Erythrocyte Calcium Metabolism

CALCIUM EXCHANGE IN NORMAL AND SICKLE-CELL-ANAEMIA ERYTHROCYTES

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Under exchange conditions (no net increase in calcium), erythrocytes incubated in iso-osmotic phosphate-buffered saline have an exchangeable calcium pool comprising about 10% of the total erythrocyte calcium. This pool reaches exchange equilibrium, for either inward-directed or outward-directed transfer of the ^{45}Ca -exchange label, with a half-time of about 20 min. The uptake of Ca^{2+} requires phosphate, even under hypo-osmotic conditions, where the calcium loading expected as the cells swell is obtained only when phosphate is present. The phosphate requirement is not due to Ca^{2+} transport as a phosphate salt. This exchangeable-calcium pool is also present in sickle-cell-anaemia erythrocytes, and comprises a similar proportion of total cellular calcium.

The human erythrocyte is known to maintain concentration gradients for several substances across its limiting membrane. Among these is Ca^{2+} , which is present in the erythrocyte at less than 1% of the serum concentration.

Values of normal human erythrocyte Ca^{2+} concentration have been determined by several workers, and are in the range from about 15 nmol/ml of packed cells (Harrison & Long, 1968; Palek *et al.*, 1971) to about 40 nmol/ml of packed cells (Schatzmann & Vincenzi, 1969).

The calcium gradient across the membrane is at least 10^4 – 10^5 , since the major part of erythrocyte Ca^{2+} is membrane-bound (Harrison & Long, 1968), with estimates of intracellular Ca^{2+} concentration in the nanomolar range (Schatzmann, 1973). This gradient is maintained both by a diffusion barrier to the bivalent cation (Schatzmann & Vincenzi, 1969) and an active outward-directed calcium pump (Schatzmann, 1975).

The relationship of erythrocyte calcium to normal cell physiology or pathophysiology is not established. There are suggestions that increased Ca^{2+} promotes the discocyte–echinocyte transformation (Weed *et al.*, 1969; Palek *et al.*, 1971; LaCelle *et al.*, 1972; Weed & Chailley, 1973), especially in senescent erythrocytes. Increased Ca^{2+} has been shown by Lew (1970, 1971) and Blum & Hoffman (1971) to modify erythrocyte potassium transport. There are at least two pathophysiological studies in which erythrocyte calcium metabolism is modified; Eaton *et al.* (1973) found that the calcium concentration in the cell is increased by a factor of 5 in sickle-cell disease, and Horton *et al.* (1970) demonstrated a decrease in active calcium transport in cystic-fibrosis erythrocytes.

We have initiated a study of erythrocyte calcium

metabolism under non-loading conditions in an attempt to evaluate steady-state exchange properties.

Experimental and Results

The Medical Sciences Sub-Committee for the Protection of Human Subjects in Research of the University of Miami has reviewed and approved this study.

Blood samples

Blood was drawn fresh daily from laboratory personnel into heparinized Vacutainer tubes, and used within 3 h of drawing. Blood from patients with sickle-cell disease was obtained from patients having electrophoretically confirmed homozygous haemoglobin S, drawn into tubes containing heparin or EDTA, and used within 3 h. All blood samples were stored at room temperature (about 24°C) so as to eliminate the possibility of the cold loading described by Long & Mouat (1973).

Cell suspensions were prepared by washing three times at room temperature in incubation medium, unless otherwise specified.

Chemicals

^{45}Ca was obtained from ICN Corp. (Cleveland, OH, U.S.A.) as $^{45}\text{CaCl}_2$ (5–25 Ci/g in 0.5 M-HCl). ^{32}P as $^{32}\text{P}_i$ (100 mCi/nmol), was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.).

All other chemicals were reagent-grade, except Bis-Tris,* which was enzyme-grade, purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

* Abbreviations: Bis-Tris, bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; EGTA, ethanedioxybis-(ethylamine)tetra-acetic acid; PPO, 2,5-diphenyloxazole.

