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DEPENDENCE ON CALCIUM CONCENTRATION AND STOICHIOMETRY OF THE CALCIUM PUMP IN HUMAN RED CELLS

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SUMMARY

1. In resealed human red cells loaded with Ca-EGTA buffer solutions it was found that the intracellular free Ca^{2+} concentration for half saturation of the Ca transport system (which pumps Ca out of the cell) is equal to or smaller than 4×10^{-6} M and thus closely agrees with the dissociation constant of the Ca + Mg activated membrane ATPase.

2. The maximal rate of Ca transport from resealed cells to medium was found to be $0{\cdot}148 \pm 0{\cdot}009$ μmole/ml. cells.min at 28° C.

3. The rate of Ca transport was unaffected by a variation of the extracellular Ca^{2+} concentration from $3{\cdot}10^{-7}$ to $5{\cdot}10^{-3}$ M.

4. Evidence is presented making it probable that the stoichiometric relation between Ca transported and ATP hydrolysed is 1:1 rather than 2:1.

5. As the Ca transport is quite rapid even at half saturation and the passive leak for Ca negligible in intact cells it can be predicted that the steady-state cellular Ca^{2+} concentration must be low, most probably less than 10^{-6} μmole/ml. cells. Transport from cells containing $5{\cdot}10^{-7}$ μmole/ml. into blood plasma is thermodynamically compatible with the normal plasma Ca^{2+} concentration and the normal cellular ATP, ADP and P_i content.

6. Treatment with the mercurial PCMBS in the cold for 15 hr allows to load red cells with 1 μmole Ca/ml. cells without destroying their ability to transport Ca after removal of the mercurial.

7. It is shown that at high cellular Ca concentrations (0·1–3 μmole/ml. cells) about 50% of the total is free Ca^{2+} on account of binding mainly to dialysable cell constituents.

INTRODUCTION

Since its first demonstration (Schatzmann, 1966) the existence of an ATP dependent uphill calcium extrusion from human red cells has been confirmed by several authors (Olson & Cazort, 1969; Schatzmann & Vicenzi, 1969; Lee & Shin, 1969; Porzig, 1970; Cha, Shin & Lee, 1971; Romero & Whittam, 1971). However, the description remained unsatisfactory in one point so far. Two studies (Romero & Whittam, 1971; Schatzmann & Rossi, 1971) seemed to indicate that Ca concentration for half saturation of the transport system lies in the order of 10^{-3} moles/l. cells, whereas disrupted membranes, when tested for ATPase activity, show a dissociation constant of the Ca-receptor complex of $1 \cdot 10^{-6}$–$4 \cdot 10^{-6}$ M-Ca (Wolf, 1970, 1972*a*, *b*; Schatzmann & Rossi, 1971). The present work attempts to demonstrate that this discrepancy is not real and was probably caused by an overestimate of the intracellular Ca concentration in the transport experiments. By loading the cells with Ca buffers a reasonable agreement between Ca affinity in transport and membrane ATPase was obtained in the experiments presented here. The improved knowledge of the Ca affinity of the transport system may serve as a clue for gaining an estimate of the upper limit of the free intracellular Ca^{+2} concentration under normal conditions. Furthermore, dependence of Ca transport on the external Ca concentration was studied and measurements of the ratio of moles of Ca transported per mole ATP split were made. The resulting figures allow to calculate whether the proposed transport of Ca is thermodynamically possible.

METHODS

Abbreviations used. ATP: adenosine-5′-triphosphate; ADP: adenosine-5′-diphosphate; P_i: inorganic phosphate; EGTA: 2,2′-ethylenedioxybis [ethyliminodi-(acetic acid)]; EDTA: ethylenediamine-tetra-acetic acid; murexide = ammonium purpurate: 5-5′-nitrilodibarbituric acid, ammonium derivative; Tris: Tris (hydroxymethyl)amino-methane; PCMBS: parachlormercuribenzene-sulphonic-acid; TCA: trichloroacetic acid. Na + K − ATPase, Ca + Mg − ATPase, Ca + Mg + K − ATPase, Mg − ATPase means adenosine-triphosphatase activated by the respective cation(s).

Blood cells, preserved in acid citrate dextrose solution, were obtained from the Swiss blood donor service 1 day after collection.

Loading cells with Ca. In most experiments reported *reversal of haemolysis* was used. Cells were washed 5 times in isotonic NaCl or KCl solution, occasionally buffered to pH 7·4 with Tris-Cl, at room temperature, 5 min were allowed before each centrifugation for glucose to equilibrate with the medium. White cells were discarded by suction. An aliquot of packed cells was squirted rapidly into a fivefold volume of water containing ATP (usually 2 mM), $MgCl_2$ (4 mM), $CaCl_2$ or Ca-EGTA buffer at the desired concentration and imidazole-Cl buffer (5 mM) at room temperature. After 135 sec the mixture was made isotonic by adding 3 M-KCl solution,

cooled to 0° C and after 10 min, 0·1 mM-$CaCl_2$ was added. The cells were then washed in the cold incubation medium and diluted into prewarmed incubation medium at a haematocrit of 0·3 – 0·5 to start the experiment.

Treatment with PCMBS (Garrahan & Rega, 1967) was an alternative method to load cells with Ca without destroying their ability to pump Ca. Starved cells were stored in a solution with (mM): 150 NaCl, 15 Tris-Cl, 1 $MgCl_2$, 5 $CaCl_2$ and 0·05 PCMBS, pH 7·2 for 15 hr at 2° C. They were then washed once in the same solution containing 2 mM cysteine instead of PCMBS and incubated at 37° C in the same medium with 2 mM cysteine, 1 mM-P_i, 10 mM inosine and 5 mM adenine. After the cold period the cellular Ca concentration was about 1 m-mole/l. cells and upon removal of the PCMBS and supplying inosine and adenine at 37° C the cells pumped Ca against a gradient. However, the rate of Ca movement was less than in resealed cells.

Isolated red cell membranes were prepared according to Garrahan, Pouchan & Rega (1969) as described earlier (Schatzmann & Rossi, 1971) or according to Wolf (1972*a*) with some modifications: Cells were washed in a solution of (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, and lysed at 2° C in a ninefold volume of (mM): 20 Tris-Cl, 20 sucrose, 10 NaCl, pH 7·45. The mixture was centrifuged at 22 000 *g* under refrigeration for 30 min and the ghosts were washed 5 times in the cold with a solution containing (mM): 1 NaCl, 1 KCl, 2 Tris-Cl, 2 sucrose, pH 7·7, frozen in solid CO_2-ethanol and kept frozen at approximately −10° C until used.

