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THE PREPARATION OF HUMAN RED CELL GHOSTS CONTAINING CALCIUM BUFFERS

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SUMMARY

1. Ca buffers may be introduced into human red cells by reversible haemolysis. The resealed ghosts retain Ca and chelating anions in the same ratio as in the haemolysing solution, enabling the intracellular Ca^{2+} concentration to be calculated simply.

2. The passive permeability of the ghosts to Na and Cl is unaffected by intracellular Ca^{2+} concentrations in the 10^{-8}–10^{-4} M range, whereas the K permeability is greatly increased at concentrations above 10^{-7} M.

3. These preparations enable Ca-dependent K movements to be studied under stable conditions. When the ghosts contain about 5×10^{-6} M–Ca^{2+}, over 96% of K transport occurs via the Ca-sensitive route.

INTRODUCTION

A wide variety of impermeant solutes may be introduced into human red cells by the process of reversible haemolysis. During resealing, the ghosts regain a considerable degree of membrane integrity, and these preparations are invaluable for the study of membrane function. This paper describes the preparation of ghosts containing Ca buffers. Ca and a chelating anion are incorporated into the ghosts at haemolysis, and subsequently keep the intracellular Ca^{2+} concentration at a stable level. Intracellular Ca is known to increase the K permeability of the human red cell (Gárdos, 1958; Lew, 1970; Romero & Whittam, 1971). The purpose of this work was to establish conditions for the study of Ca-dependent K movements in the steady state. These K movements will be considered in much greater detail in the following paper (Simons, 1976).

It was necessary to omit ATP (adenosine triphosphate) from the preparations, to maximize the retention of Ca by the ghosts. When ATP

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was present it fuelled a rapid extrusion of Ca from the ghosts (Schatzmann, 1973), which continued until either Ca or ATP was exhausted. For this reason, the ghosts were prepared from ATP-depleted red cells.

A preliminary account of some of this work has been published (Simons, 1975).

METHODS

Preparation of ghosts

4 to 7-day-old acid citrate dextrose blood was centrifuged, and after the buffy coat had been removed, the red cells were washed 3 times with saline, by resuspension and centrifuging. The red cells were depleted of ATP by 24 hr incubation at 37° C, while suspended in a medium containing 152 mM-KCl, 2 mM phosphate, 0·3 mM-EGTA, and chloramphenicol (10 μg/ml.) (pH adjusted to 7·4 with Tris base). After washing with saline, they were packed by centrifuging at up to 35,000 *g* for 10 min (MSE High Speed 17) and haemolysed by squirting a known volume of cells into 15 times that volume of haemolysing solution, with vigorous stirring. The haemolysing solutions contained 3 mM chelating anion (EGTA, HEDTA or citrate), 0–2·7 mM-$CaCl_2$, 2 mM phosphate, and ^{42}KCl and/or ^{22}NaCl, as required. They were adjusted to a pH of 7·2 at room temperature (with KOH or Tris base), but haemolysis and restoration of the ghosts was carried out at 0° C (Bodemann & Passow, 1972). Three minutes after haemolysis, each ghost suspension was restored to an osmotic pressure of 200 ideal m-osmole/l., by the addition of an appropriate volume of 2 M-KCl solution, and then incubated at 37° C for 20 min. The ghosts were collected by centrifuging at up to 35,000 *g* for 10 min, and washed 3 times by resuspension and centrifuging at 2° C. The solution for resuspension and washing of ghosts contained 98 mM-KCl, 2 mM phosphate and 0·4 mM-EGTA, and its pH was adjusted to 7·1 at 37° C, using KOH and/or Tris base. (A pH of 7·1 was chosen in order to obtain higher concentrations of Ca^{2+} in Ca/EGTA mixtures than are possible at pH 7·4.)

Analytical

Washed ghosts were resuspended in the solution described and incubated at 37° C for periods ranging from 5 to 30 min. They were then pooled, and centrifuged at 30,000 *g* for 15 min. Known volumes of packed ghosts were lysed with water and extracted with trichloroacetic or perchloric acid (final concentration about 0·15 M). Ca and Mg were estimated by atomic absorption flame photometry (Pye Unicam SP 90B, Series 2), using the acid extracts without further treatment. The results were corrected for quenching by adding known quantities of Ca or Mg to duplicate samples, either before or after extraction. Chloride was measured by conductimetric titration of perchloric acid extracts with $AgNO_3$ solution. ATP and citrate were measured using perchloric acid extracts, neutralized with $KHCO_3$ solution: ATP by a firefly method (Glynn & Hoffman, 1971), and citrate enzymically (Williamson & Corkey, 1969). Red cell Ca, Mg and ATP were determined in a similar fashion.

Uncomplexed EGTA in ghost extracts was estimated by addition of sufficient NaOH to make them strongly alkaline, and titration with 1 mM-$CaCl_2$ solution, using Patton & Reeder's indicator (2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthyl-azo)-3 naphthoic acid). The total EGTA present may be estimated by adding the free EGTA and the total Ca concentrations, because all the Ca in the ghost extracts would already be complexed to an equivalent amount of EGTA at the start of the titration. Before measuring EGTA, the ghosts were always given a final wash with an EGTA-free medium, to avoid contamination with extracellular EGTA.

All concentrations are related to litres of packed cells or ghosts, unless otherwise stated, and expressed in terms of molarity. The water content of the ghosts was 95–96 % (w/w).

