The Binding of Calcium Ions by Erythrocytes and 'Ghost'-Cell Membranes

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1. Washed human erythrocytes, suspended in iso-osmotic sucrose containing 2.5 mM-calcium chloride, bind about 400 μg -atoms of calcium/litre of packed cells. Sucrose may be replaced by other sugars. 2. Partial replacement of sucrose by iso-osmotic potassium chloride diminishes the uptake of calcium, 50% inhibition occurring at about 50 mM-potassium chloride. 3. Other univalent cations behave like potassium, whereas bivalent cations are much more inhibitory. The trivalent cations, yttrium and lanthanum, however, are the most effective inhibitors of calcium uptake. 4. An approximate correlation exists between the calcium uptake and the sialic acid content of erythrocytes of various species and of human erythrocytes that have been partially depleted of sialic acid by treatment with neuraminidase. However, even after complete removal of sialic acid, human erythrocytes still bind about 140 μg -atoms of calcium/litre of packed cells. 5. A Scatchard (1949) plot of calcium uptake at various Ca^{2+} concentrations in the suspending media shows the presence of three different binding sites on the external surface of the human erythrocyte membrane. 6. Erythrocyte 'ghost' cells, the membranes of which appear to be permeable to Ca^{2+} ions, can bind about 1000 μg -atoms of calcium per 'ghost'-cell equivalent of 1 litre of packed erythrocytes. This indicates that there are also binding sites for calcium on the internal surface of the erythrocyte membrane.

Harrison & Long (1968) used atomic-absorption spectrophotometry to determine the calcium content of fresh human erythrocytes that had been washed with iso-osmotic sodium chloride until completely free from plasma and buffy coat. The mean value was found to be about 16 μg -atoms of calcium/litre of packed cells (0.634 $\mu\text{g}/\text{ml}$).

However, investigations by Kahn (1958) and by Gent, Trounce & Walser (1964) had suggested, on the basis of radioactivity measurements with ^{45}Ca or ^{47}Ca , that erythrocytes and 'ghost' cells were capable of binding very large amounts of Ca^{2+} . In the present work, this capacity of erythrocytes and 'ghost' cells to bind Ca^{2+} ions has been measured directly by atomic absorption.

A preliminary account of some of these results has been given (Long & Mouat, 1971).

MATERIALS AND METHODS

Materials

Blood. Fresh heparinized blood from human volunteers and from several animal species was supplied by the

Department of Pharmacology, Institute of Basic Medical Sciences. Heparin was present at a concentration of 10 units/ml of blood.

Reagents. Triple-glass-distilled water was used throughout; it was calcium-free. HCl was the constant-boiling mixture (about 5.5 M), distilled from all-glass apparatus. Lanthanum chloride (0.25 M) was prepared by adding slightly more than the theoretical quantity of La_2O_3 to 2 ml of 0.75 M-HCl and shaking. Excess of undissolved La_2O_3 was removed by centrifugation. The supernatant had a pH of about 5. Yttrium chloride was prepared in the same way. Both La_2O_3 and Y_2O_3 were obtained from Johnson-Matthey Chemicals Ltd., Hatton Garden, London E.C.1, U.K., and were spectroscopically pure.

Neuraminidase (from *Clostridium perfringens*) and *N*-acetylneuraminic acid were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

The stock calcium analytical standard was prepared by dissolving CaCO_3 in a slight excess of HCl and diluting to 1 mg of calcium/ml (25 mM). The working calcium standard for atomic-absorption spectrophotometry (62.5 μM) was obtained by diluting 1 vol. of this solution to 400 vol. with ethanol-water (1:1, v/v), containing 1 M-HCl and 43.5 mM-NaCl.

For calcium-uptake experiments, 1 M- CaCl_2 (British Drug Houses volumetric standard) was diluted with iso-

osmotic sucrose solution to a final concentration of 2.5 mM- CaCl_2 .

The sucrose used was of analytical-reagent standard, and was found to contain $3.61 \mu\text{g}$ of calcium/g. Iso-osmotic sucrose, which was taken to be 256 mM (8.75% w/v), therefore contained $7.9 \mu\text{g}$ -atoms of calcium/litre.

All other chemicals were the best available commercial preparations.