To deplete cells of energy stores they were washed as mentioned above, and incubated at 37° C for 17 hr under light shaking in a fivefold volume of the following solution (mM): 130 KCl, 20 Tris-Cl, 2 or 4 $MgCl_2$, pH 7·4, with 1 mg chloramphenicol/100 ml. solution.

Short-time experiments for measuring Ca movements at 28° C were started by suspending ice-cold cells in about an equal volume of medium prewarmed to 38° C and transferring the mixture into flasks at 28° C.

Ca+Mg−ATPase activity of isolated membranes was assayed at 37° C in a medium of (mM): 70 choline-Cl, 30 imidazole-Cl, 2 $MgCl_2$, 2 Mg-ATP, pH 7·0 and $CaCl_2$ as desired, or 2·5 Ca-EGTA buffer of various Ca^{2+} concentration or 2·5 K-EGTA. Sample size was 2·5 ml. with 0·7–1·0 mg protein per sample; incubation time was 45 min or 90 min. P_i liberated was measured after deproteinization with equal volume of 10% TCA. Total ATPase of resealed cells was measured via P_i determination in the TCA filtrate of the whole suspension.

ATP was measured after deproteinization with 0·6 N cold perchloric acid (1 vol.: 4 vol. cells) by the enzymic method of Bücher (1947) using the test set of Biochimica Boehringer, calibrated with $Na_2ATP.3H_20$ (Boehringer).

Total Ca was measured by atomic absorption flame photometry in TCA filtrates or dilute haemolysates without deproteinization. Standards and samples were made up to a final $LaCl_3$ concentration of 50 mM. TCA was checked for possible Ca contamination. An EEL instrument was used with direct reading for high concentrations and with scale expansion and recorder for low concentrations. Alternatively, total Ca was measured by microtitration with EGTA: 1 ml. test solution, 1 ml. 0·1 N-NaOH and 0·3 ml. 16 mg % 2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphtylazo)-3-naphtoic acid indicator were mixed in a 1 cm cuvette, 0·05 ml. portions of 1 mM-K-EGTA were added from an AGLA micrometer syringe and the change of light absorption at 635 nm was followed. The end-point was taken as the intersection of the steep part of the curve with the plateau value. As traces of haemoglobin disturb the method, samples were prepared by ultrafiltration of appropriate dilutions of incubation medium.

Ionized free Ca in dialysates was measured with the murexide method originally

introduced by Raaflaub (1951). Ca shifts the absorption peak of the dye murexide to shorter wave-lengths. The effect depends strongly on the ionic strength but is not very sensitive to pH changes between 7·3 and 6·9. $CaCl_2$ standards were made up in a solution with (mM) 140 KCl, 20 Tris-Cl and a pH equal to that of the samples to within 0·1 pH unit. 2·5 ml. of standard, and 0·05 ml. freshly prepared 30 mg % murexide solution were mixed in a 1 cm cuvette. The difference in extinction between the standard and a 1 mM-EGTA solution in the same medium was measured at 464 and 536 nm (the latter going negative) in a Beckman DB spectrophotometer. The absolute values of the differences were added and the sum plotted against Ca^{2+} concentration. Between 0·1 and 5 mM-Ca^{2+} the value of the test samples treated identically was read from this calibration curve.

Whole cell haemolysates were dialysed for 15 hr at 0–2° C in slowly revolving tubes. 20 ml. haemolysate obtained by twice freezing and thawing packed cells were added to the tube and a Visking cellulose bag with 10 ml. (mM) KCl 140, Tris-Cl 20, pH 7·3, was immersed into it.

Ultrafiltration was carried out at room temperature in a stainless-steel cylinder through Sartorius filters no. SM 121 36 of 5 cm diameter. A pressure of 8 atm was exerted with compressed N_2 and the apparatus was shaken during the process.

^{45}Ca was used to measure the apparent extracellular space accessible to Ca during 5 min in the experiments designed to yield the Ca/ATP ratio. It was available as aqueous $CaCl_2$ solution with originally 100 μc/ml. and a specific activity of 50–100 mc/g Ca. It was 6 months old when used. 0·01 μc/ml. (original activity) was added to 2·0 ml. cell suspension; after 5 min incubation at 28° C the cells were spun down in a microcentrifuge (Ecco) and 0·3 or 0·4 ml. supernatant was spread on a brass planchet with household detergent, dried on a hot plate (approx. 60° C) and counted in a gas flow Geiger–Müller tube (Frieseke and Höpfner). The result was compared to the count obtained in the same procedure with cell free supernatant.

Haematocrit values were measured in 7 cm capillaries of 1 mm internal diameter, spun for 15 min at 11 000 rev/min (Ecco centrifuge).

Ca-EGTA buffers. The stability constant K for Ca-EGTA was taken as $10^{10\cdot65}$ which is strictly correct only for ionic strength 0·1 and temperature 37° C (G. Schwarzenbach, personal communication). The apparent stability constant K' for a given pH was calculated according to

$$K' = \frac{K}{\alpha_H},$$

with $$\alpha_H = 1 + K_{HZ}.[H^+] + K_{HZ}.K_{H_2Z}.[H^+]^2$$

and $$K_{HZ} = \frac{[HL^{3-}]}{[H^+].[L^{4-}]}, \quad K_{H_2Z} = \frac{[H_2L^{2-}]}{[H^+].[HL^{3-}]},$$

where L stands for EGTA (see Portzehl, Caldwell & Ruegg, 1964; Wolf, 1973). K' was found to be $10^{6\cdot33}$ for pH 7·0 and $10^{5\cdot94}$ for pH 6·8. The ratio r of total Ca concentration $[Ca]_T$ over total EGTA concentration $[L]_T$ for a desired Ca^{2+} concentration or the Ca^{2+} concentration for a given r was calculated according to

$$r = \frac{[Ca]_T}{[L]_T} = \frac{[Ca^{2+}]}{K'_{diss} + [Ca^{2+}]}, \quad \text{with } K'_{diss} = \frac{1}{K'}$$

which obtains on the assumption that $Ca^{2+} \ll Ca_T$ (and L_T). This approximation is satisfactory if $r \leqslant 0\cdot9$ and $K'_{diss} \leqslant 0\cdot001.L_T$. These conditions were respected in the critical range of the experiments, since comparison of Table 1 with Figs. 2 and 5 shows that the r values were below 0·9 for the resulting Ca^{2+} concentrations

and since 2·5 and 5 mM-EGTA was used. The error at $r \leqslant 0{\cdot}9$ and $K'_{diss} \leqslant 0{\cdot}001 . L_T$ is $\leqslant 13\%$ (calculated Ca^{2+} concentration too high) as can be seen when the approximation is compared to the rigorous treatment (given by Wolf, 1973). The increase of Ca^{2+} concentration due to the presence of 2–4 mM-Mg is in the order of 10% and was neglected. r was plotted on a probability scale *vs.* log Ca^{2+} and the desired values read on the resulting straight line. Buffers were made by mixing a fixed amount of 100 mM-K-EGTA solution with the amount of 100 mM-$CaCl_2$ solution required by the r value wanted; the solution was made up to 50 mM-K-EGTA by adding water and diluted into the media. When $[Ca]_T$ changed during the experiment owing to transport, the average Ca^{2+} concentration was obtained by taking the arithmetic mean of initial and final $[Ca]_T$, recalculating r and finding the corresponding Ca^{2+} concentration.

