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MAGNESIUM BUFFERING IN INTACT HUMAN RED BLOOD CELLS MEASURED USING THE IONOPHORE A23187

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SUMMARY

1. A method was developed for measuring the cytoplasmic magnesium buffering of intact red cells using the divalent cation selective ionophore A23187. Addition of A23187 to a suspension of red cells induces rapid equilibration of ionized magnesium across the cell membrane.

2. Entry of magnesium into red cells is associated with cell swelling and depolarization of the membrane potential.

3. At an external ionized magnesium concentration of about 0·15 mM corresponding to an internal ionized concentration of 0·4 mM the addition of A23187 did not produce a change in the magnesium content of the cells. This indicates that the normal ionized magnesium concentration inside the oxygenated red cell is about 0·4 mM.

4. The magnesium buffering curve for oxygenated, inosine-fed human red blood cells is adequately described by the existence of three buffer systems of increasing capacity and decreasing affinity. These are 0·15 mM with a $K_m < 10^{-7}$ M, probably structural magnesium bound within the cell proteins; 1·6 mM with a $K_m \cong 0{\cdot}08$ mM, mainly ATP and other nucleotides; and about 21–25 mM with a $K_m \cong 3{\cdot}6$ mM, a major portion of this being organic phosphates. It is suggested that the contribution of 2,3-DPG to the low affinity site involves each phosphate group acting as an independent binding site for magnesium.

INTRODUCTION

The plasma membrane of human red blood cells has such a low permeability to magnesium (Rogers, 1961; Ginsburg, Smith, Ginsburg, Reardon & Aikawa, 1962) that it was not possible until recently to alter the magnesium content of red cells in a controllable manner without disruption of the cell membrane. The dependence of fundamental physiological functions on the concentration of magnesium was therefore studied using reconstituted ghosts (Bodemann & Hoffman, 1976) and broken membrane fragments (Dunham & Glynn, 1961; Schatzmann, 1977). Recently the introduction of the divalent cation selective ionophore A23187 (Reed & Lardy, 1972), has made it possible to render the cell membrane selectively permeable to calcium and magnesium. The dependence of metabolism and ion transport on the concentration of magnesium can now be studied in the intact cell.

The red cell contains many substances which bind magnesium, thus reducing the concentration of ionized magnesium to a fraction of the total. It is the concentration

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of ionized magnesium, however, which regulates the activity of enzymes, some directly, for instance pyruvate kinase (see Wacker, 1969), and some indirectly by determining the concentration of the true substrate. Either the magnesium-bound form or the free, unbound form, may be the true substrate. In the case of creatine kinase, for instance, MgATP rather than ATP is the substrate (see Wacker, 1969). Thus to evaluate the role of magnesium in the regulation of cell processes it is not only important to control the magnesium content of the cell, but also to measure the concentration of ionized magnesium at each level of total magnesium.

In this paper we describe a method using the ionophore A23187 which enabled us to control the magnesium content of intact red cells and at the same time to measure the concentrations of ionized and bound forms. We were therefore able to measure magnesium buffering by oxygenated, inosine-fed red cells over a wide range of magnesium concentrations and to identify the major buffer systems.

A brief account of some of the work has appeared elsewhere (Flatman & Lew, 1977*a*).

METHODS

Principle of the method

Addition of the ionophore A23187 to well stirred red cell suspensions leads to rapid equilibration of magnesium between cells and medium. The cytoplasmic magnesium buffering curve for intact cells can be constructed by measuring the chloride distribution ratio and the magnesium content of cells over a wide range of magnesium concentrations.

Preparation of cells

Cells from freshly drawn, heparinized blood were washed 3 times in at least 5 volumes of medium A (75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, pH 7·7 at 37 °C) containing 0·1 mM-EGTA to chelate contaminant calcium. Care was taken to ensure that all of the buffy coat was removed. The cells were then washed twice in medium A, packed at 2500 *g* for 5 min and stored on ice until used (usually less than half an hour). The packed cells were then added to the incubation medium to give a final haematocrit of about 10%.

Composition of the media

The composition of each medium was carefully chosen to minimize changes in the sodium and potassium content or volume of the cells during the incubation. Since the addition of A23187 allows even contaminant ionized calcium to activate the calcium-sensitive potassium channel (Lew & Ferreira, 1976), 10 μM-EGTA was always added to the medium. In addition, the potassium content of the cells was clamped at a constant level by increasing the potassium content of the medium to 75 mM (see Ferreira & Lew, 1976). Recently Flatman & Lew (1977*b*) have shown that A23187 can transport sodium into red cells when the level of ionized magnesium in the medium is low (10^{-8} to 10^{-5} M). The amount of sodium transported can be drastically reduced by replacing part of the external sodium by potassium. Hence using medium A instead of a more physiological, high sodium medium, reduces by a factor of about 5 the amount of sodium transported during experiments carried out at low magnesium concentrations. Medium A containing 10 μM-EGTA was therefore chosen for most experiments. Fig. 1 shows the effect of adding 10 μM-A23187 to a 10% suspension of red cells in medium A containing 10 μM-EGTA and 0·15 or 8·0 mM-$MgCl_2$. It can be seen that in both cases, the sodium and potassium contents of the cells were virtually unaffected by the addition of the ionophore. Inosine was normally included in the medium. This is an excellent glycolytic substrate (Whittam & Wiley, 1968) which also reduces the concentration of inorganic phosphate inside the cell to a very low level (Glynn, Lew & Lüthi, 1970). Reduction of intracellular inorganic phosphate diminished the likelihood that the solubility product for magnesium phosphate would have been exceeded when large amounts of magnesium had been taken up by the cells.

Procedure

The cell suspensions were incubated at 37 °C with constant magnetic stirring in plastic or Pyrex vials. After a short equilibration period of about 10 min, 100 μl. samples were taken to measure the initial magnesium content of the cells and the haematocrit of the suspension. At a time designated $t = 0$ a small quantity of a concentrated stock solution of A23187 (1·91 mM in absolute ethanol) was added to give a final concentration of 10 μM. 5–10 sec before the indi-

