The Influx of Calcium Ions into Human Erythrocytes during Cold Storage

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1. When human erythrocytes, suspended in iso-osmotic sucrose containing CaCl_2 , are stored at 3°C , Ca^{2+} influx into the cells occurs. Simultaneously, efflux of K^+ , Na^+ , Cl^- and water takes place and cell volume diminishes. 2. The extent of Ca^{2+} influx increases with duration of cold storage and with increasing concentration of Ca^{2+} in the suspending medium. 3. Erythrocytes that have been thus loaded with Ca^{2+} exhibit Ca^{2+} efflux against a concentration gradient when subsequently incubated at 37°C . 4. Ca^{2+} influx likewise occurs when the sucrose of the medium is replaced by iso-osmotic solutions of other non-ionized compounds. 5. Replacement of sucrose by iso-osmotic KCl or NaCl greatly diminishes the rate of Ca^{2+} influx during cold storage; however, in iso-osmotic choline chloride, Ca^{2+} influx is as rapid as in sucrose. 6. Preincubation of erythrocytes in iso-osmotic sucrose at 37°C causes rapid efflux of K^+ and Na^+ and renders the cell membranes highly permeable to Ca^{2+} during subsequent cold storage. 7. Preincubation of erythrocytes in iso-osmotic NaCl at 37°C with trypsin or neuraminidase is without effect on the permeability of the membrane towards Ca^{2+} . 8. The experimental results lead to the conclusion that the main prerequisite for Ca^{2+} influx into erythrocytes is the partial depletion of the cells of their univalent cations.

Erythrocytes, obtained from freshly drawn human blood after removal of plasma and buffy coat followed by exhaustive washing with iso-osmotic NaCl, contain very little calcium, the mean value being $16\text{ }\mu\text{mol/litre}$ of packed cells (Harrison & Long, 1968). Since at least 90% was firmly bound to the external surface of the cell membranes, it follows that erythrocytes contain an almost negligible quantity of intracellular calcium.

When erythrocytes were suspended in iso-osmotic sucrose containing 2.5 mM-CaCl_2 , an immediate uptake of Ca^{2+} occurred, amounting to about $400\text{ }\mu\text{mol/litre}$ of packed cells. This uptake was due to electrostatic binding by negative charges on the external surface of the cell membranes, about two-thirds of which were accountable for as carboxyl groups of sialic acid residues (Long & Mouat, 1971). This loosely bound calcium was readily removed by EDTA.

It was briefly reported by Harrison & Long (1968) that when whole heparinized blood was stored at 3°C for several days, some of the plasma Ca^{2+} entered the cytoplasm of the erythrocytes. This was shown to be truly intracellular, since it could not be removed by washing the cells with EDTA, but was released by hypo-osmotic haemolysis.

In the present work, Ca^{2+} influx into erythrocytes under a variety of experimental conditions has been studied. All the observations are consistent with the view that the main prerequisite for Ca^{2+} influx is loss

of univalent cations (chiefly K^+) from the cells and their non-replacement by other cations.

Materials and Methods

Materials

Human erythrocytes. Freshly drawn blood was supplied by the Department of Pharmacology, Institute of Basic Medical Sciences. Heparin was added to a concentration of 10 units/ml. Washed erythrocytes were obtained as described in the Methods section.

Reagents. Triple-glass-distilled water was used throughout; it was calcium-free. The calcium analytical standard for atomic-absorption spectrophotometry was described by Long & Mouat (1971).

Sodium benzylpenicillin and streptomycin sulphate were from Glaxo Laboratories, Greenford, Middx., U.K. Neuraminidase (type VI) from *Clostridium perfringens* (EC 3.2.1.18) and trypsin (type III) from bovine pancreas (EC 3.4.4.4) were obtained from Sigma (London) Chemical Co. Ltd., Kingston upon Thames, Surrey, U.K.

EDTA (lithium salt) was prepared by treating a suspension of EDTA (free acid, 0.606 g) in 10 ml of water with 6.23 ml of 1 M-LiOH and diluting to 25 ml. The resulting solution of the trilithium salt had a pH of 7.4, was 83 mM with respect to EDTA, and was iso-osmotic with 256 mM-sucrose or 160 mM-KCl. The

lithium salt of EDTA was used rather than the sodium or potassium salt, so that determinations of Na^+ and K^+ in the cells or suspending media could be made.

All other chemicals were the best available commercial preparations.

Methods

Influx of Ca^{2+} into erythrocytes. All manipulations were carried out in a cold-room at 3°C . Fresh heparinized human blood (about 120 ml) was centrifuged for 10 min at 2500g, and the plasma and buffy coat were removed. The cells were washed once with 2 vol. of 160 mM-KCl and twice with 2 vol. of iso-osmotic sucrose solution (256 mM). The washed cells were suspended in a suitable medium (usually 256 mM-sucrose containing 2.5 mM- CaCl_2), which also contained sodium benzylpenicillin (120 mg/litre) and streptomycin sulphate (200 mg/litre). The suspension normally contained 20–30 ml of packed cells/100 ml.