Measurement of ion movements

Na and K efflux. Ghosts containing ^{42}K and/or ^{22}Na were resuspended in the solution given above, at an haematocrit of 2–5 %, and aliquots were placed in test tubes, at 0° C. These were incubated at 37° C for varying times (usually 6, 12, 18 and 24 min, each in duplicate), then plunged into melting ice for 2 min, and centrifuged at 2000 *g* for 4 min. The supernatants were removed and counted for γ-emission (Nuclear Enterprises NE 8312). Solutions containing ^{22}Na and ^{42}K were counted again after an interval of 4 days, during which most of the ^{42}K had decayed. Rate constants for the equilibration of tracer were calculated from plots of log (1-(counts in supernatant/counts in suspension)) against time (see Text-fig. 2). Note that K is the dominant cation, and its efflux is measured under equilibrium conditions, whereas Na efflux is measured from a low intracellular concentration (under 1 mM) into an Na-free medium. Any contribution from the Na pump can be ruled out for lack of ATP.

Chloride efflux was measured by the rapid filtration method of Dalmark & Wieth (1972). The temperature was 0–0·5° C. A pH of 7·3 was used, because extrapolation of stability constants suggested that the affinity of EGTA and HEDTA for Ca might be about the same at 0° C and pH 7·3 as at 37° C and pH 7·1.

Ca buffer calculations

The equations by which the Ca^{2+} (and Mg^{2+}) concentrations in buffer mixtures may be calculated are too well known to be repeated, but some comment is needed on the derivation of appropriate stability constants from the chemical literature. Normal experimental conditions were 37° C, pH 7·1, and ionic strength 0·1 M, and every effort was made to obtain values for these conditions. When this was not possible, more importance was attached to ionic strength than temperature, as disregard of the former usually produces larger errors.

EGTA. The stability constants for Ca and Mg, measured at 20° C by Schwarzenbach, Senn & Anderegg (1957), were corrected to 37° C, using the thermochemical data of Boyd, Bryson, Nancollas & Torrance (1965). (The calculated values were $K_{Ca} = 10^{10\cdot67}$ M^{-1} and $K_{Mg} = 10^{5\cdot44}$ M^{-1}.) The proton association constants of Boyd *et al.* (1965) were also extrapolated to 37° C, and corrected from activity to concentration units, giving values $K_1 = 10^{9\cdot25}$ M^{-1} and $K_{12} = 10^{8\cdot65}$ M^{-1}. Finally, the apparent stability constants at 37° C, pH 7·1, and ionic strength 0·1 M were calculated: $K'_{Ca} = 10^{6\cdot94}$ M^{-1}, $K'_{Mg} = 10^{2\cdot07}$ M^{-1}.

HEDTA. Interpolation of the data of Moeller & Ferrús (1961) gives the apparent stability constant for Ca as $10^{5\cdot66}$ M^{-1} under experimental conditions. No satisfactory value for Mg could be found: the apparent stability constant used was $10^{3\cdot2}$ M^{-1}, based on the results of Kroll (1959), quoted by Sillén & Martell (1964).

Citrate. The results of Campi, Ostacoli, Meirone & Saini (1964) give apparent stability constants for Ca and Mg of $10^{3\cdot53}$ M^{-1} and $10^{3\cdot38}$ M^{-1}, respectively, at 20° C, pH 7·1 and ionic strength 0·1 M. Use of these values at 37° C introduces an error, but no studies of the temperature dependence of the Ca-citrate equilibrium could be found. Note that the values given for citrate in Simons (1975) are wrong, because they are based on values at zero ionic strength, and there was an error in calculation.

Phosphate. No satisfactory values could be found: the apparent stability constants used were $K'_{Ca} = 10^{1\cdot7}$ M^{-1} and $K'_{Mg} = 10^{1\cdot9}$ M^{-1} (Smith & Alberty, 1956). These were obtained at 25° C, and err on the side of high ionic strength, rather than low.

Materials

Radiochemicals were from the Radiochemical Centre Ltd., Amersham, Bucks. All other chemicals were A.R. grade, where possible. $CaCl_2$ solutions were made from anhydrous A.R. $CaCO_3$ and standardized relative to EGTA and HEDTA stock solutions by titration. The indicator, 2-OH-1-(2-OH-4-sulpho-1-naphthylazo)-3-naphthoic acid, was obtained from Koch-Light Laboratories Ltd., and the water was double distilled.

Abbreviations

ATP	adenosine triphosphate.
CDTA	trans-cyclohexane 1,2-diamine-*NNN'N'*-tetra-acetic acid.
EDTA	ethylenediamine-*NNN'N'*-tetra-acetic acid.
EGTA	1,2-di(2-aminoethoxy)ethane-*NNN'N'*-tetra-acetic acid.
HEDTA	*N*-(2'hydroxyethyl)ethylenediamine-*NNN'*-triacetic acid.

RESULTS

Visual appearance

Microscopic examination of ghosts in a slide-and-coverslip preparation showed that they were mainly crenated spheroids, with greatest diameters in the range of 6–8 μm (Pl. 1). This result was found at all levels of intracellular Ca: the abnormal shape is probably due to the lack of ATP (Weed, LaCelle & Merrill, 1969).

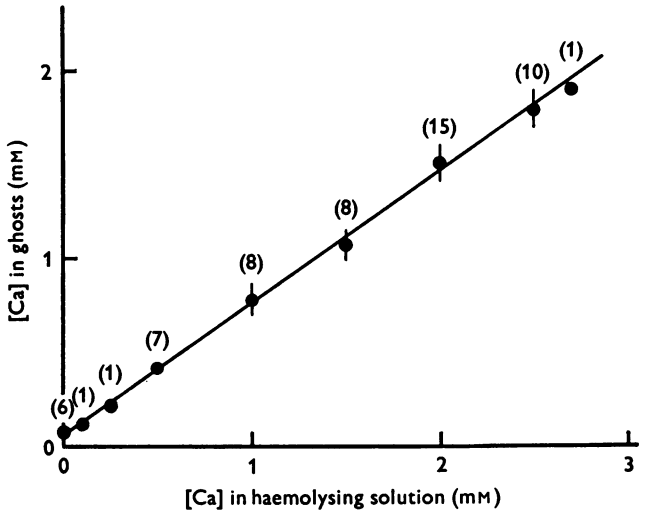
No measurements of ghost volume were made, but visual inspection of ghost pellets indicated that they had roughly the same volume as the red cells from which they were made. In some cases the ghosts were very permeable to potassium chloride, but this did not cause shrinkage because there were no gradients of K or chloride.

