The Influx of Calcium Ions into Human Erythrocytes during Cold Storage

THE INFLUENCES OF EXTRACELLULAR pH, INTRACELLULAR ADENOSINE TRIPHOSPHATE AND EFFLUX OF UNIVALENT CATIONS

By BARBARA MOUAT and CYRIL LONG

*Department of Biochemistry, Institute of Basic Medical Sciences,
Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN, U.K.*

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1. When human erythrocytes are stored at 3°C for several days as a suspension in iso-osmotic sucrose or KCl, containing CaCl_2 , the rates of cellular ATP degradation are similar. 2. During cold storage of erythrocytes in sucrose– CaCl_2 medium, Ca^{2+} influx and univalent-cation efflux occur, the pH value of the suspending medium rises and the intracellular pH falls. These pH changes correlate reasonably well with alterations in the membrane potential calculated from Cl^- distribution. 3. The presence of Ca^{2+} in the medium does not increase the rate of univalent-cation efflux from the cells. 4. When the pH of the medium is raised by addition of buffers, the rates of both Ca^{2+} influx and univalent-cation efflux increase. 5. Replacement of sucrose by KCl as the main osmotic component of the medium completely suppresses Ca^{2+} influx and univalent-cation efflux, although the pH of the KCl medium is higher than that of the sucrose medium. 6. When sucrose is replaced by choline chloride, Ca^{2+} influx and univalent-cation efflux still occur, and the pH of the medium is similar to that found in iso-osmotic KCl. 7. When valinomycin, Pb^{2+} or Cd^{2+} are added to the iso-osmotic sucrose medium, the rate of efflux of univalent cations increases as also does the influx of Ca^{2+} . 8. From these and other observations, it was concluded that it is univalent-cation efflux rather than ATP depletion or elevated extracellular pH which is the prerequisite for Ca^{2+} influx during cold storage.

The influx of Ca^{2+} into human erythrocytes from different suspending media and at different temperatures has been studied recently by three groups of workers. Lew (1970, 1971) showed that Ca^{2+} influx at 37°C from a saline medium occurred only after the cells had become depleted of their ATP content. Romero & Whittam (1971) confirmed this and further demonstrated that for ATP-depleted erythrocytes, also incubated under similar conditions, Ca^{2+} influx was strongly pH-dependent; it did not occur at pH 7.0 or below, but was appreciable at higher pH values.

Long & Mouat (1973), on the other hand, used fresh erythrocytes and worked at low temperature to minimize any interfering effects of cell metabolism. They reported that when a suspension in iso-osmotic sucrose was stored at 3°C for several days, influx of Ca^{2+} occurred only after the cells had become markedly depleted of their univalent cations. This conclusion appears to be at variance with other observations by Whittam (1968) and by Lew (1970, 1971), which indicated that at 37°C the reverse was actually the case, namely that the presence of Ca^{2+} within the cells stimulated K^+ efflux. Thus it is not at all clear whether the mechanisms of Ca^{2+} influx are similar in these three investigations.

It is a well-established fact that when erythrocytes are suspended in a non-ionic iso-osmotic medium (glucose, sucrose or lactose) and then incubated at 37°C, the medium progressively becomes alkaline (Maizels, 1935; Davson, 1939; Bolingbroke & Maizels, 1959). If this should also prove to be the case in the cold-storage procedure of Long & Mouat (1973), then it might be possible to explain their observations in terms of the pH-dependence of Romero & Whittam (1971).

On the other hand, Passow (1964) demonstrated very clearly that when erythrocytes are suspended in iso-osmotic sucrose solution containing buffers of different pH values and incubated at 22°C, the rate of efflux of K^+ from the cells is pH-dependent. Below pH 7.0, K^+ efflux was barely detectable, but as the pH was progressively raised efflux became increasingly rapid. These different observations indicate that there is a very close relationship between extracellular pH changes and univalent-cation effluxes from the cells.

