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THE MAGNESIUM DEPENDENCE OF SODIUM-PUMP-MEDIATED SODIUM–POTASSIUM AND SODIUM–SODIUM EXCHANGE IN INTACT HUMAN RED CELLS

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SUMMARY

1. The magnesium content of human red blood cells was controlled by varying the magnesium concentration in the medium in the presence of the ionophore A23187. The new magnesium levels attained were very stable, which allowed the magnesium dependence of the sodium pump to be investigated.

2. The effects of magnesium were shown to occur at the inner surface of the red cell membrane for the range of magnesium concentrations tested (10^{-7} to 6×10^{-3} M).

3. At intracellular ionized magnesium concentrations below 0·8 mM the activation of ouabain-sensitive sodium–potassium exchange by internal ionized magnesium could be resolved into two or three components: (*a*) a small component, about 5 % of the maximum flux, which is apparently independent of the ionized magnesium concentration below 2 μM, (*b*) a saturating component with a $K_{\frac{1}{2}}$ of between 30 and 45 μM, and possibly (*c*) a component which increases linearly with ionized magnesium concentration and which only becomes apparent at concentrations above 0·1 mM.

4. At intracellular ionized magnesium concentrations below 0·8 mM, activation of ouabain-sensitive sodium–sodium exchange by internal ionized magnesium could be resolved into two components: (*a*) a small component, about 6 % of the maximal flux, which is apparently independent of the ionized magnesium concentration below 2 μM, and (*b*) a saturating component with a $K_{\frac{1}{2}}$ of about 9 μM. At ionized magnesium concentrations between about 0·2 and 0·8 mM the rate of sodium–sodium exchange remained constant at the maximal level.

5. The intracellular concentration of ATP decreased and the ADP concentration increased as the magnesium content of the cells was reduced from the normal level. A small increase in ATP and a small decrease in ADP was seen when the magnesium content was increased above the normal level. The variation in the ATP:ADP ratio from 2·5 at very low magnesium levels to about 6 at normal magnesium levels can account, at least in part, for the different $K_{\frac{1}{2}}$ values of sodium–potassium and sodium–sodium exchange.

6. When the concentration of ionized magnesium was increased above about 0·8 mM both sodium–potassium and sodium–sodium exchange were inhibited. Sodium–sodium exchange was more strongly inhibited than sodium–potassium exchange.

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7. The possible sites of action of magnesium in the sodium pump cycle are discussed.

INTRODUCTION

It is well known that magnesium is necessary for Na,K-ATPase activity (Skou, 1957, 1960; Dunham & Glynn, 1961) and for many of the partial reactions of the sodium pump (see Glynn & Karlish, 1975) and yet it is not clear how magnesium affects the pump's ability to transport sodium and potassium ions. The major problem in assessing the magnesium dependence of ion transport by the pump is that the magnesium binding site which controls the pump's activity appears to be inside the cell (see Hoffman, 1978, for details). This means that methods have to be found to alter the intracellular magnesium in a controllable way while measuring the pump-mediated fluxes. Until recently the only preparations in which this was possible were resealed red cell ghosts and perfused or injected giant cells.

Bodemann & Hoffman (1976) examined the effects of three different magnesium concentrations on the rate of sodium–potassium and sodium–sodium exchange in red cell ghosts which had been resealed containing different magnesium-EDTA buffer mixtures. They showed that sodium–potassium exchange was more sensitive to changes in ionized magnesium concentration than was sodium–sodium exchange.

De Weer (1976) examined the effects of internal magnesium on active sodium extrusion from squid giant axons. He demonstrated that active sodium efflux had a parabolic dependence on the ionized magnesium in the injection solution. The flux was inhibited at low magnesium concentrations, passed through a maximum at about 10 mM ionized magnesium and was inhibited as the concentration was increased further. Having established the relationship between sodium efflux and ionized magnesium, De Weer used the rate of sodium efflux as a measure of ionized magnesium in axons and did not pursue further the effects of magnesium on the sodium pump. In both preparations described above it was difficult to assess the exact concentration of ionized magnesium at the inner surface of the membrane.

Recently it has become possible to alter the magnesium concentration inside red cells in a precise way by using the ionophore A23187 (Reed & Lardy, 1972; Flatman & Lew, 1977*a*, 1980*a*) which allows magnesium to enter or leave red cells so that internal magnesium rapidly equilibrates with external magnesium. Moreover, if the conditions are chosen correctly the presence of the ionophore causes only minor variations in the concentrations of other cell constituents. Using the ionophore A23187 to alter internal magnesium we have now been able to measure the magnesium dependence of sodium–potassium and sodium–sodium exchange through the pump in intact human red blood cells.

A preliminary report of this work has been published (Flatman & Lew, 1979).

METHODS

Principle of the method

Fluxes of sodium through the sodium pump were determined as the ouabain-sensitive components of ^{24}Na efflux from intact human red cells. The sodium–potassium exchange fluxes were measured in media containing at least 10 mM-potassium, and the sodium–sodium exchange fluxes were measured in nominally potassium-free media containing a high concentration of sodium (Garrahan & Glynn, 1967*b*). The magnesium content of the cells was changed by addition to the medium of

between 3 and 10 μM-A23187 and different concentrations of magnesium chloride. Under these conditions magnesium comes rapidly into equilibrium across the red cell membrane so that within 5 min the following relationship holds (see Flatman & Lew, 1977*a*, 1980*a*):

$$[Mg^{2+}]_i^\infty = [Mg^{2+}]_o^\infty \left(\frac{[Cl^-]_o}{[Cl^-]_i}\right)^2, \quad (1)$$

where $[Mg^{2+}]_i^\infty$ and $[Mg^{2+}]_o^\infty$ are the equilibrium concentrations of ionized magnesium in the cells and medium respectively and $[Cl^-]_i$ and $[Cl^-]_o$ are the internal and external chloride ion concentrations. Thus the concentration of ionized magnesium inside the cells can be calculated from the measured chloride concentration in the cells and the measured concentrations of chloride and ionized magnesium in the medium. The efflux of ^{24}Na was measured when the magnesium concentration had reached its equilibrium level.

A23187 will transport calcium into red cells. Care has to be taken, therefore, to ensure that all of the contaminant calcium in the medium is chelated. This prevents an A23187-mediated increase in intracellular calcium which can directly lead to inhibition of the sodium pump (A. M. Brown & V. L. Lew, unpublished observations) and to activation of a potassium channel (Lew & Ferreira, 1976). The latter would lead to loss of cell potassium chloride, cell shrinkage and a consequent alteration in transport rate constants. Excess EGTA (0·01–0·05 mM) was therefore added to all media and its concentration was increased in conditions where calcium contamination was likely to be high, for instance where high magnesium concentrations were used. The ratio of cell potassium to haemoglobin was routinely monitored during experiments and was shown not to alter, thus indicating that the potassium channel had not been activated and indirectly that cell calcium had not increased.

Mg-EDTA buffers were used when it was necessary to maintain low magnesium concentrations in the medium and thus in the cells. Normally 2 mM-EDTA was added to the medium together with the required amount of magnesium chloride. Care was taken to correct the acidification of the medium which occurred when EDTA bound magnesium. Cells lose magnesium when they are suspended in media containing A23187 and low ionized magnesium concentrations (see Flatman & Lew, 1980*a*, for details of the red cell magnesium buffer curve). It was necessary to allow for this extra magnesium in the medium when calculating the equilibrium ionized magnesium concentration. The concentration of magnesium lost from the cells under these conditions was calculated from the initial and final magnesium contents of the cells and the haematocrit of the suspension. In some experiments the magnesium content of the medium was measured directly by atomic absorption spectroscopy. The value of the dissociation constant for Mg-EDTA at pH 7·5, 37 °C and physiological ionic strength was calculated using the method of Wolf (1973) from the information given in tables of Sillén & Martell (1971). A value of $10^{-6.33}$ was obtained and this was then used to calculate the concentration of ionized magnesium in the medium and cells.

Preparation of cells

Cells from freshly drawn, heparinized blood were washed four times in medium A, which contained: 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris Cl (pH 7·7 at 37 °C) and 0·1 mM-Tris EGTA. Care was taken to ensure that all of the buffy coat was removed. The washed cells were resuspended at 20% haematocrit in medium A containing 10 mM-inosine as a glycolytic substrate (see Whittam & Wiley, 1967) and 1 mCi of $^{24}NaCl$ per 6 ml of suspension (Amersham, $^{24}NaCl$ injection, SGS 1P). The suspension was then incubated at 37 °C with constant slow agitation for at least 4 h. At the end of this period the cells were washed four times with the relevant ice-cold incubation medium.

Composition of incubation media

All solutions were prepared with double glass distilled water. EGTA, EDTA, inosine, ouabain, choline chloride, Hepes and Tris-Cl were obtained from Sigma Ltd. All other reagents were Analar grade. A23187 was a gift from the Lilly Research Centre. Choline chloride was recrystallized from boiling ethanol before use.

(*a*) For experiments at external magnesium concentrations below 0·15 mM the media contained:

10 mM-Tris-Cl (pH 7·7 at 37 °C)
10 mM-inosine
0·01–0·02 mM-Tris-EGTA

plus either 75 mM-KCl + 75 mM-NaCl for sodium–potassium exchange

or 150 mM-NaCl for sodium–sodium exchange.

(*b*) For experiments at external magnesium concentrations above 0·15 mM the media contained:

10 mM-Hepes (pH 7·7 at 37 °C)
10 mM-inosine
0·05 mM-Tris-EGTA

plus either 140 mM-NaCl + 10 mM-KCl for sodium–potassium exchange

or 140 mM-NaCl + 10 mM-choline chloride for sodium–sodium exchange.