Chemical analyses

On the simplest theory, the concentration of an impermeant solute in resealed ghosts is expected to be 15/16 of its concentration in the haemolysing solution, plus 1/16 of its concentration in the red cells used. This assumes complete equilibration at haemolysis (lysing ratio 1:15), and no volume changes or loss of solute after ghost resealing.

Ca. After pre-treatment, the red cells contained variable quantities of Ca. In three experiments 60, 30 and less than 10 μM were found. Analyses of resealed ghosts showed that their Ca contents were linearly related to the concentration in the haemolysing solution (Text-fig. 1). This result is in agreement with the simple theory proposed above, except that the fraction of Ca retained by the ghosts was 70%, instead of the theoretical 94%. Possible reasons for this will be discussed below. When the haemolysing solution was Ca-free, the ghosts contained an average of 70 μM Ca, with a range from 20 to 180 μM. The higher results must have been caused by accidental contamination with Ca, but it is also possible that red cell Ca

does not equilibrate at haemolysis, perhaps because it is partly membrane-bound. Apart from this possibility, the Ca in the ghosts in Text-fig. 1 would have been in solution in the cytoplasm, and not membrane-bound. If there were an appreciable component of membrane-bound Ca, the results in Text-fig. 1 would have been fitted by a curve, convex upwards, and not by a straight line. In addition, Forstner & Manery (1971) have



Text-fig. 1. The retention of Ca by resealed ghosts. Each point is the average of several observations whose number is indicated in parentheses. The vertical lines indicate ±1 S.D. The oblique line obeys the equation $y = 0{\cdot}065 + 0{\cdot}70\ x$, and is a least-squares fit to the points.

shown that the membranes from 1 l. of red cells bind about 200 μmole Ca at a Ca concentration of 1 mM, and an ionic strength of 0·01 M. In Text-fig. 1 the membrane-bound Ca would have been much less than 200 μM, because the free Ca concentration was always below 1 mM (and in most cases below 10 μM), and the ionic strength was 0·1 M, rather than 0·01 M (increasing ionic strength reduces Ca binding).

It was also of interest to see whether Ca-containing ghosts lose Ca during incubation in a Ca-free medium. Table 1 gives the results of two experiments, which show that they lose less than 10% of their Ca in a 20 min interval, and in many cases the loss is much smaller still. This result implies that the red cell membrane is not very permeable to the $Ca.EGTA^{2-}$ and $Ca.HEDTA^{-}$ anions, which account for most of the intracellular Ca in Table 1.

Mg. In these experiments the haemolysing solutions contained no Mg, but human red cells contain about 2 m-mole Mg/l. cells. The average Mg

concentration found in resealed ghosts (haemolysis ratio 1:15) was 112 ± 4 μM (S.E. of mean, $n = 22$).

Chloride. One would expect a Donnan equilibrium of chloride ions, as in red cells, but with a concentration ratio close to unity. In one experiment, ten separate samples of packed ghosts were each analysed in duplicate, and the average Cl content was $87{\cdot}9 \pm 1{\cdot}0$ mM (S.E. of mean). This is equivalent to 92 mM, expressed per litre of cell water, as the ghosts had a water content of 95%. The supernatant was found to contain $92{\cdot}7 \pm 0{\cdot}3$ mM-Cl (S.E. of mean, $n = 3$), which is in good agreement with the intracellular concentration, expressed in terms of cell water.

TABLE 1. Loss of Ca from resealed ghosts during incubation at 37° C in a Ca-free medium

	Ca concentration (mM)		
	In haemolysing solution	In resealed ghosts after	
		10 min	30 min
Expt. 1	2	1·52	1·52
	2·5	1·94	1·93
		5 min	25 min
Expt. 2	0·5	0·39	0·36
	1	0·77	0·72
	1·5	1·15	1·10
	2	1·43	1·42
	2·5	1·87	1·71

In Expt. 1 the haemolysing solution also contained 3 mM-EGTA, and in Expt. 2, 3 mM-HEDTA.

TABLE 2. The analysis of resealed ghosts for EGTA

Concentration in haemolysing solution (mM)		Concentration in resealed ghosts (mM)		
EGTA	Ca	Ca (1)	Free EGTA (2)	Total EGTA (1+2)
3	0	0·03	2·08	2·11
3	0	0·18	2·08	2·26
3	2	1·64	0·64	2·28
3	2	1·65	0·67	2·32
3	2	1·63	0·65	2·28
0	0	0·29	−0·21	0·08

(1) gives the total Ca found by atomic absorption flame photometry, and (2) the free EGTA, by titration with $CaCl_2$ solution. The negative result in column (2) implies that the extract contained excess Ca, which was titrated to equivalence with EGTA solution. The results in columns (1) and (2) are averages of duplicate determinations.

EGTA. Table 2 presents the results of analyses of ghosts, in which the total EGTA is estimated as the sum of the free EGTA, measured by titration with Ca, and the Ca-bound EGTA, measured as the total Ca content. The average concentration found was 2·25 mM, in ghosts intended to contain 3 mM. This result needs to be reduced by 0·05 mM to allow for the indicator blank, making an average of 2·20 mM.

HEDTA. It was not possible to estimate intracellular HEDTA by the method used for EGTA, because the end-point of the titration was too indistinct. However, extracts of ghosts resealed with Ca/HEDTA buffer mixtures, which should contain more HEDTA than Ca, did give a blue colour on treatment with NaOH and Patton & Reeder's indicator, confirming the presence of excess HEDTA.