The present experiments were largely designed to ascertain whether univalent-cation efflux or alkalinity of the extracellular medium was primarily responsible for Ca^{2+} influx into erythrocytes in cold-storage experiments. Because of their interdependence, these

two effects have been difficult to separate. However, conditions have been chosen which leave no doubt that it is univalent-cation efflux that is all important under cold-storage conditions. Results are also given that indicate that the ATP content of erythrocytes is not an important factor for Ca^{2+} influx at 3°C .

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 i.u./ml. Washed erythrocytes were obtained as described by Long & Mouat (1971).

Reagents. Triple-glass-distilled water was used throughout; it was calcium-free. The calcium analytical standard for atomic-absorption spectrophotometry was described by Harrison & Long (1968).

Sodium benzylpenicillin, streptomycin sulphate and EDTA (trilithium salt; 83 mM, pH 7.4) were as described by Long & Mouat (1973). Valinomycin was purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. A $50\text{ }\mu\text{M}$ solution was prepared by dissolving $278\text{ }\mu\text{g}$ in $55\text{ }\mu\text{l}$ of ethanol and diluting to 5 ml with 256 mM-sucrose. The final concentration used in the suspending medium was $1\text{ }\mu\text{M}$.

Methods

Influx of Ca^{2+} into erythrocytes. This was measured by the method of Long & Mouat (1973).

Analytical methods. Sucrose and calcium were determined as described by Long & Mouat (1971). Sodium, potassium, chloride, haemoglobin, ATP and dry weights were measured as described by Long & Mouat (1973).

pH. All pH measurements were made at room temp (23°C) with a glass electrode and pH meter 25 (Radiometer, Copenhagen, Denmark). The determination of the pH value of supernatants separated by centrifugation from erythrocyte suspensions in iso-osmotic sucrose was unsatisfactory. Since the only buffer present was a trace of HCO_3^- derived from the cells, the pH increased appreciably as CO_2 was lost. Instead therefore 2 ml of whole suspension was used. The measured pH value was that of the medium and any slight loss of CO_2 was compensated for by the buffering power of the cells. In those experiments in which buffers were added to the original suspending media, the suspensions and separated supernatants gave identical pH values.

The pH value of the cells was measured after centrifuging down the erythrocytes from 3 ml of suspension (2500 g for 30 min) and haemolysing the cell deposit with 3 vol. of water. No haemolytic agent was added. The addition of a trace of saponin (Bolingbroke &

Maizels, 1959) did not alter the measured pH value. Further dilution of the haemolysate with an equal volume of water was also without effect on the pH value, presumably because the cell contents were strongly buffered (haemoglobin, phosphate esters). Two standards at 23°C were used, namely pH 7.42 (Instrumentation Laboratory Inc., Lexington, Mass., U.S.A.) and pH 6.96 (Fisons Scientific Apparatus, Loughborough, Leics., U.K.).

Calculation of membrane potentials. Cells that are suspended in aqueous media exhibit a membrane potential (E_m), which is due to the uneven distribution of non-diffusible or slowly diffusible ions on the two sides of the membrane. The magnitude of this potential difference may be calculated from the ratio of the molal activities of those ions which are passively distributed across the membrane, such as Cl^- , HCO_3^- , H^+ or OH^- (Whittam, 1964). According to the Nernst equation:

$$E_m = \frac{RT}{F} \ln \frac{[\text{Cl}^-]_i}{[\text{Cl}^-]_e} = \frac{RT}{F} \ln \frac{[\text{H}^+]_e}{[\text{H}^+]_i} = \text{etc.},$$

where R is the gas constant, F the Faraday and T the absolute temperature; the values in square brackets are molal activities, the suffixes i and e referring to the internal and external media respectively. E_m is expressed in mV.

In this paper, true membrane potentials have been calculated from the Cl^- distribution. Cl^- was determined by the method of Schales & Schales (1941) on supernatants and cell haemolysates. The Cl^- content of the haemolysate was corrected for the presence of the small quantity of trapped supernatant, determined from the sucrose content (Long & Mouat, 1973). From the dry weights of measured volumes of supernatant and haemolysate (corrected for trapped supernatant), the Cl^- concentrations in mmol/kg of cell water and supernatant water were calculated. Mean molal activity coefficients of KCl at different molal concentrations, as given by Moore (1963), were used; their square roots were taken to represent the activity coefficients of the Cl^- , which is standard practice. The final molal activities were inserted in the Nernst equation, and E_m values were obtained.