Experimental procedure

Uptake of calcium by erythrocytes. All manipulations were carried out in a cold-room at 3°C . Fresh heparinized human blood (about 40 ml) was centrifuged for 7 min at 2500g, and the plasma and buffy coat were removed. The cells were washed with 160 mM-KCl and then suspended in iso-osmotic sucrose solution to a total volume of 42 ml. Samples (4 ml) were pipetted into a series of glass-stoppered 5 ml graduated centrifuge tubes. The tubes were centrifuged for 30 min and the supernatants discarded. When blood from other species was used, the procedure was similar but on a smaller total volume scale.

To the packed sample of washed erythrocytes (about 1.3 ml), the appropriate medium (usually iso-osmotic sucrose containing 2.5 mM- CaCl_2 , or a variant of this) was added to give a total volume of 4.0 ml. The cells were uniformly suspended in the medium by vigorous shaking, and the tube was centrifuged for 30 min at 2500g. Without disturbance of the sedimented cells, about 2 ml of the supernatant was taken for analysis. All visible remaining supernatant was then removed as completely as possible by suction pipette and discarded.

Calcium-free water (3 ml) was added to the tube containing the packed erythrocytes. After vigorous shaking, whereby haemolysis occurred, measured volumes of haemolysate were taken for the determination of calcium and sucrose. Calcium and sucrose were also determined in the supernatant. By assuming that all the sucrose was extracellular, the amount of trapped supernatant associated with the sedimented erythrocytes could readily be calculated. The amount of calcium in this volume of trapped supernatant was then deducted from the total calcium of the haemolysate, so giving the quantity of bound calcium.

Uptake of calcium by 'ghost' cells. A 12.5% (v/v) suspension of washed erythrocytes in 160 mM-KCl was haemolysed by dropwise addition of 4 vol. of Ca^{2+} -free water with mechanical stirring. The 2.5% (v/v) haemolysate was then centrifuged in stoppered 40 ml polycarbonate tubes for 30 min at 20 000g and 0°C . The clear supernatant was removed and the 'ghost' cells were washed once with 32 mM-KCl and twice with iso-osmotic sucrose.

An appropriate volume of the 'ghost'-cell preparation, equivalent to about 1 ml of packed erythrocytes and contained in a 5 ml thick-walled centrifuge tube, was mixed with sufficient iso-osmotic sucrose containing 2.5 mM- CaCl_2 to give a total volume of 4 ml. After centrifugation at 10 000g for 1 h at 0°C , as much as possible of the clear supernatant was removed and set aside. The supernatant and the loosely packed 'ghost'-cell deposit were analysed for sucrose and calcium.

Determination of calcium. A measured volume (0.1 ml of supernatant, 1.0 ml of haemolysate or 0.1 ml of 'ghost'

cells) in a 5 ml glass-stoppered centrifuge tube was diluted to 1.2 ml with Ca^{2+} -free water. Constant-boiling HCl (0.73 ml), 2.5 M-NaCl (0.07 ml) and ethanol (2.1 ml) were added in that order, with mixing between each addition. The final volume was exactly 4.0 ml, because of volume shrinkage caused by mixing ethanol with an aqueous medium. Supernatant, treated in this way, gave a clear solution which was used directly for calcium determination. With the haemolysate or 'ghost'-cell suspension, the precipitate was centrifuged down and the clear supernatant analysed for calcium.

Calcium was determined in the above solutions by atomic-absorption spectrophotometry, as described by Harrison & Long (1968), except that the standard contained $2.5 \mu\text{g}$ of calcium/ml ($62.5 \mu\text{M}$) instead of $5 \mu\text{g}/\text{ml}$, as in the previous work.

Determination of sucrose. To the supernatant (0.1 ml of a 20-fold dilution) or haemolysate (0.1 ml) water (1.5 ml) was added followed by 0.5 M-NaOH (0.2 ml) and 10% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 ml). After mixing and centrifuging, 0.3 ml of the protein-free supernatant was heated with 2.7 ml of the resorcinol-thiourea reagent of Roe, Epstein & Goldstein (1949) for 15 min at 75°C . The extinction at 520 nm was read against a standard ($67.5 \mu\text{g}$ of sucrose) and a blank, run simultaneously.

