*Biochimica et Biophysica Acta*, 604 (1980) 159–190


BBA 85207

# ACTIVE CALCIUM TRANSPORT IN HUMAN RED CELLS

B. SARKADI

*Department of Cell Metabolism, National Institute of Haematology and Blood Transfusion, Daróczi Út. 24, 1113 Budapest (Hungary)*

(Received May 28th, 1980)

## Contents

I. Introduction The role of calcium in biological systems . . . . . 160

II. Calcium in the red cell and its effects on cellular functions . . . . . 160
A. Effect of calcium on red cell shape and plasticity . . . . . 161
B. Calcium-induced rapid potassium transport ('Gárdos-effect') . . . . . 161
C. Effect of calcium on the ($Na^+$, $K^+$) pump . . . . . 162

III. Discovery of the red cell calcium pump. Methods for studying calcium movements in red cells . . . . . 162

IV. Basic characteristics of the red cell calcium pump . . . . . 164
A. Dependence on calcium concentration . . . . . 164
B. Dependence of active calcium transport on ATP and $Mg^{2+}$ . . . . . 165
C. Dependence of active calcium transport on temperature and pH effects of ions and drugs . . . . . 167

V. ($Ca^{2+}$ + $Mg^{2+}$)-ATPase(s) and active calcium transport . . . . . 168

VI. Calcium-stimulated *p*-nitrophenylphosphate phosphatase and active calcium transport . . 171

VII. Stoichiometry of the red cell calcium pump . . . . . 171

VIII. Calcium-dependent membrane phosphorylation and active calcium transport . . . . . 174

IX. Molecular structure of the red cell calcium pump - reconstitution experiments . . . . . 176

X. Regulation of the red cell calcium pump . . . . . 178
A. Regulation by intracellular soluble proteins . . . . . 178
B. Regulation of the calcium pump by $Mg^{2+}$, ATP, cyclic AMP and monovalent cations . 180

XI. Model for the reaction mechanism of the red cell calcium pump . . . . . 181

XII. Comparison of the red cell calcium pump with other active calcium transport systems . . 183

XIII. Summary and conclusions . . . . . 185
Acknowledgements . . . . . 186
References . . . . . 186

## I. Introduction – The role of calcium in biological systems

In the past few years, calcium has been recognized as the key ion in regulating cellular functions. A nerve action potential is triggered and modulated by calcium influx through the axon membrane [1,2], and muscle contraction is evoked by calcium release from sarcoplasmic reticulum, or from membrane-associated calcium pools, into the cytoplasm [3]. In cellular responses to different stimuli, calcium may function either by itself as an intracellular 'second messenger'; or, by affecting adenylate cyclase and/or phosphodiesterase enzyme activities, calcium may modulate the second messenger role of cyclic AMP [4]. Intracellular redistribution of calcium was shown to regulate various contractile and secretory mechanisms, membrane and other cellular events – possibly through the activation or inhibition of specific enzyme systems (for a summary of calcium effects, see the recent review by Case [5]). The proposed positive control of cell proliferation by calcium, and the loss of this control in tumorigenic cells attracted much attention [6].

All the regulatory functions of calcium depend upon its specific distribution: the calcium concentration in the cytoplasm is less than $10^{-5}$–$10^{-7}$ M, in contrast to the much higher calcium levels (about $10^{-3}$ M) in the extracellular fluids and in intracellular organelles such as mitochondria, endoplasmic reticulum, or sarcoplasmic reticulum in muscle. The non-equilibrium calcium distribution is maintained by binding and sequestration of calcium in the cell interior and by the calcium-transporting systems of the plasma membrane and the intracellular membranes. As an ultimate way to control the low cellular calcium concentration, animal cells extrude this divalent cation by the $Na^+$-$Ca^{2+}$ exchange mechanism and/or by the ATP-driven calcium pump of the plasma membrane. Human red cells may serve as a suitable model system for studying the latter calcium transport, especially because they seem to lack a system for $Na^+$-$Ca^{2+}$ exchange [7,8]. The ready availability and relative stability of the human erythrocyte, its lack of intracellular organelles or membrane elements, and the fact that the functional properties of its plasma membrane are preserved for a long time in vitro have made this cell one of the most extensively studied materials of membrane physiology and biochemistry. At present, we possibly know more about membrane functions and particularly transport in red cells than in any other biological systems.

The following sections only briefly summarize the large amount of data concerning the problems of passive calcium permeation and the effects of calcium on red cell functions. I have concentrated on the ATP-dependent 'active' calcium transport, the 'calcium pump' in the red cell membrane. The focus of the present review is on the most recent advances in this field; especially the regulation of the calcium pump is discussed (for more details, the reader should refer to the excellent review by Schatzmann [7]).

## II. Calcium in the red cell and its effects on cellular functions

The physiological calcium concentration in human red cells, as determined by careful atomic absorption measurements, is between 10 and 20 μmol/l of cells [9–13] and most

of this calcium is attached to the cell membrane [9,10]. Cytoplasmic calcium concentration is in the range $10^{-6}-10^{-7}$ M, about three orders of magnitude smaller than that in the blood plasma. Any intracellular buffering of calcium makes this large, inwardly directed gradient even steeper and maintaining this calcium distribution requires powerful defence mechanisms. These are the low passive permeability of the red cell membrane for calcium and the ATP-dependent, active calcium extrusion from the cell interior.

The rate of calcium uptake by normal, freshly drawn human red cells when incubated in iso-osmotic saline media (containing 2–10 mM calcium) is almost undetectable. The low permeability of the membrane for calcium can be shown if active calcium extrusion is inhibited by energy depletion or by incubating red cells in the cold, when calcium uptake is as slow as 1–10 μmol/l cells per h [10,14,15]. Exhaustive ATP and 2,3-diphosphoglycerate depletion of red cells increases calcium uptake to about 100 μmol/l cells per h, indicating the role of small amounts of ATP in preserving the normal, low permeability of the membrane for calcium [16,17]. Increasing the pH [18], and the osmotic concentration in the media [13], exposure of the cells to membrane-active drugs such as *p*-chloromercuribenzenesulfonic acid [19], trinitrocresol [20], propranolol [21] or the calcium ionophore A23187 [22–24] also enhance calcium influx into red cells (for details see Refs. 14 and 15).