A low-sodium, high-potassium medium was used in the study of sodium–potassium exchange at low magnesium concentrations. This reduced the amount of sodium which entered through A23187 under these conditions (see Flatman & Lew, 1977*b*) without affecting the sodium pump fluxes. Preliminary experiments showed that neither the change of buffer from Tris to Hepes nor the increase in the concentration of EGTA affected the sodium pump fluxes.

Magnesium chloride was added to these basic media as a concentrated stock solution so that dilution of the media was less than 1 %. A23187 was also added as a concentrated stock solution made up in ethanol and containing 1 mg A23187 per ml ethanol (1·9 mM). In early experiments a final concentration of 10 μM-A23187 was used. However, the A23187 concentration was reduced to 3 μM when it became apparent that A23187 could transport sodium when the concentration of magnesium was low (Flatman & Lew, 1977*b*). This reduced the sodium influx to less than one quarter of that found with 10 μM-A23187.

Procedure

In any one experiment the magnesium dependence of both sodium–potassium and sodium–sodium exchange were examined using the same batch of ^{24}Na-loaded red cells.

^{24}Na-loaded red cells were added to incubation media to give a haematocrit of between 4 and 10 %. The suspensions were incubated at 37 °C in small plastic vials under constant magnetic stirring. After 10 min to allow equilibration 100 μl samples were taken to measure the haematocrit, total ^{24}Na activity in the suspension, and the magnesium content of the cells and medium. A small quantity of A23187 stock solution was then added to give a final concentration of 3–10 μM in the medium. After 15 min to allow magnesium to come into equilibrium across the membrane a 100 μl sample of the suspension was taken to measure the ^{24}Na activity in the medium and the new magnesium content of the cells. Further samples were taken at 10 min intervals over the next 40 min. Each 100 μl sample was quickly ejected into an Eppendorf microcentrifuge tube containing 0·9 ml of an ice-cold inactivation medium (medium A + 2 mM-EDTA) and 0·4 ml of the oil di-n-butylphthalate. The tube was capped, inverted so that the contents were mixed and then spun at 8000 *g* for 10 s. This procedure quickly inactivates the ionophore-induced permeability and separates cells from medium. The tubes were then set aside and processed as a batch at the end of the experiment. During the experiment samples of the cells were taken as necessary to measure their sodium, potassium, ATP and ADP contents. The chloride distribution ratio for each magnesium level was obtained from a similar experiment where no ^{24}Na was present (see Flatman & Lew, 1980*a*, for details).

Haematocrit, and cell potassium, sodium and magnesium were measured using the methods described by Flatman & Lew (1980*a*). When necessary, the magnesium content of the medium was measured as described by Flatman (1980).

At the end of the experiment the ^{24}Na activities were measured in the aqueous supernatants above the oil and in the samples of the whole suspension. The sodium efflux rate constant was calculated from these data (see Garrahan & Glynn, 1967*a*).

Measurement of cell ATP and ADP

Cell ATP and ADP were measured using the assay kits supplied by Boehringer Ltd (ATP kit: catalogue number 123897; ADP + AMP kit: catalogue number 123820) and Sigma Ltd (ATP kit: catalogue number 366-UV). The assays were scaled down so that 1–3 ml of suspension could be used. The proportion of NADH added in the analysis of ADP was reduced to improve sensitivity.

RESULTS

The addition of 10 μM-A23187 to red cell suspensions caused magnesium to enter or leave the cells depending on the magnesium concentration in the medium, and new equilibrium levels were reached within 15 min. Fig. 1 shows the magnesium content of red cells measured over the 40 min period starting 15 min after ionophore addition. The magnesium levels were very stable for this period and the concentrations attained were not affected by the concentrations of sodium, potassium or ouabain in the medium (Table 1). Other experiments showed that the magnesium levels were insensitive to changes in the concentration of A23187 (Flatman & Lew, 1980*a*) or of

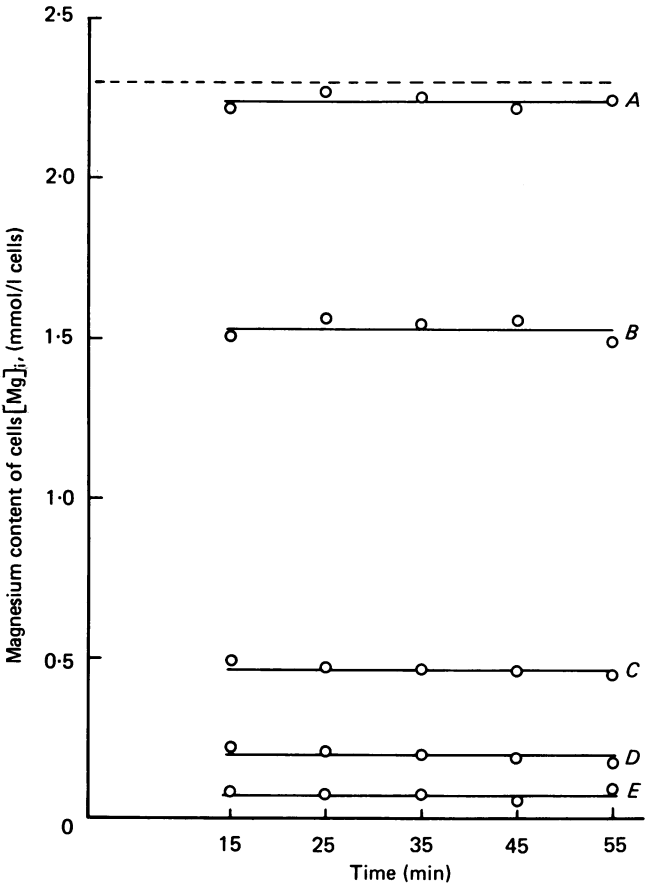


Fig. 1. The magnesium content of red cells measured during an experiment on the magnesium dependence of the sodium pump. Red cells were incubated at 37 °C and 10% haematocrit in a medium which contained: 150 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 0·01 mM-EGTA and the following additions: *A*, 2 mM-EDTA + 2·1 mM-$MgCl_2$; *B*, no additions; *C*, 2 mM-EDTA + 1·8 mM-$MgCl_2$: *D*, 2 mM-EDTA + 1·6 mM-$MgCl_2$: *E*, 2 mM-EDTA. At time $t = 0$. 10 μM-A23187 was added to the medium and 15 min later and at subsequent 10 min intervals 100 μl samples were taken to measure the magnesium content of the cells and the ^{24}Na efflux. The lines drawn through the points represent the mean magnesium content during the 40 min period. The dashed line indicates the initial magnesium content of the cells.

choline and to whether Tris or Hepes was used as pH buffer (unpublished observations).

Fig. 2 shows the total (circles) and ouabain-resistant (triangles) sodium effluxes measured during the same 40 min period mentioned above. The cells were incubated in media containing potassium (left panel) or not containing potassium (right panel) and containing either very low (top row) or normal (bottom row) ionized magnesium. It can be seen from Fig. 2 that straight lines fit the data well. This, together with

TABLE 1. The effect of changes in the composition of the medium on the magnesium content of red cells (mmol/l cells, $n = 5$) incubated in the presence of 10 μM-A23187 and 2 mM-EDTA.

	Sodium–potassium medium		Sodium–sodium medium	
Additions	Control	$+10^{-3}$ M-ouabain	Control	$+10^{-3}$ M-ouabain
—	0·060 ± 0·006	0·074 ± 0·006	0·054 ± 0·015	0·030 ± 0·004
1·5 mM-$MgCl_2$	0·221 ± 0·012	0·240 ± 0·006	0·243 ± 0·012	0·229 ± 0·007
2·1 mM-$MgCl_2$	not available	2·239 ± 0·037	2·121 ± 0·037	2·239 ± 0·010

This Table shows the results from a single experiment. The sodium–potassium medium contained: 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 0·01 mM-EGTA, 2 mM-EDTA and 10 mM-inosine. The sodium–sodium medium contained: 150 mM-NaCl, 10 mM-Tris-Cl, 0·01 mM-EGTA, 2 mM-EDTA and 10 mM-inosine. The magnesium contents are given as the mean ± S.E.M.

data not shown, indicated that it is possible to measure both sodium–potassium and sodium–sodium exchange mediated by the sodium pump for concentrations of internal ionized magnesium ranging from 10^{-7} to 6×10^{-3} M.

Table 2 gives the rate constants obtained from the data shown in Fig. 2. It can be seen that there was a low but detectable level of both sodium–potassium and sodium–sodium exchange through the pump when the medium contained 10 μM-A23187, 2 mM-EDTA and no added magnesium. When 2 mM-EDTA and 2·1 mM-magnesium chloride were present in the medium ionized magnesium was initially at electrochemical equilibrium and the addition of A23187 affected neither the magnesium content of the cells (Fig. 1) nor the rate of sodium efflux when compared with control cells to which no A23187 had been added (Table 2). It can be concluded that the presence of 10 μM-A23187 *per se* does not affect the performance of the sodium pump in either the sodium–potassium or sodium–sodium exchange mode of operation. It was also found that the ouabain-resistant fluxes were unaffected by the presence of A23187 (Table 2).

The magnesium dependence of sodium–potassium exchange at magnesium concentrations below the physiological level

In Fig. 3 sodium–potassium exchange is plotted as a function of the total magnesium content of the cells. In this form the data are not affected by the choice of MgEDTA dissociation constant or by assumptions concerning the distribution of magnesium across the membrane. However, the data are difficult to analyse when expressed in this way because total magnesium comprises ionized magnesium together with magnesium bound to a wide variety of intracellular constituents (Flatman & Lew, 1977*a*, 1980*a*). These constituents include substances such as 2,3-DPG (diphosphoglycerate) which are not thought to affect sodium pump activity.