Theoretically, E_m may also be calculated from the distribution of H^+ . However, measurements made with a pH meter do not give true thermodynamic values for H^+ molal activities for a variety of reasons. Nevertheless, approximate values may be obtained from the modified Nernst equation, namely: $E_m = 59 (\text{pH}_i - \text{pH}_e)$. When E_m values were calculated from both Cl^- distribution and pH, the agreement was very close in many cases.

Presentation of results for Ca^{2+} and univalent-cation contents of erythrocytes. Long & Mouat (1973) calculated the Ca^{2+} and univalent-cation contents of erythrocytes in cold-storage experiments as μmol or

mmol/litre of packed cells at day 0, to obtain comparable values in spite of the shrinkage undergone by cells during storage at 3°C in an iso-osmotic sucrose suspending medium. This method, however, takes no account of the haemolysis which occurs under these conditions and which may amount to 15–20% during 6 days. Values calculated in this way will clearly underestimate Ca^{2+} and univalent-cation content and Ca^{2+} influx, whereas univalent-cation efflux will be overestimated.

In the present work, the extent of haemolysis was always determined, and values have been expressed as the content of Ca^{2+} or univalent cations calculated for 1 litre of packed cells at day 0, after correction for the degree of haemolysis.

Results

Loss of ATP from erythrocytes during cold storage in iso-osmotic sucrose or KCl

Since Lew (1970, 1971) and Romero & Whittam (1971) both demonstrated that Ca^{2+} influx at 37°C was observed only with cells of low ATP content, measurements have been made of the ATP contents of erythrocytes after cold storage in iso-osmotic sucrose or KCl, containing 2.5 mM- CaCl_2 . The results are shown in Table 1. It will be seen that the ATP content of the erythrocytes falls as the period of cold storage increases and that the values at any stage are similar whether the cells are stored in either sucrose- CaCl_2 or KCl- CaCl_2 media. On the other hand, the influx of Ca^{2+} is rapid in sucrose- CaCl_2 medium, and almost undetectable in KCl- CaCl_2 , as shown by Long & Mouat (1973). It appears therefore that, in cold-storage experiments, the ATP content in the

cells is not a primary controlling factor for Ca^{2+} influx.

In Table 1, a control experiment was also carried out in which CaCl_2 was omitted from the iso-osmotic sucrose suspension medium. The rate of ATP destruction under these conditions appears to be somewhat greater than in the presence of CaCl_2 , but the effect is slight and perhaps not significant.

Effect of Ca^{2+} on the efflux of univalent cations from erythrocytes during cold storage in iso-osmotic sucrose

Whittam (1968) first reported that when ATP-depleted erythrocytes were incubated at 37°C in Ringer solution, the rate of K^+ efflux was increased sixfold when 0.2 mM- CaCl_2 was present in the suspending medium. Lew (1970) confirmed this effect of Ca^{2+} with erythrocytes incubated in a saline medium containing inosine plus iodoacetamide.

The effect of Ca^{2+} on univalent-cation efflux from erythrocytes during cold storage has now been examined. Fresh erythrocytes were used, the cells were suspended in iso-osmotic sucrose, and the final concentration of added Ca^{2+} was 2.5 mM. It was found that the presence of Ca^{2+} in the suspending medium did not increase the rate of K^+ or Na^+ efflux from the cells, and there was even a slight indication that Ca^{2+} was inhibitory. In one experiment, after 4 days of cold storage in the absence of Ca^{2+} , the Na^+ plus K^+ efflux was 62.5 mmol/litre of packed cells, whereas in the presence of Ca^{2+} it amounted to 54.2 mmol/litre. In another experiment lasting 7 days, the values were 79.0 and 71.1 respectively. Thus it is clear that the observations of Whittam (1968) and of Lew (1970) at 37°C do not apply at 3°C.