Two samples (4 ml each) of suspension were pipetted into 5 ml glass-stoppered centrifuge tubes, one of which was treated with 0.3 ml of 83 mM-EDTA (trilithium salt, pH 7.4) in order to remove those Ca^{2+} ions that were loosely bound to the external surface of the cell membranes. Both tubes were then centrifuged at 2500g for 30 min. Supernatants were removed as completely as possible by teat pipette and set aside for analysis. It was not possible to remove all the supernatant without disturbing the cell deposit, so that the latter was usually associated with about 0.1–0.2 ml of supernatant, as measured by sucrose determination.

The remainder of the erythrocyte suspension was stored at 3°C with gentle rocking. At intervals up to 7 days, samples (4 ml) were removed and treated as already described. Calcium, sucrose, potassium, sodium, chloride and haemoglobin were determined on the supernatants and on haemolysates of the cells, obtained after addition of 3 ml of water to the cell deposit. The corrected amounts of calcium and other components of the cells were determined after allowing for contamination with supernatant, as measured by the sucrose content of the haemolysates. The assumption was made that all sucrose was extracellular.

Haemolysis. Haemolysis was measured by determining the amount of haemoglobin in the supernatant, after centrifuging down the cells, and expressing this quantity as a percentage of the total haemoglobin in the suspension.

Analytical methods. Sucrose and calcium were determined as described by Long & Mouat (1971), and magnesium by atomic-absorption spectrophotometry (Harrison & Long, 1968). Sodium and potassium were measured in suitable dilutions of supernatants and cell haemolysates with a clinical flame

photometer (model 150; Evans Electroselenium Ltd., Halstead, Essex, U.K.). Chloride was determined as described by Schales & Schales (1941), and haemoglobin as cyanmethaemoglobin after treatment with Drabkin's reagent (King & Wootton, 1959).

ATP was determined by the method of Adam (1965), by using the test kit supplied by Boehringer Corp. (London) Ltd., London W.5, U.K., choline by the enneaiodide method, by the procedure of Long *et al.* (1967), and *N*-acetylneuraminic acid as described by Aminoff (1961). Water-soluble breakdown products, formed by the action of trypsin on suspensions of erythrocytes or erythrocyte 'ghosts', were measured as N after digestion with HClO_4 , by the phenol–hypochlorite method of Chaney & Marbach (1962), as modified by Sloane-Stanley (1967).

Dry weights were obtained after removal of water from 0.5 ml samples of supernatant or haemolysate at 105°C overnight.

Results

Measurement of Ca^{2+} influx into erythrocytes

The determination of intracellular calcium in cold-stored erythrocytes is not straightforward, as indicated by the results shown in Table 1. The amounts of calcium associated with the EDTA-treated erythrocytes are at all times less than those found in the absence of EDTA (Table 1, cf. columns 5 and 8). This was to be expected, since Long & Mouat (1971) showed that in the absence of EDTA treatment the outer surface of the erythrocyte readily binds Ca^{2+} . They also showed that the extent of external binding was dependent on the univalent cation concentration in the suspending medium. Since efflux of Na^+ and K^+ from erythrocytes occurs during cold storage (Maizels, 1935; Davson, 1939), their concentrations in the medium increase (columns 2 and 3). The amounts of external calcium (column 6) were calculated from Fig. 2 of Long & Mouat (1971), and the corrected values are shown in column (7). These quantities would be expected to be identical with those found after addition of EDTA before centrifugation (column 8). However, at both periods of storage, the values in column (8) are less than those in column (7), and the difference is particularly great after 6 days of cold storage.

To account for this discrepancy, the first possibility to be considered was that the amounts of calcium found after EDTA treatment would be too low, if EDTA withdrew Ca^{2+} from the cell cytoplasm. However, this appears unlikely to be the case, because EDTA removed no magnesium from the cells (cf. columns 9 and 10), and it might be expected that intracellular calcium and magnesium would behave similarly towards EDTA.

A more probable explanation is that during cold

Table 1. *Calcium and magnesium contents of erythrocytes after cold storage in a sucrose-CaCl₂ medium*

The suspension contained initially 24.4 ml of packed erythrocytes/100 ml. The suspending medium consisted of 256 mM-sucrose, 2.5 mM-CaCl₂, sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). Storage was at 3°C with gentle rocking. Analyses were made on supernatants and cell haemolysates obtained from 4 ml samples of suspension, with or without addition of 0.3 ml of 83 mM-EDTA (trilithium salt, pH 7.4) immediately before centrifugation.

