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DEOXYGENATION PERMEABILIZES SICKLE CELL ANAEMIA RED CELLS TO MAGNESIUM AND REVERSES ITS GRADIENT IN THE DENSE CELLS

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SUMMARY

1. Our findings of a low total magnesium content in the dense fraction (over 1·118 g ml^{-1}) of sickle cell anaemia (SS) red cells seemed inconsistent with the low Mg^{2+} permeability and outward Mg^{2+} gradient seen in normal red cells, and prompted studies of the Mg^{2+} permeability and equilibria in the SS cells.

2. Deoxygenation and sickling induced Mg^{2+} permeabilization in SS cells, supporting non-specificity of the sickling-induced cation permeabilization, previously described for Na^+, K^+ and Ca^{2+}. The extent of Mg^{2+} permeabilization was comparable in SS cells with normal or high density.

3. Compared with normal-density SS cells and normal red cells, the dense SS cells showed a much larger increase in the fraction of ionized magnesium ($[Mg^{2+}]_i$) on deoxygenation, resulting in $[Mg^{2+}]_i$ levels sufficient to reverse the normal inward direction of the transmembrane Mg^{2+} gradient.

4. The molar ratio of 2,3-diphosphoglycerate (2,3-DPG) to haemoglobin was markedly reduced in the dense SS cells. Since 2,3-DPG and ATP are the main cytoplasmic Mg^{2+} buffers, their further reduction upon binding to deoxyhaemoglobin accounts for the high $[Mg^{2+}]_i$ in the deoxygenated dense SS cells; the resulting outward electrochemical Mg^{2+} gradient, together with sickling-induced Mg^{2+} permeabilization, could explain the decreased total magnesium content of these cells.

5. The above findings suggested that the documented low sodium pump fluxes in dense SS cells may result from an increased Mg^{2+}:ATP ratio, which is known to inhibit Na^+–K^+ exchange fluxes through the sodium pump. If so, deoxygenation, by increasing the Mg^{2+}:ATP ratio, should inhibit the pump further, whereas increasing ATP should relieve the inhibition. Experiments designed to test this possibility showed that in these dense SS cells, the ouabain-sensitive K(^{86}Rb) influx was low in oxygenated cells, was reduced further by deoxygenation, but was substantially increased after treatment with inosine, pyruvate and phosphate to increase their organic phosphate pool. These results were thus consistent with such a mechanism for Na^+ pump inhibition in the dense SS cells.

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INTRODUCTION

The total magnesium content of normal human red cells is about 2·5 mmol (l cells)$^{-1}$, but the ionized cell magnesium concentration ($[Mg^{2+}]_i$) oscillates between about 0·3 mM in oxygenated red cells and 0·5 mM in the deoxygenated cells, due to increased binding of the main cytoplasmic magnesium buffers, ATP and 2,3-diphosphoglycerate (2,3-DPG), to deoxyhaemoglobin (Bunn, Ransil & Chao, 1971; Berger, Janig, Gerber, Ruckpaul & Rapoport, 1973; Gerber, Berger, Janig & Rapoport, 1973; Flatman & Lew, 1977; Flatman & Lew, 1980*a*; Flatman, 1980, 1988*a*; Watson, Lyon & Hilditch, 1980; Bunn & Forget, 1986). Since the plasma Mg^{2+} concentration is about 0·5 mM (Walser, 1961; Heaton, 1967) and the RBC membrane potential is about −10 mV, the electrochemical gradient for Mg^{2+} is normally inwards. This inward gradient is apparently maintained by ATP- and $[Na^+]_o$-dependent uphill magnesium extrusion balancing a minute passive influx of less than 8 μmol (l cells)$^{-1}$ h^{-1} (Gunther & Vormann, 1985, 1986, 1989; Feray & Garay, 1986; Ludi & Schatzmann, 1987; Flatman, 1988*a*).

In the course of investigations of cation transport abnormalities in sickle cell anaemia (SS) red cells, we performed pilot studies of their total magnesium content after cell fractionation on arabinogalactose density gradients (see Methods below). Our initial findings (Bookchin, Ortiz & Lew, 1987), of a normal magnesium content in the SS RBC fraction with normal morphology (discocyte) and density, but a distinctly low total magnesium in the dense SS cell fraction (comprised mostly of 'irreversibly sickled cells' (ISCs)), were surprising from two standpoints. First, even if deoxygenation-induced sickling were to permeabilize SS red cells to magnesium, the normal inward electrochemical gradient for Mg^{2+} (Bunn *et al.* 1971; Berger *et al.* 1973; Gerber *et al.* 1973; Flatman & Lew, 1980*a*; Flatman, 1980) should have resulted in an increase in cell magnesium. Secondly, the increased cell Na^+ in dense SS red cells (Tosteson, Shea & Darling, 1952; Hellerstein & Bunthrarungroj, 1974) would be expected to inhibit uphill magnesium extrusion from the cells (Gunther & Vormann, 1985, 1989; Feray & Garay, 1986; Ludi & Schatzmann, 1987). It was therefore puzzling to find a net loss of magnesium in the dense SS red cells.

In order to explain these findings, we investigated the effects of sickling on magnesium permeability and the Mg^{2+} distribution in SS red cells of different densities. In addition, since the findings suggested that the Mg^{2+}:ATP ratio in the dense red cells may exceed normal values, and since this ratio has been shown to affect cation fluxes mediated by the Na^+ pump (Dunn, 1974; Flatman & Lew, 1980*b*, 1981), we examined the effects of deoxygenation and of an increase in the RBC organic phosphate pool on the ^{86}Rb fluxes in these cells.