Incubation media

Phosphate-buffered saline, pH 7.2, was 'medium LP, salts' (Kneee & Leif, 1971), modified to increase buffer capacity (R.C. Leif, personal communication). The medium consisted of 4.52 mM-KCl, 0.86 mM-MgSO₄·7H₂O, 118.1 mM-NaCl, 11.55 mM-Na₂HPO₄·7H₂O, 4.00 mM-NaH₂PO₄·H₂O. Bis-Tris-buffered saline was prepared by replacing the phosphate in phosphate-buffered saline by NaCl to maintain the total concentration of Na⁺, and buffering by addition of Bis-Tris, adjusted to pH 7.2 with HCl at room temperature, to a final concentration of 10 mM. The composition was 4.52 mM-KCl, 0.86 mM-MgSO₄·7H₂O, 145.2 mM-NaCl, 10 mM-Bis-Tris/HCl buffer. This medium is hyperosmotic with respect to the phosphate-buffered saline, but is similar with respect to total cation concentration, and therefore similar with respect to osmotic effects on the erythrocyte (Kneee & Leif, 1971). Sucrose/Bis-Tris medium was 0.25 M-sucrose in 10 mM-Bis-Tris/HCl buffer, pH 7.2.

Determination of erythrocyte calcium

Atomic-absorption spectroscopy for calcium was carried out in an air/acetylene flame (Ramirez-Muñoz, 1968) in a Techtron AA-5 instrument with scale expansion, on a 0.5 ml total sample volume containing 0.1–0.2 ml of packed erythrocytes. The samples were digested in 1.0 ml of 6% (w/v) trichloroacetic acid in a boiling-water bath for 30 min. The precipitated residue was removed by centrifugation (10 min at 1500g), and 0.5% LaCl₃ was added as a releaser to counteract the inhibition of the Ca²⁺ response by phosphate. A standard calcium curve was run in parallel with the unknowns, and was linear from 2.5 to 37.5 μM-Ca²⁺. Under these conditions, a Ca²⁺ concentration of 1.5 nmol/0.1 ml of packed cells corresponds to a concentration of 1 μM in the measured solution, and is at the limit of detectability. All glassware for the determination was rinsed in 1 M-HCl; reagent blanks corresponded to Ca²⁺ concentrations below 1 μM.

Values of erythrocyte Ca²⁺ as obtained in our laboratory are summarized in Table 1. A brief single wash with added EGTA gave results not significantly

different from those obtained with a phosphate-buffered saline wash, indicating that the measurement does not include Ca²⁺ bound to the membrane outer surface. To evaluate possible cold loading (Long & Mouat, 1973), various combinations of cold storage and/or cold washing of cells were examined. Cells stored at 5°C for the few hours duration of an experiment did give higher Ca²⁺ concentrations whether washed cold or at room temperature, and duplicate determinations exhibited great variability. No effect of cold wash rather than at room temperature could be seen when freshly drawn (<15 min) blood or blood stored at room temperature for 2–3 h was used, nor did storage for this period affect the measurement.

It should be noted also that our population normal is weighted upward by the inclusion of three normal individuals whose erythrocyte Ca²⁺ concentration is relatively high, and by the fact that values at our detectability limit (1 μM in the analysis solution) are tabulated as 15 nmol/ml of packed cells.

Exchange incubation

Sample incubations were carried out in a shaking water bath at 37°C with a flow of moist gas (O₂+CO₂, 94:6) over the sample surface. About 2.0–2.5 ml of packed erythrocytes were diluted to a total of 10 ml with phosphate-buffered saline incubation medium, with addition of deionized bovine serum albumin, glucose and CaCl₂ containing 20 μCi of ⁴⁵Ca tracer (final concentrations 1%, 1%, 1.25 mM respectively). Zero time of incubation was taken as the addition of the cells. Duplicate 0.5 ml samples of the incubation mixture were taken at time-intervals up to 120 min, diluted to 2 ml with the stock incubation medium without additions, and washed three times by centrifugation and decantation of the supernatant. Centrifugation was for 2 min at room temperature at 3000 rev./min in a Damon/IEC HNS table-top centrifuge with an IEC 221 horizontal rotor. The final cell pellet was resuspended in phosphate-buffered saline to 1 ml total volume in a volumetric tube.