Period of cold storage (days) (1)	Concn. in supernatant (mmol/l)			Erythrocyte calcium (μmol/l of packed cells*)				Erythrocyte magnesium (μmol/l of packed cells*)	
				Without EDTA			EDTA added (8)	Without EDTA (9)	EDTA added (10)
	K ⁺ (2)	Na ⁺ (3)	Total (4)	Total (5)	External† (6)	Corrected (7)			
0	1.3	1.3	2.6	310	310	0	54	2532	2627
3	17.3	2.8	20.1	855	237	618	481	2653	2404
6	20.3	3.2	23.5	1966	226	1740	728	1960	1947

* At both periods of storage, calcium and magnesium contents were calculated for 1 litre of packed cells at day 0, after correction for sucrose space. This method eliminates the effect of shrinkage of the cells.

† The amount of external Ca²⁺ on the cell membranes is a function of the K⁺ plus Na⁺ concentration in the medium; the curve in Fig. 2 of Long & Mouat (1971) was used for calculating these quantities.

Table 2. *Time-course of Ca²⁺ influx into cold-stored erythrocytes*

The suspension contained initially 29.3 ml of packed erythrocytes/100 ml. The composition of the sucrose-CaCl₂ suspending medium and storage conditions were the same as in Table 1. At the times indicated, 4 ml samples of suspension were treated with 0.3 ml of 83 mM-EDTA (trilithium salt) immediately before centrifugation.

Period of cold storage (days)	Cation contents of erythrocytes*			Cell volume† (ml)	Cell water† (g/ml)	Haemolysis (%)
	Ca ²⁺ (μmol/l)	K ⁺ (mmol/l)	Na ⁺ (mmol/l)			
0	20	91.7	8.9	1.000	0.715	0.15
1	199	58.7	5.8	0.794	0.512	1.30
2	454	46.5	5.1	0.721	0.430	3.07
3	830	39.2	5.5	0.677	0.390	5.36
4	1084	36.0	5.6	0.634	0.373	9.77
5	1253	29.6	5.1	0.606	0.346	19.70
6	1389	25.7	4.6	0.574	0.324	26.30
7	1400	22.7	3.6	0.529	0.281	28.80

* Calculated for 1 litre of packed erythrocytes at day 0.

† To demonstrate shrinkage, values are related to 1 ml of packed erythrocytes at day 0.

storage, some change occurs in the erythrocyte membrane, whereby the amount of externally bound calcium is increased, so that the values shown in column (7) are erroneously high. It has therefore been assumed that the true intracellular calcium concentration is that amount found after treatment of the suspension with EDTA immediately before centrifugation.

In all cold-storage experiments, penicillin and streptomycin were included in the suspending media.

These antibiotics prevented the growth of rod-shaped micro-organisms, which otherwise were detected after 4 days of cold storage. In the absence of the antibiotics, the erythrocyte membrane became significantly more permeable to Ca²⁺ after 4 days and the extent of haemolysis became unacceptably high.

An alternative method of determining intracellular calcium was also examined, namely to wash the sedimented cells several times with iso-osmotic NaCl and determine the calcium content after dry ashing

(Harrison & Long, 1968). However, it was found that erythrocytes that had been cold stored in the standard sucrose-CaCl₂ medium became extensively haemolysed when later suspended in iso-osmotic NaCl, so that only 20–30% of the cells could be recovered. Although the values obtained for intracellular calcium concentration by this procedure were of the same order of magnitude (500–600 $\mu\text{mol/litre}$ of packed cells) as those given in Table 1, this method of treating the cells was regarded as unsatisfactory, and was not further used.

Time-course of Ca²⁺ influx into cold-stored erythrocytes

At an early stage in this work, it was found that Ca²⁺ entered erythrocytes at a measurable rate during cold storage in a medium containing sucrose as the main osmotic component (see Table 1). Table 2 shows the time-course of this influx together with other changes occurring in the system.

The Ca²⁺ influx increases with the duration of cold storage. Simultaneously, K⁺ and Na⁺ are progressively lost from the erythrocytes together with cell water, and the erythrocytes shrink in volume. Haemolysis also increases with duration of storage, though this is responsible for only a small fraction of the univalent-cation loss from the cells.

In Fig. 1, the concentrations of Ca²⁺ in the cell water and in the water of the suspending medium are plotted against duration of cold storage. After a short lag period, Ca²⁺ influx becomes linearly related to duration of cold storage. At the same time, the concentration of Ca²⁺ in the medium falls, partly owing to Ca²⁺ influx into the cells and partly because of dilution of the medium by outward movement of cell water. After 3 days of cold storage, the apparent concentration of Ca²⁺ in the cells is already equal to that in the medium, and by 7 days is more than twice as much. These findings do not disprove that diffusion

is the mechanism of Ca²⁺ influx, since the Ca²⁺ entering the cells will become complexed with organic phosphate esters and will also bind to the inner surface of the membrane (Long & Mouat, 1971). Thus

Fig. 1. *Time-course of Ca²⁺ influx into erythrocytes during cold storage*

Experimental details are given in Table 2. Ca²⁺ concentrations are expressed in $\mu\text{mol/kg}$ of cell water (○) or $\mu\text{mol/kg}$ of water in the suspending medium (●).

