = Ca > Ba > Sr > Na > Rb = Cs = guanidine > K can be deduced by combination of the two sets of results. EDTA (1 mM), which was included to test the effect of chelation of trace amounts of divalent cation in the NMDG medium, caused a slight increase in the flux.

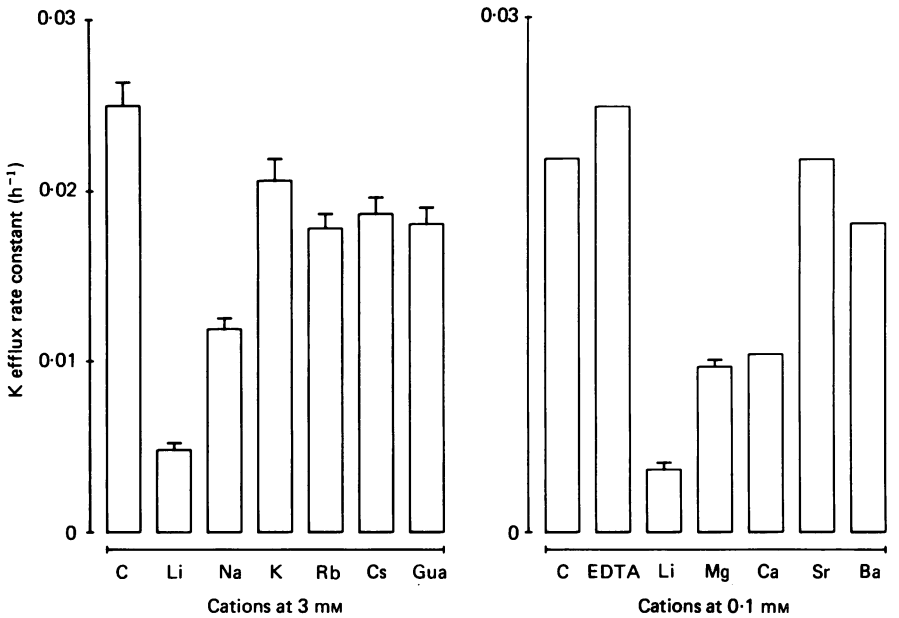

Fig. 9. K (^{86}Rb) efflux in NMDG media at 0 °C in the presence of a number of different cations. After loading, the cells were washed in ice-cold NMDG with buffer and glucose as for Fig. 1, then suspended in media containing the added amounts of cation indicated. EDTA was present at 1 mM where indicated. The error bars denote 1 S.E. of mean ($n = 6$). C, control (NMDG only).

Amino acid transport vs. temperature in Na and NMDG media

In an attempt to find out if these temperature effects were confined to inorganic cation transport, we measured L-leucine and L-lysine transport as functions of temperature in both Na and NMDG media (Fig. 10). Neither amino acid showed Na dependence at 37 °C, consistent with previous reports (Young *et al.* 1980). Hoare (1972) has shown that L-leucine transport in Na media had a simple monotonic temperature dependence, and we confirmed that finding, and demonstrated a similar pattern for L-leucine in NMDG and for L-lysine in both media, with no suggestion of a paradoxical effect in either medium. The cationic L-lysine showed a considerably greater sensitivity to temperature than L-leucine.

DISCUSSION

The principal thrust of these data is that the human red cell membrane becomes markedly permeable to Na and K when suspended in solutions of some organic cations (NMDG, choline, L-lysine, arginine and trimethylphenylammonium) at low temperatures. After exposure to organic cation media at 0 °C, this enhancement of permeability developed over a period of 30–60 min. The replacement of the organic cation by relatively smaller cations with a higher charge density could restore low permeability down to a base line, suggesting that the relative absence of the small cations was more important than the presence of the larger ones. Consistent with the