Citrate. The average concentration found in ghosts resealed with citrate was $2·13 \pm 0·06$ mM (S.E. of mean, $n = 6$). This should be compared with a concentration of 3 mM in the haemolysing solution.

ATP. The ATP content of the red cells used in these experiments was reduced to 5–20 μM by the 24 hr preincubation, and the ghosts contained less than 1 μM-ATP.

Intracellular ionized calcium concentration

The results of analyses show that, for impermeant solutes, the ratios of the concentrations found in resealed ghosts to the concentrations in the haemolysing solutions are all about the same. The average was 70% for Ca, 73% for EGTA and 71% for citrate. There are several explanations why these ratios might be less than the theoretical 94%. For example, extracellular fluid is trapped with the ghost pellets; the ghosts might swell after resealing, or lose solute during the washing procedure, and there could be a small fraction of ghosts that do not reseal to solutes and subsequently equilibrate with the external medium. Whatever the explanation, the results imply that the ratio of Ca to EGTA or citrate in the resealed ghosts is almost, if not exactly, the same as in the haemolysing solution. Now, the level of Ca^{2+} in Ca/anion buffer mixtures is determined primarily by the Ca/anion ratio, and is very insensitive to small changes in the total concentration. It follows that a reasonable estimate of intracellular Ca^{2+} may be obtained by calculating what the Ca^{2+} concentration would be if the Ca/anion mixture in the haemolysing solution were at 37° C and pH 7·1. All such estimates given in this paper are also corrected for the binding of Mg, by the anion used to buffer Ca, and for the binding of Ca, by the 2 mM phosphate present in the ghosts.

Sodium and potassium efflux

Text-fig. 2 illustrates a typical experiment, in which both ^{42}K and ^{22}Na efflux were measured. The results are quite well fitted by straight lines, suggesting that the movement of each tracer can be described by a single exponential function. Intracellular Ca has a marked effect on the rate of K loss, which increases monotonically with the estimated Ca^{2+} concentration, but there is little or no effect on Na permeability. Many similar experiments were performed, in order to investigate the effects of a wide range of intracellular Ca^{2+} concentrations, and to check the reproducibility of the results. In each experiment the rate constants for the equilibration of tracer were obtained from graphs similar to Text-fig. 2, and Table 3 provides a summary of these rate constants.

Table 3 confirms that the Na permeability of the ghosts is independent of the internal Ca^{2+} concentration. The range of rate constants is from 0·075 to 0·193 hr^{-1}, with an average of $0·119 \pm 0·007$ hr^{-1} (S.E. of mean).

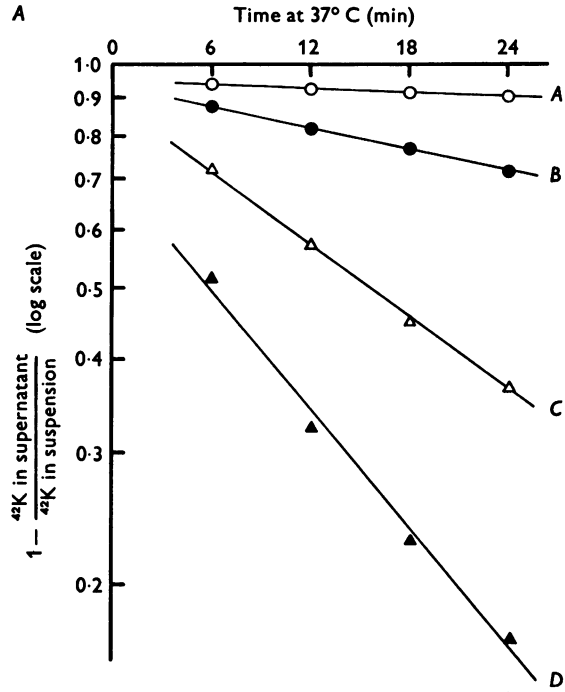
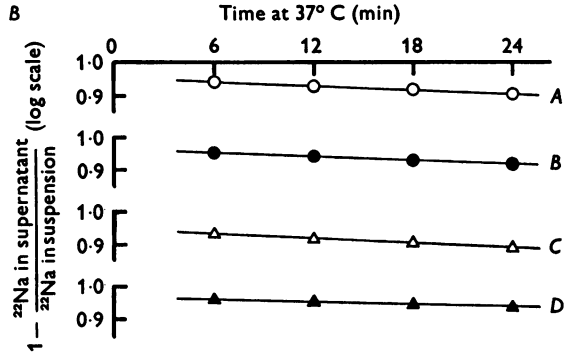


Fig. 2A. For legend see facing page.

If the results are pooled according to the anion used, the averages (and S.E. of mean) are $0·110 \pm 0·011$ for EGTA, $0·111 \pm 0·008$ for HEDTA and $0·133 \pm 0·015$ for citrate. These are not significantly different, suggesting

that the Na permeability of the ghost membrane is unaffected by the nature of the anion used. The results for K efflux are very different. Ghosts containing no Ca have an average rate constant of $0{\cdot}136 \pm 0{\cdot}010$ hr^{-1} (S.E. of mean), which is not significantly higher than the average for Na. Those containing Ca have, in most cases, much higher rate constants. The average was calculated for each level of Ca^{2+}. Text-fig. 3 shows that the results for all three Ca buffer systems fall on a single continuous curve, when plotted against the estimated intracellular Ca^{2+} concentration. Possible explanations for the shape of this curve will be discussed in the next paper (Simons, 1976). For the present, let us note that the greatest K permeability is shown by ghosts containing 2 mM-Ca and 3 mM-HEDTA, for which the average rate constant is $3{\cdot}71 \pm 0{\cdot}17$ hr^{-1} (S.E. of mean).