Haemoglobin was determined on duplicate 10 μl samples, by a micro-modification of the method of Drabkin & Austin (1932) by using $\epsilon = 11.0 \times 10^6$ litre·mol⁻¹·cm⁻¹ per haem iron (Zijlstra & van

Table 1. Human erythrocyte calcium content

Values are related to packed cell volume by haemoglobin determination, assuming 19 mm-haemoglobin iron in packed erythrocytes. The normal population mean is determined on *n* individuals; where there are multiple determinations for an individual, the mean value was used. For sickle-cell blood samples, *n* refers to number of samples and may include more than one sample per patient. The Ca²⁺ content results are given ±s.d.

	Subject	Ca ²⁺ (nmol/ml of packed cells)	<i>n</i>
Individual variability	P.S.	58.5 ± 9.9	33
	B.C.	22.9 ± 7.2	8
Population mean	Normal	34.3 ± 17.1	27
	Sickle-cell disease	149.7 ± 46.7	23

Table 2. Radioactivity-recovery experiment

Calculated radioactivity is based on a 10% contamination of the erythrocyte pellet by trapped supernatant. The c.p.m. (calculated and experimental) are per system, consisting of 0.5 ml of a cell suspension of a nominal haematocrit of 20%. The calculated value for the final pellet count is based on no exchange, i.e. a 10% residual of trapped supernatant. The corresponding experimental c.p.m. are those measured in an actual exchange study. Total c.p.m. (supernatants) is based on the full original sample volume, whereas total c.p.m. (cell) is based on the 0.1 ml cell volume. Net Ca²⁺ is calculated assuming an average pellet contamination from the experimental data (6.4%) and subtracting from the pellet count that percentage of the third wash supernatant count.

	Calculated		Experimental	
	Total c.p.m.	% of previous	Total c.p.m.	% of previous
Initial supernatant	1.65 × 10 ⁶		1.65 × 10 ⁶	
1st-wash supernatant	1.65 × 10 ⁵	10	9.18 × 10 ⁴	5.6
2nd-wash supernatant	1.65 × 10 ⁴	10	5.22 × 10 ³	5.7
3rd-wash supernatant	1.65 × 10 ³	10	4.14 × 10 ²	7.9
Final pellet	1.65 × 10 ²		3.50 × 10 ³	
			Net Ca ²⁺ (cell-associated) 3.23 × 10 ³ c.p.m.	

Kampen, 1960; Cameron, 1965). Measurements were made in a Zeiss PMQ-II spectrophotometer. Haemoglobin concentration was converted into ml of cells by assuming a nominal value of 19 mm-haemoglobin for packed erythrocytes (based on 14.5 g of haemoglobin/100ml of blood at a normal haematocrit of 45 and mol.wt. of haemoglobin per haem iron of 17000).

Calcium determination was carried out directly on a 0.5 ml sample as described above.

The balance of the incubated cell sample, 0.48 ml, was used for scintillation counting. The sample was quantitatively transferred to a scintillation vial, dried, digested with 70% HClO₄/30% H₂O₂ at 90°C (Cameron, 1965) and counted for radioactivity in a toluene/Cellosolve/PPO fluor, as described by Mahin & Lofberg (1966), in a Searle Mark III 6880 scintillation spectrometer (Searle Analytic, DesPlaines, IL, U.S.A.) in a pre-programmed variable-quench mode. Double-labelled (³²P_i, ⁴⁵Ca) samples were handled in the same way, and counted for radioactivity under a double-isotope variable-quench mode program.

The radioactivity in the washed erythrocyte pellet represents only a very small percentage (<0.2%) of the total. A radioactivity-recovery experiment, summarized in Table 2, demonstrates that these pellet counts do indeed represent Ca²⁺ uptake into cells and not merely trapped supernatant. The calculated values are based on a 10% entrapment of supernatant in the centrifuged erythrocyte pellet, an amount that is greater than any actual plasma contamination we have seen in washed erythrocytes.