METHODS

Sample preparation. Heparinized blood was obtained, with informed consent, from two normal donors and six patients in whom the SS genotype had been established by electrophoretic and family studies at our Heredity Clinic. The blood was kept at 4 °C, and all experiments on intact red cells were performed within 24 h of venesection. Density fractionation of the red cells on discontinuous gradients of Stractan II (arabinogalactan, St Regis Paper Co., Libby, MT, USA) followed the method of Corash, Piomelli, Chen, Seaman & Gross (1974) with minor modifications as previously described (Ortiz, Lew & Bookchin, 1986), using three density (δ) layers, 1·087, 1·091,

1·118, and a cushion of $\delta = 1{\cdot}170$. The cells with $\delta \leqslant 1{\cdot}087$, including most of the reticulocytes and any remaining white cells, were discarded, and two SS RBC fractions were harvested: the layer of SS red cells with $1{\cdot}087 < \delta < 1{\cdot}118$, primarily the SS discocytes, and the dense SS cell fraction, with $\delta > 1{\cdot}118$, containing most of the ISCs, as estimated by morphological criteria described previously (Ortiz *et al.* 1986). The density-fractionated red cells were washed three times using either buffer A containing (in mM): 5 KCl, 145 NaCl, 0·05 EGTA, 0·2 $MgCl_2$, 10 Na-HEPES, pH 7·40; or buffer B, containing (in mM): 75 NaCl, 75 KCl, 10 Na-HEPES, pH 7·5, with additions or modifications appropriate to the experiment, as noted below.

For measurement of the 2,3-diphosphoglycerate (2,3-DPG) content of red cells, SS and normal (AA) cells, washed in buffer A after removal of plasma and buffy coat, were layered onto 1·0 ml arabinogalactan solution with $\delta = 1{\cdot}118$ in 2·0 ml conical tubes and centrifuged for 15 min at 12000 r.p.m. in an Eppendorf microcentrifuge kept at 4 °C. Two fractions of SS cells were harvested, those remaining at the top (with $\delta < 1{\cdot}118$) and the dense cell pellet ($\delta > 1{\cdot}118$). The AA cells virtually all remained at the top. The cell fractions were suspended in buffer A to give a total haemoglobin concentration of 5–15 g dl^{-1}, and 2,3-DPG was measured using a modification of the method of Lowry, Passoneau, Hasselberger & Schulz (1964) with a Sigma No. 35-UV Kit (Sigma Chemical Co., St Louis, MO, USA). The time interval between venesection and deproteinization of the samples with trichloracetic acid never exceeded 2 h, during which they were kept at 4 °C.

Measurement of total magnesium in red cells and in solutions. RBCs from whole blood or from arabinogalactan density fractions were washed twice in buffer B containing, in addition, 0·1 mM-Na-EGTA and 2 mM-Tris-EDTA (B-EDTA) and twice more in buffer B alone from which all divalent cations had been removed by passage through a column of Chelex 100 (Biorad Laboratories, Richmond, CA, USA) (B-Chelex). Packed red cells (20 μl) were lysed in 0·5 ml deionized water and after sampling into Drabkins solution for haemoglobin concentration, 40 μl distilled trichloracetic acid (TCA) was added to 0·4 ml lysate. Following centrifugation, the clear supernatant was diluted quantitatively to give a final concentration of 2·5% TCA. Plasma samples were deproteinized in 5% TCA and the supernatants also diluted to 2·5% TCA. Magnesium concentrations were measured by atomic absorption spectroscopy (Perkin Elmer model 360, flame), using magnesium standards in 2·5% TCA. For measurements after incubations, 100 μl aliquots of RBC suspensions with haematocrit (Hct) 20–30% were delivered into chilled 1·5 ml plastic tubes containing 0·9 ml buffer B-EDTA layered onto 0·4 ml dibutylphthalate ($\delta = 1{\cdot}042$), and immediately spun in an Eppendorf microcentrifuge at 12000 r.p.m. for 15 s. After aspiration of the aqueous supernatant, the tube walls were rinsed once with B-Chelex buffer, taking care not to disturb the organic phase before aspirating it. The packed red cells were then processed as described above. All determinations were done on at least triplicate samples and the reproducibility of replicates was better than 2%.

Measurements of net movement of magnesium across RBC membranes. To study magnesium permeability, normal red cells and the discocyte and dense fractions of SS red cells were resuspended at 30% Hct in autologous plasma with the addition of 10 mM-inosine, and either (i) no further additions, (ii) addition of 5 mM-$MgCl_2$, to produce a large inward gradient of Mg^{2+}, or (iii) addition of 5 mM-Na-EDTA (from a stock solution of 400 mM, pH 7·4), to chelate the plasma Mg^{2+} and create a large outward Mg^{2+} gradient. The suspensions were incubated at 37 °C in a tonometer (Model 237, Instrumentation Laboratory Inc., Lexington, MA, USA) for at least 2 h, with stirring, under an atmosphere of either 5·6% CO_2 in air or 5·6% CO_2 in argon or nitrogen. Samples (100 μl) were taken initially and at specified times for measurements of total RBC magnesium by atomic absorption spectroscopy as described above.

Equilibrium distribution of magnesium in SS blood. The method of Flatman and Lew (Flatman & Lew, 1977, 1980*a*) was used to assess RBC magnesium buffering and equilibrium distribution under oxygenated and deoxygenated conditions, in autologous plasma and in buffers containing varied amounts of Mg^{2+}. In one series of experiments, the discocyte and dense SS red cells were suspended at 20% Hct in autologous plasma containing, in addition, 10 mM-inosine, and 2·5 mM-EGTA (to chelate plasma Ca^{2+} and avoid activation of the Ca^{2+}-sensitive K^+ channel). After 30 min equilibration at 37 °C under 5·6% CO_2 in nitrogen, the ionophore A23187 was added from a 2 mM-stock solution in dimethylsulphoxide to give a final concentration in the cell suspensions of 20–40 μM, to permit equilibration of Mg^{2+} across the RBC membranes. After 2 h, the gas was switched to 5·6% CO_2 in air. At the times indicated, 100 μl samples were delivered into chilled 1·5 ml Eppendorf tubes containing 0·9 ml of buffer A and 0·4 ml of dibutylphthalate, mixed, and immediately spun in an Eppendorf microcentrifuge at 12000 r.p.m. for 15 s to separate the red cells

from the aqueous media. After careful removal of the phthalate oil, the red cells were processed as above for determination of total magnesium.