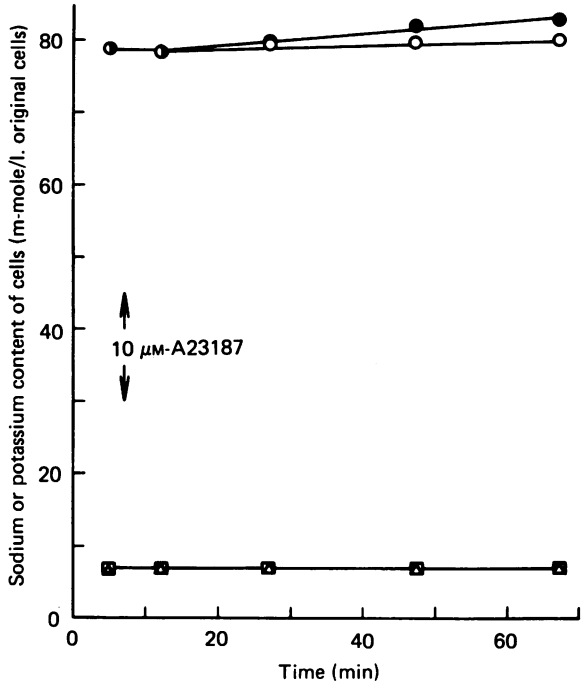

Fig. 1. The effect of A23187 on the sodium and potassium content of human red cells. Red cells were suspended at a haematocrit of 10% in medium A containing 10 μM-EGTA and the magnesium concentration indicated. 1 ml. samples were taken to measure the initial sodium and potassium contents of the cells. After the addition of 10 μM-A23187 further samples were taken as indicated to measure the new sodium and potassium contents of the cells. ○, potassium content, medium containing 0·15 mM-magnesium chloride; ●, potassium content, medium containing 8·0 mM-magnesium chloride; △, sodium content, medium containing 0·15 mM-magnesium chloride; □, sodium content, medium containing 8·0 mM-magnesium chloride. Lines were drawn by eye.

cated sampling time, a 100 μl. sample was taken to measure the new magnesium content of the cells. Since the sample was taken with a micropipette fitted with a plastic tip of low thermal conductivity it was assumed that the incubation continued in the tip right up to the indicated sampling time. At this point the sample was ejected into an Eppendorf microcentrifuge tube containing 0·9 ml. ice-cold inactivation medium (medium A + 2 mM-EDTA) and 0·4 ml. of the oil di-*n*-butylphthalate (density 1·042 g/ml.). The tube was capped, inverted to mix the contents and then centrifuged at 8000 *g* for 10 sec. The ionophore-induced permeability was quickly inactivated by dilution of the ionophore concentration and by the reduction in temperature and the cells were separated from the medium within 5 sec of the indicated sampling time. Since the inactivation medium contained EDTA, loosely bound magnesium was removed from external sites on the cell membrane. The tubes were set aside and processed in a batch at the end of the experiment.

During the new magnesium equilibrium state samples were taken where necessary to measure the haematocrit (0·1 ml.), the sodium and potassium content of the cells (1·0 ml.), the water content of the cells (1·0 ml.) and the chloride distribution ratio (1·0 ml.).

Measurement of cell magnesium

The diluted medium and oil were removed by suction. The caps and walls of the microcentrifuge tubes were cleaned with a cotton swab making sure that no aqueous drops remained and the cells were lysed in 500 μl. distilled water. 50 μl. 55% TCA was added to precipitate the protein and to release the bound magnesium. The tubes were vortexed, allowed to stand at room temperature for at least 10 min and then centrifuged at 8000 *g* for 3 min. The supernatant was diluted with 640 μl. distilled water and the magnesium concentration determined by atomic absorption spectroscopy. On a few occasions it was necessary to dilute the supernatant further with 2·5% TCA to bring the concentration of magnesium within the operating range of the spectrometer. All blanks and standards were prepared in 2·5% TCA using the same 55% stock solution which was used to prepare the cell samples. No magnesium contamination was found in the TCA or di-*n*-butylphthalate supplied by B.D.H. or in the Eppendorf microcentrifuge tubes. Since no magnesium could be detected in the TCA-cell pellets after they had been dissolved in 50% nitric acid it was assumed that all the cell magnesium is extracted by treatment with 5% TCA. It was found, however, that 5% TCA produced light scattering in the spectrometer and since no background corrector was available it was necessary to dilute the TCA concentration to 2·5% as described above.

Measurement of haematocrit

The haematocrit of each cell suspension was determined by adding a 100 μl. sample of the suspension to 5·0 ml. 0·5% NH_4OH and recording the absorption at 540 nm. The packed cell (100% haematocrit) absorbance for a 1 cm pathlength was 284.

Measurement of cell potassium and sodium

A 1 ml. sample of the cell suspension was centrifuged at 8000 *g* for 1 min. The supernatant was removed and the cells washed 4 times in a buffered choline chloride solution at 0 °C. The cells were then lysed in 3 ml. distilled water and the sodium content measured by flame photometry. A sample of this lysate was diluted twelvefold and the potassium content measured by flame photometry. The exact quantity of cells lysed was determined by measuring the absorbance of the diluted lysate at 540 nm and assuming that the packed cell absorbance was 284. In this way the sodium and potassium contents of the cells were calculated and expressed in m-mole/l. original cells. Standards for flame photometry were prepared from a stock solution containing 100 mM-potassium chloride and 10 mM-sodium chloride (Funder & Wieth, 1966).

Measurement of cell water content (f_w)

A 1 ml. sample of the cell suspension was transferred to a vial containing a small quantity of [^{60}Co(III)]EDTA, and incubated at 37 °C for 5 min. The sample was then transferred to an Eppendorf microcentrifuge tube containing 0·2 ml. di-*n*-butylphthalate and spun at 8000 *g* for 5 min. A sample of the cell pellet was weighed and then dried to constant weight at 105 °C. The size of the extracellular space in the pellet was measured by determining the activities of ^{60}Co in the dried pellet and in the supernatant. From these data the water content of the cells (f_w) was calculated in litres water per litre cells, assuming that their density was 1·1 (Bernstein, 1959).

Measurement of the chloride distribution ratio (r)

A 1 ml. sample of the suspension was transferred to a vial containing a tracer amount of ^{36}Cl (approximately 1 μc/ml. suspension) and incubated at 37 °C for 5 min. The sample was transferred to an Eppendorf microcentrifuge tube containing 0·2 ml. di-*n*-butylphthalate and spun at 8000 *g* for 5 min. The ^{36}Cl activities in the cell pellet and the supernatant were determined by liquid scintillation counting after precipitation of the protein with 5% TCA. The cell activity was corrected for the extracellular medium trapped in the pellet using the value for extracellular space determined during the measurement of water content (see above).

The chloride distribution ratio is given by the expression:

$$r = f_w [Cl^*]_o/[Cl^*]_i, \quad (1)$$

where $[Cl^*]_o$ and $[Cl^*]_i$ are the ^{36}Cl activities in a given volume of medium and cells respectively.