The data were therefore replotted as a function of ionized magnesium (Fig. 4) so that the findings can be interpreted using current models of the pump.

It was found that when the medium contained 2 mM-EDTA, a condition where there was at least ten times as much EDTA as magnesium in the suspension, the cells still contained between 0·03 and 0·08 mmol/l cells of magnesium (see Table 3 and Flatman & Lew, 1980*a*) and there was still a significant amount of sodium–potassium exchange occurring through the pump (Table 3). Under these conditions it was calculated that the intracellular ionized magnesium concentration was less than 5×10^{-7} M. As the concentration of ionized magnesium was increased to 2×10^{-6} M

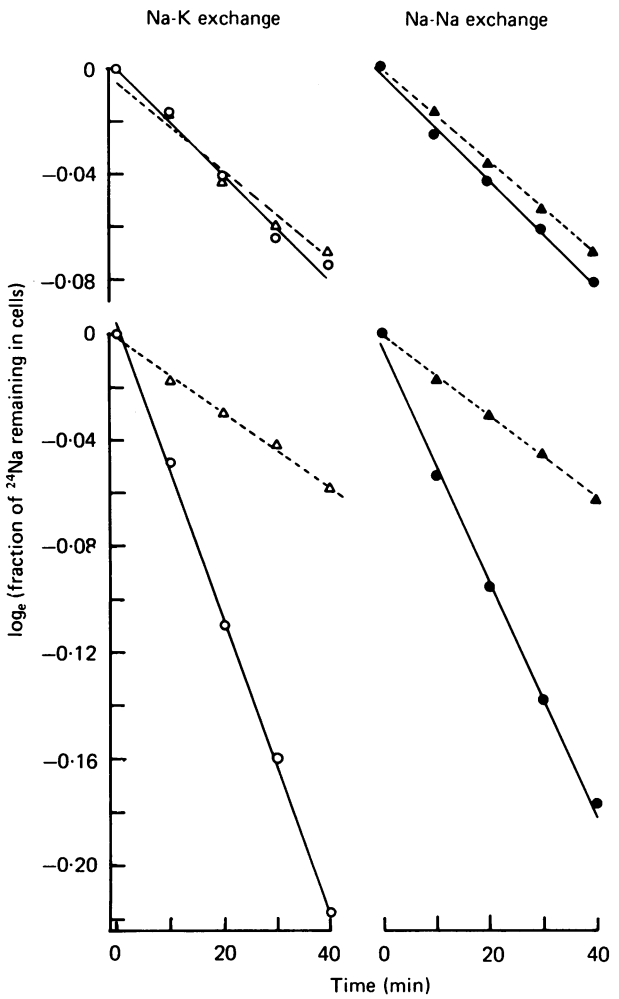


Fig. 2. Examples of ^{24}Na efflux after the addition of A23187. Red cells were incubated in the following media: left panel, open symbols (sodium–potassium exchange): 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 0·01 mM-EGTA; right panel, filled symbols (sodium–sodium exchange): 150 mM-NaCl, 10 mM-Tris, 10 mM-inosine, 0·01 mM-EGTA. The following additions were made to both media: upper row 2 mM-EDTA + 10 μM-A23187; bottom row: 2 mM-EDTA + 2·1 mM-$MgCl_2$ + 10 μM-A23187. In both cases the circles indicate the total efflux and the triangles the efflux in the presence of 10^{-3} M-ouabain. The lines were fitted through the points by regression analysis.

there was little change either in the cellular magnesium content or in the rate of sodium efflux.

Once the level of ionized magnesium increased above 2×10^{-6} M (equivalent to a total magnesium content of about 0·12 mmol/l cells), the rate of sodium–potassium exchange started to increase rapidly, reaching a maximum rate when the intracellular ionized magnesium concentration ($[Mg^{2+}]_i^{\infty}$) was about 0·6–0·8 mM (equivalent to a total magnesium content of 3–4 mmol/l cells).

TABLE 2. The effect of medium composition and 10 μM-A23187 on the rate constants for sodium efflux in the presence of 2 mM-EDTA

Medium	A23187 (μM)	Rate constant in absence of 10^{-3} M-ouabain (h^{-1})	Rate constant in presence of 10^{-3} M-ouabain (h^{-1})	Ouabain-sensitive rate constant (h^{-1})
Sodium–potassium	10	0·1196 ± 0·0038	0·1097 ± 0·0040	0·0099 ± 0·0055
Sodium–potassium + 2·1 mM-$MgCl_2$	10	0·3291 ± 0·0030	0·0851 ± 0·0017	0·2440 ± 0·0034
Sodium–sodium	10	0·1200 ± 0·0023	0·1064 ± 0·001	0·0136 ± 0·0025
Sodium–sodium + 2·1 mM-$MgCl_2$	10	0·2629 ± 0·0043	0·0921 ± 0·0011	0·1708 ± 0·0044
Sodium–potassium + 2·1 mM-$MgCl_2$	0	0·3200 ± 0·0045	0·0900 ± 0·0045	0·2300 ± 0·0061
Sodium–sodium + 2·1 mM-$MgCl_2$	0	0·2710 ± 0·0059	0·1149 ± 0·0023	0·1561 ± 0·0063

The rate constants are given with the standard deviation. The values in the presence of 10 μM-A23187 are the slopes of the curves shown in Fig. 2.

The sodium–potassium medium contained: 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 0·01 mM-EGTA, 2 mM-EDTA and 10 mM-inosine.

The sodium–sodium medium contained: 150 mM-NaCl, 10 mM-Tris-Cl, 0·01 mM-EGTA, 2 mM-EDTA and 10 mM-inosine.

Bodemann & Hoffman (1976) showed that removal of internal magnesium from red cell ghosts slowed the rate of ouabain binding to the sodium pump. It is therefore possible that the sizes of ouabain-sensitive fluxes reported in this paper for low magnesium concentrations are an underestimate of their true value, as a result of incomplete pump inhibition. However, ouabain was always present in the medium at large excess (10^{-3} M) for at least 15 min before the addition of A23187. All the sodium-pumps should therefore have irreversibly bound ouabain long before magnesium was removed from the cell interior. This view is supported by the data in Table 4, which shows that the size of the ouabain-resistant fluxes did not alter significantly as the concentration of ionized magnesium was varied over a wide range.

The dashed line in Fig. 4 was drawn assuming that ionized magnesium binds to a single site on the enzyme to stimulate sodium–potassium exchange and that Michaelis–Menten kinetics are obeyed. The $K_{\frac{1}{2}}$ and V_{max} were obtained after subtracting from the total flux the small component which was apparently independent of magnesium concentration and which was seen at the very lowest magnesium concentrations (see Table 3). The quotient, (magnesium-sensitive k_e)/$[Mg^{2+}]_i^{\infty}$, was then plotted against $[Mg^{2+}]_i^{\infty}$ and a straight line was drawn through the points using

linear regression analysis. The $K_{\frac{1}{2}}$ and V_{max} were calculated from the intercepts (Hanes plot: see Dixon & Webb, 1979). The dashed line was drawn according to the equation:

$$k_e = 0{\cdot}0117 + \frac{0{\cdot}264[Mg^{2+}]_i^{\infty}}{0{\cdot}045 + [Mg^{2+}]_i^{\infty}} \quad (h^{-1}). \tag{2}$$

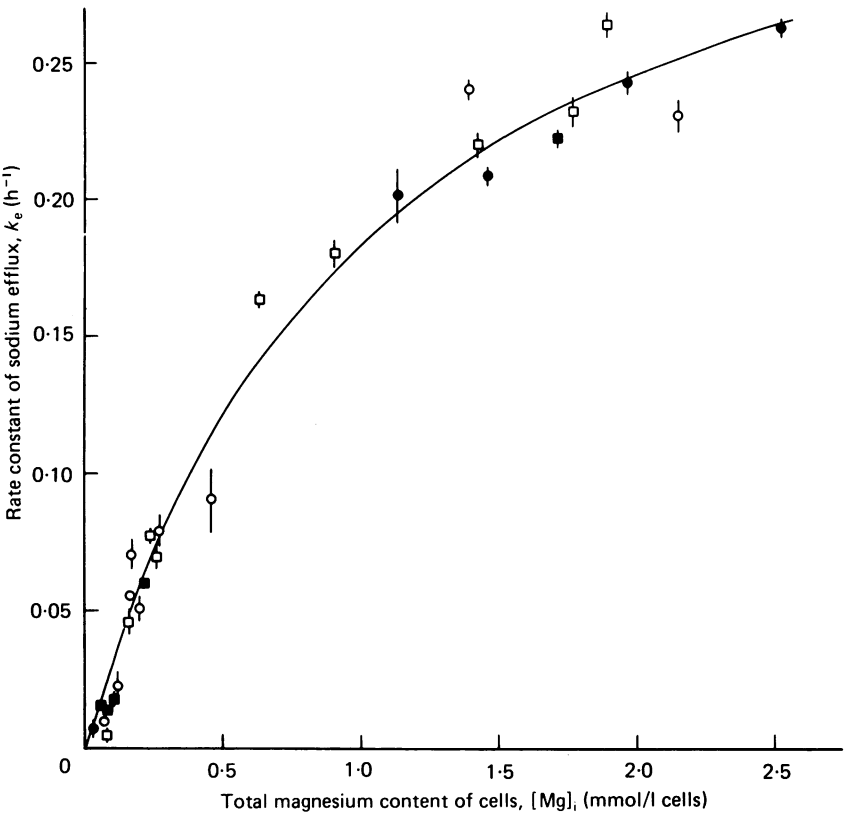


Fig. 3. Sodium–potassium exchange as a function of internal magnesium. The ouabain-sensitive sodium efflux rate constants, k_e ($\pm$standard deviation), are plotted as a function of the equilibrium magnesium content of the cells ($[Mg]_i$) in the presence of A23187. The medium contained: 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 0·01 mM-EGTA, 10 μM-A23187, varying concentrations of $MgCl_2$ and $\pm 10^{-3}$ M-ouabain. The different symbols represent separate experiments. The line through the data was drawn according to the equation:

$$k_e = \frac{0{\cdot}376\,[Mg]_i}{1{\cdot}059 + [Mg]_i}\ (h^{-1}).$$

However, it is not clear whether this equation accurately describes the data when the ionized magnesium concentration is greater than 0·1 mM. Careful examination of the results from particular experiments, for instance that denoted by the open squares or that denoted by the filled circles, suggests that there is a more or less linear dependence of k_e on $[Mg^{2+}]_i^{\infty}$ at magnesium concentrations above 0·1 mM. If a linear component is included in the description of the data a much better fit can

be obtained. The continuous line in Fig. 4 was therefore drawn according to the equation:

$$k_e = 0{\cdot}117 + 0{\cdot}14[Mg^{2+}]_i^{\infty} + \frac{0{\cdot}2013[Mg^{2+}]_i^{\infty}}{0{\cdot}0305 + [Mg^{2+}]_i^{\infty}} \quad (h^{-1}), \qquad (3)$$

where the Michaelis constants were calculated from a Hanes plot after subtraction of the constant and linear components from the data.

TABLE 3. Ouabain-sensitive sodium efflux rate constants at very low internal magnesium concentrations

Sodium–potassium exchange			Sodium–sodium exchange		
A23187 (μM)	Magnesium content (mmol/l cells) ($n = 5$)	Ouabain-sensitive sodium efflux rate constant, k_e (h^{-1})	A23187 (μM)	Magnesium content (mmol/l cells) ($n = 5$)	Ouabain-sensitive sodium efflux rate constant, k_e (h^{-1})
10	0·038 ± 0·007	0·0076	10	0·031 ± 0·007	0·0014
10	0·060 ± 0·006	0·0153	10	0·054 ± 0·015	0·0106
10	0·076 ± 0·007	0·0099	10	0·076 ± 0·007	0·0136
10	0·079 ± 0·004	0·0056	10	0·079 ± 0·004	0·0021
3·3*	0·043 ± 0·004	0·0199	3·3	0·044 ± 0·005	0·0197
Mean rate constant = 0·0117 ± 0·0028 (h^{-1})			Mean rate constant = 0·0095 ± 0·0035 (h^{-1})		

This Table summarizes the results of five separate experiments. Red cells were suspended at approximately 10% haematocrit in the following media. For sodium–potassium exchange: 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 0·01 mM-EGTA, 10 mM-inosine, except in the experiment denoted by * where the NaCl concentration was increased to 140 mM and KCl concentration was decreased to 10 mM. The medium for sodium–sodium exchange contained: 150 mM-NaCl, 10 mM-Tris, 0·01 mM-EGTA, 10 mM-inosine. All solutions contained 2 mM-EDTA. When present, the ouabain concentration was 10^{-3} M. Five samples were taken 15 min after the addition of A23187, to measure the new magnesium content (shown as the mean ± S.E.M., $n = 5$) and the rate constant of sodium efflux. The calculated ionized intracellular magnesium was less than 5×10^{-7} M in all cells.

TABLE 4. Ouabain-resistant fluxes as a function of ionized magnesium

Ionized magnesium concentration in cells (mM)	Ouabain-resistant sodium efflux rate constant in sodium–potassium medium (h^{-1})	Ouabain-resistant sodium efflux rate constant in sodium–sodium medium (h^{-1})
0·0003	0·110 ± 0·004	0·106 ± 0·001
0·003	0·101 ± 0·005	0·106 ± 0·004
0·007	0·110 ± 0·003	0·104 ± 0·001
0·008	0·095 ± 0·003	0·094 ± 0·002
0·011	0·107 ± 0·002	0·085 ± 0·002
0·02	0·102 ± 0·003	0·093 ± 0·003
0·043	0·096 ± 0·002	0·087 ± 0·003
0·20	0·085 ± 0·002	0·092 ± 0·001
0·85	0·096 ± 0·002	0·107 ± 0·001

The sodium–potassium medium contained: 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 0·01 mM-EGTA, 10 mM-inosine, 1·0 mM-ouabain, 10 μM-A23187 and varying magnesium concentrations.

The sodium–sodium medium contained: 150 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 0·01 mM-EGTA, 10 μM-A23187, 1·0 mM-ouabain and varying magnesium concentrations.

The values of the sodium efflux rate constant are given with the standard deviation.

The magnesium dependence of sodium–sodium exchange at magnesium concentrations below the physiological level

A similar strategy to the one described above was used to investigate the magnesium dependence of sodium–sodium exchange. Fig. 5 shows the values of the ouabain-sensitive sodium efflux rate constants plotted as a function of the total magnesium content of red cells and Fig. 6 shows the same data replotted as a function of the equilibrium ionized magnesium concentration in the cells.

Once again it was found that when the medium contained 2 mM-EDTA there was a significant level of sodium–sodium exchange through the pump (see Table 3). This

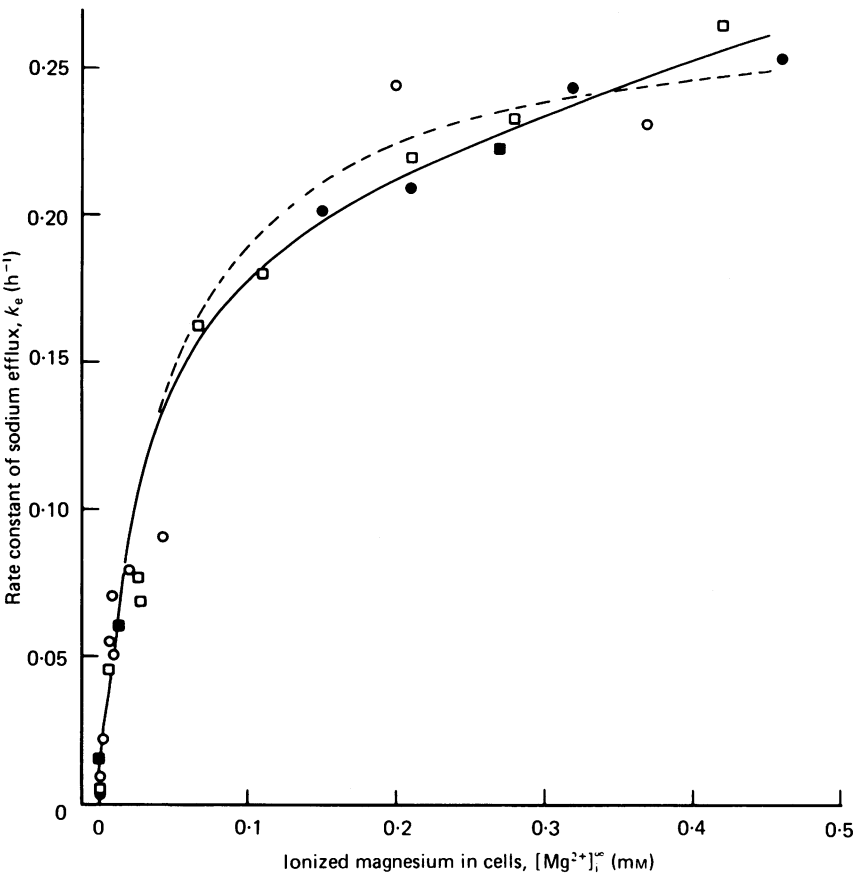


Fig. 4. Sodium–potassium exchange as a function of ionized magnesium. The data from Fig. 3 have been replotted as a function of the intracellular ionized magnesium concentration, $[Mg^{2+}]_i^{\infty}$. The dashed line through the data was drawn according to the equation:

$$k_e = 0{\cdot}0117 + \frac{0{\cdot}264[Mg^{2+}]_i^{\infty}}{0{\cdot}045 + [Mg^{2+}]_i^{\infty}} \quad (h^{-1})$$

and the continuous line according to the equation:

$$k_e = 0{\cdot}0117 + 0{\cdot}14[Mg^{2+}]_i^{\infty} + \frac{0{\cdot}2013[Mg^{2+}]_i^{\infty}}{0{\cdot}0305 + [Mg^{2+}]_i^{\infty}} \quad (h^{-1}).$$

level of activity was unaffected by increasing the ionized magnesium concentration from 5×10^{-7} to 2×10^{-6} M (see above). When the concentration of intracellular ionized magnesium ($[Mg^{2+}]_i^{\infty}$) was increased above 2×10^{-6} M the rate of sodium–sodium exchange increased, reaching a maximum level when $[Mg^{2+}]_i^{\infty}$ was about 0·2 mM (equivalent to a total magnesium content of 1·5 mmol/l cells). This maximal