In another series of experiments, the discocytes and dense red cells were suspended at 20% Hct at 37 °C in buffer C containing (in mM): 80 KCl, 60 NaCl, 20 Na-HEPES (pH 7·40 at 37 °C), 10 inosine, 0·02 EGTA, and various levels of $MgCl_2$, as indicated below, and kept in air or under humidified nitrogen gas. The ionophore A23187 was added (10 μM in the medium) and, after 20 min equilibration, samples were taken as above for measurements of total magnesium in the red cells and buffer. These values were then compared with the original levels of RBC magnesium in the same cell fractions, to determine the levels of extracellular Mg^{2+}, $[Mg^{2+}]_o$, at which the magnesium-permeabilized red cells were in equilibrium with the original cell magnesium.

At each equilibrium point in these two groups of experiments, the Donnan ratio r was assessed by measuring both the medium pH and the intracellular pH (pH_i), to determine $r = [H^+]_i : [H^+]_o$. For measurements of intracellular pH, aliquots of red cell suspensions were transferred with a gas-tight syringe to microcentrifuge tubes under oil, the centrifuged cells were frozen in the tubes with liquid nitrogen, and the thawed haemolysates were aspirated into a capillary microelectrode at 37 °C. With the deoxygenated dense SS red cells, pH_i could not be measured this way because of the high viscosity of the cytoplasm, and the chloride ratio $[Cl^-]_o : [Cl^+]_i$ was determined directly using a chloridometer, as described before (Bookchin, Lew, Balazs, Ueda & Lew, 1984). The cytoplasmic ionized magnesium levels which correspond to the measured total magnesium in each cell fraction could then be calculated, using the above equilibrium values of $[Mg^{2+}]_o$, and the equilibrium relationship $[Mg^{2+}]_i = r^2[Mg^{2+}]_o$ (Flatman & Lew, 1977, 1980*a*).

As an expression of cytoplasmic magnesium buffering, the fraction of ionized magnesium in cell water was estimated in the following manner. After incubation of the discocyte or dense RBC in the oxygenated or deoxygenated states, microhaematocrits were done on samples of the suspensions containing trace amounts of ^{60}Co-EDTA as a marker of extracellular space, to permit correction of the Hct for trapped extracellular fluid. Assuming that the RBC volume consists essentially of haemoglobin and water, then taking 0·75 as the partial specific volume of haemoglobin (Bureau & Banerjee, 1976), the volume fraction of cell water was estimated as $[1 - (MCHC/100 \times 0{\cdot}75)]$ (MCHC in units of g haemoglobin (100 ml of cells)$^{-1}$). The total RBC magnesium was considered to be distributed in the cell water, so that the fraction of ionized magnesium in cell water is $[Mg^{2+}]_i$/(total RBC Mg/volume fraction of cell water).

Measurement of ouabain-sensitive $K(^{86}Rb)$ influx in red cells. Dense SS cells ($\delta > 1{\cdot}118$) obtained as above were resuspended at 10% Hct in buffer containing (in mM): 140 NaCl, 5 KCl, 20 Na-HEPES (pH 7·40 at 37 °C), and 0·5 $MgCl_2$. To half of the RBC suspension, 10 mM-glucose was added, and to the other half, a combination of 10 mM-inosine, 10 mM-sodium pyruvate, and 5 mM-sodium phosphate, pH 7·40 (IPP), and the suspensions were incubated for 2 h at 37 °C to increase the pool of organic phosphates in the red cells suspended with IPP (Deuticke, Duhm & Dierkesmann, 1971; Duhm, 1976). Each of these suspensions was then subdivided into four 5 ml aliquots for the additional conditions of equilibration with either 100% oxygen or argon, and with or without 0·1 mM-ouabain. After a 30 min period of gas equilibration of the stirred suspensions, ouabain was added to the appropriate vials, 10 μCi ^{86}Rb ml^{-1} added to all, and after 1 h incubation at 37 °C in the same gas atmospheres, 100 μl samples were processed for ^{86}Rb content as described previously (Ortiz *et al.* 1986).

RESULTS

Magnesium content in SS and normal (AA) red cells

Measurements of total magnesium ([total Mg]$_i$) in density-fractionated SS red cells from six donors are summarized in Table 1. The magnesium content of the SS discocytes was within the range observed in normal red cells. The density fractions of SS discocytes showed no significant differences in [total Mg]$_i$. The dense, ISC-rich SS RBC fractions, however, had consistently and significantly low magnesium contents, when expressed in terms of mean cell haemoglobin (equivalent to cell number for the dense SS cells (Bertles & Milner, 1968)), indicating that these cells must have sustained a net loss of magnesium. The experiments described below explore the possible mechanisms of this loss.