Calculation of the intracellular concentrations of bound and ionized magnesium

A23187 is a carboxylic acid ionophore which causes the electroneutral exchange of one magnesium ion for two protons (Reed & Lardy, 1972; Case, Vanderkooi & Scarpa, 1974; Kafka & Holz, 1976). Hence

$$\frac{[Mg^{2+}]_i^\infty}{[Mg^{2+}]_o^\infty} = \left(\frac{[H^+]_i}{[H^+]_o}\right)^2, \quad (2)$$

where $[Mg^{2+}]_i^\infty$ and $[Mg^{2+}]_o^\infty$ are the equilibrium concentrations of ionized magnesium in the cells and medium respectively. $[H^+]_i$ and $[H^+]_o$ are the proton concentrations in the cells and medium.

In the red cell there is a rapid anion shuttle which effectively equilibrates the proton and chloride concentration gradients so that

$$\frac{[Mg^{2+}]_i^\infty}{[Mg^{2+}]_o^\infty} = \left(\frac{[Cl^-]_o}{[Cl^-]_i}\right)^2 = r^2 \quad (3)$$

and

$$[Mg^{2+}]_i^\infty = r^2 [Mg^{2+}]_o^\infty, \quad (4)$$

where $[Cl^-]_i$ and $[Cl^-]_o$ are the equilibrium concentrations of chloride in the cells and medium respectively and r is the chloride distribution ratio.

The magnesium content of the cells, expressed in m-mole/l. original cells ($[Mg]_i^\infty$) was calculated from the magnesium concentration in the cell lysate and the haematocrit of the cell suspension. Since the uptake of magnesium by the cells is accompanied by swelling (see later), the intracellular magnesium concentration, $[Mg]_T^\infty$, expressed in m-mole/l. cell water was calculated from the following expression:

$$[Mg]_T^\infty = \frac{[Mg]_i^\infty (1-f_{wi})}{f_{wi}(1-f_{wo})}, \quad (5)$$

where f_{wi} and f_{wo} are the water contents of the cells (expressed in litres water per litre cells) during the new magnesium equilibrium state and the initial unswollen state respectively. The concentration of bound magnesium inside the cell, $[Mg]_b^\infty$, can then be obtained by subtraction:

$$[Mg]_b^\infty = [Mg]_T^\infty - r^2[Mg^{2+}]_o^\infty. \quad (6)$$

The concentration of ionized magnesium in the medium at equilibrium was calculated from the initial and final magnesium contents of the cells and the initial magnesium concentration in the medium. All magnesium in the medium was assumed to be ionized except when EDTA was present. When EDTA was present in the medium, the concentration of ionized magnesium was calculated by the method of Wolf (1973). The following dissociation constants were obtained from the tables of Sillén & Martell (1971) and corrected for use at 37 °C: first proton-EDTA dissociation constant, K_1, $10^{-9.97}$ M; second proton-EDTA dissociation constant, K_2, $10^{-6.03}$ M; magnesium-EDTA dissociation constant, K_L, $10^{-8.82}$ M. The apparent magnesium-EDTA dissociation constant at 37 °C and pH 7·5 calculated from these data was $10^{-6.33}$ M.

RESULTS

Initial magnesium content of red cells incubated in the absence of A23187

Table 1 shows the results of experiments measuring the initial magnesium content of red cells using the method described above. The cells were incubated in a variety of different media containing between 10^{-8} and 8×10^{-3} M-ionized magnesium. The results in Table 1 show that the human red cell contains about 2·3–2·4 m-mole magnesium per litre of cells. This range of values coincides closely with those reported

in the literature obtained using different analytical techniques (Hunt & Manery, 1970; Stiles, Batsakis, Hardy & Briere, 1965; Ginsburg *et al.* 1962). Although a rather small sample of donors was used in this study, the magnesium contents of the cells of two subjects were measured over a long period of time. The magnesium content of the cells of V.L.L. was observed for $2\frac{1}{2}$ yr during which time the maximum range for the values was 12% of the mean. The maximum range of values for the cells of P.F. over a period of 2 yr was only 8%. Table 1 also shows that the magnesium contents

TABLE 1. The magnesium content of human red cells

Donor	Details	Magnesium content of cells $[Mg]_i$ (m-mole/l. cells)
V.L.L.	Freshly drawn, inosine-fed and oxygenated	$2{\cdot}303 + 0{\cdot}010$ ($n = 72$) Minimum value: $2{\cdot}164 + 0{\cdot}015$ ($n = 5$) Maximum value: $2{\cdot}449 + 0{\cdot}017$ ($n = 9$)
V.L.L.	Starved for 6 h, oxygenated	$2{\cdot}294 + 0{\cdot}024$ ($n = 13$)
P.F.	Freshly drawn, inosine-fed and oxygenated	$2{\cdot}412 + 0{\cdot}011$ ($n = 50$) Minimum value: $2{\cdot}340 + 0{\cdot}015$ ($n = 3$) Maximum value: $2{\cdot}485 + 0{\cdot}032$ ($n = 4$)
Blood-bank	Stored in acid-citrate-dextrose (ACD), oxygenated	
	2 days old	$2{\cdot}378 + 0{\cdot}010$ ($n = 9$)
	4 days old	$2{\cdot}355 + 0{\cdot}025$ ($n = 9$)
	4 days old	$2{\cdot}187 + 0{\cdot}032$ ($n = 10$)
	6 days old	$2{\cdot}337 + 0{\cdot}009$ ($n = 13$)

of four batches of cells which had been stored in acid-citrate-dextrose (ACD) for between 2 and 6 days were similar to those of the fresh cells. In addition, Table 1 shows that freshly drawn red cells, incubated for 6 hr at 37 °C in a medium lacking glucose or inosine have the same magnesium content as fed cells.

During the course of many experiments the magnesium content of human red cells was found to be independent of the sodium (0–150 mM), potassium (0–145 mM), choline (0–145 mM) and magnesium (10^{-8}–8×10^{-3} M) contents of the medium during 1–2 hr incubations. The presence or absence of ouabain (10^{-4} M), inosine (10 mM) or glucose (10 mM) had no effect on the magnesium content of cells. These results confirm the notion, based on ^{28}Mg tracer studies (Rogers, 1961; Ginsburg *et al.* 1962), that the red cell membrane is virtually impermeable to magnesium. Since the magnesium content of the red cells of a given individual is constant over a long period of time and since no system has yet been found which is capable of transporting magnesium across the red cell membrane, it would seem likely that the magnesium content of the red cell is a genetically programmed load designed to meet precisely the needs of the mature cell during its life span. Thus the magnesium content of the mature cell seems to be determined in a manner similar to that by which the enzyme content is determined.