The results show that the actual contamination of pellet by supernatant is always less than 10%. Also, successive washes do not release significant ⁴⁵Ca from the pellet, since at no point does experimental radioactivity in the supernatant exceed the expected value. Finally, counts in the washed pellet must be due to label fixed to the cell, since final washed cell counts exceed total counts in the 3 ml-wash supernatant.

Fig. 1. Calcium exchange of normal human erythrocytes

Washed cells were exchanged in phosphate-buffered saline with added ⁴⁵Ca, samples removed, washed and counted for radioactivity at various times. The theoretical curve (—) and error bars are obtained from the exchange kinetic analysis (Fig. 2a).

Calcium exchange

Results of a series of eight experiments in which no loading occurred (that is, final erythrocyte Ca²⁺ concentration was unchanged from the initial concentration as measured by atomic-absorption spectroscopy) are presented in Fig. 1.

The characterization of this process as exchange is based primarily on the absence of an increase in erythrocyte Ca²⁺ during the incubation. Alternatively the term exchange may be applied as defined by Schrader (1973), i.e. uptake of less than 16 nmol of ⁴⁵Ca/h per ml of packed cells. There are experimental conditions, discussed below, in which there is net increase (uptake) of Ca²⁺. However, by the standard incubation protocol the process observed was almost invariably exchange.

The experimental data have been analysed and

Fig. 2. Calcium exchange kinetics

Semilogarithmic plots (first-order exchange reaction) for Ca^{2+} exchange in erythrocytes are shown. The ordinate is $\ln [m_{\infty}/(m_{\infty} - m)]$, m for in-exchange being [^{45}Ca] within the erythrocytes and for out-exchange ^{45}Ca in the supernatant as percentage of total in the system. The value taken for m_{∞} is the average value of the respective m after equilibrium is reached, i.e. after time-periods of 1 h. (a) Normal erythrocytes, in-exchange; (b) normal erythrocytes, out-exchange; (c) sickle-cell erythrocytes, in-exchange; (d) sickle-cell erythrocytes, out-exchange. The error bars are 1 S.D. of the estimated ordinate at a given value of the abscissa, and are obtained from least-squares linear regression. The error bar at the intercept on the abscissa (time) is derived from those points at which an estimated ordinate value of 0 is 1 S.D. from the regression line.

show a first-order isotope-exchange reaction (Harris 1951; Frost & Pearson, 1953); the resulting semilogarithmic plot is presented in Fig. 2(a). For the purposes of linear least-squares regression calculations, the equilibrium concentration of intracellular ^{45}Ca is taken to be the plateau reached after 60 min of incubation.

This uptake of calcium appears to follow a first-order exchange, essentially complete in about 1 h. The data do not exclude a much slower exchange or uptake, since long-term incubations have not been done. The linearity of the plot suggests the absence of a two-stage Ca^{2+} uptake. Note that external membrane-bound ^{45}Ca , for which rapid uptake would be expected, is excluded from the analysis through wash-out in the sample handling.

Re-exchange of Ca^{2+} from labelled cells

The experiments reported above were identified as exchange on the basis of total ^{45}Ca bound and by the absence of a net increase in calcium. However, the total calcium uptake, less than 6 nmol/ml of packed cells, is near the limit of sensitivity of the atomic-absorption Ca^{2+} measurement.

To confirm that the calcium pool in the erythrocyte undergoes reversible exchange, preincubation in ^{45}Ca followed by exchange with external unlabelled Ca^{2+} was carried out.

Normal erythrocytes were incubated as described, under exchange conditions, for 90 min. Exchange was assumed to be complete (as shown in Fig. 1, exchange is essentially complete at 60 min and [^{45}Ca] remains constant between 60 and 120 min). The sample was washed in unlabelled incubation medium (three times at room temperature), and incubation was resumed in complete medium lacking ^{45}Ca . Samples were withdrawn at intervals for 60 min to determine out-exchange rate.