TABLE 1. Ionic Ca^{2+} concentration as function of the molar concentration ratio r of total Ca over total EGTA at pH 6·8 and pH 7·0. No correction for presence of Mg

	$[Ca^{2+}]$ (10^{-6} M)	
$r = Ca_{T/EGTA_T}$	pH 6·8	pH 7·0
0·9	10·0	4·0
0·8	4·6	1·8
0·7	2·8	1·1
0·6	1·8	0·7
0·5	1·2	0·46
0·4	0·78	0·31
0·3	0·49	0·20
0·2	0·29	0·12

P_i and protein were measured as before (Schatzmann & Rossi, 1971).

$CaCl_2$ and $MgCl_2$ stock solutions were titrated with EDTA and served both for making up media and standards for measuring.

Chemicals were analytical grade of Merck or Fluka, Na_2ATP, inosine, adenine and the enzymes for ATP determination were from Boehringer, PCMBS was the product of Sigma Chemicals, Na_2EDTA and the Ca indicators were obtained from Siegfried and the ^{45}Ca solution was from Reaktor A. G. Würenlingen. All solutions were made up in water from an ion exchange column, redistilled from an all glass still (Scorah).

RESULTS

A. Dependence of Ca-transport and (Ca + Mg) − ATPase on intracellular Ca^{2+} concentration

(*a*) *Transport experiments with Ca buffers inside the cells.* Fig. 1, curve *A* shows the type of experiment used in this study. When the cells are loaded by reversal of haemolysis with a high concentration (5 mM) of Ca-EGTA buffer with a Ca^{2+} concentration of $2{\cdot}2 \times 10^{-5}$ M (initial concentration inside the cells), the rate of Ca extrusion into a solution with 10^{-4} M-$CaCl_2$ concentration declines only slowly with time, whereas in cells loaded with $CaCl_2$ ($0{\cdot}3 \times 10^{-3}$ M) the rate declines sharply at the latest after 5 min (Fig. 1, curve *B*). The insert in Fig. 1 shows that for the

buffered cells the rate falls off at an intracellular Ca^{2+} concentration near 10^{-6} M. This is borne out by the experiments shown in Fig. 2 with cells loaded with different Ca-EGTA mixtures in order to measure initial rates (5 min) at different Ca^{2+} concentrations. It may be seen that half saturation obtains at about $4 \cdot 10^{-6}$ M Ca^{2+}.

In these experiments a technical difficulty arose. It was noticed that at the low Ca^{2+} concentrations the cells did not seal as well to ATP and Ca-EGTA as at the high Ca^{2+} concentrations. The possibility exists,

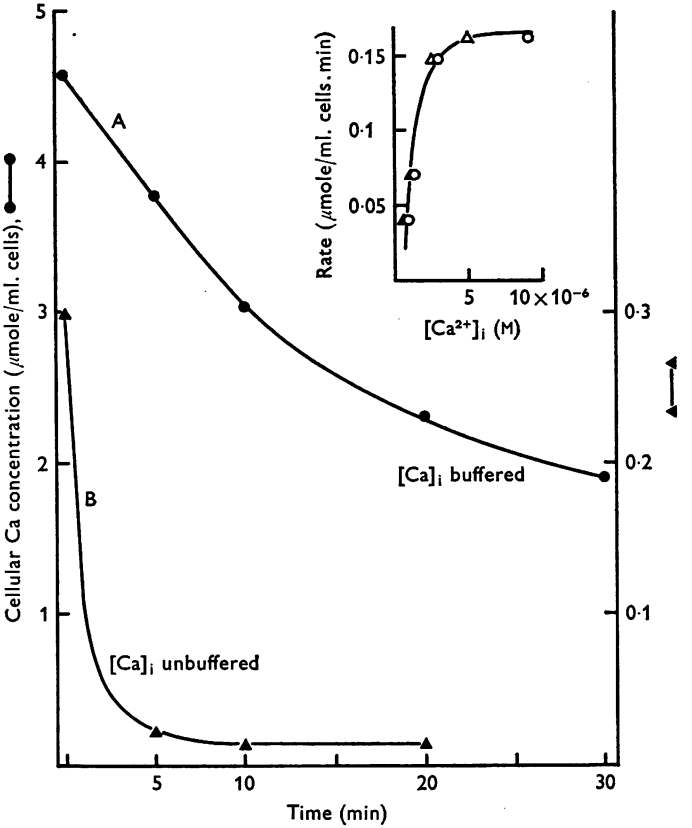

Fig. 1. Single experiment. Resealed cells prepared from fresh cells in the presence of 2 mM-Na_2-ATP, 2 mM-$MgCl_2$, 5 mM imidazole-Cl, resealed with imidazole-KCl (1:2) pH 6·8; curve *A* with 5 mM-Ca-EGTA buffer (Ca^{2+} concn. = $2 \cdot 2 \times 10^{-5}$ M), curve *B* with 0·75 mM $CaCl_2$. Incubation at 28° C in: 100 mM-KCl, 50 mM imidazole-Cl, 4 mM-$MgCl_2$, 0·1 mM-$CaCl_2$, pH 6·8. Haematocrit 0·385. Cells washed twice in Ca-free medium before analysis. Notice difference in scale of ordinate for *A* and *B*. Insert: rate of transport from curve *A* *vs.* calculated mean intracellular Ca^{2+} concentration in each time interval. ○, calculation based on 4·85 mM-EGTA) corresponding to initial Ca concn.); △, based on 5 mM-EGTA (concn. added).

therefore, that at low Ca^{2+} concentration they were also less tight to Ca ions during the experiment and some leakage of Ca back into the cells might in part account for the reduced rate of net extrusion observed and also for the curve obviously not being a rectangular hyperbola through the origin. Consequently it can only be stated that the concentration for half saturation is 4×10^{-6} M *or less.*