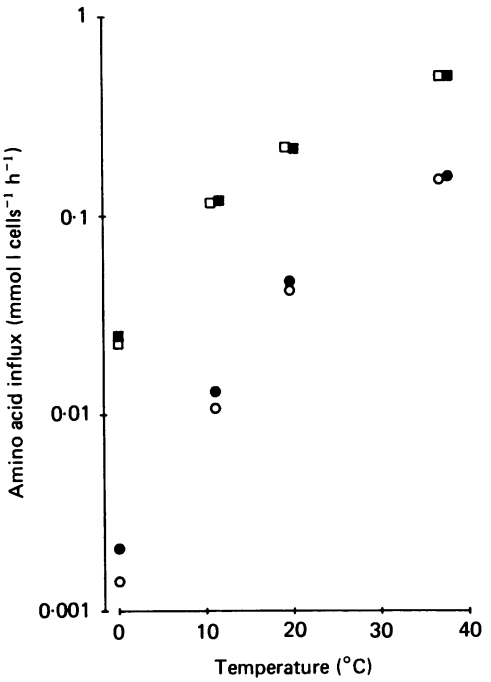

Fig. 10. Amino acid transport *vs.* temperature in Na and NMDG media. The media contained 150 mM-Na or NMDG and buffers and glucose as for Fig. 1, in addition to ^{14}C-labelled L-lysine or L-leucine (0·2 mM each). The symbols denote: ○, L-lysine in Na; ●, L-lysine in NMDG; □, L-leucine in Na; ■, L-leucine in NMDG. The points denote the mean of six observations. The errors (1 S.E. of mean) were all smaller than the points.

effects of Li, Na and the divalents, the fluxes were markedly reduced by increasing [H^+]. The effect was common to influxes and effluxes of Na and K but did not apply to the amino acids tested, one of which (L-lysine) was cationic. The symmetry of the effect on influx and efflux of Na and K argues against a major shift in membrane potential.

It seems most likely that the increased fluxes at low temperatures are mediated by a simple passive diffusional process: a 'leak'. Such a process would be consistent with the lack of selectivity shown either for direction of transport or for cation. It would be surprising if cold activation were a property of a carrier-mediated system (e.g. see Ellory & Willis, 1981, for a review). The fluxes at 0 °C were increased in EDTA and inhibited by external Ca, making activation of the Gardos channel an unlikely mechanism (Lew & Ferreira, 1977). Inhibitor and anion replacement studies (Table 1) excluded contributions from the Na–K pump and Na–K co-transport systems at low temperatures. A simple 'leak' process should obey Fick's law

$$\text{flux} = P_M \cdot [M],$$

(where M is a univalent cation and P_M is a constant) and be a linear function of the external cation concentration (e.g. ouabain plus furosemide-insensitive K influx in human red cells at 37 °C: Wiley & Cooper, 1974). This condition was not obeyed by K influx in NMDG at 0 °C, suggesting a mediated process. However, given the

inhibitory effects of increasing external K on Na influx and vice versa (Fig. 4*A* and *B*), it could be that the 'permeability constant' for K, P_K, in Fick's law is not in fact a constant but is itself a decreasing function of the external K concentration, giving rise to the apparent saturable behaviour.

One possible underlying mechanism for these effects could be a phase transition. Some methods, e.g. Raman spectroscopy (Verma & Wallach, 1976), ^{31}P-nuclear magnetic resonance (Cullis, 1976), and viscometry (Zimmer & Schirmer, 1974) reveal discontinuities in human red cell membrane ordering at about 15–20 °C, while electron paramagnetic resonance (Minetti, Ceccarini & di Stasi, 1984) shows discontinuities at 8, 20 and 40 °C. Trauble & Eibl (1974) showed that phase transitions in simple phospholipid systems can be modified by pH and divalent cations, but in general, the divalents (or decreasing pH) increase the temperature of phase transition (e.g. see Sacre, Hoffman, Turner, Tocanne & Chapman, 1979), opposite to the effects described here. However, the effects are complex, especially if phospholipid mixtures are present, when divalents can influence domain formation amongst the components (Jacobsen & Papahadjopoulos, 1975; Tanaka & Ohnishi, 1976). In the erythrocyte membrane, not only multiple lipids but also proteins are present, and the precise mechanism could be complex. Protein-induced packing faults (Pringle & Chapman, 1981) could be present, for instance. It would be interesting if electron spin resonance or nuclear magnetic resonance studies of the erythrocyte membrane in solutions comparable to those employed here showed differences in temperature dependence.