Table 3. *Efflux of Ca²⁺ from calcium-loaded erythrocytes during incubation at 37°C*

The suspension contained initially 21.1 ml of packed erythrocytes/100 ml. The composition of the sucrose-CaCl₂ suspending medium and storage conditions were the same as in Table 1. After 4 days of cold storage, a sample of the suspension was incubated at 37°C for 2 h. At the times indicated, 4 ml samples of suspension were treated with 0.3 ml of 83 mM-EDTA (trilithium salt) immediately before centrifugation.

Period of cold storage (days)	Cell volume (ml/4 ml of suspension)	Cell Ca ²⁺ ($\mu\text{mol/l}$ of packed cells*)	Cell ATP (mmol/l of packed cells*)	Cell K ⁺ (mmol/l of packed cells*)	Cell Na ⁺ (mmol/l of packed cells*)	Haemolysis (%)
0	0.845	89	1.17	93.8	19.5	0.08
4	0.549	464	0.31	35.4	8.3	5.60
4†	0.466	279	0.18	30.3	5.9	8.35

* Calculated for 1 litre of packed cells at day 0.

† Cell suspension, cold-stored for 4 days, then incubated for 2 h at 37°C.

the concentration of free Ca^{2+} in the cell water will be much less than the total calcium concentration.

The absolute rates of Ca^{2+} influx into erythrocytes during cold storage varied considerably between experiments carried out under identical conditions. In 17 experiments, the mean rate of Ca^{2+} influx was $95 \mu\text{mol/litre}$ of initial packed erythrocytes per day, with extreme values of 17 and 232. It seems probable that erythrocytes from different donors exhibit a marked biological variation.

Efflux of Ca^{2+} from calcium-loaded erythrocytes during incubation at 37°C

When erythrocyte suspensions, the cells of which had accumulated intracellular Ca^{2+} as a result of being cold-stored in a sucrose- CaCl_2 medium, were incubated at 37°C , a marked efflux of Ca^{2+} occurred. This is illustrated in Table 3.

In this experiment, the water content of the cells was not measured but, from the mean value of 0.450 g of cell water/ ml of packed cells found in similar experiments after 4 days of cold storage, the maximum concentration of intracellular Ca^{2+} would be $1031 \mu\text{mol/kg}$ of cell water. Since the Ca^{2+} concentration of the medium at this stage was found to be $1806 \mu\text{mol/litre}$, it follows that Ca^{2+} efflux occurred

against the concentration gradient. This behaviour indicates an 'active' mechanism, and is probably to be explained by the existence on the internal surface of the cell membrane of the 'non-specific' ATPase, which requires Mg^{2+} and is stimulated by Ca^{2+} (Wins & Schoffeniels, 1966). Its activity as a 'calcium pump' has been demonstrated by Schatzmann (1966) in erythrocytes artificially loaded with Ca^{2+} and ATP by the procedure of 'reversed haemolysis' (Whittam, 1962). Table 3 also shows that during 4 days of cold storage in sucrose- CaCl_2 , about 73% of the ATP of the cells was lost, but sufficient remained to act as substrate for the enzyme. The ATP concentration in the cells decreased further during 2 h incubation at 37°C .

Because of the activity of the 'calcium pump' at 37°C , Ca^{2+} influx by diffusion into erythrocytes had necessarily to be studied at low temperature. It is not known whether the Ca^{2+} -activated ATPase is totally or only partially inhibited at 3°C .

Ca^{2+} influx into erythrocytes from media containing pentoses or hexitols; the non-relationship between influx and cell shrinkage

It was shown in Table 2 that the influx of Ca^{2+} into erythrocytes, during cold storage in a sucrose- CaCl_2

Table 4. Ca^{2+} influx into cold-stored erythrocytes suspended in media containing different sugars or their derivatives

Washed human erythrocytes were suspended in media containing either 256 mM-sucrose or 224 mM-D-sorbitol, -D-mannitol, -D-arabinose or -L-arabinose together with 32 mM-sucrose to act as extracellular marker. All suspending media also contained 2.5 mM- CaCl_2 , sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). Immediately before centrifuging, 4 ml of suspension was treated with 0.3 ml of 83 mM-EDTA (tri-lithium salt).

Expt. no.	Main osmotic component of medium	Period of cold storage (days)	Cell volume* (ml)	Ca^{2+} influx ($\mu\text{mol/l}\dagger$)	Haemolysis (%)	$\text{K}^+ + \text{Na}^+$ efflux (mmol/l)
1	Sucrose	3	0.719	328	—	—
		6	0.633	479	—	—
	D-Sorbitol	3	0.766	245	—	—
		6	0.713	389	—	—
	D-Arabinose	3	0.991	351	—	—
		6	1.074	512	—	—
2	Sucrose	4	0.757	136	2.30	41.6
	D-Mannitol	4	0.785	158	1.24	39.9
	D-Arabinose	4	1.114	147	0.78	32.6
	L-Arabinose	4	1.326	197	1.28	32.6
3	Sucrose	3	0.701	428	2.75	42.8
		6	0.550	675	17.25	67.9
	D-Arabinose	3	0.987	313	1.44	31.9
		6	0.952	534	4.82	54.8

* Cell volumes are related to 1 ml of packed erythrocytes at day 0.