Magnesium content of red cells after the addition of A23187

Fig. 2 shows the effect of adding 10 μM-A23187 to a 10% suspension of red cells incubated in medium A containing 10 μM-EGTA, 10 mM-inosine and the indicated initial magnesium concentrations. Magnesium usually entered or left the cells rapidly,

depending on the concentration of magnesium in the medium. However, when the medium contained between 0·12 and 0·15 mM-ionized magnesium the addition of the ionophore did not cause a change in the magnesium content of the cells. Under these conditions the concentration of ionized magnesium in the cells was in electrochemical equilibrium with that in the medium before the addition of A23187.

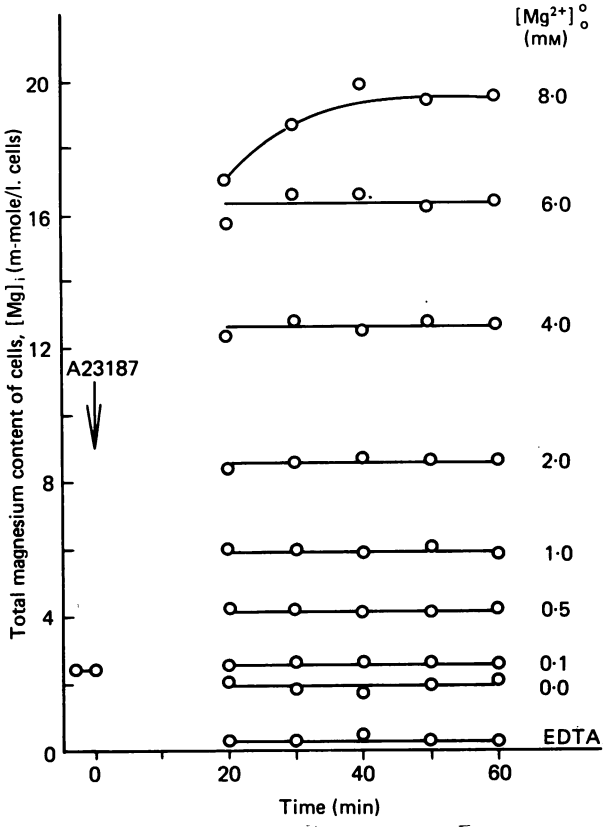

Fig. 2. The magnesium content of red cells after the addition of 10 μM-A23187. Ten per cent suspensions of freshly drawn red cells were incubated at 37 °C in medium A containing 10 μM-EGTA, 10 mM-inosine and the indicated initial magnesium concentrations ($[Mg^{2+}]^{o}_{o}$). Two samples were taken to measure the initial magnesium content of the cells. After the addition of 10 μm-A23187 further samples were taken as indicated to measure the new level. The lines represent the mean magnesium content of the cells during the new equilibrium state except at the highest magnesium level where the line was drawn by eye.

In the experiment shown in Fig. 2 new magnesium equilibrium levels had been attained within 20 min when the medium contained less than 6 mM-magnesium chloride, but took up to 40 min to reach equilibrium if the medium contained 8 mM-magnesium chloride. In separate studies (not shown) it was found that new equilibrium levels were reached within 5–10 min if the medium contained between 10^{-8} and 2×10^{-3} M-magnesium chloride. Once attained, the new magnesium levels were maintained for the duration of the experiment, which was 40 min in the experiment shown in Fig. 1, and in some cases up to 2 hr (not shown). During these incubations the degree of cell

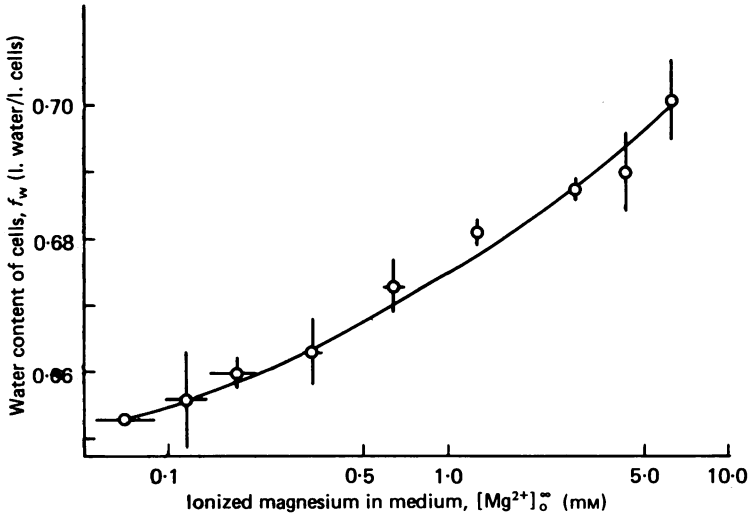

Fig. 3. The water content of the cells, f_w, plotted as a function of the new equilibrium magnesium concentration in the medium ($[Mg^{2+}]_o^\infty$). Ten per cent suspensions of red cells were incubated in medium A containing 10 μM-EGTA, 10 mM-inosine and between 0 and 8 mM-$MgCl_2$. 10 μM-A23187 was added to the suspensions and at least 20 min later 1 ml. samples were taken to measure the water content of the cells, f_w. Each point represents the mean and S.E. of mean of four experiments. The curve was drawn by eye.

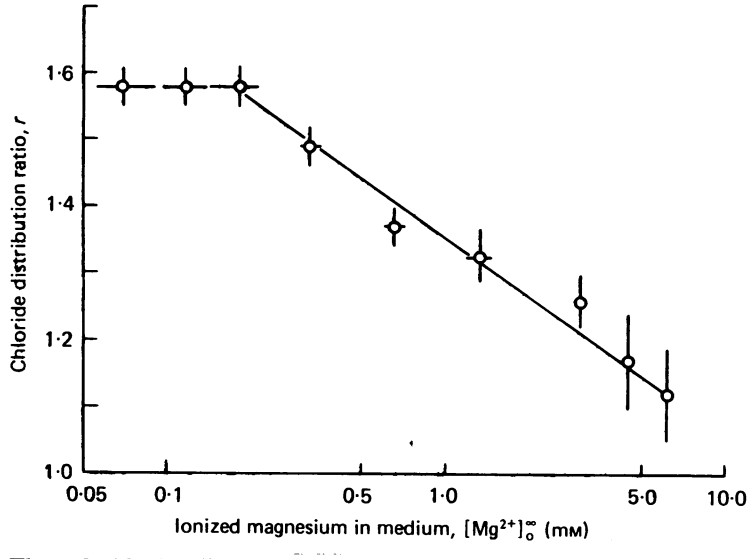

Fig. 4. The chloride distribution ratio, r, plotted as a function of the new equilibrium magnesium concentration in the medium, $[Mg^{2+}]_o^\infty$. Ten per cent suspensions of red cells were incubated at 37 °C in medium A containing 10 μM-EGTA, 10 mM-inosine and between 0 and 8 mM-$MgCl_2$. At least 20 min after the addition of 10 μM-A23187 1 ml. samples were taken to measure the chloride distribution ratio, r. Each point represents the mean and S.E. of mean of four experiments. The curve was drawn by eye.

lysis was usually less than 2% of the cell volume. The separate values of the magnesium contents of the cells sampled at 10 min intervals during the new magnesium equilibrium state were averaged and the mean value used to calculate the new equilibrium concentration of ionized magnesium in the medium ($[Mg^{2+}]_o^\infty$). Figs. 3 and 4 show the water content (f_w) and chloride distribution ratio (r) for cells, plotted as a function of the concentration of ionized magnesium in the medium at equilibrium.