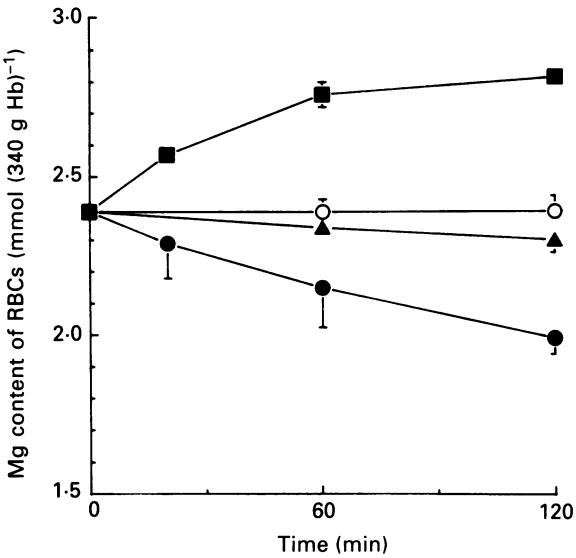

Fig. 1. Effects of a magnesium gradient and periods of deoxygenation on the magnesium content of SS discocytes. The density fraction of SS red cells containing most of the discocytes ($1{\cdot}087 < \delta < 1{\cdot}118$), suspended in autologous plasma supplemented with 10 mM-inosine, was equilibrated with either 5·6% CO_2 in air (open symbols, oxygenated) or 5·6% CO_2 in argon (closed symbols, deoxygenated). Each symbol represents replicate measurements of total RBC magnesium, $[\text{total Mg}]_i$, by atomic absorption spectroscopy, following incubation in plasma whose total Mg was either normal (0·5 mM; ▲), raised to 5·0 mM by addition of $MgCl_2$ (■), or chelated by addition of 5 mM-EDTA (●). Under oxygenated conditions, RBC Mg was unchanged by addition of $MgCl_2$ or EDTA, so that a single symbol (○) represents all three conditions. The error bars in all the figures indicate standard error of the mean of replicates, and are shown when their dimensions exceed those of the symbols.

TABLE 1. Total magnesium content of SS discocytes and dense RBCs

	Total RBC magnesium		
Donor	Discocytes	Dense RBC (mmol (340 g Hb)$^{-1}$)	D*
M.M.	2·24	1·83	0·41
J.E.	2·15	2·04	0·11
E.M.	2·17	1·44	0·73
W.R.	2·55	2·26	0·29
T.N.	2·18	1·74	0·44
K.W.	2·06	1·74	0·32
T.N.	1·67	1·31	0·36
Mean	2·15±0·11**	1·77±0·14**	0·38±0·19***

* [Discocyte−dense RBC] total magnesium.

** Standard error of the mean.

*** Standard deviation of the mean difference; $P < 0{\cdot}005$ (paired t statistic).

Magnesium content of density-fractionated SS RBCs was measured by atomic absorption spectroscopy. A sample of normal RBCs similarly exposed to Stractan had a total magnesium of 2·26 mmol (340 g Hb)$^{-1}$ which was well within the reported normal range. In view of excellent reproducibility of replicate original samples (see Methods), the different values obtained for donor T.N. several months apart probably reflect a genuine change.

Effect of sickling on magnesium permeability

To examine the possible effects of deoxygenation-induced sickling on RBC membrane permeability to magnesium, SS discocytes and dense red cells were resuspended in autologous plasma with and without addition of either EDTA or

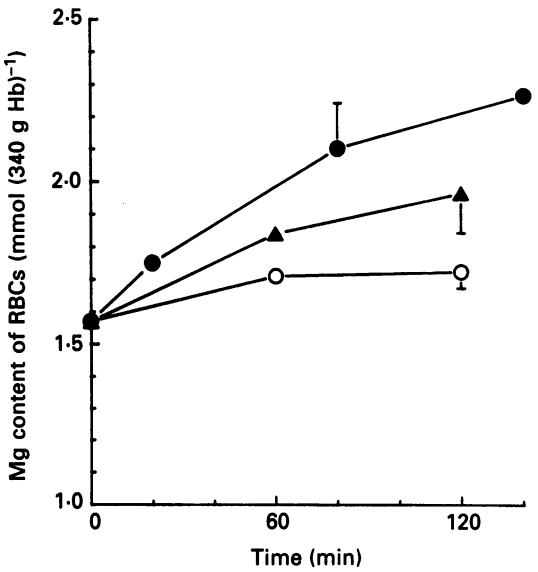

Fig. 2. Effects of deoxygenation and of an inward magnesium gradient on the magnesium content of dense SS red cells. SS red cells with $\delta > 1{\cdot}118$ were suspended at 30% Hct in autologous plasma with 10 mM-inosine, whose total Mg was raised to 5·0 mM by addition of $MgCl_2$. The symbols represent $[\text{total Mg}]_i$ (see Fig. 1 legend) following periods of equilibration either oxygenated (○), or deoxygenated (▲, ●), using the gas mixtures described in Fig. 1. Deoxygenation was either continuous (●), or by alternating cycles of 20 min deoxygenation and 5 min oxygenation (▲).

$MgCl_2$ to produce a large outward or inward Mg^{2+} gradient across the membranes, and their magnesium content was assessed after incubation for various times in oxygenated or deoxygenated conditions. When the SS discocytes (Fig. 1) were incubated at normal plasma levels of magnesium, either oxygenated or deoxygenated, there was no significant change in their $[\text{total Mg}]_i$. When 5 mM-$MgCl_2$ or EDTA was added to the plasma, the red cells incubated in oxygenated conditions showed no change in magnesium content but when the cells were kept deoxygenated, there was a substantial rise or fall in $[\text{total Mg}]_i$, according to the direction of the Mg^{2+} gradient. Thus, deoxygenation of the SS discocytes increases their magnesium permeability. The extent of change in $[\text{total Mg}]_i$ in physiological or experimental conditions will therefore depend on the size of the Mg^{2+} gradients and on the extent and duration of deoxygenation.

A similar experiment was performed with the dense SS RBC fraction (Fig. 2) but, because of the limited quantity of cells, was done only with the single plasma

condition of a large inward Mg^{2+} gradient. In this condition, the cells also showed an increase in [total Mg]$_i$ only when they were deoxygenated; the extent of the increase was similar to that seen with the SS discocyte fraction (about 0·5 mmol Mg (340 g haemoglobin)$^{-1}$ h^{-1}).