It is simple to hypothesize that the nature of the association between the cations and the membrane is electrostatic. However, a simple association with a negatively charged binding site would not permit explanation of both our results and those of Wieth (1970), in which anion substitution led to substantially identical effects on cation permeability. It may be that small charge-dense ions of whatever nature can exert a polarizing influence on the membrane, analagous to the common effects of anions and cations on salting-in and salting-out of proteins in solution (Conway, 1981), in which small ions are thought to reduce the quantity of available water for dissolution of protein molecules.

The flux at 0 °C depended strongly on the buffer used and was in fact least in MOPS. It is possible that these differences were due to variations in ionized organic anion or cation concentration or charge density, reflecting Wieth's observations (Wieth, 1970). Alternative mechanisms, e.g. effects due to binding of trace amounts of divalent cations by different buffers (Perrin & Dempsey, 1974), or due to partitioning of buffer molecules in the membrane, cannot be excluded.

The effectiveness of the smaller cations in inhibiting ^{86}Rb efflux at 0 °C was not a simple function of ionic radius and charge. The notable exceptions were K, which was (repeatedly) less effective than Rb, Cs and guanidine, and Li, which was more effective than the divalents. The K anomaly is consistent with Eisenmann's series IV (Eisenmann & Horne, 1983), which can describe, for example, the ionic selectivity of the skeletal muscle K channel (Gay & Stanfield, 1978).

The transport of the amino acids L-leucine and L-lysine showed monotonic temperature dependence in both media. While the major part of these fluxes is attributable to saturable systems at 37 °C, both have linear components probably reflecting passive diffusion (Young *et al.* 1980) and if marked paradoxical effects were

present, they would be expected to be evident at low temperatures. The absence of such effects argues that the amino acids do not share the same passive diffusional pathway as inorganic cations, at low temperatures at least. Interestingly, L-leucine transport, which is transported by the 'L' system (Young *et al.* 1980) was considerably less temperature sensitive than L-lysine (which uses the 'Ly^+' system). This result could simply represent the effect of temperature on substrate affinity (e.g. see Ellory & Willis, 1981, for such effects on the Na–K pump).

From a methodological standpoint, these studies highlight the difficulty of finding a truly 'inert' replacement for Na and K in human red cell cation flux studies. It is clear that termination of flux experiments by washing in ice-cold $MgCl_2$ is preferable to washing in choline to prevent undue loss of isotope from the cells during washing. Prior to or during the flux measurement, cell washing or incubation in ice-cold choline or NMDG might lead to unsuspected changes in intracellular electrolyte concentration, notably K loss and cell shrinkage, and our results show that K fluxes at 37 °C can be modified by the temperature of the cell preparation. However, for Na or K replacement in experiments at 37 °C, NMDG and choline remain preferable to Mg, which is inhibitory (Ellory *et al.* 1983). One compromise would be to use choline or NMDG as ionic replacement and to have a low and constant concentration (about 0·25–0·5) of Mg present in all media, such that the present effects were prevented at low temperatures while the inhibitory effects of variable concentrations of Mg were avoided. Guanidine is a possible alternative to choline and NMDG, but it shares structural features with the transport inhibitor amiloride (Benos, 1982).

Our measurements at 37 °C indicated that the residual, ouabain plus bumetanide-insensitive K influxes in NMDG were influenced by the temperature at which the cells were washed prior to the flux measurement. The residual K influx in choline or NMDG is usually found to be greater than or equal to that in Na: e.g. see Figs. 1*A* and 2 here; Wiley & Cooper (1974), Fig. 4; Wiater & Dunham (1983), Table 2. In Na replacement experiments, this exaggeration of the passive diffusional component could lead to underestimates of the Na-dependent fraction of the total ouabain-insensitive K flux. Although the residual fluxes can be greater in NMDG than in Na, it should be noted that our data confirm the presence of both Na-dependent and Na-independent fractions within the ouabain-insensitive K fluxes (Table 1; Wiley & Cooper, 1974, Fig. 4). These technical points may help to elucidate some contradictions at present evident in the literature dealing with erythrocyte cation transport and hypertension (e.g. see Parker & Berkowitz, 1983).

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