Table 1. Ca^{2+} influx and ATP content of erythrocytes cold-stored in iso-osmotic sucrose or iso-osmotic KCl

The suspensions contained initially 19.1 ml of packed erythrocytes/100 ml. All suspending media contained sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). The sucrose media contained 256 mM-sucrose; the KCl medium contained 140 mM-KCl plus 32 mM-sucrose. Where indicated, the final concn. of CaCl_2 was 2.5 mM. The amounts of trapped supernatant in the cell deposits were determined from their sucrose contents. Where Ca^{2+} was determined, 4 ml of suspension was treated with 0.3 ml of 83 mM-EDTA (trilithium salt, pH 7.4) immediately before centrifuging to remove externally bound membrane Ca^{2+} . The cellular Ca^{2+} and ATP concentrations were calculated as μmol or mmol per litre of packed cells at day 0, after correction for haemolysis.

Period of cold storage (days)	Suspension media				
	KCl- CaCl_2		Sucrose- CaCl_2		Sucrose only
	Cell Ca^{2+} ($\mu\text{mol/l}$)	Cell ATP (mmol/l)	Cell Ca^{2+} ($\mu\text{mol/l}$)	Cell ATP (mmol/l)	Cell ATP (mmol/l)
0	0	0.87	33	1.16	1.01
3	11	0.40	578	0.37	0.29
5	2	0.29	895	0.30	0.22
7	34	0.28	1415	0.22	0.18

Fig. 1. pH changes during storage at 3°C of suspensions of erythrocytes in iso-osmotic sucrose, containing 2.5 mM- CaCl_2

(a) pH of cells, (b) pH of suspending media. Results are given for six separate experiments, which are indicated by different symbols.

pH changes associated with cold storage of erythrocytes in unbuffered sucrose media

Washed human erythrocytes were suspended in iso-osmotic sucrose, containing 2.5 mM- CaCl_2 together with penicillin and streptomycin, and the suspension was stored at 3°C. At intervals, the pH values of the haemolysed erythrocytes (cell pH) and of the untreated suspension (medium pH) were measured at 23°C. Fig. 1 shows the results of six experiments.

Fig. 1 shows that the suspending medium had an initial pH of 6.3–6.7 (average 6.46); during cold storage the pH increased to 7.4–7.5 in the course of 10 days. The variations between experiments are largely due to differences in the cells/medium volume ratio. Thus erythrocytes suspended in a sucrose medium behave on cold storage in the same way as during incubation at 37°C (e.g. Maizels, 1935). Simultaneously, there was a slight fall in the pH value of the cells during storage from about 7.4 to 7.25. This decrease was probably caused by slow enzymic hydrolysis of intracellular phosphate esters.

The pH changes occurring in the cells and suspending media during cold storage can be explained fairly satisfactorily in terms of changes in the membrane potential, as shown in Table 2. The true membrane potentials were calculated from the distribution of Cl^- between cells and media. These are to be compared with the approximate values of E_m calculated from pH differences. Table 2 shows that the correspondence is good at zero time and for the early stages of cold storage. After longer periods, however, E_m values calculated from pH are consistently less

Table 2. Membrane potentials of human erythrocytes cold-stored in unbuffered iso-osmotic sucrose media

The suspending medium consisted of 256 mM-sucrose, 2.5 mM- CaCl_2 , sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). Storage was at 3°C.

Expt. no.	Period of cold storage (days)	$10^3 \times$ Molal activities of Cl^-		pH values		E_m (mV) calculated from	
		Cells	Medium	Cells	Medium	Cl^-	pH*
1	0	88.15	8.54	7.32	6.32	+59.8	+59.0
	1	62.15	14.15	7.32	6.70	+38.0	+36.6
2	0	84.87	8.43	7.31	6.31	+59.3	+59.0
	4	50.38	19.49	7.33	7.09	+23.8	+14.2
	8	29.80	21.37	7.23	7.31	+8.5	-4.7
3	0	81.85	10.55	7.48	6.70	+52.5	+46.0
	2	67.63	22.97	7.42	7.02	+27.7	+23.6
	4	63.28	27.14	7.42	7.15	+21.7	+15.9
	7	47.85	30.35	7.38	7.30	+11.7	+4.7
	10	42.40	31.88	7.30	7.43	+7.3	-7.7
4	0	83.03	7.54	7.41	6.39	+64.8	+60.2
	3	51.13	16.44	7.43	7.08	+29.1	+20.7
	6	22.63	21.25	7.31	7.50	+1.6	-11.2

* Approximate values (see under 'Methods').