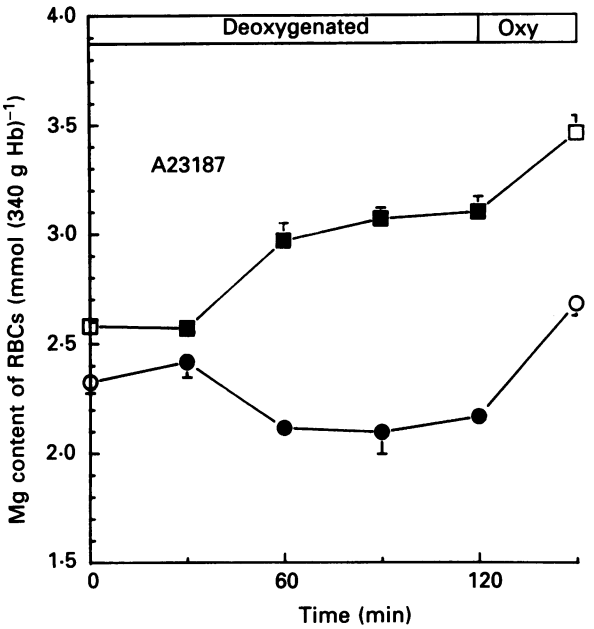

Fig. 3. Effect of Mg^{2+} permeabilization on the magnesium content of oxygenated (Oxy) and deoxygenated SS RBC density fractions equilibrated with plasma Mg^{2+}. SS discocytes (■, □) and dense cell fractions (●, ○) were suspended at 30 % Hct in autologous plasma containing 10 mM-inosine and 2·5 mM-EGTA to reduce $[Ca^{2+}]_o$ without significantly affecting $[Mg^{2+}]_o$. After 30 min deoxygenation equilibration, samples were taken for [total Mg]$_i$ before and after addition of A23187, 40 μM (l cell suspension)$^{-1}$. This concentration of ionophore was found to be sufficient to overcome the binding capacity of plasma albumin (Simonsen, 1981) and to permeabilize the membranes to divalent cations. After 2 h, the cells were equilibrated with 5·6 % CO_2 in oxygen for 30 min and again sampled for [total Mg]$_i$ (○, □).

When the SS discocytes or dense red cells were suspended in the Mg^{2+}-supplemented plasma and exposed to cycles of alternating deoxygenation (20 min) and oxygenation (5 min), their [total Mg]$_i$ also increased, to values intermediate between those of the cells kept oxygenated and cells kept continuously deoxygenated. Normal red cells subjected to the same conditions showed no change in [total Mg]$_i$ (not shown).

These results indicate that the deoxygenation-induced increase in magnesium permeability occurs to a similar extent in SS discocytes and dense red cells in physiological conditions (*in vitro*), and that the permeability increase occurs during the period of deoxygenation, and not primarily during the initiation or reversal of the sickling process. In order for such an increased permeability to account for a loss of

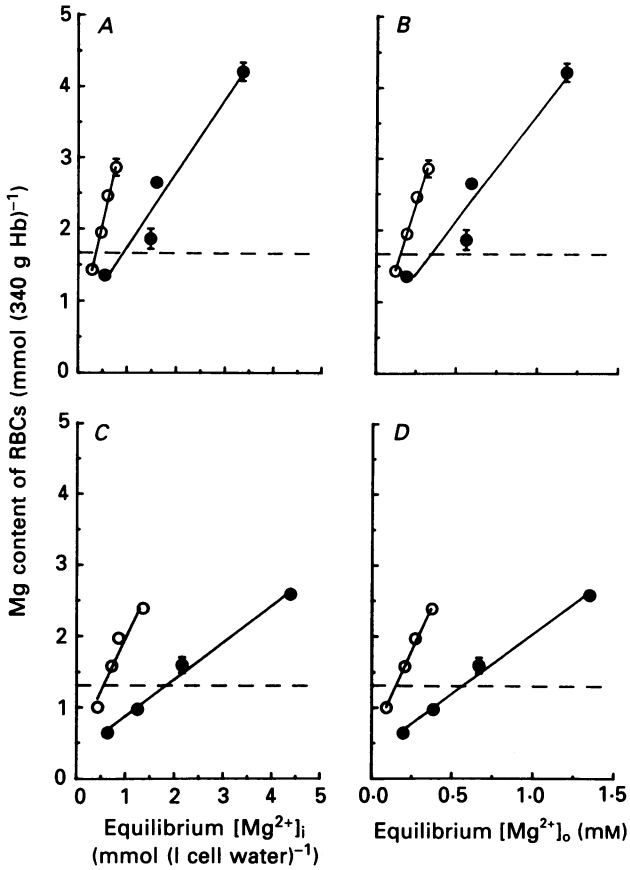

Fig. 4. Equilibrium levels of total magnesium in oxygenated and deoxygenated SS discocytes and dense red cells. [Total Mg]$_i$ was measured after equilibration of the cells, in buffers containing the ionophore A23187 and various concentrations of Mg^{2+} (see Fig. 1 legend) with either air (○) or nitrogen (●). Data for the SS discocytes are shown in *A* and *B*, and for the dense SS red cells in *C* and *D*. In each equilibrium, the values of [Mg^{2+}]$_i$ are calculated (see Methods), and the corresponding equilibrium data are shown as functions of [Mg^{2+}]$_i$ (in mmol (l cell water)$^{-1}$) in *A* and *C*, and of [Mg^{2+}]$_o$ in *B* and *D*. The continuous lines are derived by linear regression analysis of the data. The dashed horizontal lines represent the original measured [total Mg]$_i$ levels in the RBC fractions; the intersection of these lines with the linear regression (continuous lines) indicates the values of [Mg^{2+}]$_i$ and [Mg^{2+}]$_o$ at which the original RBC [total Mg]$_i$ would be in equilibrium. The data from these experiments are also included in Table 2.

magnesium from dense SS cells, however, the normal inward gradient of Mg^{2+} would have to be reversed.