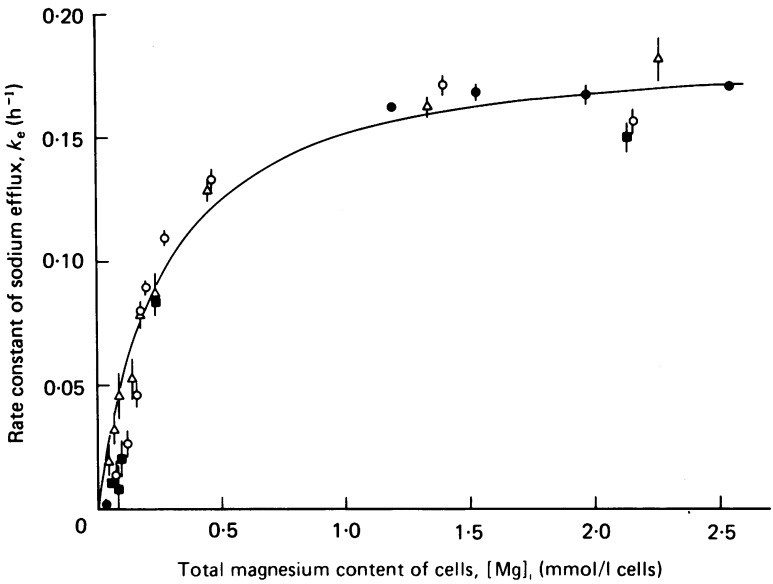


Fig. 5. Sodium–sodium exchange as a function of internal magnesium. The ouabain-sensitive sodium efflux rate constants, k_e (±standard deviation), are plotted as a function of the equilibrium magnesium contents of the cells ($[Mg]_i$) in the presence of A23187. The medium contained: 150 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 0·01 mM-EGTA, varying concentrations of $MgCl_2$, $\pm 10^{-3}$ M-ouabain and 10 μM-A23187 (○, ●, ■) or 3·3 μM-A23187 (△). The different symbols represent separate experiments. The line through the points was drawn according to the equation:

$$k_e = \frac{0{\cdot}189\,[Mg]_i}{0{\cdot}250 + [Mg]_i} \quad (h^{-1}).$$

rate of efflux was maintained until $[Mg^{2+}]_i^{\infty}$ was between 0·6 and 0·8 mM, whereupon further increase in the magnesium concentration led to an inhibition of sodium–sodium exchange.

The line through the data in Fig. 6 was drawn assuming that sodium–sodium exchange was stimulated by ionized magnesium binding to a single site on the enzyme. The line was drawn according to the equation:

$$k_e = 0{\cdot}0095 + \frac{0{\cdot}158\,[Mg^{2+}]_i^{\infty}}{0{\cdot}009 + [Mg^{2+}]_i^{\infty}} \quad (h^{-1}), \tag{4}$$

which takes into account the small, apparently magnesium-independent component of sodium efflux seen at low magnesium concentrations. The line drawn in Fig. 6 is an excellent fit to the data, which indicates that two efflux components are sufficient to describe the observations. Unlike sodium–potassium exchange there is no component of efflux which increases linearly with ionized magnesium concentration.

Comparison of the magnesium dependence of sodium–potassium and sodium–sodium exchange

It is possible to compare the magnesium dependence of sodium–potassium and sodium–sodium exchange since in each experiment both fluxes were examined using the same stock of cells. The cells would have been in the same metabolic state but may have had some minor difference in their sodium and potassium contents due to the washing and storage afer ^{24}Na-loading.

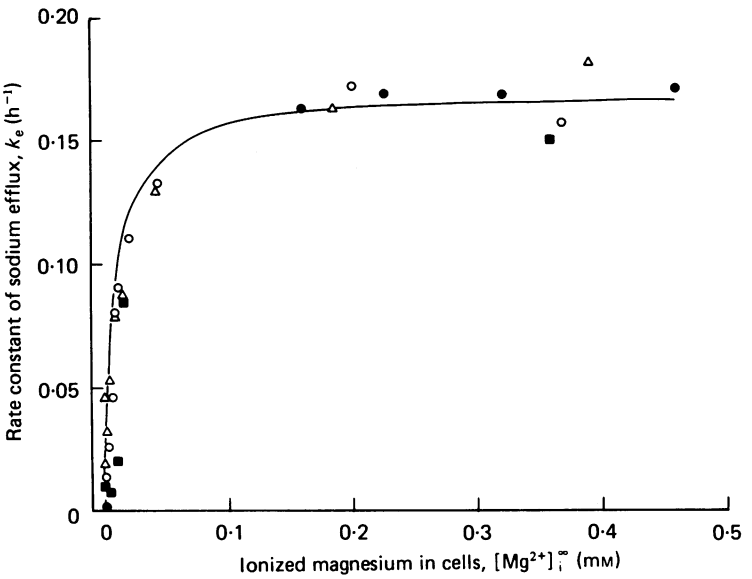


Fig. 6. Sodium–sodium exchange as a function of ionized magnesium. The data shown in Fig. 5 have been replotted as a function of the intracellular ionized magnesium concentration, $[Mg^{2+}]_i^\infty$. The continuous line through the data was drawn according to the equation:

$$k_e = 0{\cdot}0095 + \frac{0{\cdot}158[Mg^{2+}]_i^\infty}{0{\cdot}009 + [Mg^{2+}]_i^\infty} \quad (h^{-1}).$$

In each individual experiment (the same symbol has been used for each experiment throughout Figs. 3–6) it was found that the rate constants for sodium–sodium and sodium–potassium exchange were more or less equal at the very low magnesium concentrations (see Table 3). In the range of ionized magnesium concentrations between 2×10^{-6} and 6×10^{-5} M (equivalent to total magnesium contents between 0·12 and 0·05 mmol/l cells) the rate constants for sodium–sodium exchange were usually larger than those for sodium–potassium exchange, though the difference was not always significant. At higher magnesium levels the rate constants for sodium–potassium exchange were very significantly greater than those for sodium–sodium exchange so that at physiological ionized magnesium levels for the oxygenated red cell (0·4 mM: see Flatman, 1980) sodium–potassium exchange was 60 % greater than sodium–sodium exchange, as is found in cells from this donor not treated with A23187 (see Table 2). A summary of results is given in Fig. 7, where the three-component curve fit to the data of Fig. 4 (sodium–potassium exchange) and the curve fit to the data in Fig. 6 (sodium–sodium exchange) have been plotted on the same graph.

Sodium–potassium and sodium–sodium exchange as a function of high internal magnesium concentration

The effects on the sodium pump of internal magnesium concentrations higher than the normal physiological level were examined in a separate series of experiments. Media containing 140 mM-sodium chloride were chosen for experiments on sodium–potassium exchange as well as for experiments on sodium–sodium exchange since sodium entry through A23187 does not occur at the magnesium concentrations used

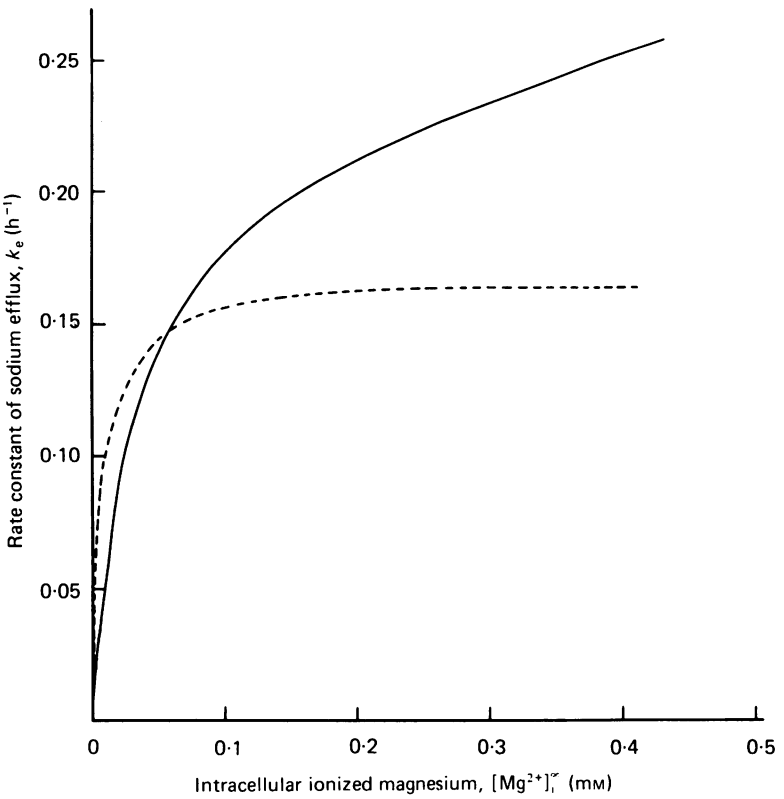


Fig. 7. Comparison of sodium–potassium and sodium–sodium exchange as functions of ionized magnesium. The three-component curve fit to the sodium–potassium exchange data from Fig. 4 (continuous line) and the curve fit to the sodium–sodium exchange data from Fig. 6 (dashed line) are plotted together as a function of ionized intracellular magnesium.

here. Measurement of the sodium and potassium content of the cells during these experiments showed that the contents did not change significantly.

Fig. 8 shows the results of two experiments where the rate constants have been normalized relative to the value obtained when the medium initially contained 0·15 mM-magnesium chloride. These normalized values were then plotted as a function of the ionized intracellular magnesium concentration in the cells at equilibrium. Fig. 8 clearly shows the progressive inhibition of sodium–sodium and sodium–potassium exchange as the ionized magnesium concentration was increased

above 0·6–0·8 mM. It also shows that sodium–sodium exchange is more strongly inhibited than sodium–potassium exchange. 50 % inhibition of sodium–sodium and sodium–potassium exchange occurred when the ionized magnesium concentrations in the cells were about 2·7 and 7·7 mM respectively (corresponding to magnesium contents of 9·3 and approximately 20 mmol/l cells).