If passive calcium influx is not entirely compensated for by active calcium extrusion, an increase in cellular calcium leads to pathological consequences.

*IIA. Effect of calcium on red cell shape and plasticity*

Small increases in intracellular calcium evoke crenation and increased rigidity in red cells [12,25–28]. Establishing an exact relationship to cell calcium concentration and the molecular explanation for these alterations still requires much work. Current theories emphasize the role of contractile proteins of the spectrin-actin network attached to the internal surface of the red cell membrane. The presence of calcium in micromolar concentrations may alter the configuration of this network, thus evoking cell shape changes [29,30]. Calcium-dependent cross-linking of the above proteins may contribute to the irreversible stiffening of the red cell membrane under certain conditions [31]. On the other hand, Allan and Michell [32,33] provided evidence for calcium-dependent changes in the lipid composition of the red cell membrane, especially for the accumulation of 1,2-diacylglycerol during crenation. Whether these changes in the lipid composition induce the shape changes or are just concomitant phenomena, is yet to be elucidated. In a 'coupled bilayer' hypothesis, Sheetz and Singer [34,35] tried to combine the role of proteins and lipids in the red cell shape regulation.

*IIB. Calcium-induced rapid potassium transport ('Gárdos-effect')*

In metabolically depleted human red cells, in the presence of calcium, the rate of downhill potassium transport across the plasma membrane increases by several orders of magnitude [36,37]. The loss of cellular potassium and the concomitant shrinkage of the cells are evoked by intracellular calcium [38,39], and the basic role of metabolic depletion is to enhance calcium influx [15,40]. It is of interest to mention that the data in the literature concerning the intracellular calcium concentration-dependence of this phenomenon are contradictory. In red cells made leaky to calcium by low concentrations of the ionophore A23187 [41] and in calcium-loaded intact red cells [42], the maximum rate of

potassium movement is evoked by 1–2 mM calcium. However, if high concentrations of A23187 are applied [41], or a CaEGTA buffer is introduced into metabolically depleted, resealed erythrocyte ghosts, the free $Ca^{2+}$ concentration eliciting the same response is 1–5 $\mu$M [43,44]. A similar discrepancy is also observed when studying the kinetics of active calcium transport (see Section IV). The possible relation of the calcium-induced potassium transport of red cells to molecular events in stimulus-secretion coupling in different cell types has recently attracted much attention [45].

*IIC. Effect of calcium on the ($Na^+$, $K^+$) pump*

Dunham and Glynn [46] first showed that calcium concentrations above 0.1 mM strongly inhibit the ouabain-sensitive, ($Na^+ + K^+$)-stimulated ATPase activity in isolated red cell membranes. The inhibition of active sodium-potassium transport by 0.1–0.5 mM intracellular calcium has been demonstrated in resealed ghosts [47,48], in intact red cells [12,49] and in inverted membrane vesicles [50].

## III. Discovery of the red cell calcium pump. Methods for studying calcium movements in red cells

In 1961, Dunham and Glynn [46] demonstrated an ouabain-insensitive, ($Ca^{2+} + Mg^{2+}$)-stimulated ATPase activity in hemoglobin-free red cell membranes, at that time with an unknown physiological role. It was Schatzmann [51] who, 5 years later, discovered the presence of an ATP-consuming active calcium extrusion from resealed red cell ghosts. Since that time, numerous studies have shown that calcium efflux from human red cells is faster by several orders of magnitude than inward calcium movement, and that rapid calcium efflux requires the splitting of cellular ATP in the presence of magnesium [7,8,10, 52,53]. The possibility that rapid calcium efflux is only caused by an increase in the calcium permeability of the membrane by ATP + $Mg^{2+}$, was excluded by demonstrating net calcium extrusion against large, inwardly directed calcium concentration gradients [7, 10,53].

For investigating the characteristics of active calcium transport in red cells, one possible way is to introduce calcium into the cell interior and then measure the extrusion of this divalent cation. However, prolonged incubation of red cells in high-calcium media causes only very little calcium uptake, and if calcium loading is forced by metabolic depletion of the cells, alterations in the membrane structure are most likely to occur [7,10]. The preparation of resealed ghosts, the most frequently used technique for calcium-pump studies, facilitates adjustment of the intracellular ion and nucleotide contents as well as the introduction of calcium-chelator buffers into the cells. The disruption of the cell membrane during ghost preparation, however, may cause certain artifacts in such studies [7]. The use of calcium-loaded intact red cells minimizes these artifacts and provides a relatively homogeneous cell population with nearly physiological intracellular milieu. The treatment of red cells with *p*-chloromercuribenzenesulfonic acid [7,19], with trinitrocresol [12,20], or with salicylate and thiocyanate [55,56] results in an effective calcium loading during a few hours incubation period, without considerable alterations in the cell metabolism. The use of ionophore A23187 provides a gentle way of introducing calcium into intact red cells. This ionophore is presumed to function as a lipid-soluble carrier for divalent cations [57], and the time required to load red cells even with millimolar concentrations of calcium is less than 1 min. After calcium loading, the ionophore