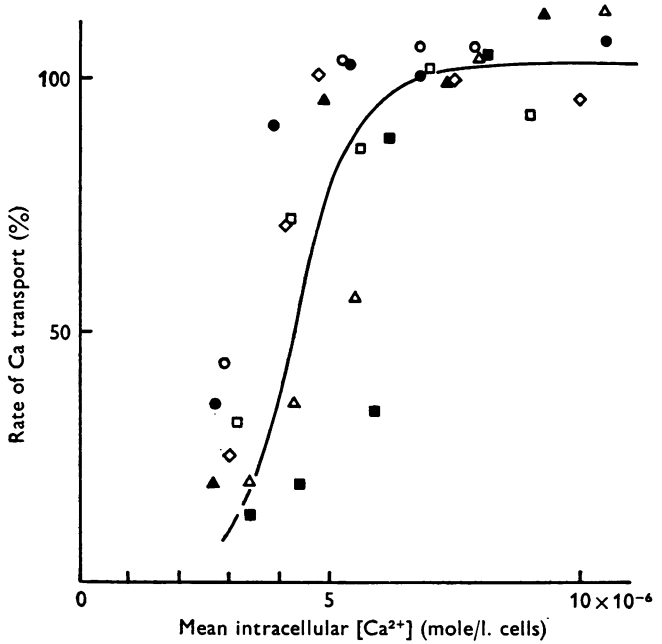

Fig. 2. Seven experiments as in Fig. 1*A*. Initial rate measured during 5 min. Different initial cellular Ca concentrations set by different Ca: EGTA ratios. Ca measured in medium. Haematocrit 0·4–0·5. Maximal average rate (100 % on ordinate) = 0·148 μmole/ml. cells.min. Mean Ca^{2+} concn. calculated from initial Ca-EGTA concn. and amount transported per 5 min. Curve drawn by eye, no theoretical significance.

Intracellular ATP concentration was measured in two similar experiments and found to be reduced in cells of very low Ca^{2+}, but certainly not to the extent of being rate limiting in view of the K_m for Mg-ATP being 0·04 mM (Wolf, 1970) (at zero Ca^{2+} in cells loaded with EGTA (1 mM) cellular ATP concentration went from 0·148 μmole/ml. cells to 0·138 μmole/ml. cells and in cells with an initial Ca^{2+} concentration of $4{\cdot}55 \times 10^{-6}$ M it went from 0·39 to 0·185 and from 0·19 to 0·092 μmole/ml. in 5 min in two experiments respectively).

Fig. 3 shows two necessary controls for the experiments of Fig. 2. Fig. 3*A* demonstrates that the calcium appearing outside the cells is Ca^{2+} rather than Ca-EGTA and Fig. 3*B* indicates that the process is ATP dependent also under the chosen conditions. The small loss of Ca

in the absence of ATP could easily be accounted for by cells not resealing to Ca-EGTA and/or to traces of ATP still present in starved cells.

The average rate at the highest intracellular Ca^{2+} concentrations achieved in the experiments of Fig. 2 is $0{\cdot}148 \pm 0{\cdot}0092$ μmole/ml. cells.min at 28° C and may be taken as the maximal transport rate at that temperature. In three of the experiments of Fig. 2 one sample was loaded with high Ca^{2+} concentrations (1 mM and 5 mM-$CaCl_2$). In all three samples

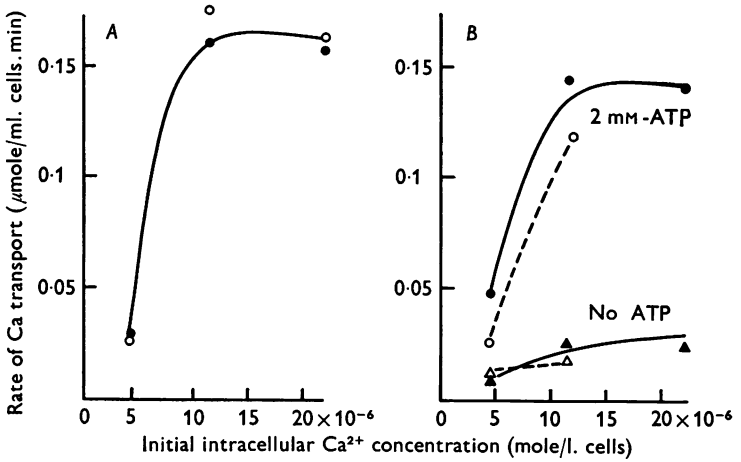

Fig. 3. Three experiments of the type shown in Fig. 2, plotted as in Fig. 2. *A*, single expt. ●, Ca measured by flame photometry; ○, Ca measured by EGTA titration. This experiment shows that the Ca appearing outside cells is free Ca^{2+}. *B*, two different expts. (filled and open symbols) showing that the transport observed in experiments of Fig. 2 is dependent on the presence of ATP inside the cells (starved cells, with and without ATP added during haemolysis).

the rate was well below 0·148 μmole/ml. cells.min which shows that the cells were leakier than cells with Ca buffers (Romero & Whittam, 1971) or that high Ca concentrations are inhibitory (Dunham & Glynn, 1961; Schatzmann & Rossi, 1971).

(*b*) *Ca+Mg activated membrane ATPase.* In earlier work dealing with isolated membranes prepared in the presence of 1 mM-EDTA (Schatzmann & Rossi, 1971) it was shown that the Ca+Mg stimulated ATPase seemed to exhibit two active sites with different affinity for Ca, which was at variance with Wolf's (1972*b*) finding of a homogeneous ATPase with the high affinity site only.

The demonstration by Wolf (1972*a*) and Scharff (1972) that Ca deprivation is detrimental to the enzyme led us to check this point. Fig. 4 confirms that the method of preparation with a rather short exposure to EDTA

during haemolysis and centrifugation is the cause for the reduction of activity at low Ca concentration and the appearance of low affinity sites. On omitting EDTA during preparation simple kinetic behaviour (curve *B*) and a very low value for the dissociation constant for the Ca receptor complex is obtained ($2{\cdot}35 \times 10^{-6}$ M-Ca). Consequently there is only one membrane ATPase which can account for the Ca transport and its dissociation constant lies in the micromolar range.