Effect of temperature. In one experiment the efflux of Na and K from resealed ghosts was measured over a range of temperatures. This was done both for Ca-free ghosts and for those containing Ca, and Text-fig. 4 gives



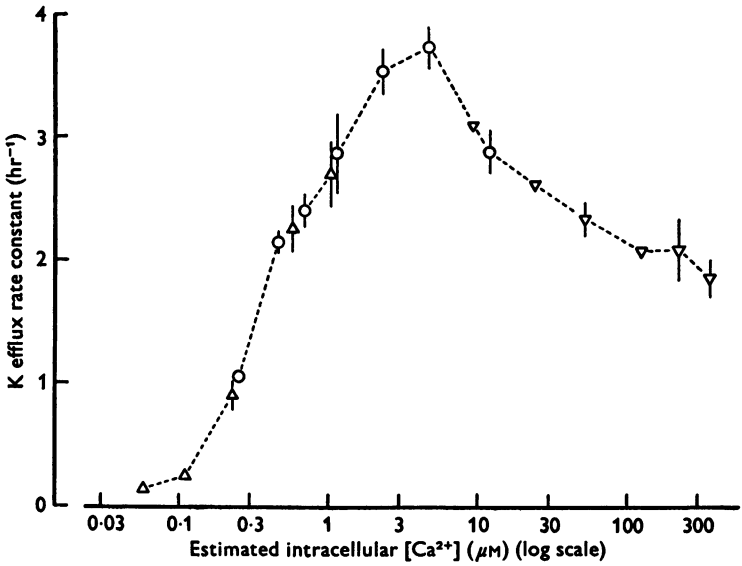
Text-fig. 2. Simultaneous measurements of ^{42}K efflux (Fig. 2*A*) and ^{22}Na efflux (Fig. 2*B*) from resealed ghosts. Each point is the average of two results, and the lines are least-squares fits. The table below gives details of the ghost contents, and the rate constants (± S.D.), calculated from the slopes of the lines.

Ghosts	Ca buffer in haemolysing solution	Estimated intracellular [Ca^{2+}] (μM)	Rate constants (hr^{-1}) ^{42}K	^{22}Na
A (○)	1 mM-Ca + 3 mM-EGTA	0·058	0·114 ± 0·007	0·125 ± 0·004
B (●)	2 mM-Ca + 3 mM-EGTA	0·23	0·669 ± 0·018	0·106 ± 0·005
C (△)	0·5 mM-Ca + 3 mM-HEDTA	0·46	2·27 ± 0·04	0·146 ± 0·004
D (▲)	1·5 mM-Ca + 3 mM-HEDTA	2·3	3·71 ± 0·16	0·083 ± 0·002

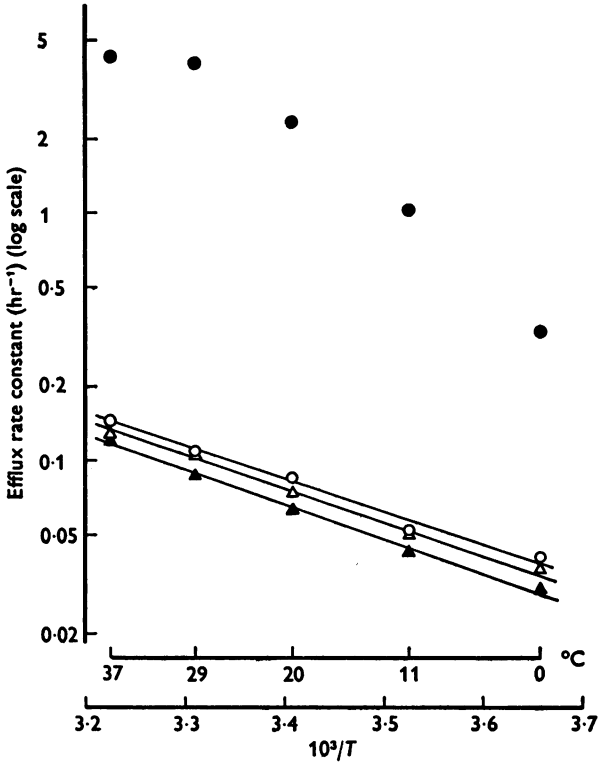
TABLE 3. Na and K permeabilities of ghosts containing Ca buffers

Haemolysing solution contents		Estimated intracellular		
Anion (concn. = 3 mM)	Ca (mM)	$[Ca^{2+}]$ (μM)	^{42}K efflux rate constant (hr^{-1})	^{22}Na efflux rate constant (hr^{-1})
(EGTA*)	0	—	0·103, 0·113, 0·122, 0·144, 0·185	0·128
EGTA	1	0·057	0·114, 0·144, 0·169	0·125
EGTA	1·5	0·11	0·205, 0·215, 0·314	—
EGTA	2	0·23	0·669, 0·969, 1·07	0·106
EGTA	2·5	0·57	1·87, 2·34, 2·50	0·082
EGTA	2·7	1·0	2·41, 2·94	—
HEDTA	0	—	0·150	—
HEDTA	0·3	0·25	1·03, 1·04, 1·07	0·142
HEDTA	0·5	0·46	1·93, 2·04, 2·27, 2·28	0·146
HEDTA	0·7	0·70	2·26, 2·52	0·143
HEDTA	1	1·2	2·28, 2·88, 3·39	—
HEDTA	1·5	2·3	2·64, 3·45, 3·67, 3·71, 3·72, 3·91	0·083, 0·086, 0·121, 0·132
HEDTA	2	4·7	3·37, 3·45, 3·57, 3·84, 4·32	0·080, 0·093, 0·121
HEDTA	2·5	12	2·45, 2·74, 3·11, 3·15	0·075
Citrate	0	—	0·132	—
Citrate	0·1	9·5	3·05, 3·08	—
Citrate	0·25	24	2·59, 2·59	—
Citrate	0·5	53	2·06, 2·34, 2·52	0·080, 0·131
Citrate	1	130	2·03, 2·06	0·094, 0·177
Citrate	1·5	230	1·81, 2·31	0·117, 0·171
Citrate	2	370	1·65, 1·71, 2·13	0·104, 0·193

* Some of the 0-Ca experiments used EDTA or CDTA in place of EGTA. They bind Ca slightly more strongly than EGTA does.