The cells were centrifuged, and ^{45}Ca was determined in a portion of the supernatant as well as in the cells washed once in phosphate-buffered saline. Qualitatively, the process behaved as expected for an equilibrium exchange, i.e. ^{45}Ca increasing in the medium as incubation proceeded at the expense of intracellular ^{45}Ca . The data are plotted in semilogarithmic form in Fig. 2(b). The half-time ($t_{1/2}$) for the out-exchange (appearance of ^{45}Ca in the medium from labelled cells) is essentially the same as for the in-exchange

Table 3. *Calcium-exchange properties of erythrocytes*

Exchangeable Ca^{2+} (%) is defined as the ratio of labelled Ca^{2+} to total Ca^{2+} in nmol/ml of packed cells. Reaction half-time ($t_{1/2}$) is obtained from the kinetic plots in Fig. 2, and represents the time from the intercept on the abscissa to the half-reaction point, i.e. $m = (m_{\infty}/2)$, an ordinate value of $\ln(1/2)$ or 0.69. Exchangeable Ca^{2+} is given $\pm$ s.d.

Samples	Exchange	Exchangeable Ca^{2+} (%)	$t_{1/2}$ (min)
Normal erythrocytes	In	10 ± 2	27
Normal erythrocytes	Out	*	28
Sickle-cell erythrocytes	In	11 ± 2	30
Sickle-cell erythrocytes	Out	*	18

* The 'exchangeable fraction' is not defined for out-exchange (see the text).

(appearance of ^{45}Ca in erythrocytes incubated in medium containing tracer).

Sickle-cell disease

The exchange properties of a few blood samples from sickle-cell-anaemia patients were examined. The kinetic plots are presented in Figs. 2(c) and 2(d), and kinetic data for both normal and haemoglobinopathic erythrocytes are summarized in Table 3.

Calcium loading and effects of the medium

The exchange of Ca^{2+} into normal erythrocytes corresponds to an exchangeable pool of only about 10% of the total cell calcium (Table 3). To evaluate the accessibility of the remaining Ca^{2+} pool to exchange or uptake of ^{45}Ca , a series of studies were carried out under conditions of presumptive osmotic loading.

The first experiments comprised adjusting total osmolarity of the standard phosphate-buffered saline incubation medium, by dilution of a stock solution of twice normal concentration. Incubation was carried out on cells washed in the normal medium and resuspended for incubation in control, hypo-osmotic or hyperosmotic medium with ^{45}Ca . All incubation mixtures contained 1% albumin, 1% glucose and 1.25 mM- CaCl_2 .

The relative ^{45}Ca uptake did vary with osmolarity of the medium, but directly rather than inversely as expected (Table 4). Also, even in the hypo-osmotic incubation, there was no net increase in Ca^{2+} .

Experiments were then carried out in which osmolarity of the incubation medium was varied by increasing or decreasing only the Na^+ . These results are also presented in Table 4. In this case, uptake of ^{45}Ca had the expected relation to the osmotic properties of the medium. In hypo-osmotic medium (125 mM- Na^+) the uptake of Ca^{2+} was greatly enhanced, with calcium loading (final Ca^{2+} concentration shown by atomic absorption to be 15 times the initial value). The difference between normo-osmotic and hyperosmotic solutions given is consistent with

the expected 15% shrinkage of erythrocytes in the more concentrated medium (Leif & Vinograd, 1964), since Ca^{2+} is measured per ml of packed cells.

The effect of phosphate was confirmed by incubation in Bis-Tris medium (phosphate-free). In this case no loading occurred, even at 125 mosmol, and the Ca^{2+} uptake was greatly decreased with respect to the phosphate-buffered saline control (Table 4).