Fig. 5 shows that the discrepancy observed in transport (Schatzmann & Rossi, 1971; Romero & Whittam, 1971) between cells loaded with 1 mM free Ca^{2+} and those loaded with Ca buffer is also reflected by the ATPase

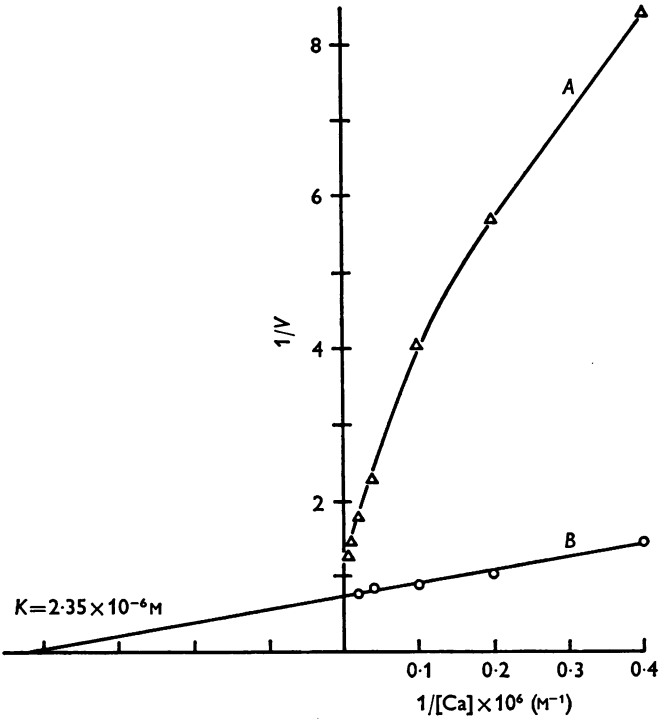

Fig. 4. Lineweaver–Burk plot of dependence of rate (v) of Ca + Mg – ATPase reaction on Ca^{2+} concentration in isolated membranes. v in μmole/mg protein.hr. A (△), membranes according to Garrahan and Rega (haemolysis in 1 mM-EDTA, 30 mM-Tris), *B* (○), membranes according to Wolf (haemolysis in (mM) 20 Tris-Cl, 20 sucrose, 10 NaCl, no EDTA). Both preparations from same batch of cells. Both types of membranes frozen and thawed once. Time 45 or 90 min, temp. 37° C. Incubation medium identical for both expts. (mM): 30 imidazole-Cl, 70 choline-Cl, 4 $MgCl_2$, 2 MgATP, 0·5 Ca-EGTA-buffer (up to 10^{-5} M Ca^{2+}) or $CaCl_2$, pH 7·0 (measured at 37° C). Blank: 0·5 mM-EGTA instead of Ca. Notice: in *A* the K_{diss} increases with increasing Ca^{2+} concn. whereas in *B* a well defined $K_{diss} = 2{\cdot}35 \times 10^{-6}$ M is obtained. v_{max} seems to be identical in both cases.

if resealed cells are used and if comparatively long observation intervals (20 min) are chosen. Disrupting the cells by freezing and thawing abolishes the apparent low affinity of the enzyme.

The obvious explanation for the discrepancy is the assumption that the rapid pumping of Ca removes 1 μmole of Ca from 1 ml. cells in a few minutes so effectively that the intracellular Ca^{2+} concentration is below the saturation region during most of the time of an observation lasting 10–20 min. However, when measuring Ca inside the cells one may obtain

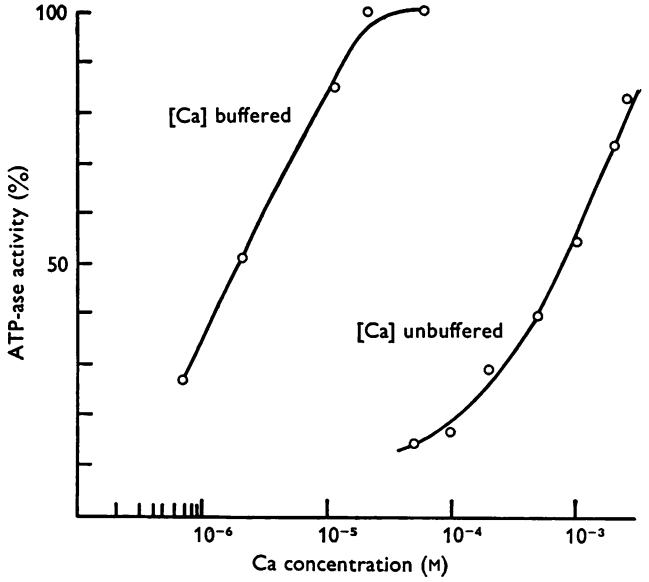

Fig. 5. Ca-Mg-ATPase in resealed cells as a function of added Ca^{2+} concn. Cells starved in glucose-free medium (KCl 130 mM, Tris 20 mM, $MgCl_2$ 2 mM) for 17 hr at 37° C. Haemolysis (mM) 4 $MgCl_2$, 5 imidazole-Cl, 3 Na_2ATP, 2·5 Ca-EGTA or variable concn. of $CaCl_2$; Ca-free blank with 2·5 mM-K-EGTA. Isotonicity restored with KCl-imidazole (2:1), pH 6·8 at 24° C. Incubation 20 min at 37° C. Mean of two experiments. Abscissa: Ca^{2+} concn. Ordinate: P_i liberated in % maximum in buffered cells. Notice: apparent dissociation constant differs by a factor of approx. 1000 between buffered and unbuffered cells.

curves such as the one shown in Fig. 6, where the rate starts falling at 2 or 1×10^{-4} μmole/ml. intracellular total Ca (if the final steady concentration is completely ascribed to extracellular Ca, or to extracellular Ca plus Ca in leaky cells), which is at least one order of magnitude above the region of unsaturation. The question of intracellular calcium binding therefore, became of some interest.

(c) *Ca binding inside the cells.* Gent, Trounce & Walser (1964) found a dissociation constant for Ca binding to isolated red cell membranes of

$2{\cdot}8 \times 10^{-4}$ M and a binding capacity of 5·9 μmole/g dry wt. under conditions of physiological pH and ionic strength. Assuming 10 g membrane dry weight per litre cells this would mean that at 0·1 mM-Ca concentration 14% could be bound to the membrane material, provided that this binding occurs essentially on the inside, which is not certain at all. This small amount can be neglected here, because it does not offer a solution for our problem. As other cell constituents might also bind Ca we carried