Fig. 2. Comparison of membrane potential (E_m) values, calculated from Cl^- distribution and pH, for erythrocytes suspended in iso-osmotic sucrose, containing 2.5 mM- CaCl_2 together with penicillin and streptomycin, and stored at 3°C for several days

----, Theoretical equivalence between E_m values determined by the two methods. Results are given for five separate experiments, represented by the different symbols.

positive than those based on Cl^- distribution. Fig. 2 compares the E_m values calculated by the two methods. There appears to be no clear explanation for this discrepancy.

Ca^{2+} influx in relation to pH of iso-osmotic sucrose medium and univalent-cation efflux from cells

In the experiments shown in Table 2, the Ca^{2+} and univalent-cation contents of the cells were also determined. In confirmation of Long & Mouat (1973), Ca^{2+} influx was associated with the efflux of Na^+ and K^+ from the cells. However, because of these univalent-cation movements, there was also the expected correlation between Ca^{2+} influx and pH of the medium. Such experiments clearly do not distinguish between univalent-cation efflux and rise in pH value as being the prerequisite for Ca^{2+} influx.

Effect of preincubating cells at 37°C in iso-osmotic sucrose on subsequent Ca^{2+} influx and pH changes during cold storage

Long & Mouat (1973) demonstrated that incubation of erythrocytes at 37°C in iso-osmotic sucrose resulted in rapid loss of K^+ and Na^+ and rendered the cell membranes more permeable to Ca^{2+} during subsequent cold storage in sucrose- CaCl_2 medium.

A repetition of this experiment was made in which the associated pH changes were also measured.

It was found that the initial pH value of the medium depended on the period of preincubation at 37°C, being higher after longer periods. Thus after incubation at 37°C for 0, 20, 40 and 60 min, the initial pH values after resuspending the cells in sucrose- CaCl_2 media were 6.32, 6.90, 7.22 and 7.60 respectively. In all cases there was a rise in pH value during cold storage for 24 h. Thus the extent of Ca^{2+} influx was again associated with both a lowering of the intracellular univalent-cation concentration and with an increase in pH of the suspending medium.

Ca^{2+} influx during cold storage of erythrocytes suspended in buffered iso-osmotic sucrose

It was decided to determine whether adjustment of the pH value of the medium by means of added buffers had any effect on Ca^{2+} influx into erythrocytes during cold storage in iso-osmotic sucrose. The buffers used were Tris-HCl at a final concentration of 20 mM in the suspending media, and the pH values varied between 6.4 and 7.8. A positive correlation was found between Ca^{2+} influx and pH of the medium. Thus after 8 days of cold storage in sucrose containing the pH 6.4 buffer, Ca^{2+} influx was 460 $\mu\text{mol/litre}$ of packed cells; in the pH 7.8 buffer it amounted to 1471 $\mu\text{mol/litre}$. However, there was also a clear correlation between Ca^{2+} influx and efflux of Na^+ plus K^+ , because in the pH 6.4 buffer the efflux of Na^+ plus K^+ was 67.1 mmol/litre, whereas in the pH 7.8 buffer the value was 86.0 mmol/litre. The probable explanation of these observations is that at higher pH values, the greater loss of univalent cations from the cells renders them more permeable towards Ca^{2+} .