† Calculated for 1 litre of packed cells at day 0.

Table 5. Ca^{2+} influx into cold-stored erythrocytes suspended in media containing sucrose, KCl, NaCl or choline chloride

The suspensions contained initially 21.2 ml of packed erythrocytes/100 ml. All suspending media contained 2.5 mM- $CaCl_2$, sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). The sucrose medium also contained 256 mM-sucrose; the KCl medium, 140 mM-KCl plus 32 mM-sucrose; the NaCl medium, 140 mM-NaCl plus 32 mM-sucrose; the choline chloride medium, 140 mM-choline chloride plus 32 mM-sucrose. Immediately before centrifuging, 4 ml of suspension was treated with 0.3 ml of 83 mM-EDTA (trilithium salt).

Main osmotic component of medium	Period of cold storage (days)	Cell volume (ml)	Cell composition				Ca^{2+} influx (μ mol/l*)	Effluxes†		Haemolysis (%)
			Ca^{2+} (μ mol/l*)	K^+ (mmol/l*)	Na^+ (mmol/l*)	Cl^- (mmol/l*)		$K^+ + Na^+$ (mmol/l*)	Chloride (mmol/l*)	
Sucrose	0	0.845	89	93.8	19.5	58.8				0.08
	4	0.553	464	35.4	8.3	8.6	375	69.6	50.2	6.00
KCl	0	0.836	15	99.9	19.3	69.4				0.44
	4	0.891	144	112.7	15.2	77.8	129	-8.7	-8.4	2.65
NaCl	0	0.803	82	95.8	22.2	62.7				0.36
	4	0.771	167	52.1	58.7	62.5	85	7.2	0.2	4.60
Choline chloride	0	0.818	49	96.1	18.5	59.2				0.34
	4	0.622	559	48.7	13.9	34.6	510	52.0‡	24.6	6.50

* Calculated for 1 litre of packed erythrocytes at day 0.

† Negative sign indicates influx.

‡ Influx of cholinium ion was 12.8 mmol/l of packed cells.

medium, was accompanied by cell shrinkage. Shrinkage is clearly due to the efflux of K^+ and Na^+ from the cells together with cell water and to the fact that the cell membrane is impermeable to sucrose. To test whether cell shrinkage was a prerequisite for Ca^{2+} influx, experiments were carried out in which the sucrose of the medium was largely replaced by some other non-ionized compound that would slowly penetrate the membrane and so prevent shrinkage, without, however, causing excessive haemolysis. A low concentration of sucrose (32mM) was always included in the suspending medium to act as an extracellular marker. Various monosaccharides and their derivatives were examined, but many had to be rejected because they interfered with the determination of sucrose. The following, however, were satisfactory: D-arabinose, which is slightly diffusible and in which the volume change is negligible, and L-arabinose, which is much more diffusible and in which there is marked swelling of the cells. The results are shown in Table 4, together with observations on D-mannitol and D-sorbitol, which, being non-diffusible, cause shrinkage.

Table 4 shows clearly that, within individual experiments, rates of Ca^{2+} influx were similar, whether the cells exhibited shrinkage (sucrose, D-mannitol, D-sorbitol), little or no volume change (D-arabinose) or swelling (L-arabinose). Thus Ca^{2+} influx during cold storage of erythrocytes is not dependent on change in cell volume. Volume changes merely reflect the permeability characteristics of the main osmotic component of the medium. Table 4 also shows that the diffusible monosaccharides, D- and L-arabinose, slightly decrease the extent of haemolysis by comparison with sucrose.

Relationship between univalent cation content of erythrocytes and Ca^{2+} influx

The above experiments suggested that during cold storage of erythrocytes in a non-ionic medium containing $CaCl_2$, the efflux of univalent cations from the cells might be responsible for enabling Ca^{2+} influx to occur. Evidence in favour of this has been obtained from experiments in which erythrocytes were suspended in media the main osmotic component of which consisted of KCl, NaCl or choline chloride, together with just sufficient sucrose to act as an extracellular marker. Results of one such experiment are given in Table 5.

Table 5 shows that Ca^{2+} influx from the sucrose medium (375 μ mol/litre of packed cells) is much greater than from the KCl or NaCl media (129 and 85 μ mol/litre respectively). In the sucrose medium, the net effluxes of univalent cations and of chloride from the cells are large, whereas in the KCl or NaCl media they are negligible, and these differences are reflected in the cell-volume changes.

In the experiment of Table 5, results are also shown for erythrocytes cold-stored in iso-osmotic choline chloride containing $CaCl_2$. Their behaviour was similar to that observed in the sucrose medium, and quite different from that found in the KCl or NaCl media. Ca^{2+} influx was large, as also was the efflux of univalent cations and of chloride, and there was extensive shrinkage of the cells. This is clearly due to the fact that the cholinium ion is too large to penetrate readily the erythrocyte membrane, and confirms the idea that Ca^{2+} influx is closely associated with a net univalent cation efflux.