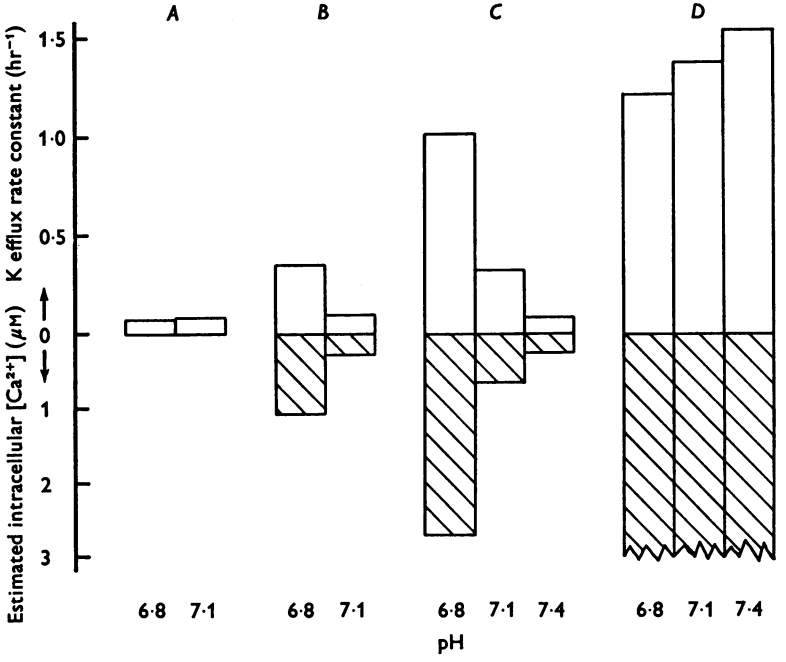
Text-fig. 3. The dependence of K permeability on intracellular Ca^{2+}. The ghosts contained Ca and either EGTA (△), HEDTA (○) or citrate (▽). The points give the average of the results in Table 3, ± 1 S.E. of mean.



Text-fig. 4. Effect of temperature on resealed ghost Na and K permeabilities. The efflux of ^{22}Na (△, ▲) and ^{42}K (○, ●) was measured over a range of temperatures from 0 to 37° C from ghosts containing either 0-Ca and 3 mM-CDTA (○, △) or 2 mM-Ca and 3 mM-HEDTA (●, ▲). Straight lines, fitted by the least-squares method, correspond to apparent activation energies of 24·7 ± 1·7 (○), 25·1 ± 0·8 (△), and 26·3 ± 1·3 (▲) kJ/mole.

the result. The Na and K effluxes from Ca-free ghosts, and the Na efflux from Ca-containing ghosts, all show the same pattern, with linear Arrhenius plots over the range from 0 to 37° C, and low apparent activation energies, of about 25 kJ/mole. In contrast, the Arrhenius plot for the K efflux from Ca-containing ghosts cannot be fitted by a straight line. It might be fitted by two intersecting straight lines, with a discontinuity at about 25° C, but there are insufficient data to be sure of this interpretation. In any case, it is clearly much steeper than the other three below 20° C.

Effect of pH. In another experiment, the ghosts were resuspended in solutions of differing pH, after they had been resealed, and K efflux was



Text-fig. 5. Comparison of the effects of pH changes on intracellular Ca^{2+} concentration and K efflux from resealed ghosts. The haemolysing solutions contained 3 mM-EGTA and 2 mM-Mg in all cases, and either no Ca (*A*), 2 mM-Ca (*B*), 2·5 mM-Ca (*C*) or 3 mM-Ca (*D*). After resealing, the ghosts were washed 3 times with a solution of normal pH (7·1 at 37° C), then washed and incubated in solutions of different pH, as indicated in the Figure. For further details see text.

measured. Ca buffers are very sensitive to pH: acidification increases the Ca^{2+} concentration, and alkalinization reduces it. A small pH change should have a large effect on K efflux if it alters the internal Ca^{2+} concentration in a sensitive range, for example around 1 μM (see Text-fig. 3). Thus, in Text-fig. 5, pH changes have little effect on K efflux from ghosts

A and *D*, containing no Ca and excess Ca, respectively, while they have a large effect on ghosts *B* and *C*. In these cases the changes in the K efflux rate constant correlate well with the calculated changes in the intracellular Ca^{2+} concentration. This experiment demonstrates that the Ca buffers do not have an irreversible effect on the K permeability of the red cell membrane at the time of haemolysis, for the ghosts continue to respond to changes in internal Ca^{2+} after resealing has occurred.

The experimental conditions in Text-fig. 5 were unusual. The haemolysing solutions contained 2 mM-$MgCl_2$, in addition to the usual ingredients. This has been allowed for in calculating Ca^{2+} concentrations, but it does also affect K movements (see Simons, 1976). In ghosts *D*, Ca was not buffered properly, and the Ca^{2+} concentrations were probably in the 5–100 μM range. Furthermore, the monovalent cations were 12 mM-K, 12 mM-Na and 76 mM choline, in all the ghost preparations, and in the suspending medium. These factors make a quantitative comparison with other experiments impossible.