Treatment of erythrocytes with neuraminidase. An erythrocyte sample, previously washed with 160 mM-KCl, was suspended in iso-osmotic sucrose to give an haematocrit of 0.30–0.35. Neuraminidase, dissolved in iso-osmotic sucrose, was added in appropriate amount to give a final enzyme concentration of $5 \mu\text{g}/\text{ml}$ of suspension and the reaction mixture was incubated at 37°C for 15 min. The tube was then cooled in ice-water and centrifuged at 2500g for 30 min at 3°C . The supernatant was discarded and the packed cells were resuspended in iso-osmotic sucrose containing 2.5 mM- CaCl_2 for determination of calcium-binding activity.

Determination of N-acetylneuraminic acid. Samples of haemolysate or 'ghost'-cell suspension, corresponding to about 0.15 ml of packed erythrocytes, were diluted with water to 5 ml and centrifuged at 10 000g for 1 h at 0°C . The supernatant was removed by decantation and the cells were washed once with water. This procedure removed not only 95% of the haemoglobin but also most of the sucrose, which would otherwise interfere with the analysis by reacting with the periodic acid.

Water was added to the cells, followed by 0.016 ml of 72% (w/w) HClO_4 , to give a total volume of 2.0 ml. The tube was heated in a water bath at 84°C for 90 min, cooled and centrifuged; 0.5 ml of the supernatant was taken for N-acetylneuraminic acid determination by the method of Aminoff (1961). The standard consisted of $0.030 \mu\text{mol}$ of N-acetylneuraminic acid.

Determination of cholesterol. A volume of haemolysate or 'ghost'-cell preparation, equivalent to about 0.10 ml of packed erythrocytes, was diluted with water to 5 ml and centrifuged for 1 h at 10 000g and 0°C . The 'ghost' cells were washed once with water and then extracted twice with 2 ml portions of acetone at room temperature. An appropriate volume of the combined extract was evaporated to dryness and the lipid residue was analysed for cholesterol by the method of Zlatkis, Zak & Boyle (1953).

RESULTS

Binding of Ca^{2+} ions by human erythrocytes

Effect of concentration of Ca^{2+} in the suspending medium. Fig. 1 (curve *a*) shows the effect of varying the concentration of calcium chloride in the iso-osmotic sucrose suspending medium on the uptake of Ca^{2+} ions by human erythrocytes. Even at the highest concentration of calcium chloride used (about 7 mM), saturation of the binding sites was not completely achieved. However, it was determined graphically that the theoretical maximum uptake of Ca^{2+} in this experiment would have been 448 μg -atoms/litre of packed cells. Half-maximum calcium binding occurred at 0.2 mM-calcium chloride in the suspending medium. At 2.5 mM-calcium chloride, which was used in all subsequent experiments, the binding sites were 85% saturated with calcium.

Effect of calcium-complexing agents. In twelve experiments with 2.5 mM-calcium chloride in the suspending medium, the mean calcium uptake was 403 μg -atoms/litre of packed cells (range 364–434). When a threefold excess of EDTA (potassium salt, pH 7.4) over calcium was also present in the suspending medium, the uptake of Ca^{2+} by the erythrocytes was decreased to an average of 28 μg -atoms/litre (range 10–39). This has been

Fig. 1. Effect of concentration of Ca^{2+} ions in an iso-osmotic sucrose suspending medium on the uptake of calcium by mammalian erythrocytes: (a) human; (b) rabbit. Experimental details are given in the text.

interpreted as showing that it is the bivalent Ca^{2+} ions that are bound to the erythrocyte membrane. A threefold excess of citrate (potassium salt, pH 7.4) lowered the uptake of Ca^{2+} to an average of 45 μg -atoms/litre (range 43–47). In all experiments in which a comparison was made, EDTA was more effective than citrate as an inhibitor of uptake of Ca^{2+} .

Effect of osmolarity. Alterations in osmolarity of the suspending medium, within the permissible limits, had little effect on the uptake of Ca^{2+} ions, as shown in Table 1.

Replacement of sucrose by other non-ionic compounds. When 95% of the sucrose was replaced by other sugars or related compounds, the uptake of Ca^{2+} by the erythrocytes was not significantly changed, as shown by the following comparison (uptake in μg -atoms of Ca^{2+} /litre): sucrose, 398; maltose, 374; lactose, 380; melibiose, 390; D-glucose, 336; D-fructose, 398; D-galactose, 370; L-sorbose, 354; D-mannose, 390; mannitol, 349; sorbitol, 355; inositol, 369; D-arabinose, 363; L-arabinose, 347; D-ribose, 367; D-xylose, 368; L-rhamnose, 387.