In Fig. 2 are plotted the values for Ca^{2+} influx against K^+ plus Na^+ effluxes for seven experiments in which these parameters were measured. The curve is clearly biphasic, and indicates that Ca^{2+} influx is slow until about one-half of the univalent cations have been lost from the cells. Thereafter, Ca^{2+} influx is rapid.

Fig. 2. Relationship between univalent cation efflux from erythrocytes and Ca^{2+} influx

Experimental details are given in Table 2. Points are taken from seven experiments in which erythrocytes were cold-stored for 1–7 days in 256mM-sucrose containing 2.5mM- $CaCl_2$, together with penicillin and streptomycin. The Ca^{2+} and univalent cation (K^+ plus Na^+) contents of the cells are given in terms of 1 litre of packed erythrocytes at day 0.

Table 6. *Effects of preincubation of erythrocytes at 37°C on Ca²⁺ influx during cold storage*

Washed human erythrocytes were suspended in 256mM-sucrose or 160mM-KCl (29.3ml of packed cells/100ml of suspension). The KCl suspension and one-half of the sucrose suspension were incubated at 37°C for 1 h. The cells were then centrifuged down and resuspended in the standard sucrose-CaCl₂ medium (Table 1) to the original volume. The non-incubated control suspension was also centrifuged and the cells were resuspended in the sucrose-CaCl₂ medium. All three suspensions were stored at 3°C, and 4ml samples were removed each day for 7 days, and treated with 0.3ml of 83mM-EDTA (trilithium salt) immediately before centrifugation. The preincubation procedure resulted in the following cell-volume changes of 4ml samples of the suspensions: non-incubated control, 1.170ml; incubated in sucrose, 0.800ml; incubated in KCl, 1.264ml.

Period of cold storage (days)	Non-incubated control cells		Cells incubated 1 h in sucrose†		Cells incubated 1 h in KCl	
	Ca ²⁺ influx (μmol/l*)	K ⁺ +Na ⁺ content (mmol/l*)	Ca ²⁺ influx (μmol/l*)	K ⁺ +Na ⁺ content (mmol/l*)	Ca ²⁺ influx (μmol/l*)	K ⁺ +Na ⁺ content (mmol/l*)
0	—	100.6	—	38.4	—	107.0
1	179	64.5	4404	16.7	174	71.3
2	434	51.5	—	—	388	59.4
3	810	44.7	—	—	597	50.4
4	1064	41.6	—	—	772	46.9
5	1233	34.7	—	—	820	40.8
6	1369	30.3	—	—	988	34.8
7	1380	26.2	—	—	958	29.4

* Calculated for 1 litre of non-incubated packed cells at day 0.

† After 1 day of cold storage, haemolysis was so extensive as to render results meaningless.

Effect of preincubation of erythrocytes in different media on Ca²⁺ influx during cold storage

The close relationship between efflux of univalent cations from erythrocytes and Ca²⁺ influx was confirmed and extended by the following experiment. Erythrocytes suspended in iso-osmotic sucrose were incubated at 37°C for 1 h, whereby K⁺ and Na⁺ were lost from the cells and shrinkage occurred. The cells were centrifuged down, resuspended in sucrose-CaCl₂ and cold-stored. As shown in Table 6, they exhibited a much more rapid Ca²⁺ influx than did non-incubated cells. (Ca²⁺ influx is given for only 1 day of cold storage, because of later extensive haemolysis.) Further, as Table 6 also indicates, when erythrocytes were incubated at 37°C in iso-osmotic KCl, a slight influx of K⁺ into the cells occurred, and after resuspension in sucrose-CaCl₂ there was a somewhat slower influx of Ca²⁺ during cold storage than occurred in non-incubated control cells.

In Table 7 is shown an experiment in which erythrocytes were incubated at 37°C for different periods in iso-osmotic sucrose. The cells were then centrifuged down, resuspended in sucrose-CaCl₂ and cold-stored. As the period of preincubation was increased from 0 to 120min, there was a progressive loss of univalent cations from the cells, and when these cells were subsequently cold-stored for 24 h in

sucrose-CaCl₂ the rates of Ca²⁺ influx increased in parallel. The extents of haemolysis are also recorded in Table 7.

Ca²⁺ influx into erythrocytes from media containing different concentrations of CaCl₂; relationship between influx and haemolysis

In most of the experiments described above, the extents of haemolysis of the erythrocytes were also measured, and are given in the tables. When erythrocytes were cold-stored in the standard sucrose-CaCl₂ medium, both Ca²⁺ influx and extent of haemolysis increased progressively with period of storage. The possibility was first considered that during cold storage both Ca²⁺ influx and haemolysis simply reflected some generalized increase in the permeability of the erythrocyte membrane caused by loss of cations from the cells. If this were so, then one would expect a linear relationship between these two variables. In Fig. 3, observations from 15 experiments are plotted, and the curve is non-linear. At cell Ca²⁺ concentrations below 1000 μmol/litre, haemolysis is moderate in extent, but at higher cell Ca²⁺ concentrations haemolysis becomes very extensive. The curve suggests that the presence of a high concentration of Ca²⁺ in the cells might cause increased haemolysis.