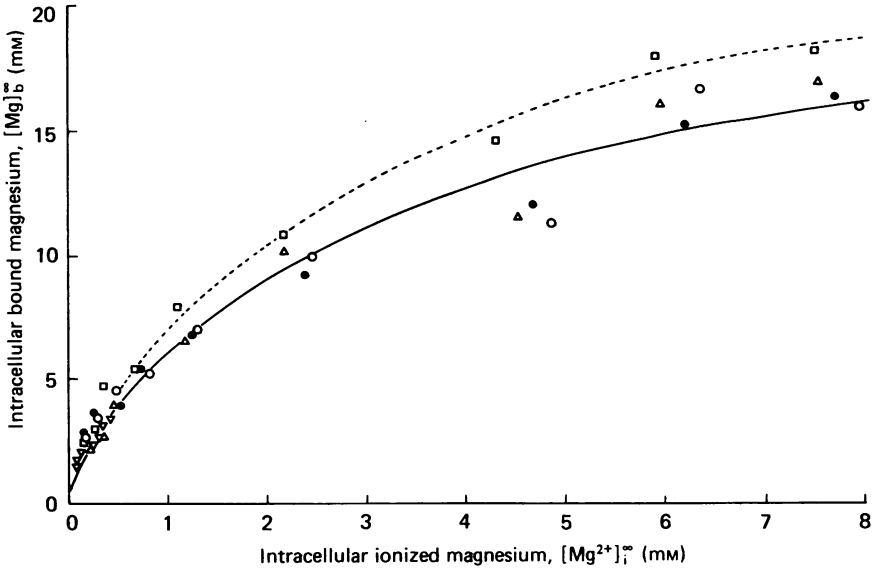

Fig. 5. The magnesium-buffering curve for freshly drawn, inosine-fed, oxygenated red cells. This Figure shows the result of five experiments, each represented by a different symbol, using the cells from a single donor (V.L.L.). In four of the experiments the cells were incubated in medium A containing 10 μM-EGTA and 10 mM-inosine. In the fifth, represented by the open circles, the medium also contained 10^{-4} M-ouabain. Each point represents the mean and S.E. of mean of five measurements. The continuous line drawn through the points was calculated from eqn. (7) using the following parameters: $K_0 = 10^{-7}$M, $C_0 = 0{\cdot}15$ mM; $K_1 = 0{\cdot}08$ mM, $C_1 = 1{\cdot}6$ mM; $K_2 = 3{\cdot}6$ mM, $C_2 = 21$ mM. The dotted line was also calculated from eqn. (7) using the same parameters except that $C_2 = 25$mM.

It can be seen that as $[Mg^{2+}]_o^\infty$ increases the cells gain water and the chloride distribution ratio falls. The decline of the chloride distribution ratio from 1·58 in cells containing a normal magnesium content ($[Mg^{2+}]_o^\circ = 0{\cdot}12$ mM) to about 1·2 in those which contain about 19 m-mole magnesium/l. cells ($[Mg^{2+}]_o^\circ = 8$ mM) indicates that the cells progressively depolarize as they take up magnesium. The decline in the ratio is caused by magnesium binding to the phosphate portion of the fixed anion inside the cell thus reducing their net negative charge. This reduction in charge results in a depolarization of the cell membrane, and an increase in the chloride content of the cell. The data suggest that the increase in chloride content is larger than that predicted from the entry of magnesium. Since all the chloride that enters remains in an ionized form whereas the magnesium is mainly bound (see later), there is an increase in water content and consequent cell swelling.

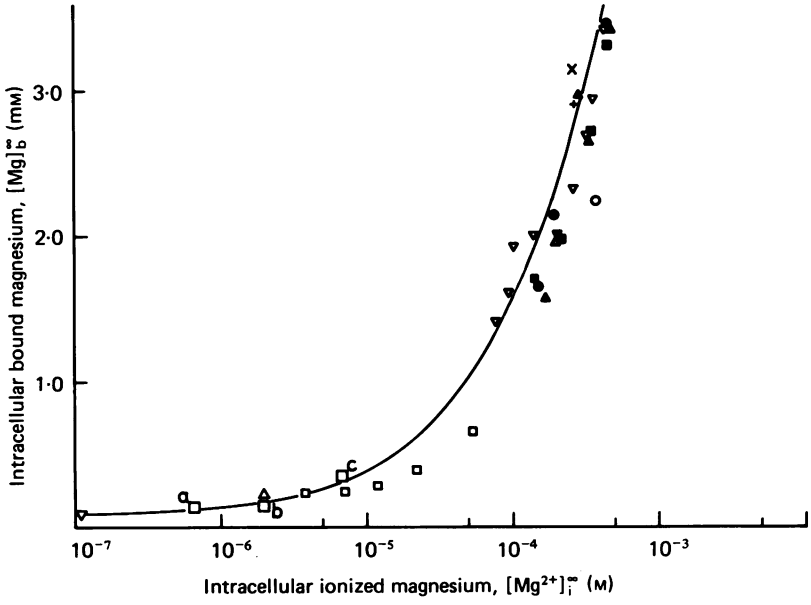

Fig. 6. Magnesium-buffering curve for freshly drawn, inosine-fed, oxygenated red cells at low magnesium concentration. The figure shows the results of nine separate experiments using both low added concentrations of magnesium and magnesium-EDTA buffer mixtures to examine the magnesium binding by red cells at very low ionized magnesium concentrations. The Figure includes data from experiments carried out in a high sodium medium (see text) and from experiments where the medium contained 10^{-4} M-ouabain. All media contained 10 μM-EGTA and 10 mM-inosine.