Effects of cations. When the sucrose in the suspending medium was partially replaced by iso-osmotic (160 mM) potassium chloride, the concentration of calcium chloride being maintained constant at 2.5 mM, there was a marked diminution of the uptake of Ca^{2+} by human erythrocytes. Fig. 2 shows the effect of increasing the fraction of the osmolarity caused by potassium chloride. At 50 mM-potassium chloride there was a 50% inhibition of uptake of Ca^{2+} .

Other univalent chlorides have also been examined, at 50 mM concentration in the suspending medium, for their effects on uptake of Ca^{2+} . The

Table 1. *Effect of osmolarity of sucrose medium on uptake of Ca^{2+} by human erythrocytes*

The initial sucrose concentrations in the suspending media varied between 165 mM and 330 mM (256 mM is iso-osmotic). All suspending media contained 2.5 mM- CaCl_2 . Identical numbers of cells were taken for each measurement of calcium uptake. The volumes of the packed cells reflect the osmolarity of the suspending media.

Concn. of sucrose in suspending medium (mM)	Packed cell volume (ml)	Calcium uptake	
		Total (μg -atom)	(μg -atoms/litre)
330	1.333	0.562	421
292	1.413	0.567	402
256	1.469	0.553	377
219	1.564	0.597	380
183	1.702	0.600	353
165	1.792	0.630	352

Fig. 2. Effect of partial replacement of iso-osmotic sucrose in the suspending medium by iso-osmotic (160 mM) KCl on the uptake of Ca^{2+} by human erythrocytes. The concentration of Ca^{2+} in the suspending medium was maintained constant at 2.5 mM. Experimental details are given in the text.

experiment described in Table 2 shows that all were inhibitory, the chlorides of rubidium and caesium having the greatest effect. These latter cations, being relatively non-hydrated, have smaller diameters than the highly hydrated Na^+ and Li^+ ions, which are less inhibitory.

Table 3 shows the results of an experiment obtained when the normal suspending medium, containing sucrose and calcium chloride, was supplemented with a bivalent chloride at a final concentration of 2.5 mM, i.e. the same as the final concentration of calcium. There was a generalized inhibition of uptake of Ca^{2+} , Cd^{2+} being the most effective bivalent cation.

The choice of tervalent cations is limited by the stability of their salts in aqueous solution. However, it has been possible to study the effects of the

Table 2. *Effect of univalent chlorides on the binding of Ca^{2+} ions by human erythrocytes*

All suspending media contained 175 mM-sucrose, 2.5 mM- CaCl_2 and 50 mM univalent chloride, and were iso-osmotic.

Univalent chloride	Calcium uptake ($\mu\text{g-atoms/litre}$)	Inhibition (%)
None*	434	—
NaCl	273	37
$(\text{CH}_3)_4\text{NCl}$	267	39
LiCl	252	42
Choline chloride	247	43
KCl	241	44
$(\text{C}_2\text{H}_5)_4\text{NCl}$	217	50
Tris chloride	213	51
RbCl	186	57
CsCl	185	57

* Medium consisted of 256 mM-sucrose and 2.5 mM- CaCl_2 .

Table 3. *Effect of bivalent chlorides on the binding of Ca^{2+} ions in human erythrocytes*

All suspending media contained 256 mM-sucrose, 2.5 mM- CaCl_2 and 2.5 mM bivalent-metal chloride.

Bivalent chloride	Calcium uptake ($\mu\text{g-atoms/litre}$)	Inhibition (%)
None	434	—
NiCl_2	368	15
MnCl_2	345	21
BaCl_2	326	25
HgCl_2	325	25
CuCl_2	301	31
CoCl_2	301	31
MgCl_2	293	32
SrCl_2	292	33
ZnCl_2	290	33
CdCl_2	197	55

chlorides of chromium, yttrium and lanthanum, at final concentrations in the suspending media of 2.5 and 0.5 mM, on the uptake of Ca^{2+} ions by human erythrocytes. The results of this experiment are shown in Table 4. Chromic chloride had about the same inhibitory effect as that of the bivalent chlorides, but lanthanum and yttrium were much more effective.

Binding of Ca^{2+} ions in relation to the sialic acid content of erythrocytes

The results presented above show that the external surface of the erythrocyte membrane has an appreciable capacity for binding cations, and that when two or more cations are simultaneously present, competition between them will occur. Thus

in the experiment shown in Fig. 2, it may be calculated that the affinity of the binding sites is about ten times greater for one Ca^{2+} ion than for two K^+ ions.