Magnesium equilibration of SS red cells in plasma

The experiment shown in Fig. 3 examines the direction of the Mg^{2+} gradients for SS discocytes and dense cells in their own plasma. Upon addition of the ionophore A23187 after a period of deoxygenation, the discocytes gained magnesium from the plasma, and upon subsequent reoxygenation, gained additional magnesium. These

findings are consistent with the inward Mg^{2+} gradient described for normal red cells, and with the larger cytoplasmic magnesium buffering of the oxygenated cells (Bunn *et al.* 1971; Berger *et al.* 1973; Gerber *et al.* 1973; Flatman & Lew, 1977, 1980*a*). The dense SS red cells, however, lost magnesium to the plasma while in the deoxygenated state, and gained magnesium only upon oxygenation. This indicates that only when deoxygenated must the dense SS cells have had a sufficiently high cytoplasmic Mg^{2+} level to maintain an outward Mg^{2+} gradient.

Cytoplasmic magnesium buffering and equilibrium levels of external Mg^{2+} for SS red cells

The total magnesium content of SS discocytes and dense red cells, equilibrated with various levels of Mg^{2+} in the oxygenated and deoxygenated states, are shown in Fig. 4, expressed as a function of $[Mg^{2+}]_i$ and $[Mg^{2+}]_o$. From these data, the values of $[Mg^{2+}]_i$ and $[Mg^{2+}]_o$ were determined at which the original [total Mg]$_i$ of the red cells would be in electrochemical equilibrium, as defined by $[Mg^{2+}]_i = r^2[Mg^{2+}]_o$ (Flatman & Lew, 1977, 1980*a*). The results of several samples studied in this way (Table 2) show that in the SS discocytes, the cytoplasmic magnesium buffering (which determines the fraction of ionized magnesium) in both the oxygenated and deoxygenated cells was normal or modestly increased, and the $[Mg^{2+}]_i$ levels did not differ significantly from normal in this small sample. In the dense SS cells, however, despite consistently lower levels of total magnesium than in the corresponding discocyte fractions (Table 1), the levels of $[Mg^{2+}]_i$ were considerably higher than in the discocytes or in normal red cells. The higher fractions of ionized magnesium in these dense SS cells indicated that cytoplasmic magnesium buffering was reduced. Nevertheless, with the oxygenated dense SS cells, as well as with the oxygenated and deoxygented SS discocytes, the equilibrium levels of $[Mg^{2+}]_o$ were far below plasma Mg^{2+} levels, and an inward Mg^{2+} gradient would be maintained. Only in the deoxygenated dense SS cells, in which magnesium buffering was lowest (48 and 89% ionized Mg^{2+}), was the equilibrium level of $[Mg^{2+}]_o$ in the same range as that in plasma.

It should be noted that in the suspension media used with the present experiments, buffered NaCl and KCl, Flatman (1980) found slightly higher magnesium buffering in deoxygenated red cells than when they were suspended in bicarbonate-buffered media with lower Cl^- content. Therefore, the equilibrium values of Mg^{2+} in the cells and media in plasma could be expected to be slightly higher than those found here, and consistent with the clearly outward Mg^{2+} gradient observed with deoxygenated dense SS red cells suspended in plasma (Fig. 3).

Measurements of 2,3-diphosphoglycerate concentrations in density-fractionated SS red cells

Earlier measurements (Seakins, Gibbs, Milner & Bertles, 1973; Kaperonis, Bertles & Chien, 1979) of RBC 2,3-DPG in 'top, middle and bottom' thirds of centrifuged columns of SS and AA cells density fractions, indicated that the increase in the total RBC 2,3-DPG : haemoglobin ratio in the SS cells could be attributed to the upper and middle fractions. The bottom third of SS red cells showed a low normal 2,3-DPG : haemoglobin ratio. Since our current studies employed different methods of

TABLE 2. Cytoplasmic magnesium buffering and equilibrium distribution in RBCs

Density fraction	Magnesium content: RBC total	Magnesium content: Plasma Total	Magnesium content: Plasma $[Mg^{2+}]$**	Magnesium levels at equilibrium with measured total RBC values: $[Mg^{2+}]_i$ Oxy	$[Mg^{2+}]_i$ Deoxy	$[Mg^{2+}]_o$ Oxy	$[Mg^{2+}]_o$ Deoxy	Fraction of ionized magnesium in cell water*: Oxy	Fraction of ionized magnesium in cell water*: Deoxy
SS discocytes									
T.N.	1·67	0·77	0·42	0·36	0·83	0·16	0·28	0·12	0·27
K.W.	2·06	—	—	0·49	—	0·16	—	0·18	—
M.M.	2·20	1·01	0·56	0·36	0·56	0·12	0·24	0·12	0·18
SS dense									
T.N.	1·31	0·77	0·42	0·58	1·88	0·15	0·54	0·22	0·92
W.R.	2·26	0·80	0·44	0·88	1·72	0·20	0·45	0·26	0·48
Normal									
O.O.	2·26	—	—	0·46	0·72	0·16	0·28	0·15	0·24
Refs***	2·1–2·5	0·65–0·98	0·36–0·54	0·45	0·62	0·12–0·15	0·27	0·17–0·19	0·26

The total magnesium content of RBCs is given in units of mmol (340 g Hb)$^{-1}$. Total and ionized magnesium concentrations in plasma and in cell water are in mM.

* The measured total RBC magnesium is assumed to be distributed in cell water; the mean volume fraction of cell water is calculated for each RBC sample in the oxygenated and deoxygenated states (see Methods), and the fraction of ionized magnesium in cell water is $[Mg^{2+}]_i$/(total RBC magnesium/volume fraction of cell water).

** The level of ionized magnesium in plasma is an approximation based on the measured total plasma magnesium and an average value of 0·55 for the ionized fraction (Walser, 1961), and is shown for comparison with the levels of $[Mg^{2+}]_o$ in equilibrium with the measured total RBC magnesium. Figure 4 illustrates the derivation of the above 'equilibrium' values in RBCs from donor T.N.