Symbol	Experiment	Medium
▽	6A	A
●	6B1	high sodium
■	6B2	high sodium + 10^{-4} M-ouabain
▲	6C1	A
×	7A1	high sodium
+	7A2	high sodium + 10^{-4} M-ouabain
○	7B1	A
△	7B2	A + 10^{-4} M-ouabain
□	8	A

The three large squares represent the results of several experiments where the points overlap:

a points from experiments, 7A1, 7A2, 7B1, 7B2, 8
b points from experiments, 7A1, 7A2, 7B1, 8
c points from experiments, 7A1, 7A2, 7B1, 7B2

The continuous line was drawn according to eqn. (7) using the following values: $K_0 = 10^{-7}$ M, $C_0 = 0{\cdot}15$ mM; $K_1 = 0{\cdot}08$ mM, $C_1 = 1{\cdot}6$ mM; $K_2 = 3{\cdot}6$ mM, $C_2 = 21$ mM. Each point represents the mean and S.E. of mean of five measurements.

Magnesium buffering in inosine-fed red cells

Fig. 5 shows the concentration of bound magnesium ($[Mg]_b^\infty$) plotted as a function of the intracellular ionized magnesium, both of which were calculated from data similar to that presented in Figs. 2, 3 and 4. The figure shows the results of five

experiments carried out on oxygenated, inosine-fed cells from a single donor (V.L.L.). Fig. 6 shows the results of nine experiments that examined the magnesium-buffering of fed red cells (V.L.L.) at low magnesium concentrations. These ranged from 10^{-7} to 5×10^{-4} M in the cells at equilibrium ($[Mg^{2+}]_i^\infty$), and were achieved by adding EDTA (up to 2 mM) and $MgCl_2$ to the medium. Under these conditions magnesium left the cells and added to that already in the medium. The concentration of ionized magnesium in the medium, and hence in the cells, was calculated according to the method of Wolf (1973, see Methods). Fig. 6 includes data from experiments in which the cells were incubated in the normal high potassium medium (medium A) containing 10 mM-inosine, 10 μM-EGTA, with and without 10^{-4} M-ouabain. It also contains data from experiments carried out in a high sodium medium containing 150 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 10 μM-EGTA, with and without 10^{-4} M-ouabain. All the data fell on the same curve and no consistent difference could be detected between cells incubated in the different media.

The experimental method described measures the total acid-labile magnesium content of red cells. It cannot distinguish between magnesium which is bound to a single site on each buffer molecule and magnesium which is bound to sites present in many copies on a single buffer molecule. Assuming that the magnesium-buffer interactions obey the mass law, the concentration of magnesium bound to the cell should be described by the following equation

$$[Mg]_b^\infty = \sum_{j=1}^{n} \frac{[Mg^{2+}]_i^\infty}{([Mg^{2+}]_i^\infty + K_j)} C_j, \tag{7}$$

where C_j is the concentration of the j_{th} magnesium buffer with a dissociation constant of K_j (mM). If the site is present p times on a given buffer molecule the concentration of the buffer in the cell is C_j/p mM.

The data in Figs. 5 and 6 have been fitted with eqn. (7) for one, two or three different species of magnesium buffer. A minimum of three classes of site with different magnesium affinities gave a reasonable fit (by eye).

For convenience, the three classes of binding site have been given the following names, in descending order of magnesium binding affinity: K_0 system, K_1 system and K_2 system. The continuous line in Figs. 5 and 6 and the dotted line in Fig. 5 are the curves obtained from eqn. (7) with the parameters given in the legend to Fig. 5. The data obtained in most experiments could be fitted, assuming that the concentration of the K_2 system was 21 mM. In one experiment, however, (open squares in Fig. 5) all the data points were elevated relative to the rest and were better fitted if the concentration of the K_2 system was assumed to be 25 mM. Although there were insufficient data at high magnesium concentrations to make a detailed examination of the uniqueness of fit, it was found that changing the parameter chosen for the K_1 system by more than 10% caused a deterioration in the quality of the fit to the data shown in Fig. 5. However, the fit was relatively insensitive to small (about 10%) concurrent changes in the parameters of the K_2 system. Hence, increasing K_2 to 3·9 mM whilst increasing C_2 to 23 mM gave a fit very similar to the solid line in Figs. 5 and 6.

Magnesium buffering in starved cells

Fig. 7 shows the magnesium buffering curve for red cells (V.L.L.) which had been starved for 6 hr at 37 °C before starting the experimental incubation. At this point the ATP concentration in the cells was 0·2 m-mole/l. cell water (measured by the fire-fly method, see Glynn & Hoffman, 1971). The continuous line through the points was drawn using eqn. (7) with the parameters given in the legend to Fig. 7.

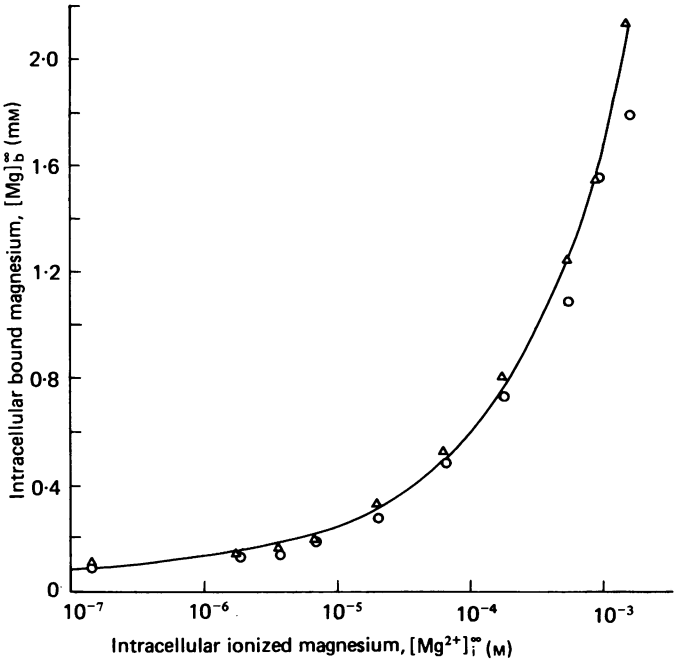

Fig. 7. The magnesium-buffering curve for cells which had been starved for 6 h before measurement. The cells were incubated at 37 °C and 30% haematocrit in medium A containing 10 μM-EGTA for 6 hr. The cells were then washed 3 times and resuspended in either medium A (Δ) or in a high sodium medium (○). Both media contained 10 μM-EGTA. The continuous line was drawn according to eqn. (7) using the following parameters: $K_0 = 10^{-7}$ M, $C_0 = 0{\cdot}15$ mM; $K_1 = 0{\cdot}08$ mM, $C_1 = 0{\cdot}6$ mM; $K_2 = 3{\cdot}6$ mM, $C_2 = 5$ mM. Each point represents the mean and S.E. of mean of five measurements.