Chloride exchange

Table 4 contains the results of two experiments to measure chloride efflux from ghosts containing Ca buffers. The rate constants lie in the range

TABLE 4. ^{36}Cl exchange in ghosts containing Ca buffers

Haemolysing solution contents			
Anion (concn. = 3 mM)	Ca (mM)	Estimated intracellular $[Ca^{2+}]$ (μM)	Rate constant for ^{36}Cl efflux (min^{-1})
EGTA	0	—	1·6, 1·3
EGTA	1	0·06	1·7
EGTA	2	0·2	1·7, 1·4
HEDTA	1	1	1·3
HEDTA	2	5	1·6, 1·7
Citrate	1	100	2·0
Citrate	2	400	1·4, 1·6

^{36}Cl exchange was measured at 0° C and pH 7·3, using a rapid filtration method (Dalmark & Wieth, 1972).

from 1·3 to 2·0 min^{-1}, but there is no obvious trend, suggesting that intracellular Ca^{2+} has no effect on the chloride exchange rate. The average, 1·6 min^{-1}, may be compared with 2·3 min^{-1} obtained by Dalmark & Wieth (1972) with intact red cells, under slightly different conditions.

DISCUSSION

Intracellular calcium

The results show that the ghost preparations retain Ca very well. Cation fluxes can be measured over a period, while the intracellular Ca^{2+}

concentration remains effectively constant. The intracellular Ca^{2+} concentrations were estimated from theoretical calculations of the Ca^{2+} concentrations which the haemolysing solutions would have, if they were at 37° C and pH 7·1. These calculations may be criticized on a number of grounds. They make use of the observation that the ratio of Ca to EGTA or citrate is the same in the ghosts as in the haemolysing solution; but the discrepancy between the concentrations found in the ghosts and the concentrations present in the haemolysing solutions has not been discussed fully. The concentrations in the ghosts would be expected to be 6% below those in the haemolysing solution, because of the dilution which occurs at lysis; another 5% lower because the water content of the ghosts is only 95%; and perhaps a further 7% lower because of trapped extracellular fluid (see Simons, 1974). This reduces the theoretical concentration in the ghosts to 82% of the haemolysing solution, which is still higher than the 70–73% observed. One is left with the possibility that up to 15% of the ghosts might be highly permeable to solutes. This would not matter, because they would lose tracer Na and K during the washing procedure, and not contribute to cation efflux measurements. However, if this high permeability were distributed unevenly, with some ghosts permeable to Ca and others to EGTA, this would upset the ratio of Ca to EGTA in an appreciable fraction of the ghosts, while keeping the overall ratio constant. This seems unlikely, but cannot easily be rejected. Another objection is that the composition of the ghost cytoplasm is not well defined. Anything in the red cells would also be present in the ghosts, but at a reduced concentration. There might be substances which interact with Ca or its chelating agents, thereby affecting the Ca^{2+} concentration. It is often claimed that EGTA, and other chelating agents, may have different stability constants for Ca under 'biological' conditions from those measured by chemists. These claims seem to rest on the work of Ogawa (1968), who observed a considerable reduction in the stability constant of EGTA for Ca, in solutions buffered with histidine, maleate or imidazole, but not with phosphate, the pH buffer used in this work. The calculations also assume an equality of internal and external pH. This is unlikely to be a serious source of error, because measurements show that chloride has about the same concentration inside and outside the ghosts, and it is reasonable to expect the hydroxide ion ratio to be the same as the chloride ratio.

These criticisms can be answered in part by the K efflux measurements. Text-fig. 3 shows that all the results lie on a single curve, when plotted against the calculated intracellular Ca^{2+} concentration. There are no discontinuities between results at a high Ca/anion ratio with one chelating agent, and a low Ca/anion ratio with the next. This implies that the cal-

culations are correct, relative to one another, though not necessarily absolutely. The alternative, that the calculations are wrong, relative to one another, but the chelating agents affect K movements differently, so that the result is a smooth curve, seems improbable. It is also hard to see how the result could be a smooth curve if there were variations in Ca/anion ratio resulting from an uneven relative distribution of Ca and anions; for such variations would be much more serious at high Ca/anion ratios than at low ones.

Cation fluxes

There must be at least two pathways for cation transport across the ghost membrane, in order to explain the different temperature dependences of Na and K effluxes in ghosts with and without Ca (Text-fig. 4). An economical theory is that the first pathway does not select between Na and K and is unaffected by Ca, while the second is Ca-dependent and selective for K. More complicated theories can be constructed, but this one explains the results adequately. The unselective pathway accounts for all Na movements, and the K movements in Ca-free ghosts. These fluxes, with rate constants of 0·12–0·14 hr^{-1}, are considerably faster than the ouabain-insensitive (passive) cation fluxes of human red cells, which have rate constants of 0·01–0·02 hr^{-1}. The ghost fluxes also have low temperature coefficients, and these facts suggest they may be largely an artifact of the ghost-making process, perhaps reflecting permanent damage at the sites of membrane rupture during haemolysis.

K movements in the presence of internal Ca are very much faster, and show a different temperature dependence. At optimal Ca levels, 96% of them occur via the Ca-dependent route, assuming that the basal flux via the Ca-independent pathway remains unaltered. There is an extensive literature on the stimulation of K movements by internal Ca in both red cells (Romero & Whittam, 1971; Blum & Hoffman, 1971) and resealed ghosts (Blum & Hoffman, 1972; Colombe & Macey, 1974). Nearly all experiments with red cells have measured K loss during or following Ca-loading induced by metabolic depletion in Ca-rich solutions. They tend to show smaller K fluxes than the present work, but an exact comparison is impossible, because experimental conditions are so different. For example, Blum & Hoffman (1971) observed a K efflux rate constant of 0·7 hr^{-1} (into a 100 mM-K medium, their fig. 1), but the average intracellular Ca concentration would have been increasing throughout the period of measurement, while there might have been a population of cells which resisted Ca entry, and this would have led to an underestimate of K efflux. Romero & Whittam (1971) observed a K efflux of similar magnitude, but also found a Ca-dependent increase in Na *influx*. This was rather small, equivalent

to a rate constant of less than 0·1 hr^{-1}. No increase in Na efflux was seen in the present work, perhaps because it was obscured by the larger leak fluxes associated with the use of ghosts. Another possibility is that the increased Na *influx* might be the result of an increased electrochemical gradient for Na, associated with a membrane hyperpolarization, which probably existed in the experiments of Romero & Whittam.