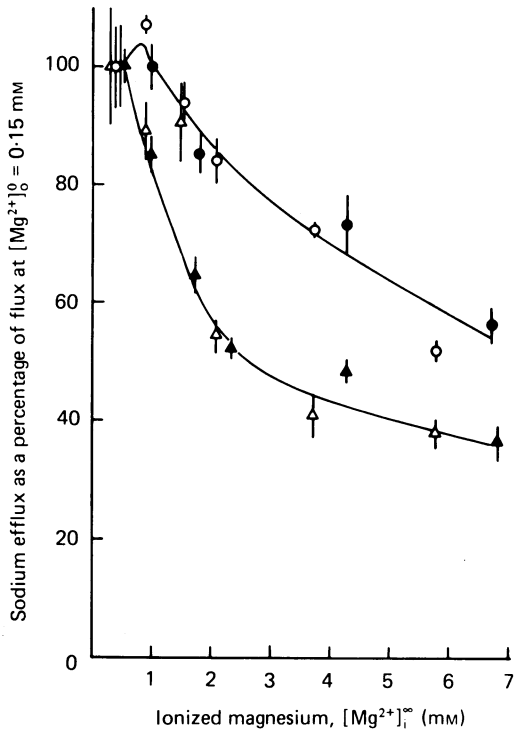


Fig. 8. Normalized rates of sodium–potassium and sodium–sodium exchange as a function of high intracellular ionized magnesium concentrations. Sodium efflux rate constants for sodium–potassium (○, ●) and sodium–sodium (△, ▲) exchange were measured using cells from two donors (open and filled symbols). The rate constants were normalized relative to the values obtained when the medium initially contained 0·15 mM-$MgCl_2$. These initial values were as follows: ●, 0·3303; ○, 0·2073; ▲, 0·4003; △, 0·1580 (h^{-1}). The normalized values were then plotted as a function of the ionized magnesium concentration in the cells at equilibrium ($[Mg^{2+}]_i^\infty$). The medium for sodium–potassium exchange experiments contained: 140 mM-NaCl, 10 mM-KCl, 10 mM-Hepes, 10 mM-inosine, 0·05 mM-EGTA, between 0·15 and 5 mM-$MgCl_2$, with and without 10^{-3} M-ouabain. A23187 at 3·3 μM was added to the medium and 15 min later the sodium fluxes were measured. The medium for sodium–sodium exchange experiments was similar but 10 mM-choline chloride replaced the KCl. Each point is given with the standard deviation and the lines were drawn by eye.

The Figure includes data from a donor whose initial rate of sodium–sodium exchange was higher than the rate of sodium–potassium exchange under the conditions of the experiments (140 mM-NaCl ± 10 mM-KCl). The pattern of inhibition of the sodium pump by magnesium was, however, identical to that found with the cells from the usual donor. The high rate of sodium–sodium exchange compared with sodium–potassium exchange in this donor may be due to the very low internal sodium content of these cells (4 mmol/l cells).

Sidedness and reversibility of magnesium's effect on the sodium pump

So far it has been assumed that magnesium acts at the inner surface of the membrane. This was confirmed in experiments where sodium–potassium and sodium–sodium exchange were measured in the absence of A23187. There was no significant change in the rate of either process as the external magnesium concentration was

TABLE 5. Sidedness and reversibility of magnesium's effect on the sodium pump

	Experimental groups of cells			Control cells
Time when flux was measured (min)	Additions	Magnesium content of cells (mmol/l cells) ($n = 5$)	Ouabain-sensitive sodium efflux rate constant, k_e (h^{-1})	Ouabain-sensitive sodium efflux rate constant, k_e (h^{-1})
(i) Sodium–sodium				
0–20	—	2·304 ± 0·025*	0·1691 ± 0·0030	0·16191 ± 0·005
44–64	3 μM-A23187 at $t = 24$ min	0·025 ± 0·006	−0·0270 ± 0·0011	0·1524 ± 0·0140
88–108	2·1 mM-$MgCl_2$ at $t = 68$ min	2·283 ± 0·0013	0·0583 ± 0·0073	0·1303 ± 0·0065
(ii) Sodium–potassium				
0–20	—	2·304 ± 0·025*	0·1957 ± 0·0045	0·1925 ± 0·0044
44–64	3 μM-A23187 at $t = 24$ min	0·026 ± 0·003	−0·0107 ± 0·0107	0·1915 ± 0·0107
88–108	2·1 mM-$MgCl_2$ at $t = 68$ min	2·452 ± 0·019	0·2519 ± 0·0182	0·2171 ± 0·0102

^{24}Na-loaded red cells were incubated at 4–5% haematocrit in media which contained: 140 mM-NaCl, 10 mM-Hepes, 10 mM-ionosine, 0·02 mM-EGTA and either 10 mM-KCl for experiments on sodium–potassium exchange or 10 mM-choline chloride for experiments on sodium–sodium exchange. EDTA at 2 mM was added to the medium of the experimental group of cells and 0·15 mM-$MgCl_2$ was added to the control group. Ouabain at 1 mM was added when necessary. Samples were taken at 5 min intervals to measure ^{24}Na efflux over the 20 min periods indicated. The following additions were made to the experimental group: at $t = 24$ min, 3 μM-A23187, and at $t = 68$ min, 2·1 mM-$MgCl_2$ followed gy sufficient NaOH to maintain pH. No additions were made to the control group. Rate constants are given with the standard deviation.

The potassium content of the experimental cells remained constant throughout the 108 min incubation period. However, the sodium content of the experimental cells increased from 9 to 16 mmol/l cells after the addition of A23187. The increase in sodium content stopped when 2·1 mM-$MgCl_2$ was added.

* $n = 9$.

increased from 10^{-7} to 5×10^{-3} M (Table 5 also includes the effect of changing external magnesium from 10^{-7} to $1{\cdot}5 \times 10^{-4}$ M in the absence of A23187).

The reversibility of the effects of magnesium is shown in Table 5. An extreme condition, where 2 mM-EDTA was present in the medium, was chosen. It can be seen that before the addition of A23187 the rates of both sodium–potassium and sodium–sodium exchange were similar to control values. After the addition of A23187 the magnesium content of the cells fell to a very low value and both modes of transport were inhibited. A small quantity of magnesium chloride (final concentration in the medium was 2·1 mM) was added to the medium followed by a small quantity

of sodium hydroxide. The magnesium content of the cells was restored to normal and the protons released by the binding of magnesium to EDTA were neutralized thus maintaining pH. The restoration of cellular magnesium content resulted in an increase in the rate constants for both sodium–potassium and sodium–sodium exchange. However, neither returned to the values seen in the control groups, probably because the sodium contents had increased from 9 to 16 mmol/l cells during the treatment with A23187 and EDTA (see Flatman & Lew, 1977*b*). Such an increase in sodium content can lead to an increase in the rate constant of sodium–potassium exchange while decreasing the rate constant of sodium–sodium exchange (Sachs, 1970), as was seen here. The results in Table 5 therefore show that the effects of changing the magnesium content of cells are not irreversible and that when the magnesium content has been restored to normal the cells can respond as usual to other changes in their internal environment.

The effects of changing intracellular magnesium on the concentration of other cell constituents

There have been recent reports that A23187 can directly alter the sodium content of cells (Flatman & Lew, 1977*b*) and indirectly alter the potassium content via a rise in internal calcium (see Lew & Ferreira, 1976). Changes in the concentrations of ATP and ADP might be expected to occur as a result of alterations in intracellular magnesium concentration because magnesium is an important co-factor for many glycolytic enzymes (see Rapoport, 1968). The cellular contents of potassium, sodium, ATP and ADP were therefore monitored during the experiments in order to ascertain whether they changed and whether these changes could account for some of the effects ascribed to magnesium. It has already been pointed out in the Methods section that significant changes in potassium content were not detected. Changes in sodium, ATP and ADP, however, were seen under some circumstances.

A23187 can transport sodium into red cells when the concentration of magnesium is low (Flatman & Lew, 1977*b*). The amount of sodium transported increases with the sodium content of the medium and the A23187 concentration in the suspension. It is reduced by raising the haematocrit and by increasing the ionized magnesium concentration in the medium. Half-maximal inhibition of sodium transport occurs when the ionized magnesium concentration is about 3 μM in the medium, which is equivalent to about 8 μM in the cells. A summary of the amounts of sodium transported by A23187 under various conditions is given in Table 6. It is clear that sodium gain via the ionophore is not a problem during the experiments on sodium–potassium exchange but it could complicate the interpretation of the sodium–sodium exchange data at low magnesium levels, especially when 10 μM-A23187 was used (see later).

Fig. 9 shows the ATP content of cells measured under conditions where the pump performs sodium–potassium exchange and ATP consumption is greater than during sodium–sodium exchange. It can be seen that there was a decline of about 30 % in the ATP content measured over a 45 min period at the two lowest magnesium concentrations, which are conditions where the pump is barely operating. As the intracellular magnesium concentration increased the fall in ATP content became smaller but was seen even when the magnesium content was not altered by the

addition of A23187 (condition *D*). This latter effect is presumably due to the ionophore itself. When the magnesium levels were increased above normal, small increases in ATP content were observed (conditions *A* and *B*). Almost identical patterns of changing ATP content as a function of internal magnesium were seen when cells were incubated in potassium-free media. The changes in ATP content must be due to a differential effect of magnesium on the ATP-consuming and ATP-producing

TABLE 6. Sodium transport in the presence of A23187

A23187 (μM)	Haematocrit (%)	Sodium content of medium (mM)	Ionized magnesium concentration in cells (mM)	Sodium gain through A23187 (mmol/l cells × h)
10	8–10	150	~ 0·0005	18
10	8–10	150	0·002	12
10	8–10	150	0·01	8
10	8–10	150	0·1	5
10	8–10	150	1·0	< 1
10	8–10	75	~ 0·0005	4
10	8–10	75	0·4	< 1
3·3	8	140–150	~ 0·0005	4
3·3	8	140–150	0·01	2
3·3	8	140–150	0·4	< 1
3·3	4–5	140	~ 0·0005	13
3·3	4–5	140	0·4	< 1
3·3	4–5	140	1·0	< 1
3·3	4–5	140	6·6	< 1

This table summarizes the increase in red cell sodium content measured after an hour's incubation in the presence of A23187. The cells were incubated at various haematocrits at 37 °C in media with widely ranging compositions containing 10^{-3} M-ouabain. The sodium content of the cells increased linearly with time.

reactions in the cell. Removal of internal magnesium probably inhibits inosine metabolism while allowing ATP breakdown to continue. Only a small fraction of this ATP breakdown can be due to the sodium pump since not only is there little pump activity at low magnesium levels, but also the fall in ATP content was similar in the presence and absence of external potassium. The rate at which the ATP content fell at the lowest magnesium concentrations was similar to the rate seen when red cells are starved at 37 °C. The increase in ATP content seen at the high magnesium concentrations probably reveals a rate-limiting effect of magnesium on inosine metabolism and cannot be due to the inhibition of the sodium pump sparing ATP since it was seen with cells incubated in the presence and absence of external potassium.