Since the zeta potential is known to be largely due to the presence of ionized carboxyl groups of sialic acid residues on the external surface of the erythrocyte membrane (Eylar, Madoff, Brody & Oncley, 1962), a comparison was made of the binding of Ca^{2+} ions and the *N*-acetylneuraminic acid content of erythrocytes. Fig. 3 shows the results obtained on twelve samples of normal human erythrocytes, seven samples of human erythrocytes pretreated with neuraminidase so as partially to deplete them of sialic acid, and seven samples of erythrocytes from other mammalian species, namely cat, dog, guinea pig and rabbit. These points tend to lie along a straight line.

In Fig. 3 the regression line, determined by the method of least squares, does not pass through the

origin, but intersects the ordinate at about $143 \mu\text{g}$ -atoms of bound calcium/litre of packed cells. Further, the slope of the line is 0.377, and not 0.50 which would have been expected if every two *N*-acetylneuraminic acid residues had bound one Ca^{2+} ion.

Fig. 3 also shows that the lowest content of sialic acid was found in rabbit erythrocytes. In a separate experiment, the uptake of Ca^{2+} by rabbit erythrocytes was examined as a function of the concentration of Ca^{2+} in the suspending medium. The result obtained is shown in curve (b) of Fig. 1. Curves closely similar to this were obtained with samples of human erythrocytes from which most of the sialic acid had been removed by preincubation with neuraminidase.

Two specimens of rat erythrocytes were also examined. Both were active in binding Ca^{2+} ions, the uptakes being 434 and $533 \mu\text{g}$ -atoms/litre of packed cells under the standard experimental conditions. However, it was not possible to determine the sialic acid content of rat erythrocytes because the cells did not readily haemolyse when suspended in water, and the presence of the haem pigment interfered with the analytical procedure.

Table 4. *Effect of trivalent chlorides on the binding of Ca^{2+} ions by human erythrocytes*

All suspending media contained 256 mM-sucrose, 2.5 mM- CaCl_2 and either 2.5 mM or 0.5 mM trivalent chloride, as indicated.

Tervalent chloride	Final concn. (mM)	Calcium uptake (μg -atoms/litre)	Inhibition (%)
None	—	439	—
CrCl_3	0.5	338	23
	2.5	276	37
LaCl_3	0.5	184	58
	2.5	118	73
YCl_3	0.5	199	55
	2.5	59	87

Binding of Ca^{2+} ions by human erythrocyte 'ghost' cells

'Ghost' cells, prepared as described by dilution-haemolysis, were suspended in media containing iso-osmotic sucrose and 2.5 mM-calcium chloride. After centrifugation, the supernatant and loosely packed 'ghost' cells were separately analysed for sucrose and calcium. The 'ghost' cells proved to be completely permeable to sucrose. When 'ghost' cells were suspended in 160 mM-potassium chloride instead of sucrose, they were also found to be completely permeable to K^+ ions. It has therefore

Fig. 3. Relationship between uptake of Ca^{2+} ions and *N*-acetylneuraminic acid content of mammalian erythrocytes: ○, human; ■, dog; □, cat; ▲, guinea pig; △, rabbit; ●, human erythrocytes pretreated with neuraminidase. Experimental details are given in the text. The correlation coefficient (r) of the regression line was 0.928.

been assumed that the 'ghost'-cell membrane is completely permeable to Ca^{2+} ions. Since the volume and total calcium content of the sedimented 'ghost' cells were measured, deduction of the calcium content of an equal volume of supernatant gave the quantity of bound calcium. The erythrocyte equivalent of the 'ghost' cells was determined from the cholesterol or *N*-acetylneuraminic acid content, so that the calcium bound by the 'ghost' cells could be expressed in $\mu\text{g-atoms/litre}$ of equivalent erythrocytes. The results are shown in Table 5.

The capacity of 'ghost' cells to bind Ca^{2+} ions is two or three times greater than that of erythrocytes. This indicates that there must be binding sites for Ca^{2+} on the internal surface of the erythrocyte membrane as well as on the outside. When EDTA was also present in the system, the binding of Ca^{2+} ions was lowered to 10% or less. Replacement of sucrose by iso-osmotic potassium chloride (Table 5, Expt. 2) greatly inhibited the uptake of Ca^{2+} ions, presumably through competition by K^+ ions for the binding sites, as was found with intact erythrocytes (Fig. 2). 'Ghost' cells prepared from neuraminidase-treated erythrocytes (Table 5, Expt. 3) had only about one-half the normal capacity to bind Ca^{2+} ions.