Several previous studies of Ca-dependent K movements have used resealed ghosts. Some have followed the usual red cell procedure of measuring K loss following Ca entry: they will not be considered here as they seem to combine the disadvantages of red cells with those of resealed ghosts, without any compensating advantages. Blum & Hoffman (1972) incorporated Ca into ghosts and measured Na and K fluxes. Their results are broadly in agreement with those in this paper. An exact comparison is not possible because they did not use Ca-buffers. Colombe & Macey (1974) also incorporated unbuffered Ca into ghosts, but they failed to deplete their red cells of ATP before starting, and did not measure how much Ca was actually in their ghosts. They observed a biphasic effect of Ca on K permeability, but in the opposite sense to that seen in Text-fig. 3, small amounts of Ca producing a minimum rate of K efflux. This minimum disappeared when the red cells had been stored for 10–14 days before use. These results can probably be explained in terms of an ATP-dependent extrusion of Ca from the ghosts during incubation. More recently, Porzig (1975) made ghosts with Ca/EGTA buffers. His results are in agreement with those in this paper, in that he observed a rise in K efflux over the 0·1–1 μM range, but he measured net K loss, rather than tracer exchange, and observed a much smaller overall stimulation than in this paper.

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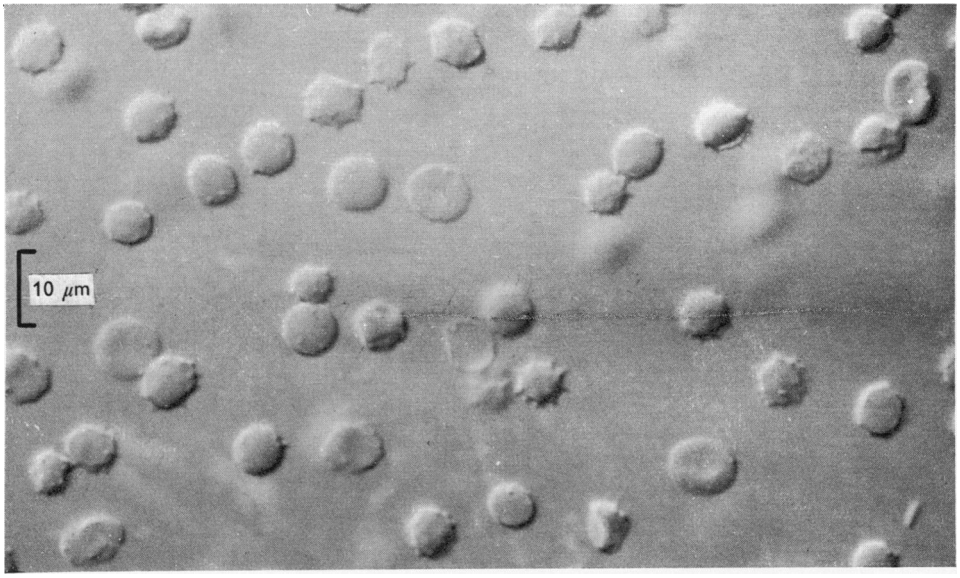

Fig. 1

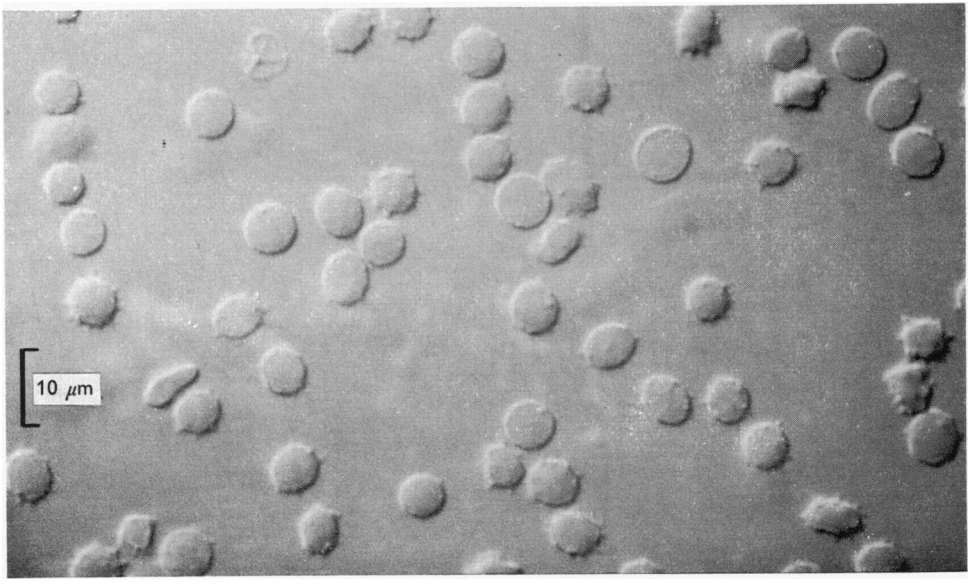

Fig. 2

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EXPLANATION OF PLATE

Resealed ghosts, suspended in wash medium, viewed by Nomarski differential interference contrast microscopy (Zeiss Universal). Total magnification = 1024 ×.

Fig. 1. Ghosts containing 1 mM-Ca and 3 mM-EGTA (estimated $[Ca^{2+}] = 0{\cdot}06\ \mu M$).

Fig. 2. Ghosts containing 0·5 mM-Ca and 3 mM citrate (estimated $[Ca^{2+}] = 50\ \mu M$).