Ca^{2+} influx into erythrocytes during cold storage in iso-osmotic KCl and the associated pH changes

To distinguish between the effects of extracellular pH and univalent-cation efflux on Ca^{2+} influx, experiments were performed in which the non-ionic sucrose medium was replaced by KCl or choline chloride. Long & Mouat (1973) showed that when erythrocytes were suspended in iso-osmotic KCl, containing 2.5 mM- CaCl_2 , and cold-stored, Ca^{2+} influx was barely detectable. This observation was confirmed (see also Table 1) and pH measurements were made simultaneously, as shown in Table 3. The pH of the sucrose suspending medium rises during cold storage as in Fig. 1, whereas in iso-osmotic KCl, the pH of the suspending medium is already high initially (pH 7.53) and falls slightly during storage. If Ca^{2+} influx were dependent primarily on the pH of the suspending medium, then it would be expected to be much greater in KCl than in sucrose. Since the

Table 3. Ca^{2+} and univalent-cation content of cells and pH changes occurring during cold storage of erythrocytes in iso-osmotic sucrose or KCl

The suspensions contained 21.1 ml of packed erythrocytes/100 ml. Both media included 2.5 mM- CaCl_2 , sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). The sucrose medium contained 256 mM-sucrose; the KCl medium contained 150 mM-KCl and 16 mM-sucrose. Storage was at 3°C. The cellular Ca^{2+} and $\text{Na}^+ + \text{K}^+$ concentrations were calculated as μmol or mmol/litre of packed cells at day 0, after correction for haemolysis.

Medium	Period of cold storage (days)	Cell composition		pH values		E_m (mV) calculated from	
		Ca^{2+} ($\mu\text{mol/l}$)	$\text{Na}^+ + \text{K}^+$ (mmol/l)	Cells	Medium	Cl^-	pH
Sucrose	0	59	105.4	7.41	6.39	+64.8	+60.2
	3	352	65.0	7.43	7.08	+29.1	+20.7
	6	629	38.7	7.31	7.50	+1.6	-11.2
KCl	0	15	109.7	7.38	7.53	-7.5	-8.8
	3	27	118.7	7.35	7.49	-9.7	-8.3
	6	2	111.1	7.28	7.43	-10.4	-8.9

Table 4. Concentrations of univalent ions and of Ca^{2+} in erythrocytes during cold storage in iso-osmotic KCl or choline chloride

Both suspensions contained 21.2 ml of packed erythrocytes/100 ml. The KCl medium contained 150 mM-KCl plus 16 mM-sucrose; the choline chloride medium contained 150 mM-choline chloride plus 16 mM-sucrose. Also present were 2.5 mM- CaCl_2 , sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). The cholinium ion is represented by Ch^+ . The ion concentrations were calculated as μmol or mmol/litre of packed cells at day 0, after correction for haemolysis.

Medium	Period of cold storage (days)	Cell composition			Total univalent cations (mmol/l)	Cl^- (mmol/l)	pH of		E_m (mV) calculated from	
		Ca^{2+} ($\mu\text{mol/l}$)	$\text{Na}^+ + \text{K}^+$ (mmol/l)	Ch^+ (mmol/l)			Cells	Medium	Cl^-	pH
Choline chloride	0	0	119.8	2.7	122.5	70.1	7.48	7.57	-12.5	-5.3
	3	258	71.9	9.5	81.4	41.7	7.40	7.65	-16.5	-15.0
	6	248	56.9	10.9	67.8	30.0	7.32	7.60	-21.5	-16.8
	9	262	43.4	17.5	60.9	27.5	7.23	7.53	-22.8	-17.7
KCl	0	0	117.7	0	117.7	70.0	7.44	7.60	-12.0	-9.4
	3	31	127.1	0	127.1	83.2	7.40	7.50	-8.7	-5.9
	6	13	133.9	0	133.9	87.0	7.31	7.44	-8.5	-7.7
	9	57	127.0	0	127.0	86.6	7.29	7.40	-7.9	-6.8

reverse is the case, it is apparent that pH is not a controlling factor for Ca^{2+} influx into erythrocytes under cold-storage conditions.

Ca^{2+} influx into erythrocytes during cold storage in iso-osmotic choline chloride

Long & Mouat (1973) showed that when erythrocytes were suspended in iso-osmotic choline chloride and cold-stored, there was an appreciable Ca^{2+} influx into the cells. This result was attributed to the rather slow inward diffusion of the cholinium ion through the cell membrane as compared with the rates for outward diffusion of Na^+ and K^+ , so that a net efflux of univalent cations occurred. Table 4 confirms these findings and shows also the simultaneous pH changes involved. For comparison, the behaviour

of an identical sample of erythrocytes, suspended in iso-osmotic KCl is given.