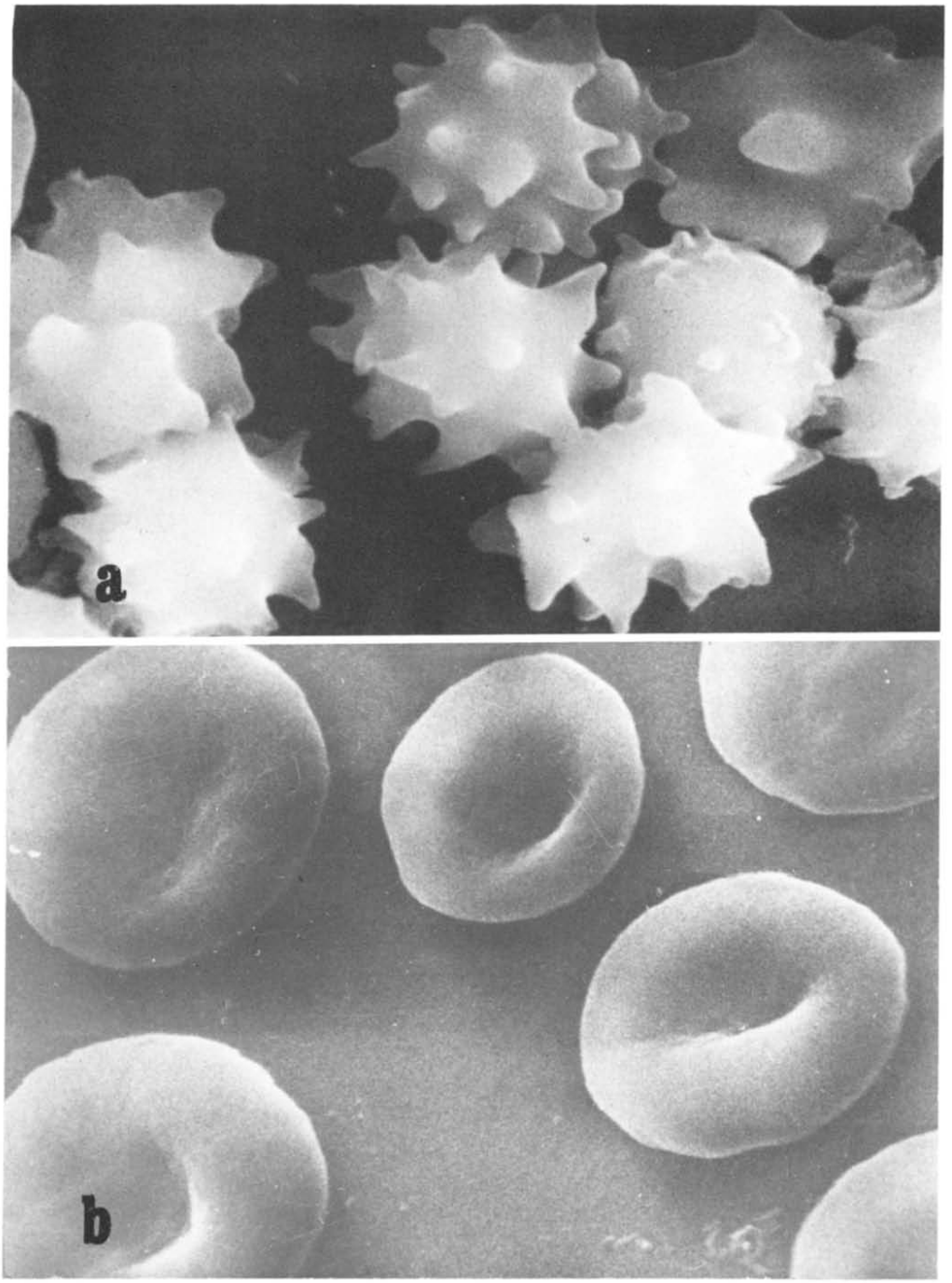


Fig. 1. Scanning electron micrographs of intact human red cells loaded with calcium by the ionophore A23187 [28]. Echinocyte formation by calcium loading (a) and regeneration of the biconcave shape during active calcium extrusion (b). Magnification, ×5320. (a) Calcium-loaded human red cells after the elimination of calcium ionophore A23187. Intracellular calcium concentration 160 μM. (b) The same cell population as in a, after 10 min incubation in a medium containing 120 mM KCl, 10 mM inosine, 10 mM Tris-HCl (pH 7.4), and 2 mM $CaCl_2$, at 37°C. Intracellular calcium concentration – in spite of the inwardly directed calcium concentration gradient – is decreased to less than 5 μM. Red cells were fixed in iso-osmotic phosphate buffer containing 1% glutaraldehyde, and dehydrated in increasing concentrations of ethanol. Electron micrographs were kindly prepared for us by Drs. J. Kovács and J. Vigh, Institute of Animal Physiology, Eötvös Loránd University of Science, Budapest, Hungary (Sarkadi, B. and Gárdos, G., unpublished data).

can be washed away from the membrane in cold, albumin-containing solutions [28], and thus membrane injury due to calcium loading is reduced to a minimum (Fig. 1). The red cell calcium pump has also been investigated in the presence of ionophore A23187, under conditions in which the ionophore-catalysed calcium influx was compensated for by the active calcium pumping [13,58].

Studies on the molecular basis of the calcium pump, on ($Ca^{2+}$ + $Mg^{2+}$)-stimulated ATPase, are mostly carried out in hemoglobin-free, fragmented red cell membrane preparations. The results for the characteristics of this enzyme depend considerably on the method of membrane preparation, especially on the presence of calcium and/or calcium-chelating agents [50,59–66]. In broken membranes, ATPase measurements cannot be connected to transport studies, therefore, the presence of any ($Ca^{2+}$ + $Mg^{2+}$)-dependent ATP splitting not related to the calcium pump may cause misleading results. In order to avoid this problem, several attempts were made for the simultaneous investigation of active calcium transport and ($Ca^{2+}$ + $Mg^{2+}$)-dependent ATPase activity in resealed ghosts [7,10,67,68], in intact red cells [49], and in inside-out membrane vesicles [69,70].

A new possibility for investigating red cell calcium transport was opened up by the method of Steck and his coworkers [71,72] for preparing and separating sealed, inside-out red cell membrane vesicles. In such vesicles, an (ATP + $Mg^{2+}$)-dependent calcium uptake was first described by Weiner and Lee [73]. The uphill calcium transport in inverted membrane vesicles undoubtedly proves the role of the red cell plasma membrane in active calcium transport. Furthermore, whilst in calcium-loaded ghosts or intact cells the calcium-, magnesium- and ATP-binding sites are in the cell interior, in inside-out vesicles the 'active centre' of the transport enzyme is on the external membrane surface. In the past few years, several laboratories have developed methods for preparing inside-out vesicles and for studying active calcium uptake by rapid separation of the vesicles from the incubation medium [69,74–80].