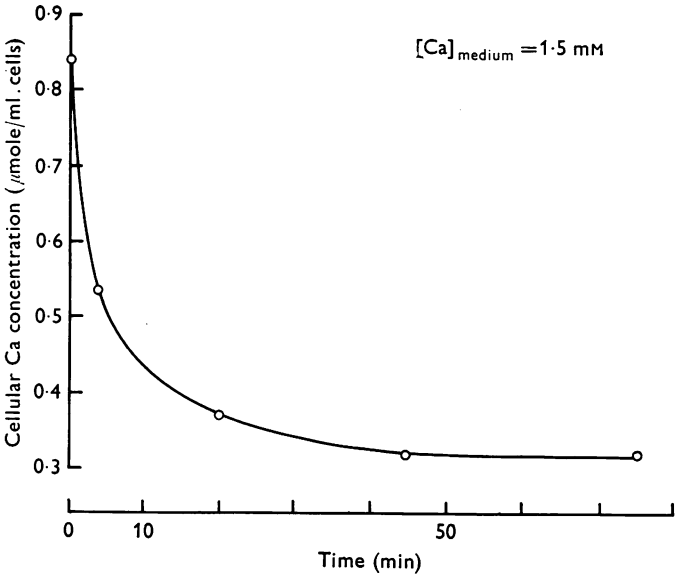

Fig. 6. Cells loaded with Ca in PCMBS 0·05 mM, $CaCl_2$ 5 mM. Medium (mM): 150 NaCl, 15 Tris-Cl, 1·5 $CaCl_2$, 2 cysteine, 10 inosine, 5 adenine, pH 7·2. Haematocrit 0·2, temp. 37° C. Ordinate total cellular Ca concn., abscissa, time. Transport is uphill from beginning. Notice final steady Ca concn. = 0·3 mM, probably due to leaky cells (tube with cells washed without stirring cells in Ca-free medium before analysis). Pumping is slower than in resealed cells.

out equilibrium dialysis with cells disrupted by twice freezing and thawing, which previously had been incubated with 10 mM inosine and 5 mM adenine at 37° C for 60 min in order to fill up the energy stores and to have similar conditions as in the transport experiment of Fig. 6. Ca concentration in the haemolysate was measured by absorption flame photometry and in the dialysate Ca^{2+} concentration was assayed by the murexide method. After correction for the water movement during dialysis bound Ca was calculated per unit water space in the haemolysate. The result is shown in Fig. 7. The average bound Ca from three experiments is plotted against the average free Ca^{2+} concentration. It may be seen that in the

range studied the bound Ca expressed as concentration per litre of water is an apparently linear function of the free Ca^{2+} concentration, and that about half of the total is bound. The binding was mainly due to dialysable compounds rather than to proteins. Membranes isolated according to Wolf suspended in an amount of cold KCl-Tris medium corresponding to the original cell volume at pH 7·4 did not bind any measurable fraction of Ca present between 0·1 and 5 mM both in the presence and absence of

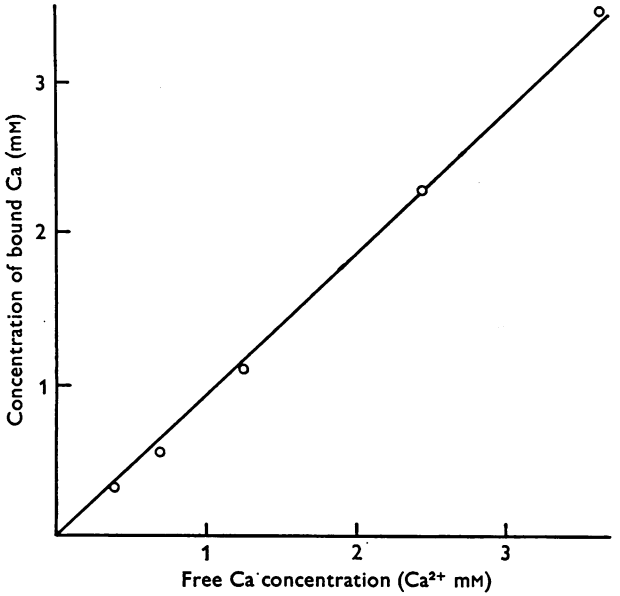

Fig. 7. Equilibrium dialysis of whole cells treated with 10 mM inosine and 5 mM adenine for 60 min at 37° C and lysed by freezing-thawing twice. Mean of three experiments. Total Ca in haemolysate measured by flame photometry, free Ca^{2+} in dialysate measured by murexide method. Ordinate: bound Ca expressed as concentration in the water space of haemolysate (assuming 35% solids in intact cells and correcting for H_2O movement during dialysis). Abscissa: ionized Ca. Temp. 2° C, time 15 hr, 20 ml. haemolysate, 10 ml. medium (140 mM-KCl, 20 mM-Tris, pH 7·3).

ATP without Mg. Thus, if the free intracellular Ca^{2+} concentration is to be approximated from the total Ca measured chemically, over-all intracellular binding of Ca can account only for a reduction by a factor of 2.

Dialysis was carried out at 2° C, and calcium was added to the cold material (between the two freezing periods). The possibility exists, of course, that at elevated temperature a large number of new binding sites might appear, for instance by membrane phosphorylation. However, no information in this respect can be offered at the present time.

Whatever answer may be found for this problem the experiments with

Ca-buffers inside the cells are quite straightforward. If the free Ca^{2+} concentration inside the cells is controlled both the transport system and Ca + Mg stimulated ATPase show similar dissociation constants in the micromolar range.

B. Dependence of Ca transport on external Ca concentration

In seven experiments starved cells loaded with ATP and 5 mM-Ca-EGTA buffer, giving $2{\cdot}2 \times 10^{-5}$ M Ca^{2+} concentration initially, were incubated in a medium with either 1 mM-EGTA or 1 mM of the same Ca-EGTA buffer of $2{\cdot}2 \times 10^{-5}$ M for 5 min at 28° C. Table 2 gives the average external Ca^{2+} concentration and the corresponding rate of transport. The difference in rate is statistically not significant. In the first case the Ca movement was downhill and in the second case it was uphill.

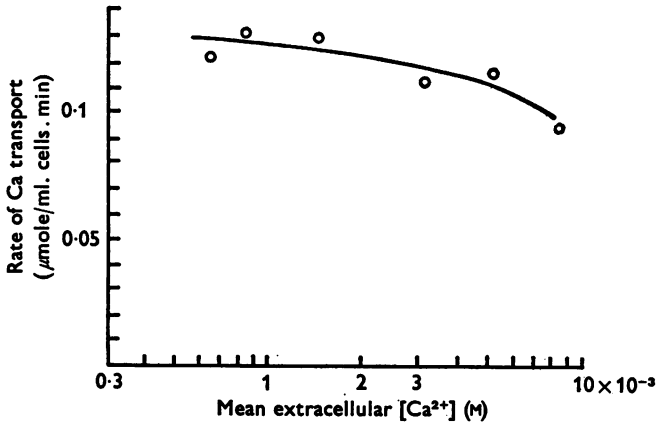

Fig. 8. Single experiment as described in Fig. 1*A* (cells loaded by reversal of haemolysis with 5 mM-Ca-EGTA buffer ($2{\cdot}2 \times 10^{-5}$ M-Ca^{2+}). Medium: 100 mM-KCl, 50 mM imidazole-Cl, 4 mM-$MgCl_2$, 0·1 mM-$CaCl_2$. Temp. 28° C, incubation 7·5 min in medium supplemented with different $CaCl_2$ concn. Mean extracellular Ca concn. calculated from added and final concn. Notice that transport rate of Ca does not change between 6×10^{-4} M and 6×10^{-3} M external Ca concn.