Table 7. Effect of period of preincubation at 37°C of erythrocyte suspensions in iso-osmotic sucrose on Ca²⁺ influx during cold storage

Washed erythrocytes were suspended in 256mm-sucrose (33 ml of packed cells/100ml). Eight 10ml samples were incubated at 37°C for the times indicated. The cells were then centrifuged down and resuspended to 10ml in the standard sucrose-CaCl₂ medium (Table 1). At zero time and after 1 day of cold storage at 3°C, 4ml samples were treated with 0.3ml of 83mm-EDTA (trilithium salt) immediately before centrifugation.

	Period of cold storage (days)	Preincubation at 37°C (min)									
		0	15	30	45	60	80	100	120		
Cell volume (ml/4ml of suspension)	0	1.319	1.196	1.183	1.121	1.017	0.980	0.940	0.928		
Cell univalent cations (mmol/l of packed cells*)	1	1.130	0.914	0.932	0.901	0.867	0.864	0.795	0.768		
Cell Ca ²⁺ (μmol/l of packed cells*)	0	102.5	86.4	75.2	64.4	55.4	47.8	40.9	37.5		
	1	77.2	42.3	36.5	33.2	26.9	26.3	21.2	19.8		
	0	52	16	58	56	69	142	159	180		
	1	189	843	1090	1319	1761	2085	2139	2051		
Haemolysis (%)	0	0.13	0.23	0.14	0.19	0.30	0.55	0.59	0.59		
	1	1.48	3.59	4.85	2.88	4.17	5.86	7.78	9.46		

* Calculated for 1 litre of non-incubated packed cells at day 0.

Fig. 3. Relationship between Ca²⁺ content of erythrocytes and extent of haemolysis

Experimental details are given in Table 2. Points are taken from 15 experiments in which erythrocytes were cold-stored for 1–7 days in 256mm-sucrose containing 2.5mm-CaCl₂, together with penicillin and streptomycin. The Ca²⁺ concentration of the cells is expressed as μmol/l of packed erythrocytes at day 0.

Support for this view was obtained from an experiment in which Ca²⁺ influx and haemolysis were measured during cold storage of erythrocytes in iso-osmotic sucrose containing different concentrations of CaCl₂. The losses of cations and water from the cells and the extents of shrinkage were identical for the different media and were only dependent on the duration of storage. Figs. 4(a) and 4(b) show the rates of Ca²⁺ influx and extents of haemolysis. After any given period of cold storage, both Ca²⁺ influx and haemolysis increased with increasing concentration of CaCl₂ in the suspending medium. Thus haemolysis is not simply a function of cation loss from the cells. Further, it cannot be a function of the Ca²⁺ concentration in the suspending medium, since in the experiment of Table 5, in which the Ca²⁺ concentration in the medium was maintained constant at 2.5mm, the degree of haemolysis varied with the nature of the main osmotic component. It follows, therefore, that the extent of haemolysis must be largely dependent on the concentration of Ca²⁺ within the cells. This conclusion is supported by the finding, referred to above, that washing with iso-osmotic NaCl of Ca²⁺-loaded erythrocytes is accompanied by extensive haemolysis.

Fig. 4. Ca^{2+} influx into erythrocytes and extent of haemolysis as a function of duration of cold storage and Ca^{2+} concentration in the suspending medium

Experimental details are as in Table 2, except that the concentration of CaCl_2 in the suspending medium was varied. Ca^{2+} concentrations in media: ■, 0.5 mM; □, 1.0 mM; ●, 2.0 mM; ○, 2.5 mM.

Lack of effect of preincubation of erythrocytes with trypsin and neuraminidase on Ca^{2+} influx during cold storage

It was decided to determine whether modification of the erythrocyte membrane by the action of enzymes would have any effect on its permeability to Ca^{2+} . Preliminary experiments with erythrocyte 'ghosts', prepared as described by Long & Mouat (1971), suspended in iso-osmotic KCl or NaCl, showed that incubation at 37°C with trypsin caused maximal solubilization of about 55% of the membrane protein and about 90% of the *N*-acetylneur-

aminic acid. When washed erythrocytes were incubated with trypsin in 160 mM-KCl, the same result was obtained, and no haemolysis occurred. Incubation with neuraminidase of erythrocytes suspended in 160 mM-KCl led to release of about 90% of the *N*-acetylneuraminic acid (Long & Mouat, 1971).