Probably the most effective and promising way to investigate the molecular characteristics of active calcium transport is to isolate the pump protein in a native form, and reconstitute the system into artificial lipid membranes. The inherent difficulties of this program and the first achievements in it are discussed in Section IX.

## IV. Basic characteristics of the red cell calcium pump

### *IVA. Dependence on calcium concentration*

Active calcium transport is an intrinsic property of the red cell plasma membrane and the direction of the pump can be changed by changing the orientation of the cell membrane. If the low, physiological, passive permeability of the membrane for calcium is preserved (by using the energy of ATP splitting), the pump is able to establish a 1000- to 10000-fold oppositely directed concentration gradient for $Ca^{2+}$. Calcium at the '*cis*-side' (i.e., at the internal membrane surface) activates the transport system, whilst there is almost no effect of calcium at the '*trans*-side' [10,12,28,49,52,54,73,76–78]. The calcium pump does not carry monovalent cations; no divalent cations other than $Ca^{2+}$ and $Sr^{2+}$ can be transported by the system [7,10,49,52,54]. The maximum rate of active calcium transport is between 2 and 10 mmol/l cells per h, i.e., about 0.5–2 mmol/g membrane protein per h, as measured in ghosts, intact red cells, or inside-out membrane vesicles [18,49,54,68,76,80,81].

The early experiments of Schatzmann [51] and Schatzmann and Vincenzi [10] indi-

cated that saturation of the calcium extrusion from resealed ghosts occurred at intracellular calcium concentrations of about 1 mmol/l cells. The difficulties in correctly estimating the intracellular $Ca^{2+}$ concentration, however, hindered exact characterization of the kinetics. Calcium binding to the cell membrane and to cell constituents such as hemoglobin, ATP or 2,3-diphosphoglycerate makes such an estimation uncertain. In order to address this problem, two alternative methods have been used and they have led to completely different conclusions.

The first method entails measuring the calcium-buffering capacity of the red cell interior in separate experiments and then calculating from these data the concentration of free $Ca^{2+}$ during transport studies. Schatzmann [54], using equilibrium dialysis of red cell homogenate, and Ferreira and Lew [58,82], using the ionophore A23187 to allow passage of calcium through the red cell membrane, concluded that the free $Ca^{2+}$ is about 20 - 40% of the total in a wide concentration range examined (1 $\mu$M to 5 mM). Since half-saturation of the calcium extrusion by intracellular calcium ($K_{Ca}$) occurs at 600 -500 $\mu$mol/l red cells or ghosts [18,49,56,81], the estimated value of $K_{Ca,free}$ is 120 -600 $\mu$M.

The second method involves the incorporation of a CaEGTA buffer into resealed ghosts and then calculating the free calcium concentration from the known stability constants of this buffer. By using this method, Schatzmann [54] found a $K_{Ca,free}$ value of about 5 $\mu$M, i.e., about two orders of magnitude smaller than that reported by himself from parallel experiments with unbuffered calcium. It was also demonstrated [54] that the transported species from the CaEGTA-loaded ghosts is the uncomplexed, free calcium, and that the activation of uphill calcium extrusion parallels the activation of ATP splitting in these ghosts.

The calcium concentration dependence of the calcium pump could be analyzed in detail in inside-out red cell membrane vesicles, which accomplish an ATP + $Mg^{2+}$-dependent, uphill, net calcium accumulation (Fig. 2). The maximum calcium transport rate in inside-out vesicles depends strongly on the presence or absence of a low molecular weight, water-soluble, heat-stable cytoplasmic activator protein, recently termed as 'calmodulin' (for details see Section X). If the incubation media contain unbuffered calcium, $K_{Ca}$ for active calcium accumulation in inside-out vesicles is about 40--50 $\mu$M, and the saturation of the uptake occurs at 300–500 $\mu$M [78]. Calmodulin increases the maximum transport rate [75–78] and decreases $K_{Ca}$ to 10 15 $\mu$M [78]. If we investigate the kinetics of the transport by using CaEGTA buffers, $K_{Ca,free}$ is 0.5 1.0 $\mu$M, independent of the presence or absence of calmodulin [79]. A striking observation is that the maximum transport rate is determined by the total calcium concentration in the CaEGTA buffer; thus, an interaction of calcium, in its EGTA-chelated form, with the calcium pump is postulated [79]. A possible explanation for the above results is that the calcium pump in red cells has at least two binding sites for calcium: one of these has a dissociation constant of about 0.5 $\mu$M for calcium, whereas the second binding site has a dissociation constant between 10 and 50 $\mu$M, depending on the presence or absence of calmodulin. In intact red cells, the $K_{Ca}$ values are still greater than 50 $\mu$M, and the maximum transport rate is usually smaller than in ghosts or in inside-out vesicles. This is most probably the consequence of the complex regulation of active calcium transport, as discussed in Section X.

### *IVB. Dependence of active calcium transport on ATP and $Mg^{2+}$*

Uphill calcium extrusion from resealed ghosts or intact red cells requires intracellular ATP, whilst calcium accumulation in inside-out vesicles requires ATP in the incubation