In this experiment (Table 4), the pH values of the suspending media in the two cases are very similar, which supports the idea that pH value in itself is not important in promoting Ca^{2+} influx. It will be noted that the rate of influx of Ca^{2+} into cells suspended in the choline chloride medium is rather slow compared with the rapid influx of Ca^{2+} in sucrose media (Tables 1 and 3) and reached a limiting value after 3 days of cold storage. This behaviour has been found consistently.

Dependence of rate of Ca^{2+} influx into erythrocytes on cell density of the suspension

The foregoing cold-storage experiments strongly suggest that univalent-cation efflux from erythrocytes

Table 5. *Effects of valinomycin, Pb²⁺ and Cd²⁺ on univalent-cation efflux and Ca²⁺ influx during cold storage of erythrocytes in iso-osmotic sucrose*

Suspensions contained initially 24.0 ml (Expt. 1), 22.8 ml (Expt. 2) or 24.3 ml (Expt. 3) of packed erythrocytes/100 ml. All suspending media contained 256 mM-sucrose, 2.5 mM-CaCl₂, sodium benzylpenicillin (120 mg/l) and streptomycin sulphate (200 mg/l). Where present, the final concns. were: valinomycin, 1 μ M; Pb²⁺, 1 mM; Cd²⁺, 2.5 mM. The ion concentrations and fluxes were calculated as μ mol or mmol/litre of packed cells at day 0, after correction for haemolysis.

Expt. no.	Medium	Period of cold storage (days)	Cell composition		Na ⁺ +K ⁺ efflux (mmol/l)
			Ca ²⁺ (μ mol/l)	Na ⁺ +K ⁺ (mmol/l)	
1	Sucrose	0	36	115.4	—
		5	473	45.4	70.0
	Sucrose+valinomycin	0	42	110.1	—
		5	648	26.1	84.0
2	Sucrose	0	74	102.5	—
		4	1513	30.2	72.3
	Sucrose+Pb ²⁺	0	31	99.3	—
		4	4280	12.8	86.5
3	Sucrose	0	31	108.6	—
		4	264	56.6	52.0
	Sucrose+Cd ²⁺	0	8	106.3	—
		4	748	27.7	78.6

suspended in iso-osmotic sucrose is the main prerequisite for Ca²⁺ influx, rather than dependence on the pH value of the suspending medium. This conclusion is supported by an experiment in which Ca²⁺ influx was determined on the same sample of erythrocytes suspended in iso-osmotic sucrose at two cell concentrations differing by a factor of three, namely 29.4 and 9.8 ml of packed cells/100 ml.

After cold storage for 6 days, the extent of Ca²⁺ influx was much greater with the dilute suspension (1005 μ mol/l) than with the concentrated suspension (461 μ mol/l). This difference in behaviour is most readily explained by the simultaneous observation that univalent-cation efflux was also greater in the dilute suspension of erythrocytes (87.1 mmol/l) than in the concentrated suspension (63.0 mmol/l), as would be expected for a diffusion process.

Effect of valinomycin on influx of Ca²⁺ into erythrocytes during cold storage in iso-osmotic sucrose

The neutral ionophore, valinomycin, has been shown to stimulate the transfer of K⁺ across the erythrocyte membrane, although it does not induce any increase in permeability towards Na⁺ (Tosteson *et al.*, 1967; Henderson *et al.*, 1969).

In a preliminary experiment with human erythrocytes suspended in iso-osmotic sucrose and stored at 3°C, it was found that 1 μ M-valinomycin produced a marked stimulation of K⁺ efflux, whereas the effect of 0.1 μ M-valinomycin was just detectable. The higher concentration was therefore used in an experiment to examine the effect on Ca²⁺ influx. Table 5 shows that valinomycin increased both the rates of Ca²⁺ influx

and univalent-cation efflux when erythrocytes were cold-stored in iso-osmotic sucrose. Only the rate of K⁺ efflux was stimulated, the rate of Na⁺ efflux being unaffected. By contrast, it was found in a parallel experiment that valinomycin had no effect on Ca²⁺ influx when the erythrocytes were cold-stored in iso-osmotic KCl.