The initial ADP content of the red cells was about 0·2 mmol/l cells. An hour's incubation with A23187 and different magnesium concentrations led to an increase of between 0·05 and 0·1 mmol/l cells at the lowest magnesium concentrations. The ADP content returned towards the control level as the magnesium concentration was increased to the normal level, and fell slightly at the very highest magnesium concentrations. The changes in ADP content were also similar for cells incubated in the presence and absence of external potassium.

The ratio of ATP to ADP in the cells after an hour's incubation was between 5·5 and 6 for cells with a normal magnesium concentration and fell with magnesium concentration, reaching a minimum value of about 2·5 when $[Mg^{2+}]_i^{\infty}$ was less than 8 μM. The equilibrium constant for adenylate kinase, which catalyses the reaction

$$ATP + AMP \underset{}{\overset{K_{AK}}{\rightleftharpoons}} 2\,ADP$$

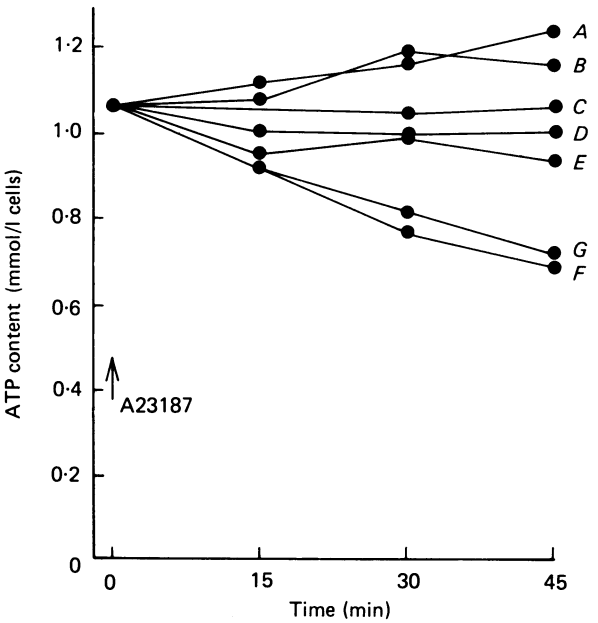


Fig. 9. The ATP content of red cells containing different magnesium concentrations. Red cells were incubated at 10 % haematocrit in a medium containing 75 mM-KCl, 75 mM-NaCl, 10 mM-Tris-Cl, 10 mM-inosine, 0·05 mM-EGTA, 10 μM-A23187 and the following additions: *A*, 5 mM-$MgCl_2$; *B*, 1 mM-$MgCl_2$; *D*, 0·15 mM-$MgCl_2$; *E*, no additions; *F*, 2 mM-EDTA + 1·5 mM-$MgCl_2$; *G*, 2 mM-EDTA. Condition *C* was a control to which no A23187 was added. The ionized intracellular magnesium concentrations at equilibrium were: *A*, 4·6 mM; *B*, 1·2 mM; *C*, about 0·4 mM; *D*, 0·36 mM; *E*, 0·22 mM; *F*, 0·008 mM; *G*, less than 10^{-3} mM. The lines were drawn by eye.

and which is highly active in red cells, is sensitive to changes in magnesium concentration (Bowen & Kerwin, 1956; Rose, 1968). The equilibrium constant is given by the expression

$$K_{AK} = \frac{[ATP][AMP]}{[ADP]^2}$$

and is about 0·37 at very low magnesium concentrations increasing to about 1·2 at physiological magnesium concentrations (Bowen & Kerwin, 1956; Rose, 1968). The changes in the concentrations of ATP and ADP would be consistent with changes in the adenylate kinase equilibrium if the level of AMP had remained constant. Unfortunately, the AMP concentrations were not measured systematically, so further speculation is not justified at present.

DISCUSSION

The results presented here show that magnesium interacts reversibly with the sodium pump at the inner surface of the membrane, altering the rates of pump-mediated sodium–potassium and sodium–sodium exchange. Changes in the concentration of external magnesium within the range 10^{-7} to 5×10^{-3} M do not affect the pump unless A23187 is present in the medium. Under these conditions internal magnesium concentration changes to a new equilibrium level within about 5 min of A23187 addition and the pump fluxes are duly altered. A23187 does not appear to have a direct pharmacological effect on the sodium pump since its addition to red cells suspended in a medium containing 0·12 to 0·15 mM-magnesium neither changes the magnesium content of the cells nor alters the pump-mediated fluxes.

Pump fluxes as functions of ionized magnesium

Certain characteristic features become apparent when sodium–potassium and sodium–sodium exchange are plotted as functions of ionized magnesium (Figs. 4, 6, 7 and 8).

(i) *Low magnesium concentrations.* At $[Mg^{2+}]_i^\infty$ levels below 1–2 μM there are low but significant rates of both sodium–potassium and sodium–sodium exchange (Table 3). There are also significant amounts of tightly bound magnesium at these low $[Mg^{2+}]_i^\infty$ concentrations and both the fluxes and the concentration of bound magnesium are fairly insensitive to changes in $[Mg^{2+}]_i^\infty$ in the submicromolar concentration range (see also Flatman & Lew, 1980*a*). It is possible that at least a part of this tightly bound magnesium is involved in the activation of the sodium pump. A similar phenomenon has been seen with membrane preparations of the Na,K-ATPase where a low level of ATPase activity can be detected in the absence of added magnesium and this is abolished by the addition of EDTA. This activity is even seen when the enzyme has been washed with an EDTA-containing solution but EDTA is omitted from the final incubation medium (Skou, 1974). Moreover, very large concentrations of EDTA or CDTA are necessary to prevent phosphorylation of the Na,K-ATPase by ATP (see for instance Mårdh, 1975). The available data therefore suggest that extremely low magnesium concentrations can partially activate the Na,K-ATPase in membrane preparations and the data presented here suggest they can also support a small amount of sodium–potassium and sodium–sodium exchange in intact red cells.

(ii) *Stimulation of pump fluxes by ionized magnesium.* When $[Mg^{2+}]_i^\infty$ was increased above 2 μM both sodium–potassium and sodium–sodium exchange were stimulated. Magnesium appeared to act at a single binding site to promote sodium–sodium exchange ($K_{\frac{1}{2}} = 9$ μM). Magnesium acting at a single binding site ($K_{\frac{1}{2}} = 45$ μM) could also reasonably explain the sodium–potassium exchange data (dashed line in Fig. 4). However, the data are explained better if magnesium acts at two sites (solid line in Fig. 4). One site has a high affinity for magnesium ($K_{\frac{1}{2}} = 30$ μM) whereas the other site has a very low affinity so that the activity appears to increase linearly with ionized magnesium concentration.

Bodemann & Hoffman (1976) measured the effects of two or three magnesium concentrations on the ouabain-sensitive sodium efflux from resealed red cell ghosts

and concluded that sodium–potassium exchange was more sensitive to changes in internal magnesium concentration than was sodium–sodium exchange. Despite uncertainties in the estimate of ionized magnesium present in the experiments (see Hoffman, 1978), their results can be explained by the behaviour shown in Fig. 7. In intact red cells their conclusion is only valid for ionized magnesium concentrations between 0·06 and 0·8 mM.

Magnesium also stimulates the Na,K-ATPase and ATP–ADP exchange reactions which are the biochemical counterparts of sodium–potassium and sodium–sodium exchange respectively. The Na,K-ATPase is stimulated by concentrations of magnesium which are less than those of ATP (Skou, 1960; Dunham & Glynn, 1961; Hexum, Samson & Himes, 1970; Skou, 1974; Robinson, 1974; Flatman & Lew, 1980*b*) and ATP–ADP exchange is stimulated by very low concentrations of magnesium (Fahn, Koval & Albers, 1966; Beaugé & Glynn, 1979).

(iii) *Inhibitory effects of high magnesium concentrations.* When $[Mg^{2+}]_i^\infty$ was increased above about 0·6–0·8 mM both sodium–potassium and sodium–sodium exchange were inhibited. Sodium–sodium exchange was inhibited to a greater extent at each magnesium level. Inhibitory effects of high magnesium concentrations on sodium–potassium and sodium–sodium exchange have been reported previously by De Weer (1970, 1976), who examined the sodium efflux from giant squid axons after injecting magnesium into them, and by Eilam & Stein (1973), who examined the effects of magnesium loading on potassium influx into red cells. Moreover excess magnesium is known to inhibit the Na,K-ATPase reaction (Skou, 1960; Dunham & Glynn, 1961; Hexum *et al.* 1970; Skou, 1974; Robinson, 1974; Flatman & Lew, 1980*b*) and ATP–ADP exchange (Fahn *et al.* 1966; Robinson, 1976; Beaugé & Glynn, 1979).