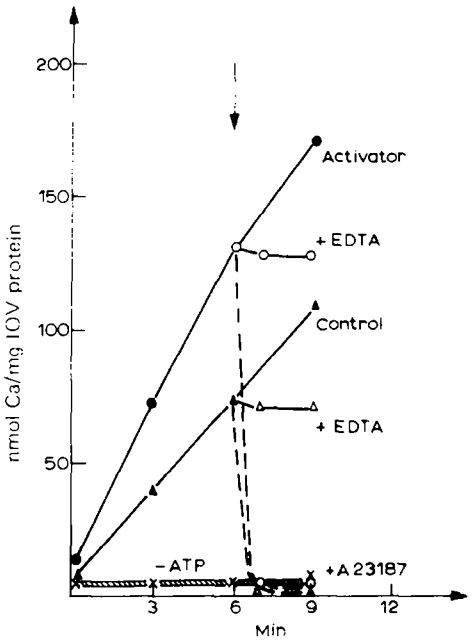


Fig. 2. Uphill calcium accumulation in inside-out red cell membrane vesicles in the presence of ATP + $Mg^{2+}$. 100 nmol calcium/mg inside-out vesicle (IOV) protein equals about 10 mM intravesicular calcium concentration. Calcium uptake is blocked by EDTA but no calcium release from the vesicles occurs in the presence of this non-penetrating chelator. Calcium ionophore A23187 releases vesicular calcium. The incubation media contained 120 mM KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 $\mu$M $CaCl_2$ ($+^{45}Ca$ tracer), and 0 or 1 mM ATP. At the time indicated by the arrow, the media were supplemented with 1 mM EDTA or 5 $\mu$M A23187. The 'activator' is a partially purified calmodulin from human red cells. Temperature 37°C. Calcium uptake into the vesicles is measured by a rapid filtration method. (Reproduced from results of Sarkadi et al. [80], by courtesy of Biochimica et Biophysica Acta.)

media. By now it is well documented that the splitting of ATP to ADP and $P_i$ is necessary to produce active calcium transport [7,8]. Inorganic phosphate is released in the cell interior and there is no movement of inorganic phosphate or phosphate esters accompanying calcium extrusion [83]. ADP, AMP, pyrophosphate or acetylphosphate cannot support active calcium transport [10,53,70]. GTP, ITP, CTP or UTP, when incorporated into resealed ghosts, can substitute for ATP [52,53], although a higher specificity for ATP was found in membrane fragments [84] and in inside-out vesicles [70].

In addition to ATP, $Mg^{2+}$ must also be present in resealed ghosts or intact cells to obtain active calcium extrusion, although magnesium is not transported by the calcium pump [10,52,53]. In inside-out vesicles, active calcium uptake requires magnesium in the medium [73–75,78,84]. All these results show a similar sidedness of the ATP- and $Mg^{2+}$-binding sites on the calcium-transport system.

In inside-out red cell membrane vesicles at low $Mg^{2+}$ levels (0.1 mM), the ATP concentration producing half-maximum rate of calcium uptake ($K_{ATP}$) is 5–6 $\mu$M, whereas in the presence of 4.0 mM $Mg^{2+}$ this value increases to about 25 $\mu$M. The maximum rate of calcium uptake is about three times greater at the higher magnesium concentration than at the lower one (Ref. 78 and Fig. 3). Thus, magnesium stimulates the overall calcium transport rate but decreases the apparent affinity of the pump to ATP (a similar finding

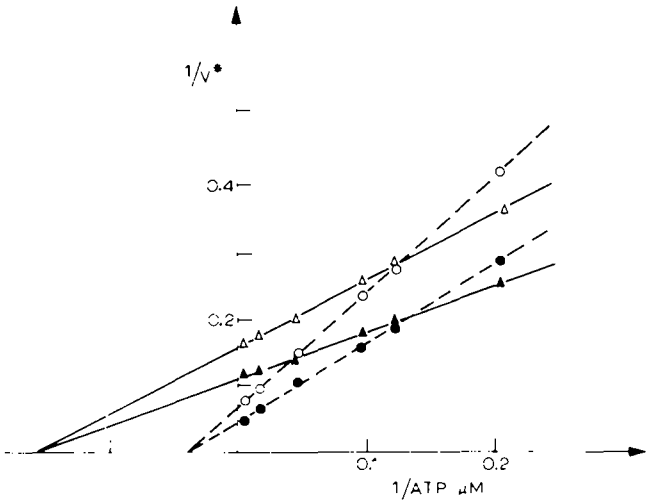


Fig. 3. Effects of total ATP and $Mg^{2+}$ concentrations on active calcium uptake in inside-out red cell membrane vesicles (IOV). Higher $Mg^{2+}$ concentrations increase the maximum transport rate and the value for $K_{ATP}$ both in the absence and in the presence of calmodulin. Lineweaver-Burk plot of the data. $V$ = nmol calcium/mg IOV protein per min. Incubation medium: 120 mM KCl, 20 mM imidazole-HCl (pH 7.0), 100 μM $CaCl_2$ ($+^{45}Ca$ tracer). Temperature 37°C. ○——○, 0.1 mM $MgCl_2$, control; ▲ - - -▲, 0.1 mM $MgCl_2$ + calmodulin; - - - - - -, 5.0 mM $MgCl_2$, control; ●- - - - -●, 5.0 mM $MgCl_2$ + calmodulin. Calmodulin is partially purified from red cells as described in Ref. 80, inside-out vesicles are prepared and calcium uptake is measured as in Refs. 78 and 80.

for ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity was reported by Schatzmann [85]). Since high magnesium concentrations decrease the level of free ATP, one possibility to explain the above results is to suppose that the substrate of the calcium pump is free ATP. The calculated value of $K_{ATP,free}$ is between 1 and 2 μM both at low and high $Mg^{2+}$ concentrations.

According to the experiments by Muallem and Karlish [86] on resealed ghosts, ATP activates calcium extrusion along a biphasic curve, that is a 'high-' and a 'low-affinity' component for ATP is observed ($K_{ATP,1}$ = 2 μM, $K_{ATP,2}$ = 200 μM). The authors suggest that at the high-affinity binding site ATP acts as a substrate of the calcium pump, while higher ATP concentrations may regulate calcium transport, by acting in a non-phosphorylating role. Data on ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity and calcium-dependent membrane phosphorylation seem to support this view (see below), although in inside-out red cell vesicles no biphasic effect of ATP on calcium uptake was observed [78].