*** Flatman & Lew (1980); Flatman (1980).

TABLE 3. Content of 2,3-diphosphoglycerate in normal and density-separated SS red cells

Donor	2,3-Diphosphoglycerate content in RBCs			
	RBCs with $\delta < 1{\cdot}118$		RBCs with $\delta > 1{\cdot}118$	
	μmol (g Hb)$^{-1}$	mol (mol Hb)$^{-1}$	μmol (g Hb)$^{-1}$	mol (mol Hb)$^{-1}$
Sickle				
M.M.	22·1	1·38	11·5	0·72
B.H.	22·4	1·40	9·8	0·61
L.M.	15·5	0·97	5·7	0·36
Normal				
S.P.	19·2	1·20	—	—
C.M.	17·7	1·11	—	—
S.S.	13·8	0·86	—	—

Each of the values listed is the mean of duplicate measurements, which differed by no more than 2%. Morphological examination of the SS RBCs after density separation on Stractan gradients showed 1–5% ISCs among the cells with $\delta < 1{\cdot}118$, and 55–86% ISCs among those with $\delta > 1{\cdot}118$.

TABLE 4. Effect of increased organic phosphates on active and passive K(^{86}Rb) influx in dense SS RBCs

Conditions			K(^{86}Rb) influx (mmol (340 g Hb)$^{-1}$ h^{-1})		
Pre-incubation	Incubation	Atmosphere	Total	Ouabain present	Ouabain sensitive
Glucose	Glucose	Oxygenated	2·71 ± 0·06*	0·74 ± 0·01*	1·97 ± 0·06**
Glucose	Glucose	Deoxygenated	2·08 ± 0·03	0·66 ± 0·01	1·42 ± 0·03
IPP	Inosine	Oxygenated	2·90 ± 0·02	0·69 ± 0·005	2·21 ± 0·02
IPP	Inosine	Deoxygenated	3·00 ± 0·03	0·69 ± 0·01	2·31 ± 0·03

* Standard error of the mean ($n = 4$). ** Standard error of the mean difference.

A fraction of SS RBCs with density > 1·118 was pre-incubated in the presence of inosine, pyruvate and inorganic phosphate (IPP) to raise the RBC content of organic phosphates (including ATP and 2,3-DPG), and K(^{86}Rb) influx was measured during incubation in an oxygen or argon atmosphere, in the presence or absence of 0·1 mM-ouabain.

density fractionation, several measurements were made to help assess the contribution of 2,3-DPG to the observed levels of cytoplasmic Mg^{2+}-buffering in the SS cell fractions studied here, particularly the dense cells. The results (Table 3) show that, in agreement with Kaperonis *et al.* (1979), the increased 2,3-DPG:haemoglobin ratio in SS red cells is limited to the less-dense cells; but the cells with $\delta > 1{\cdot}118$, the majority of which were morphologically ISCs, had a moderate to markedly low 2,3-DPG:haemoglobin ratio.

Effects of deoxygenation and increased organic phosphates on active and passive K(^{86}Rb) influx in dense SS red cells

The results summarized in Table 4 show that the ouabain-sensitive component of K(^{86}Rb) influx in the dense SS red cells, incubated in glucose-supplemented media, was about 2 mmol (l cells)$^{-1}$ h^{-1} in the oxygenated cells. From the known high-Na^+, low-K^+ content of these cells, the expected normal value was 2·5–3·5 mmol (l cells)$^{-1}$ h^{-1} (Post & Jolly, 1957; Garay & Garrahan, 1973). The relatively low flux observed with oxygenated SS cells was further reduced by deoxygenation. Following

a period of pre-incubation with inosine, pyruvate and phosphate to raise the levels of RBC organic phosphates, and with incubation in inosine-supplemented media, K(^{86}Rb) influx was substantially higher in both the oxygenated and deoxygenated states. Interpretation of these findings will be discussed below.

DISCUSSION

It is well established that deoxygenation-induced sickling is accompanied by an increased membrane permeability for the cations Na^+, K^+ and Ca^{2+}. The present finding that magnesium permeability, which is extremely low in normal red cells (Ginsburg, Smith, Ginsburg, Reardon & Aikawa, 1962; Watson *et al.* 1980; Gunther & Vormann, 1985, 1989; Feray & Garay, 1986; Ludi & Schatzmann, 1987; Flatman, 1988*a*), is considerably increased during deoxygenation of SS red cells, extends the evidence that the cation leak associated with sickling is not selective.

In contrast to the reported effects of SS RBC deoxygenation on Na^+ and K^+ permeability, that dense SS red cells were less permeabilized to these cations than the discocyte fraction (Mohandas, Rossi & Clark, 1986), the increase in magnesium permeability observed here was similar with both cell density fractions. In separate studies, we have found that the increase in Ca^{2+} permeability induced by deoxygenation is also similar in the discocyte and dense fractions of SS red cells (R. M. Bookchin, O. E. Ortiz & V. L. Lew, unpublished observations). The reason for this apparent difference in permeabilization to divalent and monovalent cations, and its relevance to the suggested association between mechanical distortion of SS cells and deoxygenation-induced cation permeability (Mohandas *et al.* 1986), have yet to be determined.

The present studies reveal that the levels of ionized magnesium in dense, deoxygenated SS cells are so high as to generate an outward electrochemical Mg^{2+} gradient, opposite to that in normal red cells. Together with the sickling-induced magnesium permeabilization, this outward gradient may account for the low total magnesium content of the dense SS red cells. To understand the origin of this abnormal state, we must consider the factors which control the level of $[Mg^{2+}]_i$.

Most of the cytoplasmic magnesium buffering in normal red cells can be attributed to organic phosphates, particularly 2,3-DPG, and to a lesser extent, ATP (Bunn *et al.* 1971; Berger *et al.* 1973; Gerber *et al.* 1973; Flatman & Lew, 1980*a*). In SS red cells, the mean levels of 2,3-DPG (per volume of cells) are increased by about 40% (Charache, Grisolia, Fiedler & Hellegers, 1970). Kaperonis *et al.* (1979) reported that after simple density fractionation, the ratio of 2,3-DPG to haemoglobin, equivalent to a measure of 2,3-DPG per SS cell of any density, was increased in the lightest and middle thirds but normal in the denser third. Our present measurements, however, show that the SS cells with $\delta > 1{\cdot}118$ (mostly ISCs), which are probably a dense subset of the 'lower third' of a centrifuged column of SS cells, have low 2,3-DPG:haemoglobin ratios.