One possible explanation for the results would be transport of Ca^{2+} as a phosphate complex. This was ruled out in two ways. First, P_i was determined in erythrocytes by the method of Leloir & Cardini (1957) after 90 min incubation in Bis-Tris-buffered saline with added P_i . Incubation media were prepared by addition of phosphate to Bis-Tris-buffered saline. Control medium contained no added P_i , whereas experimental media included 0.5–30 mM- H_3PO_4 titrated to pH 7.2 with Bis-Tris at room temperature. Such a procedure alters total osmolarity but maintains cation osmolarity, the more important factor affecting erythrocyte volume (Leif & Vinograd, 1964). In these experiments, phosphate uptake was independent of calcium uptake, increasing linearly with P_i added, whereas Ca^{2+} uptake was negligible at very low phosphate concentrations and reached a plateau at phosphate concentrations of 15 mM or above. Secondly, double-labelling experiments were performed in which the normal phosphate-buffered saline incubation medium contained tracer $^{32}\text{P}_i$ as well as ^{45}Ca . The time-course of uptake of $^{32}\text{P}_i$ was not parallel to that of ^{45}Ca . At the start of incubation the rate of ^{32}P uptake was over twice that of ^{45}Ca , whereas beyond 60 min, when ^{45}Ca uptake had reached a plateau, ^{32}P continued to increase in the cells.

Membrane binding of calcium

It is well established that the erythrocyte can bind calcium to the outer surface. Experiments were carried out in sucrose/Bis-Tris medium, in which this binding can be readily demonstrated, as shown by Long & Mouat (1971). The binding of calcium to the membrane on incubation in this iso-osmotic sucrose

Table 4. *Effects of incubation medium on erythrocyte Ca^{2+} exchange and loading*

For the experimental series with phosphate-buffered saline or constant phosphate, control uptake is specified as 100. The value relative to which the uptake in Bis-Tris is specified is the phosphate-buffered saline control. Measured uptake was taken as nmol of Ca^{2+} /ml of packed cells in 1 h, and is expressed as a relative value to summarize experiments carried out on erythrocytes with varying Ca^{2+} concentration (see Table 1).

Sample	Medium		Total ions (mequiv.)	Uptake
	Na^+ (mm)	PO_4^- (mm)		
Control; phosphate-buffered saline	145	15.5	303	100
Hypo-osmotic; phosphate-buffered saline	125	13.3	260	60
Hyperosmotic; phosphate-buffered saline	165	17.7	345	150
Control; constant phosphate	145	15.5	303	100
Hypo-osmotic; constant phosphate	125	15.5	263	2000
Hyperosmotic; constant phosphate	165	15.5	343	130
Control; Bis-Tris	145	0	—	~30
Hypo-osmotic; Bis-Tris	125	0	—	~20
Hyperosmotic; Bis-Tris	165	0	—	~20

was clearly distinguishable phenomenologically from the exchange uptake in phosphate-buffered saline, the total uptake being approximately doubled. Also, the uptake was complete instantaneously insofar as the experiment was concerned ($t_{1/2} < 5$ min). Finally, most of the Ca^{2+} taken up could be removed by one EGTA wash, which is not the case with the exchange calcium uptake.

Discussion

The data presented here are consistent with the presence, in the normal erythrocyte, of a rapidly exchangeable calcium pool comprising approx. 10% of the total. The rate of exchange of this pool, with a half-time of 15–25 min, is certainly much higher than the overall permeability of the intact erythrocyte membrane, in which even after 1 week in the cold intracellular ^{45}Ca is only 1.8% of the extracellular concentration (Schatzmann & Vincenzi, 1969). In metabolically depleted cells, under conditions in which Ca^{2+} can load to intracellular concentrations ten times normal or greater, uptake rates may well exceed the 3–5 nmol/h per ml of packed cells reported here (Passow, 1961; Lew, 1971), but this is with a dramatic net increase in Ca^{2+} . Care was taken in the present studies to exclude such loading conditions, so as to evaluate specifically steady-state exchange in erythrocytes.