*IVC. Dependence of active calcium transport on temperature and pH — effects of ions and drugs*

The Arrhenius plots obtained by measuring the calcium-pump activity in resealed ghosts [52,67,68], or in calcium-loaded intact red cells [49] gave values for the activation energy between 13.6 and 15.5 kcal/mol. In inside-out red cell membrane vesicles, the activation energy of active calcium uptake was 18–19 kcal/mol, both in the absence and presence of the cytoplasmic activator protein, calmodulin [80]. A rapid inactivation of the calcium pump at 46–50°C was reported in several studies [49,52,80,84]. The optimum pH for active calcium transport is between 7.3 and 7.6, as measured under various conditions [49,52,80,81]. At pH values higher than 8.0, calmodulin has almost no effect

on active calcium uptake in inside-out vesicles [80].

The presence of cellular magnesium is necessary to obtain active calcium transport in ghosts or in intact red cells. The possibility of a mechanism which couples calcium extrusion to the counter-transport of any divalent cation was ruled out by experiments in which chelators were added to the incubation media. EDTA or EGTA, in concentrations which decrease the extracellular free divalent cation concentrations to virtually zero, do not affect active calcium extrusion. The addition of $Mg^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Mn^{2+}$, or $Cu^{2+}$ to the incubation medium is also without effect on the calcium pumping [7,10,49,53]. By introducing divalent cations into resealed ghosts, it was shown that magnesium, barium, manganese, and copper are not transported by the calcium pump, whilst strontium can substitute for calcium in a competitive manner [7,10,53,87]. Strontium is actively transported into inside-out red cell vesicles, and calmodulin stimulates this uptake [50].

Active calcium extrusion from human red cells does not depend upon active sodium-potassium transport, nor upon a transmembrane gradient for sodium or potassium. Thus, the contribution of an $Na^+$-$Ca^{2+}$ exchange in this process can be excluded. According to the experiments of Schatzmann [7], active calcium transport is present if red cell ghosts are prepared free of sodium or potassium. A significant stimulation of active calcium uptake is observed in inside-out vesicles by $Na^+$ or $K^+$, as compared to choline$^+$, Tris$^+$, or $Li^+$ [78,80]. Half-maximum stimulation is obtained by 30–40 mM of either sodium or potassium and this stimulation is independent of that produced by calmodulin [80].

Ouabain and oligomycin, both potent inhibitors of active $Na^+$-$K^+$ transport, do not inhibit the calcium pump. The same is true for several metabolic inhibitors (if ATP is provided) such as NaF and iodoacetate [7,10,49,52]. Considerable inhibition of the calcium pump is produced by some SH reagents such as mersalyl, ethacrynic acid, *N*-ethylmaleimide and *p*-chloromercuribenzoate [10,49]. Quercetin and its chemical relative, phloretin, inhibit active calcium uptake in inside-out vesicles, whilst these drugs have no effect on the calcium pump in intact red cells [88]. Ruthenium red, a potent inhibitor of the mitochondrial calcium uptake, inhibits the calcium pump in inside-out vesicles [80], but has only a partial effect in ghosts or intact red cells [7,49].

Schatzmann and Tschabold [89] first reported the inhibition of calcium extrusion from resealed ghosts by the lanthanides, holmium and praseodymium. A 95% inhibition of active calcium transport by $1-2 \cdot 10^{-4}$ M lanthanum was reported in resealed ghosts [68] and in intact red cells [49]. $La^{3+}$ does not penetrate into intact red cells [49] and does not inhibit the sodium-potassium pump under these conditions [28]. Thus, lanthanum can be used as a selective inhibitor of the calcium pump in intact erythrocytes. However, when lanthanum penetrates into ghosts or acid/citrate/dextrose-stored red cells, it inhibits several cellular functions [90,91]; therefore, a true specific inhibitor of the calcium pump is still not available.

## V. ($Ca^{2+}$ + $Mg^{2+}$)-ATPase(s) and active calcium transport

The molecular basis of active calcium transport in human red cells is a ($Ca^{2+}$ + $Mg^{2+}$)-dependent ATPase, through which the energy released in the hydrolysis of ATP is converted to oriented calcium movement. However, it is difficult to clarify the exact relationship between calcium transport and the ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity which survives membrane preparation as measured under various experimental conditions.

As mentioned in Section III, Dunham and Glynn [46] first demonstrated that in hemoglobin-free red cell membranes calcium stimulates an ouabain-insensitive ATP

splitting. Calcium-activated ATPase requires the presence of magnesium, and the optimum concentration for calcium is about 0.5–0.8 mM. Higher calcium concentrations result in enzyme inhibition. Following these experiments, Nakao et al. [92] and Wins and Schoffeniels [93] reported similar results. The characteristics of red cell calcium-activated ATPase can be summarized as follows:

Rosenthal et al. [94] and Clarke and Griffith [95] described a calcium-stimulated, magnesium-inhibited (!) ATPase in the water-soluble, spectrin- and actin-containing protein extract of human red cell ghosts. This ATPase activity required high calcium concentrations (up to 6 mM), and the maximum rate of ATP splitting was relatively small (0.02 mmol/g membrane protein per h). Results from various laboratories could not unequivocally confirm these observations (see Ref. 96); although, in a recent report, White and Ralston [97] described the partial purification of a calcium-stimulated, magnesium-inhibited ATPase from water-soluble membrane extracts. In all probability, this type of ATPase activity has no direct role in calcium pumping.

Active calcium transport involves the functioning of certain integral membrane proteins, the strongly membrane-bound ($Ca^{2+} + Mg^{2+}$)-ATPases. However, these enzymes have significantly different characteristics in various membrane preparations. If membrane fragments are prepared in the presence of EDTA or other calcium-chelators, 500–800 $\mu$M calcium is required to produce maximum ATPase activity [46,54,81]. In membranes prepared with EDTA- or EGTA-free washing solutions, only a high-affinity ($Ca^{2+} + Mg^{2+}$) activity is observed ($K_{Ca}$ is about 1–5 $\mu$M) and the calcium-induced ATP splitting has a high maximum rate (1.5–2.0 mmol/g membrane protein per h) [54,59,98]. Based on these results, the low-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase was claimed to be an artifact caused by calcium chelators present during preparation [54,59]. In contrast, Quist and Roufogalis [61], using a variety of membrane preparations, found the 'high-calcium affinity' ATPase easily removable from the membrane, and suggested that the 'low-calcium affinity' enzyme plays the basic role in active calcium transport.