Effect of repeated ionophore addition on the magnesium content of red cells

It is important to establish whether the ionophore A23187 affects the way in which intracellular buffers bind magnesium and whether it can contribute significantly to the magnesium binding capacity of the cells. Fig. 8 shows the effect of repeated addition of A23187 to a suspension of 6-day-old blood-bank red cells suspended in a medium containing 2 mM-magnesium chloride and 10 mM-inosine. The magnesium content of the cells rose from an initial level of $2{\cdot}35 \pm 0{\cdot}03$ to $6{\cdot}19 \pm 0{\cdot}06$ m-mole/l. cells after the first addition of 3·2 μM-A23187. Subsequent addition of 3·7, 4·3 and 15·9 μM-A23187 had little effect on the magnesium content of the cells measured 10 min after the new addition. The respective magnesium contents were $6{\cdot}17 \pm 0{\cdot}06$, $6{\cdot}13 \pm 0{\cdot}05$ and $6{\cdot}07 \pm 0{\cdot}06$ m-mole/l. original cells ($n = 4$ for each addition).

Similar results were found when cells were incubated in a medium containing no added magnesium. Addition of A23187 caused a loss of magnesium from the cells in this case, but the new equilibrium concentration was not affected by further ionophore additions.

All batches of freshly drawn red cells, whether fed (Fig. 6) or starved for 6 hr (Fig. 7) were found to contain between 30 and 100 μmole bound magnesium per

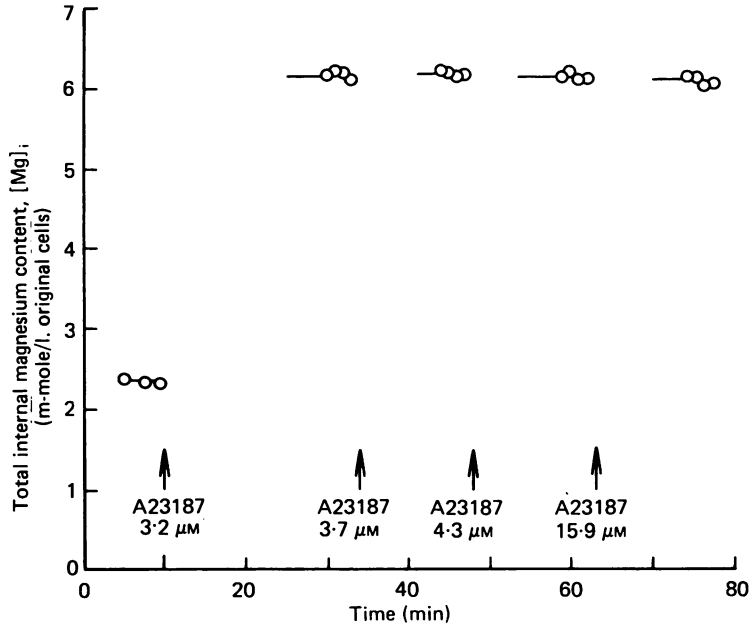

Fig. 8. The effect of repeated additions of A23187 on the magnesium content of human red cells. Red cells were incubated at 37 °C in a medium containing 20 μM-EGTA, 10 mM-inosine and 2 mM-magnesium chloride. Three samples were taken to measure the initial magnesium content of the cells. A23187 was then added as follows: 3·2 μM at t = 10 min, 3·7 μM at t = 34 min, 4·3 μM at t = 48 min and 15·9 μM at t = 62 min. 20 minutes was allowed to elapse after the first addition before four samples were taken at 1 min intervals to measure the new magnesium content. After the subsequent additions of A23187 only 10 min passed before sampling. The lines represent the mean magnesium content during the new equilibrium states.

litre of cells when the medium contained submicromolar concentrations of ionized magnesium. In order to account for the magnesium bound inside red cells at very low levels of ionized magnesium and to obtain reasonable fits to the data shown in Figs. 6 and 7 it was necessary to postulate a buffer with a very high affinity for magnesium ($K_0 = 10^{-7}$ M, $C_0 = 150$ μM). It is interesting that starvation of the cells did not affect the quantity or properties of this high affinity site. It could be argued that the high affinity magnesium binding site is in fact A23187 itself, since it has been shown to accumulate inside cells (Chandler & Williams, 1977). Recently, Simonsen & Lew (1980) have shown that at least 90% of the A23187 added to a red cell suspension is taken up by the cells. Hence if 10 μM-A23187 were initially added to a 10% suspension of red cells, the concentration in the cells could reach a level of about

90 μM/l. cells. To see whether the high affinity magnesium binding was to A23187, a 10% suspension of red cells was incubated in a medium containing 2 mM-EDTA and 10 μM-A23187 for 30 min, then the cells were separated from the medium by centrifugation and washed 5 times in at least 20 vols. ice-cold medium containing 5 mM-sodium chloride, 145 mM-choline chloride, 10 mM-Tris-Cl and 10 μM-Tris-EGTA. Under these conditions the ionophore rapidly partitions out of the cell. The magnesium content of these washed cells was $0{\cdot}062 \pm 0{\cdot}01$ m-mole/l. cells ($n = 10$) which is within the range (0·03–0·10 m-mole/l. cells) of values found for cells still containing the ionophore. To prove that the ionophore had been removed by the washing procedure the washed cells were divided into two groups; one was incubated in a medium containing 100 μM-Mg^{2+} and the other in a similar medium but also containing 10 μM-A23187. The cells incubated in the absence of A23187 contained $0{\cdot}092 \pm 0{\cdot}019$ m-mole magnesium/l. cells ($n = 4$) after a 1 hr incubation whereas those incubated in the presence of A23187 contained $1{\cdot}272 \pm 0{\cdot}048$ m-mole magnesium/l. cells ($n = 4$). The very low magnesium permeability of the washed cells shows that most of the ionophore had been effectively removed.

DISCUSSION

The method used to measure and alter the magnesium content of red cells is an adaptation of that originally developed by Ferreira & Lew (1976, 1977) to measure the calcium buffering by red cells in the presence of A23187. The modified version described here can be used not only to measure the capacity of red cells to buffer magnesium, but also to estimate the concentration of ionized magnesium in the unperturbed, oxygenated red cell. It was found that when the medium contained between 0·12 and 0·15 mM-ionized magnesium, the addition of A23187 did not cause a change in the magnesium content of the cells. It seems likely that the concentration of ionized magnesium inside the red cells under these conditions, which is about 0·4 mM, is the same as that inside the oxygenated red cell when no ionophore is present (see Flatman, 1980). Since the concentration of ionized magnesium in human serum is about 0·5 mM (Walser, 1961; Heaton, 1967), there is both an electrical and a chemical gradient favouring the entry of magnesium into red cells whilst they are in the peripheral circulation. If anything, however, red cells seem to lose magnesium as they age (Bernstein, 1959). Rogers (1961) and Ginsburg *et al.* (1962) have shown that ^{28}Mg exchanges extremely slowly between medium and red cells which suggests that the membrane has a low permeability to magnesium. Moreover, it has been shown here that red cell magnesium content does not depend on the composition of the media, at least in the short term. This also suggests a low membrane permeability. It is not clear whether this low permeability alone can explain the maintenance of a constant magnesium content below electrochemical equilibrium for the 120 day life span of a mature red cell. It is conceivable, however, that small amounts of magnesium can be actively extruded through the calcium pump.