DISCUSSION

In the experiments described above, the average uptake of calcium ions by human erythrocytes, suspended in iso-osmotic sucrose containing 2.5 mM-calcium chloride, was $403 \mu\text{g-atoms/litre}$ of packed cells. Because the binding sites were not completely saturated under these conditions, however, the mean maximum calcium-binding capacity has been calculated to be about $475 \mu\text{g-atoms/litre}$. The only published report on calcium binding by erythrocytes appears to be that of Kahn (1958), whose values for the uptake of ^{45}Ca from plasma by cells, after making certain reasonable assumptions, have been calculated to lie in the range 800–1200 $\mu\text{g-atoms/litre}$ of packed cells. These elevated values are surprising in view of the high concentration in the plasma of Na^+ ions, which would be expected to inhibit uptake of Ca^{2+} by the erythrocytes very substantially.

The capacity to bind Ca^{2+} ions was appreciably increased after erythrocytes had been converted into 'ghost' cells. In five experiments, the mean value was $1025 \mu\text{g-atoms}$ of calcium bound per 'ghost'-cell equivalent of 1 litre of packed erythrocytes. This increase is presumably due to the ability of Ca^{2+} ions to penetrate the 'ghost'-cell membrane and become bound to the inner surface. Gent *et al.* (1964), who used $^{47}\text{Ca}^{2+}$, reported calcium uptakes of between 3.8 and $48 \mu\text{g-atoms/g}$ dry weight of

Table 5. Binding of Ca^{2+} ions by human erythrocyte 'ghost' cells

'Ghost' cells, prepared as described under 'Experimental procedure', were suspended in 256 mM-sucrose containing 2.5 mM- CaCl_2 , to a total volume of 4 ml. Where indicated, 0.3 ml of 83 mM-EDTA (potassium salt), pH 7.4, was added. The 'ghost' cells were assumed to be completely permeable to Ca^{2+} ions in order to calculate the amount of bound calcium. The erythrocyte equivalent of the 'ghost' cells was determined by determination of *N*-acetylneuraminic acid or cholesterol content. Wherever the values were measured, calcium uptake by the corresponding erythrocytes is shown.

Expt. no.	Addition	Calcium uptake by 'ghost' cells ($\mu\text{g-atoms/litre}$)	Calcium uptake by erythrocytes ($\mu\text{g-atoms/litre}$)
1	Nil	1145	374
	EDTA	29	25
2	Nil	1194	377
	EDTA	0	—
	KCl*	97	—
3	Nil	853	424
	EDTA	84	—
	Nil†	484	222
	EDTA†	24	—

* 'Ghost' cells suspended in 160 mM-KCl containing 2.5 mM- CaCl_2 .

† 'Ghost' cells prepared from erythrocytes pretreated with neuraminidase.

membrane, depending on the experimental conditions. If it be assumed that the 'ghost' cells contained 4% of the original haemoglobin of the erythrocytes together with all the membrane protein and lipid, then these values would correspond to between 100 and $1300 \mu\text{g-atoms}$ of calcium per 'ghost'-cell equivalent of 1 litre of packed erythrocytes. The upper value is thus of the same order of magnitude as that found in the present work. However, Gent *et al.* (1964) have suggested that by extrapolation to zero ionic strength, the binding of calcium could be as high as $169 \mu\text{g-atoms/g}$ dry wt. of membrane, i.e. $4560 \mu\text{g-atoms}$ per 'ghost'-cell equivalent of 1 litre of packed erythrocytes.

Certain complexing agents have been found to cause a powerful inhibition of calcium uptake. From the work of Harrison & Long (1968), it would have been expected that in the presence of EDTA there would have been no calcium at all associated with the erythrocytes, whereas with citrate the quantity would be about $16 \mu\text{g-atoms/litre}$, namely the value found for the calcium content of washed human erythrocytes. The experimentally determined mean values were 28 and $45 \mu\text{g-atoms/litre}$, in the presence of EDTA and citrate respectively. These are significantly higher than predicted and

there seems to be no satisfactory explanation for this discrepancy.