An exchange process for erythrocyte Ca^{2+} has apparently not been suggested, possibly because the steady-state Ca^{2+} uptake in fresh blood is so low compared with the loading phenomenon. It has, however, been suggested that the erythrocyte membrane is actually impermeable to net Ca^{2+} flow rather than poorly permeable and that studies reporting uptake of less than 16 nmol/h per ml of packed cells indicate Ca^{2+} exchange. Such impermeability is suggested to apply even under hypo-osmotic conditions, although the present experiments indicate that failure to obtain

Ca^{2+} loading at lower ionic strength is related to phosphate concentrations rather than absolute permeability, at least in the pre-haemolytic state.

These data also indicate at least a kinetic compartmentalization of erythrocyte calcium, the rapidly exchangeable fraction comprising only a small part of the total. This exchangeable compartment cannot be anatomically localized, except that it presumably does not correspond to cytosol as distinct from membrane-associated calcium, since ^{45}Ca is found in both cytosol and pellet after high-speed centrifugation of exchange-labelled cells haemolysed by freeze-thawing.

Several other workers have reported at least two classes of calcium, distinguishable by either uptake rate or equilibrium concentration. Schatzmann & Vincenzi (1969) describe a fast component of Ca^{2+} efflux from intact starved erythrocytes, comprising about 30% of the total with a $t_{1/2}$ of 7.5 min. The effects of Ca^{2+} on K^+ efflux from erythrocytes have been suggested by Kahn (1958) to involve several classes of Ca^{2+} binding or interaction, but since this study measured loss of Ca^{2+} from serum it may reflect external membrane binding, which involves multiple sites with differing binding constants.

In an extensive series of investigations on erythrocyte 'ghosts', Porzig (1970, 1973a,b) described two mechanisms for Ca^{2+} efflux, ATP-dependent (the Ca^{2+} -activated adenosine triphosphatase) as well as an ATP-independent net Ca^{2+} movement. This latter could be characterized as Ca–Ca exchange or diffusion, and rates were comparable with the exchange rate that we have found for Ca^{2+} exchange in the intact erythrocytes, $t_{1/2}$ for influx being of the order of 30–60 min depending on Ca^{2+} concentration (Porzig, 1973a). The overall Ca^{2+} transport could not only be characterized by a dual pathway with respect to mechanism, but for both ATP-dependent and ATP-independent transport a fraction of the total Ca^{2+} was

not exchangeable. This non-exchangeable Ca^{2+} pool was always present and amounted to 10–50% of the total Ca^{2+} . This phenomenon at least indicates a compartmentalization or pool system for Ca^{2+} in the erythrocyte 'ghost', although it would be difficult to relate this inexchangeable pool specifically to the exchangeable pool that we find in the intact cell.

The functional significance of the rapidly exchanged Ca^{2+} pool in the erythrocyte is of course unknown. LaCelle *et al.* (1972) have theorized that changes in Ca^{2+} content are related to aging of erythrocytes, and that senescent erythrocytes have a second Ca^{2+} compartment. The active (rapidly exchanging) Ca^{2+} fraction we report may relate to this process, but studies of separated erythrocytes would be required to evaluate this point.

One possibility suggested by the theory of LaCelle *et al.* (1972) is that the Ca^{2+} pool that we see might be due to rapid exchange of all or most of the Ca^{2+} in a fraction of the erythrocytes. This cannot be ruled out by the present studies, although results of crude radioautography are not consistent with the ^{45}Ca being localized in only a small fraction of the cells.

The studies of Rummel *et al.* (1962) are most provocative. They found ^{45}Ca -uptake rates in intact cells incubated at 37°C of about 2–3 nmol/h per ml of packed cells, but interpretation of these data is unclear. Only a single 1 h measurement of uptake was made, so the phenomenon may not represent equilibrium exchange. The magnitude of the 1 h uptake was linearly related to external Ca^{2+} , although at the Ca^{2+} concentrations used (up to 8 mM, in the incubation medium) this may be due in part to calcium precipitation as noted by Schrader (1973). This effect of Ca^{2+} concentration is in contrast with our studies, in which at least for Ca^{2+} concentrations between about 0.8 and 1.5 mM, at constant ^{45}Ca specific radioactivity, there was no significant dependence of either exchange fraction or rate on external Ca^{2+} concentration.

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