Thus a 280-fold change in external Ca^{2+} concentration and reversal of the gradient did not affect the rate of transport. Fig. 8 shows one experiment with cells loaded with Ca-EGTA as before, incubated in media of different $CaCl_2$ concentrations. There is no significant fall in rate of transport when the extracellular Ca concentration rises from 6×10^{-4} M to 8×10^{-3} M (the last point in Fig. 8 may not be very reliable because the solubility of Ca-phosphate may have been reached). The maximal average gradient from outside to inside against which unabated transport

took place in this experiment was at least 700-fold, which shows that the resealed cells are very tight and that any 'slip' in the pump mechanism is amazingly small.

C. Stoichiometric relation between Ca transport and ATP hydrolysis

In order to measure Ca transport and Ca+Mg activated ATPase activity simultaneously in resealed cells to obtain the Ca/ATP ratio other ATPases must be arrested or measured and subtracted. The difficulty is that rigorously Ca-free cells do not reseal well, such that conditions in a Ca-free (EGTA) control might differ from the test sample. Na+K−ATPase was minimized by using high K (100 mM) and low Na (4mM) concentrations. Ca+Mg+K−ATPase (Schatzmann & Rossi, 1971; Bond & Green, 1971) was not in operation because the Ca concentrations used were too low to activate it.

In a first series of seven experiments Ca transport was estimated from the concentration change in the medium bathing Ca-EGTA loaded cells and simultaneous extra ATP splitting due to Ca transport was computed from P_i liberation in Ca-EGTA loaded and K-EGTA (1 mM) loaded cells. The result was Ca/ATP = $0{\cdot}856 \pm 0{\cdot}055$, which differs significantly from one ($P < 0{\cdot}001$). Here the contribution made by leaky cells to ATPase activity may have biased the result, making the ratio too small.

To get another estimate of the Mg-activated ATPase, and to correct for leaky cells, the following procedure was adopted. Resealed cells loaded with $2{\cdot}2 \times 10^{-5}$ M-Ca^{2+} (5 mM-Ca-EGTA buffer) were washed thoroughly in the cold with 0·1 mM-$CaCl_2$-medium, then with 2 mM-EGTA-medium and finally with Ca-free, EGTA-free medium. One sample was incubated in the standard medium with 2 mM-EGTA to keep extracellular Ca concentration low (*a*), one sample was incubated in the medium with 2 mM-EGTA plus 1 mM-ATP (*b*) and one sample was incubated in the medium with $2{\cdot}2 \times 10^{-5}$ M-Ca^{2+} (5 mM-EGTA) (*c*). A small aliquot of *a* and *c* was simultaneously incubated with ^{45}Ca labelling in the medium. From the labelling experiment the space in the sample accessible to Ca (Ca-EGTA) was calculated and from this and the haematocrit the fraction of leaky cells was obtained. The difference of ATPase activity between sample *a* and *b* gives the Mg-activated ATPase of the leaky cells. From this the Mg-ATPase activity of the tight cells was calculated. With the '^{45}Ca-space' and the change in extracellular Ca concentration during incubation the amount of Ca escaping from the cells could be calculated both in the uphill and downhill transport. The ATPase correction from *b* minus *a* was also applied to *c*. In this way the Ca/ATP ratio was obtained for downhill and uphill transport. Table 2 summarizes the result. From column *e* and *f* in Table 2 it may be seen that both for uphill and downhill transport the

ratio is very significantly different from 2. In the uphill experiment the ratio is not significantly different from 1 but in the downhill experiment it is ($P = 0{\cdot}037$). Although the excess of leaky cells in column *a* and the difference in rate between columns *c* and *d* are not statistically significant

TABLE 2. Seven experiments. Measurement of the number of molecules of Ca transported per molecule ATP hydrolysed (Ca/ATP)

Mean extracell. Ca^{2+} (M)	Expt. no.	Tight cells %	Rate of Ca-transport (μmole/ml. cells.min)	Ca/ATP
(*A*) Downhill expt.				
$9{\cdot}4 \times 10^{-7}$*	1	75·1	0·167	1·38
$3{\cdot}275 \times 10^{-7}$†	2	50·7	0·169	1·00
	3	82·3	0·114	1·03
	4	84	0·123	1·075
	5	83	0·122	1·97
	6	67	0·144	1·67
	7	76·7	0·127	1·60
Mean		74·1[a]	0·138[c]	1·39[e]
Probability				p_e *vs.* 1 = 0·037 p_e *vs.* 2 = 0·005
(*B*) Uphill expt.				
$9{\cdot}2 \times 10^{-5}$	1	94·4	0·125	1·05
	2	66·5	0·093	0·83
	3	87	0·11	1·01
	4	83·9	0·14	1·26
	5	79·2	0·145	2·42(?)
	6	87·5	0·117	1·36
	7	85·9	0·074	0·93
Mean		83·5[b]	0·115[d]	1·27[f]
Probability		$p_{b-a} \simeq$ 0·11	$p_{c-d} \simeq$ 0·09	p_f *vs.* 1 = 0·25 p_f *vs.* 2 = 0·011

Method 2 (see Discussion) as described in text. Tight cells: % cellular space measured by haematocrit not accessible to Ca-EGTA during expt. (5 min) at 28° C. Figures in *A* and *B* from paired experiments on the same cells loaded in the same procedure with Ca-EGTA-buffer of $2{\cdot}2 \times 10^{-5}$ M-Ca^{2+}. Level of significance: p_{b-a}, probability for difference between *b* and *a* to arise by chance. p_e and p_f, probability for deviation from 1 or 2 to arise by chance in *e* or *f* respectively.* 1 mM-EGTA in medium, †2 mM-EGTA in medium.

it might be possible that some passive Ca release has contributed to the Ca movement in *A*. The conclusion therefore seems to be that the Ca/ATP ratio is more likely 1 than 2.