In the past few years, several investigators devoted considerable effort to clarifying the effects of membrane preparation on the ($Ca^{2+} + Mg^{2+}$)-ATPase activity [60,62–66,99, 100]. According to Scharff and Foder [63,64], red cell membrane ($Ca^{2+} + Mg^{2+}$)-ATPase may occur in two different states, depending on the calcium concentration during preparation. State A is obtained in the absence of calcium and shows a low maximum rate of ATP splitting, no cooperative behavior in calcium activation and a $K_{Ca}$ value greater than 30 $\mu$M. State B (membranes prepared in the presence of calcium) is characterized by a high rate of ATP splitting, positive cooperativity in calcium activation and a $K_{Ca}$ value of about 1 $\mu$M. Farrance and Vincenzi [65,66] demonstrated significant changes in $K_{Ca}$ of ATPase activity in membranes prepared in various buffers with various ionic strength. Membranes showing similar characteristics to Scharff's 'state B' enzymes were obtained in iso-osmotic imidazole buffers. According to our present understanding, the reversible shifts between low- and high-calcium affinity states of ($Ca^{2+} + Mg^{2+}$)-ATPase are caused by the calcium-dependent binding of a low molecular weight cytoplasmic activator protein, calmodulin, to the red cell membrane, and state A and B correspond to the calmodulin-free and calmodulin-associated form of the enzyme, respectively [64,99].

Both low- and high-calcium affinity ($Ca^{2+} + Mg^{2+}$)-ATPases are activated by calcium and strontium, whilst other divalent cations are much less effective [7,10,101]. Calcium-stimulated ATP splitting in both types of membranes is insensitive to ouabain and oligomycin, but is inhibited by lanthanum, ruthenium red, and some SH reagents [10,61, 102]. $Na^+$, $K^+$, and $NH_4^+$, as compared to $Li^+$, choline$^+$ or $Cs^+$, stimulate ($Ca^{2+} + Mg^{2+}$)-

ATPase in both A and B type membranes [81,102–105], as well as in partially purified enzyme preparations [106]. These findings further support the view that binding of soluble proteins is responsible for the changes in the kinetic parameters of the calcium-pump enzyme.

As to the role of ATP and $Mg^{2+}$ in the $(Ca^{2+} + Mg^{2+})$-ATPase activity: from measurements at different pH values, Wolf [98] concluded that the substrate of the enzyme is MgATP, and estimated a $K_{MgATP}$ value of about 50 $\mu$M at pH 7.0. In contrast, Schatzmann [85], based on experiments carried out at variable ATP, but fixed magnesium and calcium concentrations, suggests that the substrate is free ATP and the $K_{ATP,free}$ value is 1–2 $\mu$M. The latter data are in good agreement with those obtained measuring active calcium transport in inside-out vesicles (Ref. 78 and Fig. 3), and with findings in calcium-induced membrane phosphorylation by ATP (Section VIII).

A biphasic activation of $(Ca^{2+} + Mg^{2+})$-ATPase by ATP ($K_{ATP}$ values of 1–3 $\mu$M, and 200–300 $\mu$M, respectively; Fig. 4) was observed by Richards et al. [107] and by Muallem and Karlish [86,108], indicating a regulatory role of higher ATP concentrations in enzyme activity (a similar role of ATP in $(Na^+ + K^+)$-ATPase activity has been demonstrated [109]).

Parallel with the above studies in broken membrane preparations, several attempts were made to investigate $(Ca^{2+} + Mg^{2+})$-ATPase activity in ghosts and in intact red cells. As previously mentioned, both calcium-pump rate and calcium-stimulated ATP splitting were half-maximal at 300–500 $\mu$M internal calcium [49,54,56], except when resealed ghosts were loaded with a CaEGTA buffer, which decreased this value to about 5 $\mu$M [54]. If calcium-dependent ATPase activity in intact red cells was examined in the presence of high concentrations of the ionophore A23187 [110,111], the enzyme also exhibited the high-calcium affinity character. Since the presence of ionophore A23187 considerably increases the apparent calcium affinity of ATP splitting in broken membranes [64], as well as for a partially purified $(Ca^{2+} + Mg^{2+})$-ATPase [112], the above findings may not reflect a physiological state of the calcium-pump enzyme.

In conclusion, at present we do not know the in vivo and in situ calcium, magnesium, and ATP affinity of the red cell $(Ca^{2+} + Mg^{2+})$-ATPase enzyme, but we certainly are aware of the role of regulatory effects in their actual values. Apart from these effects, in the

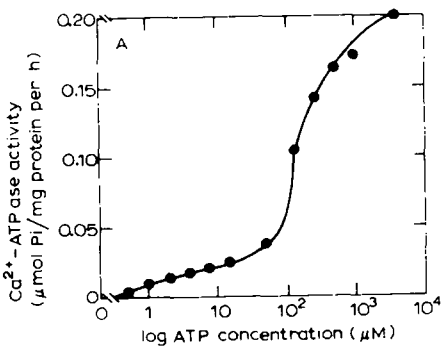


Fig. 4. $(Ca^{2+} + Mg^{2+})$-ATPase activity in fragmented red cell membranes as a function of ATP concentration. The response of ATPase activity to ATP concentration is biphasic, yielding $K_{ATP}$ values of 2.5 and 145 $\mu$M, respectively. The incubation medium contained 1 mM $MgCl_2$, 0.5 mM EGTA, 0.7 mM $CaCl_2$, 150 mM Tris-HCl (pH 7.8), at 37°C. (Reproduced from results of Richards et al. [107], by courtesy of Biochimica et Biophysica Acta.)

author's opinion, the variations in the experimental findings with $(Ca^{2+} + Mg^{2+})$-ATPase activity can be traced back to the following additional problems:

1. The human red cell membrane contains significant amounts of protein kinase and phosphatase activities [113–117]. Micromolar concentrations of calcium stimulate protein kinase [118] and/or phosphatase [119] activity. We do not know the physiological function of these membrane phosphorylation and dephosphorylation processes in the red cell, and can only assume from parallel studies in other systems that they may regulate the catalytic properties of some membrane enzymes (see Ref. 120). The distinction between classical ATPases and protein kinase + phosphatase systems can be made only on a chemical basis. Thus, when measuring (calcium + magnesium)-dependent inorganic phosphate liberation from ATP, we do not distinguish between these two processes. Since we lack a specific inhibitor of the calcium pump, any ATP splitting not related to active calcium transport may introduce a considerable error in characterizing the pump enzyme.