Effects of Pb²⁺ and Cd²⁺ on the influx of Ca²⁺ into erythrocytes during cold storage in iso-osmotic sucrose

Joyce *et al.* (1954) reported that Pb²⁺, Hg²⁺ and Au³⁺ increased the permeability of rabbit erythrocytes towards K⁺ when rabbit blood was incubated in a saline medium at 37° or 7°C.

Expt. 2 of Table 5 confirms this effect of Pb²⁺ on human erythrocytes cold-stored in the standard sucrose-CaCl₂ medium. The increased rate of univalent-cation efflux was accompanied by a stimulation of the rate of Ca²⁺ influx.

Expt. 3 of Table 5 shows that Cd²⁺ at a final concn. of 2.5 mM produced the same effect, stimulating both univalent-cation efflux and Ca²⁺ influx.

Discussion

The experiments reported in this paper allow certain conclusions to be drawn concerning the diffusion of Ca²⁺ from suspending media into erythrocytes under cold-storage conditions. First, the ATP content of the cells appears to be unimportant. When erythrocytes were cold-stored in iso-osmotic sucrose or iso-osmotic KCl, the rates at which the cells became depleted of ATP were similar (Table 1). Yet Ca²⁺

influx occurred readily from the sucrose medium, but not from the KCl medium. The amount of ATP did not fall to zero and, as previously shown (Long & Mouat, 1973), enough remained to function as substrate for the Ca^{2+} -activated adenosine triphosphatase and to cause efflux of Ca^{2+} from Ca^{2+} -loaded cells when these were subsequently incubated at 37°C . In this respect the results differ from those of Lew (1970, 1971) and Romero & Whittam (1971), who worked at 37°C and found the ATP contents of the cells to be of prime importance. This apparent discrepancy is most simply explained in terms of temperature. At 37°C the 'calcium pump', which requires Ca^{2+} -activated adenosine triphosphatase (Schatzman & Vincenzi, 1969) will not permit a net influx of Ca^{2+} to occur until the ATP content falls to a very low value. In our work at 3°C , the enzyme would be expected to have only slight or even zero activity.

With regard to the influence of extracellular pH on Ca^{2+} influx into erythrocytes under cold-storage conditions, experiments carried out in a sucrose medium did not clearly distinguish between the effects of pH and univalent-cation efflux from the cells. This is because univalent-cation efflux is accompanied by Cl^- efflux, and the membrane potential becomes less positive as required by the Nernst equation. This fall in membrane potential is accompanied by a redistribution of H^+ and OH^- , and has the effect of raising the pH of the extracellular medium.

It is only when the non-ionic sucrose medium is replaced by KCl or choline chloride that the effects of extracellular pH and univalent-cation efflux can be distinguished (Tables 3 and 4). In both ionic media, the extracellular pH value is higher than in the sucrose medium, because of the quite different cation distribution, which establishes a negative value for the membrane potential. At this negative value, the pH value of the extracellular medium becomes high. Even so, Ca^{2+} influx is virtually absent in the KCl medium, which indicates that pH itself is not an important factor under cold-storage conditions. In the choline chloride medium, however, the large cholinium ion diffuses into the cells less rapidly than K^+ and Na^+ diffuse outwards, so that there is a net efflux of univalent cations from the cells and Ca^{2+} influx therefore occurs.

Positive evidence supporting the dependence of Ca^{2+} influx on univalent-cation efflux has been

obtained by selecting conditions under which the latter is artificially raised. Thus dilution of the suspension of erythrocytes in the sucrose medium raises the rate of univalent-cation efflux and the rate of Ca^{2+} influx increases simultaneously. Similarly, addition of valinomycin, Pb^{2+} or Cd^{2+} also increase univalent-cation efflux and thus augment the rate of Ca^{2+} influx.

The results in this paper therefore strongly support our earlier conclusion (Long & Mouat, 1973) that under conditions of cold storage, where cell metabolism is largely suppressed, the loss of univalent cations from the erythrocytes is the main factor promoting Ca^{2+} influx.

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