The binding of Ca^{2+} ions by the external surface of the erythrocyte membrane must primarily be regarded as due to electrostatic forces. However, it also seems necessary to postulate the subsequent formation of co-ordinate linkages, because otherwise it is difficult to explain the greater affinity of the binding sites for Ca^{2+} as compared with univalent cations (Fig. 2; Table 2). Among all the various cations examined, trivalent yttrium has the greatest affinity for the binding sites (Table 4). When calcium and yttrium were present together in the suspending medium at the same concentration (2.5mM), the binding sites had about seven times as great an affinity for yttrium as for calcium. This observation accords well with the report by Williams & Peacocke (1967) that the affinity of a bone sialoprotein for yttrium was much greater than for calcium.

It is also noteworthy that Williams & Peacocke (1967) concluded that each binding site on bone sialoprotein consisted of three carboxyl groups. In the present work the slope of the regression line in Fig. 3 is 0.377, which suggests that the binding sites containing sialic acid have a mean negative charge of 2.7. It therefore seems possible that negative charges may normally occur in groups of three in situations where sialic acid residues are involved. In this connexion it should be mentioned that Gent *et al.* (1964) had concluded that the binding sites on erythrocyte 'ghost' cells were multivalent, with a mean value of 3.2. In this particular case, however, it is probable that negatively charged groups other than sialic acid residues were also involved.

As pointed out above, the regression line in Fig. 3 does not pass through the origin, but intersects the ordinate at $143 \mu\text{g}$ -atoms of calcium/litre of packed erythrocytes. Of this amount, about $16 \mu\text{g}$ -atoms/litre is present as firmly bound calcium (Harrison & Long, 1968), leaving $127 \mu\text{g}$ -atoms/litre bound to sites not involving sialic acid residues. The nature of these binding sites is unknown but it is possible that they could be provided by the membrane phospholipids. The acidic phospholipid phosphatidylserine, which possesses a single net negative charge, is present in human erythrocytes to the extent of about $800 \mu\text{mol}$ /litre of packed cells (Weed, Reed & Berg, 1963). Such a quantity is more than sufficient to supply the required binding sites, and is just enough to account for the increased binding of calcium by 'ghost' cells.

Among the limited number of species studied, rabbit erythrocytes present a rather special case, in that although they contain virtually no sialic acid, they still bind an appreciable amount of calcium. Abramson & Moyer (1936) reported that

rabbit erythrocytes exhibited a definite and measurable electrophoretic mobility, and from their data on net charges per cell together with values cited by Whittam (1964) for cell volume, it may be calculated that the total surface negative charges on 1 ml of packed erythrocytes of man and rabbit amount to 84×10^6 e.s.u. and 36×10^6 e.s.u. respectively. If the capacity to bind Ca^{2+} ions is a geometrical function of the surface charge, then rabbit erythrocytes would be expected to bind about $173 \mu\text{g}$ -atoms/litre of packed cells, compared with $403 \mu\text{g}$ -atoms/litre for human erythrocytes. The two experimental values found were 205 and $167 \mu\text{g}$ -atoms/litre, which are in fair agreement with the calculated value.

In an attempt to obtain further information about the binding sites for calcium, the principle of graphical analysis proposed by Scatchard (1949) has been applied. In Fig. 4 (curve *a*) the data of Fig. 1 (curve *a*) have been re-plotted with bound

Fig. 4. Scatchard (1949) plots of the results in Fig. 1. Curve (a) (human erythrocytes) has three linear portions, indicating three binding sites (I, II and III). Curve (b) (rabbit erythrocytes) has two linear portions, corresponding to sites I and III.

calcium as abscissa and the bound calcium/free calcium ratio as ordinate. The composite curve consists of three linear portions, which indicates three different types of binding site. Site I, which represents the firmly bound calcium of Harrison & Long (1968), has a stability constant of about 6×10^4 . Sites II and III have stability constants of 3.6×10^3 and 6×10^2 respectively. These values are to be compared with that found by Gent *et al.* (1964) for calcium bound to 'ghost' cells, namely 2.8×10^4 . When the results of Fig. 1 (curve *b*) for rabbit erythrocytes were plotted in the same way (curve *b*, Fig. 4), the portion corresponding to site II virtually disappeared, leaving sites I and III unaffected. This strongly suggests that site II involves the sialic acid residues.

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