How different are the magnesium requirements of sodium–potassium and sodium–sodium exchange?

Whichever model is used to describe the activation of sodium–potassium exchange by $[Mg^{2+}]_i^\infty$ the magnesium concentration needed to give half-maximal activation of the saturating component of the fluxes ($K_{\frac{1}{2}} = 30$ or 45 μM) is greater than the concentration needed to give half-maximal stimulation of the saturating component of the sodium–sodium exchange fluxes ($K_{\frac{1}{2}} = 9$ μM). In addition sodium–potassium exchange may have a component which shows a linear dependence on ionized magnesium concentration, and sodium–sodium exchange is more strongly inhibited by excess magnesium. It is possible, however, that some of these differences are due to changes in the concentration of other factors which also affect sodium pump activity and which are produced by the alteration of cell magnesium content – for instance, sodium, ATP and ADP.

The increase in sodium content seen during the experiments on sodium–sodium exchange at low magnesium concentrations (Table 6) probably produced, if anything, an over-estimate of the $K_{\frac{1}{2}}$ of the exchange, because the rate constants of sodium efflux are reduced when intracellular sodium increases above 10 mmol/l cells (Sachs, 1970). This conclusion is supported by the data shown in Fig. 5, where experiments were performed with 10 and 3 μM-A23187. The efflux rate constants at low magnesium concentrations were smaller and the sodium gains were four times larger with 10 than with 3 μM-A23187. At higher magnesium concentrations where the sodium gains were

small in both cases, the rate constants were not affected by the concentration of A23187.

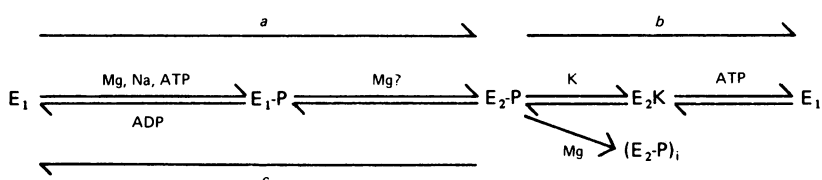
The changes in the ATP and ADP contents of cells with low intracellular magnesium concentrations probably also caused changes in the apparent $K_{\frac{1}{2}}$ values of the two reactions. The rate of sodium–sodium exchange shows a more or less linear dependence on the concentration of ADP whereas the rate of sodium–potassium exchange depends on the concentration of ATP (Glynn & Hoffman, 1971). The increase in the concentration of ADP and decrease in the concentration of ATP at low magnesium levels would have caused an apparent decrease in the $K_{\frac{1}{2}}$ of sodium–sodium exchange while apparently increasing the $K_{\frac{1}{2}}$ of sodium–potassium exchange. Thus the true $K_{\frac{1}{2}}$ values of the two reactions are closer than those reported above, but how much closer is difficult to assess because magnesium-induced changes in red cell metabolism are unavoidable with the method used here.

The changes in ATP content are too small to explain the component of sodium–potassium exchange which apparently increases linearly with $[Mg^{2+}]_i^{\infty}$, unless the concentration of ATP needed for half-maximal stimulation of sodium–potassium exchange is about 2 mM, and this is extremely unlikely (see Hexum *et al.* 1970; Robinson, 1974; Karlish & Glynn, 1974).

Sodium–sodium exchange is more strongly inhibited by excess magnesium than is sodium–potassium exchange. Probably only a small fraction of this differential effect of magnesium is due to the increase in ATP and decrease in ADP seen at high magnesium concentrations, since the changes in nucleotide concentrations were very small at high magnesium levels.

Interpretation of the effects of magnesium in terms of current models of the sodium pump

The stimulation and inhibition of the sodium pump fluxes by ionized magnesium may be analysed in terms of a simplified model of the pump's reaction sequence given below (see Karlish, Yates & Glynn, 1978, for details and nomenclature).



The native form of the enzyme, E_1, is phosphorylated by ATP in the presence of sodium and magnesium to give E_1-P which undergoes a conformational change to E_2-P (route *a*). E_2-P can revert to E_1 along either of two pathways under the conditions described in this paper, depending on whether or not potassium is present in the medium. In the presence of potassium route *b* is followed (sodium–potassium exchange) and in its absence route *c* is followed (sodium–sodium exchange). Magnesium which is tightly bound during phosphorylation is thought to be necessary for the overall sodium pump reaction (Fukushima & Post, 1978) although high magnesium concentrations may convert E_2-P into a non-reactive form, $(E_2\text{-P})_i$, thus reducing the amount of enzyme which can participate in the main reaction sequence (Post, Toda & Rogers, 1975; Forgac, 1980).

The stimulation of both sodium–potassium and sodium–sodium exchange by

magnesium seen in this paper probably reflects the magnesium requirement of phosphorylation. Post, Sen & Rosenthal (1965) showed that magnesium was required for phosphorylation and that the optimum concentration was roughly equal to that of ATP. Later, Klodos & Skou (1977) measured the ionized magnesium dependence of the reaction and obtained a $K_{\frac{1}{2}}$ of about 8 μM, which is similar to the values found here for activation of both modes of transport. However, in this paper sodium–potassium and sodium–sodium exchange were shown to have different values of $K_{\frac{1}{2}}$. This was probably due, at least in part, to the changes in ATP:ADP ratio, although the exact extent of this effect was difficult to assess. A small difference in $K_{\frac{1}{2}}$ can, however, be explained by the scheme shown above without requiring magnesium to act on steps other than phosphorylation. More ionized magnesium would be needed to maximally stimulate sodium–potassium exchange than to half-maximally stimulate sodium–sodium exchange if the level of E_1 were higher in the presence than in the absence of external potassium. The latter is probably true since E_2-P can revert to E_1 faster by route *b* than by route *c* under the conditions described above. The argument can be understood intuitively by considering extreme cases. When route *b* is extremely fast almost all of the enzyme will be in the E_1 form whereas when route *c* is extremely slow virtually none of the enzyme will be in this form. The apparent $K_{\frac{1}{2}}$ values of the overall reactions will change in parallel with the level of E_1 since it is this form of the enzyme which binds magnesium.

At least two of the experiments suggest the existence of a component of sodium–potassium exchange which increases linearly with ionized magnesium concentration and which is not observed with sodium–sodium exchange. This suggests that magnesium can activate a step during or after the enzyme's reaction with potassium, but this is controversial (see Klodos & Skou, 1977).

The inhibitory effects of excess magnesium on sodium–potassium and sodium–sodium exchange are probably due to stabilization of E_2 forms of the enzyme by magnesium. This inhibitory role has been suggested by experiments using membrane preparations of the Na,K-ATPase to investigate the effects of magnesium on the reactions between the enzyme and ouabain (Sen, Tobin & Post, 1969), the reversal of the ATPase cycle (Post, Toda, Kume & Taniguchi, 1975), and the potassium-stimulated phosphatase (Flatman & Lew, 1980*b*). Inhibition may also be caused by magnesium binding to the enzyme at a low-affinity site to produce $(E_2\text{-P})_i$, which is insensitive to dephosphorylation by ADP or potassium (Post *et al.* 1975; Forgac, 1980). This would be an attractive way of explaining inhibition since it also explains why sodium–sodium exchange is more strongly inhibited than sodium–potassium exchange. The level of E_2-P, which is the precursor to $(E_2\text{-P})_i$, is higher during sodium–sodium exchange than during sodium–potassium exchange, resulting in more enzyme being trapped in the non-transporting form. Alternatively, the extra inhibition of sodium–sodium exchange could be due to an inhibition of the reactions which result in the inward translocation of sodium and which are not involved in sodium–potassium exchange. The inhibition of these steps could be due to a direct action of ionized magnesium, could be secondary to a reduction in the concentration of free ADP which may be the true substrate for the exchange (Beaugé & Glynn, 1979), or could be due to an increase in the concentration of MgADP which may be an inhibitor of exchange (Robinson, 1976).

Is internal ionized magnesium a physiological regulator of sodium pump activity?

Normally it is not easy to change the magnesium content of mature human red cells because of the very low magnesium permeability of the membrane (see Flatman & Lew, 1980*a*). The concentration of ionized magnesium inside the cells can, however, be altered by changing the magnesium-buffering characteristics of the cell cytoplasm, for instance by changes in pH, metabolism or the charge on haemoglobin. The concentration of ionized magnesium increases when red cells are deoxygenated because deoxyhaemoglobin can bind more 2,3-DPG than oxyhaemoglobin, thus altering the magnesium-buffering characteristics of the cytoplasm (see Flatman, 1980). The precise magnitude of the increase in ionized magnesium is disputed but the experiments of Flatman (1980) suggest that it increases from 0·38 mM in oxygenated to 0·54 mM in deoxygenated red cells (final p_{O_2} = 12–15 mmHg). It can be estimated from the data presented here that the change in ionized magnesium on deoxygenation would result in no more than a 9% increase in sodium–potassium exchange. Smaller changes in ionized magnesium resulting from more physiological degrees of deoxygenation would stimulate the pump less.

Very little is known about magnesium in cells other than the red cell, though it appears that many cells have specific magnesium transport systems in their membranes and are capable of regulating their magnesium content (see Walser, 1967; Gupta & Moore, 1980). Changes in the concentration of intracellular ionized magnesium have been suggested as a means of co-ordinating the responses of the cell to changes in its environment or to the presence of hormones (see Walser, 1967; Rubin, 1977). Quite large changes in ionized magnesium would be necessary to produce substantial changes in pump activity if the magnesium dependence of the sodium pump in red cells is used as a model. As yet, such changes have not been observed. However, small changes in the concentration of ionized magnesium may be important in the long-term coordination of pump activity and metabolism.

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