Table 2 shows that the mean extracellular Ca^{2+} concentration ($3{\cdot}10^{-7}$ M) was not kept low enough to quench Ca + Mg – ATPase activity completely. Any error stemming from this would tend to make the Ca/ATP ratio too

large, which does not invalidate the conclusion. Further there is some uncertainty about the assumption that cells leaky to Ca-EGTA are also accessible for Mg-ATP.

DISCUSSION

There is little room for doubt that in human red cells Ca ions are extruded against a large gradient at the expense of ATP hydrolysis. Since it was shown earlier (Schatzmann, 1969) that the Ca transport requires Mg, a Ca + Mg activated ATPase in the membrane must account for it. We could confirm Wolf's (1972*b*) finding that only one such ATPase activity is found if one avoids using Ca complexing agents in the preparation of the membranes (Fig. 4). It is highly probable that the appearance of two activities with different affinity for Ca found previously (Schatzmann & Rossi, 1971) was an artifact because it is generally true that substrates or co-factors stabilize enzyme proteins and because Scharff (1972) has shown that drastic treatment with EDTA can strongly reduce Ca + Mg − ATPase in red cell membranes. The Ca + Mg activated ATPase found by Wolf and by us with a dissociation constant of $1–4 \times 10^{-6}$ M fits the requirement quite well because studying the dependence of transport on cellular Ca^{2+} concentration led to the result that the dissociation constant for the transport system is 4×10^{-6} M or less (Fig. 2) which is in close agreement.

Over a 15 000-fold range of concentration (from 3×10^{-7} to 5×10^{-3} M) no dependence of transport rate on external Ca^{2+}-concentration was detected. This seems to indicate that in the resealed cells used any leakage through channels in parallel to the pump is small. However, we found that 20–25% of the cells remain leaky for Ca-EGTA and that in transport experiments the final Ca concentration reached in packed unwashed cells is quite high (0·3–0·5 μmole/ml. cells). The explanation for this probably is that roughly 80% of the cells seal well for Ca and the rest seals not at all and thus simply contributes to the extracellular space. A similar explanation seems to hold true for PCMBS-treated cells, too (Fig. 6).

In the present experiments the pump was essentially unaffected by a concentration ratio for Ca^{2+} of 700 across the membrane (Fig. 8). If the energy supply exerts its directional effect on the transport system by imparting a change in affinity to the Ca binding site between the state facing the inside and the outside of the membrane, this means that the jump in affinity during translocation must be at least as large, that is three orders of magnitude.

In view of the extremely low Ca permeability of intact cells at 0° C, when the pump is arrested (Schatzmann & Vincenzi, 1969) and the rather

high rate of Ca transport found at 50% saturation (0·07 μmole/cells.min at 28° C) it is very likely that under normal conditions the steady-state Ca^{2+} concentration inside the cell will be far below the half-saturation concentration. It may be predicted with some confidence that it will be below 10^{-6} M.

The fact that a rise in internal Ca concentration increases the K permeability is well documented (Hoffman, 1962; Lew, 1970; Romero & Whittam, 1971; Porzig, 1973). Lew found that a rise by 10^{-6} mole/l. cells was sufficient to bring about a large increase in K permeability, and that the effect could be counteracted by complexing Ca with phosphate. Ca (1 μmole/l. cells) will to a large extent be membrane bound, considering the binding properties found by Gent *et al.* (1964) mentioned above. If, for the argument's sake, an increase in cellular Ca^{2+} concentration by a factor as small as 2 is postulated for the effect on K permeability seen by Lew (1970), it follows that the resting Ca^{2+} concentration is below 10^{-6} M, which is in agreement with the present contention.

In order to obtain an estimate of the number of Ca ions transported per molecules of ATP hydrolysed two approaches were used. (1) In seven experiments the rise in external Ca concentration and P_i liberation was measured in resealed cells loaded with Ca buffer. ATPases not involved were corrected for by running a sample loaded with EGTA. No correction was made for the space within leaky cells accommodating some external Ca. For this reason the method most certainly underestimates the Ca/ATP ratio. It was found to be $0{\cdot}856 \pm 0{\cdot}055$. (2) In seven more experiments a correction for leaky cells was introduced and the Mg-ATPase of leaky cells was measured by adding ATP in the medium. From this the Mg-ATPase of the tight cells was calculated and subtracted. Here a systematic error was introduced by the fact that the mean extracellular Ca^{2+} concentration was sufficiently high to activate some Ca + Mg − ATPase in leaky cells, which would tend to make the Ca/ATP ratio too large. The assumption was made that entry of ATP into cells leaky for Ca-EGTA is rapid and not rate-limiting. The result is shown in Table 2. The Ca/ATP ratio is 1·27 and 1·39 for uphill and downhill transport respectively. In both methods the ratio is statistically significantly different from 2, in method (1) it is significantly smaller than 1 and in one series of method (2) it is significantly larger than 1, probably for the reasons mentioned. Therefore it seems more likely that the true stoichiometric relation between Ca and ATP is 1:1 than 2:1; this seems to distinguish the red cell membrane from the sarcoplasmic reticulum for which excellent evidence indicates that the relation is 2:1 (Makinose & Hasselbach, 1971).

On the basis of the present results it might be warranted to compute a possible figure for the work done by the Ca pump under normal conditions and to relate it to the change in free energy of the ATP hydrolysis

reaction in order to see whether the proposed Ca transport is thermodynamically sound.

On the assumption that intracellular ATP concentration is $1{\cdot}5 \times 10^{-3}$ M, ADP concentration $0{\cdot}32 \times 10^{-3}$ M and P_i concentration $0{\cdot}36 \times 10^{-3}$ M (Bartlett *et al.* 1953) and that the standard free energy change of ATP hydrolysis under physiological conditions is 7·2 kcal (Benzinger *et al.* 1959) the change of free energy is found to be 13·038 kcal/mole at 37° C. With a possible value for cellular Ca^{2+} concentration of 5×10^{-7} M and for plasma concentration of $1{\cdot}5 \times 10^{-3}$ M the sum of the osmotic and electric work amounts to 5·349 kcal/mole; therefore, if the Ca/ATP ratio is taken as 1, there is no objection against the transport because the chemical energy available is larger than the work done. On the assumption of the same intracellular concentrations but with a Ca/ATP ratio of 2 the work at 10 mM external Ca^{2+} concentration would become 13·036 kcal/mole, that is at external Ca^{2+} concentrations exceeding 10 mM the Ca-movement would reverse direction and the cells would gain Ca in spite of metabolic activity. As this is contrary to experience it may be used as another argument against a Ca/ATP ratio of 2.

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