Magnesium buffering in red cells

Magnesium buffering in fed (Figs. 5, 6) and starved (Fig. 7) red cells was measured and the data fitted with a curve calculated assuming that three classes of binding sites exist within the cell.

K_0 system

Examination of Figs. 6 and 7 shows that both fed and starved cells contain a considerable amount of bound magnesium when the ionized concentration is submicromolar. The site is present even when all the A23187 has been removed from magnesium-depleted cells. This indicates that A23187 which has been absorbed by the cell is not the high affinity binding site. Since the site is revealed in experiments where the medium contains EDTA to draw magnesium out of the cells and bind it, it could be argued that the high affinity site is merely magnesium trapped in the extracellular space of the cell pellet. However, when a 10% suspension of cells is incubated in the presence of 2 mM-EDTA and 10 μM-A23187, the medium finally contains about 220 μM-magnesium. Trapped extracellular fluid could therefore only account for 2–3 μmole/l. cells out of the 30–100 μmole/l. cells detected.

The data in Figs. 6 and 7 have been fitted by assuming that the cells contain about 150 μM of a buffer with a magnesium dissociation constant of 10^{-7} M. It is possible, however, that this is not magnesium bound to a classical, fast-dissociating binding site but magnesium trapped within proteins or within the cell membrane. Ferreira & Lew (1977) failed to find a similar high affinity site for calcium using a ^{45}Ca tracer technique but magnesium was present in all their experiments. The literature suggests that sites which bind magnesium with a high affinity will probably have an even higher affinity for calcium (see Williams, 1972). If magnesium can exchange between the cytoplasm (or medium) and the site, then it is likely that calcium can do the same. Since no such exchange has been found it seems that the magnesium is probably trapped within a protein or membrane and is not therefore exchangeable. Such trapped magnesium has been observed before. Wacker & Vallee (1964) report metallo-enzymes which bind stoichiometric amounts of magnesium strongly enough to isolate the enzymes complete with bound magnesium. Welt (1964) found that red cell membranes contained about 1 μmole magnesium per gram dry weight (equivalent to about 10 μmole/l. cells) which could not be removed by repeated washes. Similarly, Sanui (1970) found magnesium (0·3 m-mole/g nitrogen) bound to rat liver membranes. This could not be removed by washing with a medium containing 5 mM-EDTA and 3·6 mM-magnesium. The concentration of bound magnesium was constant over a pH range from 6 to 8 indicating that it was not sensitive to changes in the level of ionized magnesium in the medium.

K_1 system

Freshly drawn, oxygenated, fed red cells contain about 1·6 m-mole/l. cell water of a magnesium binding site with a dissociation constant of 0·08 mM (Figs. 5 and 6). Moreover, it can be concluded from the studies of Ferreira & Lew (1977) that, if this site binds calcium, then it does so with a lower affinity. The site is probably ATP which was found in the cells at a concentration of between 1·3 and 1·5 mM, (measured by the fire-fly assay, see Glynn & Hoffman, 1971). Under cellular conditions the dissociation constant for Mg-ATP is 0·083 mM (Berger *et al.* 1973), whereas the calcium dissociation constant is about 0·217 mM (Collier & Lam, 1970). Hence the concentration and properties of the K_1 system coincide closely with those of ATP. The small amount of the K_1 system not accounted for by ATP (about 0·1–0·3 mM) probably

comprises binding sites on other cellular constituents including those on the inside of the membrane itself (see Carvalho, Sanui & Pace *et al.*, 1963).

K_2 system

Most magnesium in the red cell at high $[Mg^{2+}]_i^\infty$ is bound to the K_2 system, which is present at a concentration of between 21 and 25 mM and has a dissociation constant of about 3·6 mM. The low affinity, high-capacity buffer is probably made up of a whole range of phosphate groups in the cell together with a small contribution from binding sites on amino acids and proteins. The major red cell constituent, haemoglobin, has been shown not to bind magnesium either in the oxygenated or deoxygenated state (Carr & Woods, 1955; Rose, 1968; Berger *et al.* 1973).

The total concentration of organic phosphate in the red cell is about 20 mM, of which 12 mM is contributed by 2,3-DPG, 4·5 mM by ATP and 3·5 mM by unspecified intermediates of the glycolytic and pentose shunt pathways (Deuticke, Duhm & Dierkesmann, 1971). In order to account for the high concentration of the K_2 system it is therefore necessary to assume that both phosphate groups of 2,3-DPG can bind magnesium. Previously, however, it has been assumed that 2,3-DPG only binds one magnesium ion to a site between the phosphate groups. This assumption was based on measurements made on a space filling model (Collier & Lam, 1970). The magnesium dissociation constant obtained by electrode and filtration methods and by making the above assumption was 1·44 mM (Collier & Lam, 1970). Reanalysis of the data, assuming that 2,3-DPG can bind two magnesium ions, one to each phosphate group, and that each phosphate group acts as an independent binding site, gives a magnesium dissociation constant of 3·3 mM. This value is close to the dissociation constant of the K_2 system obtained from the curve fit (3·6 mM). 2,3-DPG could therefore account for about 12 mM of the K_2 system. At least another 3·5 mM could be provided by the other phosphate esters and the remainder by minor cell constituents (possibly glutathione or RNA).

Magnesium binding in starved cells

Fig. 7 shows magnesium buffering by starved cells. As expected, 6 hr of starvation considerably reduced the number of available magnesium binding sites but the data could still by fitted by assuming that there were three classes of site with the same dissociation constants used for fed cells. Whereas the concentration of the K_0 system was unaffected by starvation, the concentration of the K_1 system was reduced to 0·6 mM and that of the K_2 system to only 5 mM. 0·2 mM of the K_1 system can be accounted for by the ATP, the remaining sites being provided by the membrane and perhaps by the break-down products of 2,3-DPG.

The large reduction in the amount of K_2 system agrees well with the disappearance of 2,3-DPG from cells which have been starved for 6 h. Whittam (1958) showed that the concentration of 2,3-DPG would have been less than 0·5 mM in these cells. The remaining K_2 system probably comprises the hydrolytic products of 2,3-DPG breakdown together with cellular constituents which bind magnesium and are unaffected by starvation.

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