ATP levels in the normal-density, discocyte fraction of SS red cells were reported as normal or only modestly increased, expressed per cell volume (Clark, Ungar & Shohet, 1978*b*). In the dense ISCs, although the 'functional' concentration of ATP in the reduced cell water of these cells was not low (Clark, Ungar & Shohet, 1978*b*),

it appeared that the level of ATP per cell (and therefore the ATP:haemoglobin molar ratio) was decreased.

The observed increase in $[Mg^{2+}]_i$ when normal red cells are deoxygenated can be attributed to the enhanced binding of the major intracellular Mg^{2+} buffers, 2,3-DPG and (to a much smaller extent) ATP, to deoxyhaemoglobin (Bunn *et al.* 1971; Berger *et al.* 1973; Gerber *et al.* 1973; Flatman & Lew, 1980*a*). The predominant Mg^{2+} buffer in red cells is 2,3-DPG. When normal red cells, with a ratio of 2,3-DPG:haemoglobin of 0·8–0·9, are deoxygenated, a large fraction of the 2,3-DPG binds to deoxyhaemoglobin.

Our findings of low 2,3-DPG:haemoglobin ratios in the dense SS RBC fractions, amounting to about 40–75% of normal values, together with the reported low ATP:haemoglobin ratio, could account for the low Mg^{2+} buffering and high Mg^{2+} in deoxygenated, dense SS cells. With these low levels, the increased binding of both Mg^{2+} buffers to deoxyhaemoglobin could deplete the cytoplasm of a much larger fraction of its Mg^{2+} buffers.

The above considerations suggest that the low Mg^{2+} buffering of the dense SS red cells increases the fluctuation of their Mg^{2+} levels with the state of haemoglobin oxygenation. Whereas normal red cells are nearly fully saturated with oxygen in arterial blood, and are ordinarily well over half-saturated in mixed venous blood, SS red cells have different patterns. In addition to a low whole-blood oxygen affinity, due in part to high mean levels of 2,3-DPG, the oxygen affinity of SS cells is extremely sensitive to the intracellular haemoglobin concentration (Seakins *et al.* 1973; Bookchin, Balazs & Landau, 1976). Polymerization of deoxyhaemoglobin S is interrelated with the other ligand-linked functions of haemoglobin, including haemoglobin interaction with protons, 2,3-DPG, carbon dioxide and oxygen (Benesch, Benesch & Yu, 1969; Bunn *et al.* 1971; Deuticke *et al.* 1971; Berger *et al.* 1973; Gerber *et al.* 1973; Duhm, 1976; Van Beek & De Bruin, 1979). The polymer itself exhibits a very low oxygen affinity and, since the extent of polymerization at any given level of oxygen desaturation is highly dependent on the cell haemoglobin concentration (Hofrichter, Ross & Eaton, 1976), dehydration of SS red cells results in extremely low oxygen affinities (Seakins *et al.* 1973; Bookchin *et al.* 1976). At haemoglobin concentrations within the dense fraction of SS cells (with $\delta > 1{\cdot}118$, cell haemoglobin is over 44 g dl^{-1}), the low oxygen affinity results in a significant haemoglobin desaturation, with intracellular polymerization of haemoglobin S and sickling, even at arterial oxygen tensions (Bookchin *et al.* 1976; Noguchi, Torchia & Schechter, 1983). Thus it is likely that they will spend considerable time in the circulation with Mg^{2+} levels high enough to maintain an outward gradient during periods of Mg^{2+} permeabilization.

One caveat to bear in mind, however, is that within the SS discocyte density fraction itself there may be considerable heterogeneity of cell magnesium contents. For example, this fraction may include a variable proportion of reticulocytes of increased density, whose magnesium levels may be high, while other cells at this density may already have undergone some of the changes observed in the dense fraction which result in a low magnesium content. Therefore, the extent to which the observed difference in magnesium content between the discocyte and dense fractions is due to magnesium permeabilization will require further study.

Mg^{2+} ions are essential intracellular co-factors for main cation transporters present in red cells (Dunn, 1974; Flatman & Lew, 1981; Lauf, 1985; Brugnara & Tosteson, 1987; Canessa, Fabry & Nagel, 1987; Flatman, 1988*a*, *b*). The dense fraction of SS red cells was found by Clark, Morrison & Shohet (1978*a*) to have decreased Na^+ pump activity despite normal concentrations of ATP (per volume of cells or cell water) and normal Na^+–K^+-ATPase activity in the isolated membranes. The Na^+ pump inhibition in the dense SS cells was confirmed by Ortiz *et al.* (1986), who found a lowered maximal pumping capacity (V_{max}) but normal activation by external K^+. They excluded SS cell Ca^{2+} as the cause of the inhibition (Brown & Lew, 1983*a*, *b*), since the abnormality persisted after the increased calcium was extracted from the cells. The finding of a high Mg^{2+} concentration in the dense SS cells raised the possibility that, despite their normal ATP concentrations, a high ratio of Mg^{2+}:ATP in cell water might account for the Na^+ pump inhibition, as observed for the Na^+–K^+-ATPase (Dunn, 1974; Flatman & Lew, 1980*a*, 1981). If this were the case, then the Na^+ pump inhibition should be aggravated by deoxygenation, which would further raise the Mg^{2+} levels (by depleting available Mg^{2+} buffers), but should be relieved by raising the cell concentrations of ATP and 2,3-DPG, since the Mg^{2+}:ATP ratio would be normalized by increasing both ATP and Mg^{2+} buffering. The present findings of a large increase in Na^+ pump activity following incubation of the dense SS cells under conditions known to increase their contents of these organic phosphates is consistent with this mechanism.

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