Erythrocytes incubated with either trypsin or neuraminidase have been studied in relation to Ca^{2+} influx during cold storage, with the results shown in Table 8. Neither of these enzymes had any pronounced effect on the influx of Ca^{2+} during cold

Table 8. *Effect of preincubation of erythrocytes with trypsin or neuraminidase on Ca^{2+} influx during cold storage*

A suspension of washed erythrocytes in 160 mM-NaCl (32.6 ml of packed cells/100 ml) was divided into four equal portions (20 ml each). Sample A was untreated; B was incubated without addition; C was incubated with 112 mg of trypsin; D was incubated with 137.5 μg of neuraminidase. Incubation was for 1 h at 37°C. The suspensions were centrifuged and the cells were resuspended to the original volume in the standard sucrose- CaCl_2 medium (Table 1). At day 0 and after 5 days of storage at 3°C, 4 ml samples were treated with 0.3 ml of 83 mM-EDTA (lithium salt) immediately before centrifugation.

Sample	Treatment	Period of cold storage (days)	Cell volume (ml/4 ml of suspension)	Cell Ca^{2+} ($\mu\text{mol/l}$ of packed cells*)	Cell univalent cations (mmol/l of packed cells*)	Haemolysis (%)
A	Non-incubated	0	1.304	49	94.2	—
		5	0.876	380	41.0	1.70
B	Incubated without enzyme	0	1.257	41	90.5	—
		5	0.801	540	35.5	1.55
C	Incubated with trypsin	0	1.320	65	91.3	—
		5	0.847	536	29.4	1.13
D	Incubated with neuraminidase	0	1.286	82	89.5	—
		5	0.814	406	36.1	2.00

* Calculated for 1 litre of non-incubated packed cells at day 0.

storage in the standard sucrose- CaCl_2 medium. The extents of shrinkage, effluxes of univalent cations and degrees of haemolysis were similar in all cases.

Discussion

Freshly drawn human erythrocytes contain only a trace of calcium, almost all of which is present in the membranes (Harrison & Long, 1968), whereas plasma is relatively rich in calcium. The question therefore arises as to how the erythrocyte membrane functions so as to prevent a net influx of Ca^{2+} from the plasma under physiological conditions. On the basis of the experiments reported in the present paper, there are probably two explanations.

In the first place, cold-storage experiments have shown that a rapid influx of Ca^{2+} only occurs when erythrocytes are suspended for several days in a medium in which the main osmotic component is either non-ionized (sucrose, D-mannitol, D-sorbitol, D- or L-arabinose) or contains a very slowly penetrating cation (choline chloride). In all these instances, a net efflux of K^+ and Na^+ from the cells occurs. By contrast, when the suspending medium consists of iso-osmotic KCl or NaCl there is no net efflux of univalent cations from the cells and Ca^{2+} influx is low. The extent of efflux of univalent cations may be increased by preincubation at 37°C of a cell suspension in iso-osmotic sucrose and this treatment results in a much higher rate of Ca^{2+} influx during subsequent cold storage in a sucrose- CaCl_2 medium. Under physiological conditions, plasma would appear to be a very unsuitable suspending medium

for permitting Ca^{2+} influx, since the high concentration of Na^+ would effectively prevent a net loss of univalent cations from the cells. This would still be the case, even in the absence of the 'sodium pump', because intracellular Na^+ would be just as effective as K^+ .

The second reason why Ca^{2+} ions do not accumulate in erythrocytes under physiological conditions is because of the operation of the 'calcium pump'. Even in the presence of plasma as the suspending medium, it is probable that a small quantity of Ca^{2+} will enter the cells, but at body temperature such intracellular Ca^{2+} would be expelled rapidly by the action of the 'non-specific' ATPase, which is stimulated by internal Ca^{2+} .

It has been reported that removal of the sialic acid groups from the outer surface of the cell membrane is without effect on Ca^{2+} influx. Perhaps even more remarkable is the fact that removal of more than 50% of the membrane protein (presumably the external layer) by preincubation with trypsin is also without influence on the permeability characteristics.

It is not yet clear why loss of univalent cations from the erythrocytes is associated with an alteration in permeability of the membrane towards Ca^{2+} . It is well established that when suspensions of erythrocytes are incubated in iso-osmotic solutions of non-electrolytes, the efflux of univalent cations is accompanied by a rise in pH of the media into the range pH 7.3–8.0 (Maizels, 1935; Davson, 1939; Bolingbroke & Maizels, 1959). Romero & Whittam (1971) showed that the influx of Ca^{2+} into ATP-depleted cells was pH-dependent; Ca^{2+} influx did not occur

at or below pH 7.0, but was appreciable at pH 7.5 and 8.0. These observations, taken together, suggest that the Ca^{2+} influx observed in the present work might be due to increased alkalinity of the sucrose medium during cold storage. For this explanation to be universally valid, it follows that a similar rise in the pH of the medium must occur when erythrocytes are cold-stored in iso-osmotic choline chloride solution. Such measurements have not been made, but on general grounds choline chloride might be expected to behave like KCl or NaCl so far as pH changes are concerned, although the extents of Ca^{2+} influx are very different in the two cases.

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