2. The use of calcium-chelating substances, such as EDTA or EGTA [79] or the calcium ionophore A23187 [64,112] during ATPase or transport assay may alter the kinetic behavior of the calcium pump.

## VI. Calcium-stimulated *p*-nitrophenylphosphate phosphatase and active calcium transport

Pouchan et al. [121], Garrahan et al. [122], and Rega et al. [123] described a calcium stimulated, ouabain-insensitive *p*-nitrophenylphosphate phosphatase activity in human red cell membrane. This phosphatase activity requires the presence of ATP and $Mg^{2+}$, and calcium stimulation is enhanced by $K^+$ or $Na^+$. Half-maximum activation of the phosphatase is produced by low concentrations of calcium ($K_{Ca} \approx 8\ \mu M$), and only if calcium has access to the inner membrane surface. On the basis of these data, the authors suggested that this phosphatase activity is a property of the red cell calcium pump. Their conclusion is further supported by the observation that *p*-nitrophenylphosphate inhibits $(Ca^{2+} + Mg^{2+})$-ATPase and active calcium extrusion to a similar extent [123]. On the analogy of the $(Na^+, K^+)$ pump, it was proposed, that in the multi-step reaction of the calcium pump, some of the intermediate forms can be replaced by *p*-nitrophenylphosphate and the enzyme catalyzes the hydrolysis of the latter compound. The strict ATP and calcium dependence of phosphatase activity indicates that, in order to catalyze *p*-nitrophenylphosphate splitting, the enzyme first has to be combined with calcium and ATP. The experiments of Richards et al. [124] with the pump-inhibitor, *N*-ethylmaleimide indicated that sites on the enzyme with high affinity for calcium exist also in the absence of ATP.

The analysis of the data for $K^+$ stimulation of the phosphatase activity revealed that K is required for the hydrolysis but not for the binding of *p*-nitrophenylphosphate. A dephosphorylation of the calcium-induced phosphorylated intermediate of the calcium pump is also accelerated by $K^+$ (see Section VIII), the identity of the two systems is further supported. Until now, no report has been presented on the effect of calmodulin on *p*-nitrophenylphosphate phosphatase activity of the red cell membrane.

## VII. Stoichiometry of the red cell calcium pump

Under physiological conditions, the ratio of active calcium transport to its ATP consumption is most probably a fixed number since, in a system working against large concentration gradients, a strong coupling of the elementary steps is necessary. However

there is some controversy over the stoichiometry of the red cell calcium pump. Several investigators find a 1 : 1 ratio of calcium transported:ATP consumed [10,54,67,125], whilst others argue for a calcium : ATP ratio of 2 : 1 [49,68,69,90]. One could say that such a discussion is not very different from that in Lilliput, over the problem of where to break an egg: on its large or small end? This is not really true, as the question of stoichiometry implies significant consequences to the nature of the calcium pump. It is hoped such studies will help to define the exact relationship between membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity and calcium transport, and to relate red cell calcium transport to that in different membranes in different tissues.

In order to determine the ATP consumption of the calcium pump, Schatzmann and coworkers [10,54,125] compared the rate of inorganic phosphate liberation from ATP to the rate of calcium extrusion in calcium-loaded resealed ghosts. In these experiments, ATP consumption in the absence of calcium was substracted and the $(Na^+, K^+)$ pump was inhibited by ouabain or by sodium- and potassium-free solutions. The molar ratio of the calcium-sensitive $P_i$ liberation to the active calcium transport was approx. 1 : 1. In contrast, when ATP splitting in calcium-loaded resealed ghosts was blocked by lanthanum (which also inhibits the calcium pump), the ratio for the lanthanum-sensitive calcium efflux to lanthanum-sensitive ATP consumption was 2 : 1 [68].

In intact red cells, ATPase activity can be estimated by measuring $P_i$ liberation, ensuring that metabolic reactions are blocked by depletion of substrates and addition of appropriate inhibitors [49]. Under these conditions, in normal red cells, inorganic phosphate liberation has an ouabain-sensitive fraction (about 25–30%), which reflects the ATP consumption by the $(Na^+, K^+)$ pump. In such cells, lanthanum-sensitive ATP consumption is less than 5% of the total. Increasing the intracellular calcium concentration by using the ionophore A23187 (the ionophore is eliminated from the membrane after calcium loading [28]), increases the rate of $P_i$ liberation, and ATP splitting is sensitive no more to ouabain, but sensitive to external lanthanum. As lanthanum blocks calcium pumping without penetrating intact red cells [28], the lanthanum-sensitive fraction of ATP splitting is probably associated with active calcium transport. The molar ratio of lanthanum-sensitive calcium transport to lanthanum-sensitive ATPase activity was 2 : 1 [49]. As the lanthanum-inhibited ATP splitting is only about 50% of the total, these results may explain the 1 : 1 calcium : ATP ratios obtained by Schatzmann and his coworkers, and suggest that there is also a calcium-stimulated ATPase activity not associated with the calcium pump.

Recently, Larsen et al. [67] using calcium-loaded resealed ghosts did not find a significant lanthanum-insensitive ATPase activity, and thus the calcium : ATP ratio obtained was 1 : 1. However, Larsen et al. [67] used ghosts of outdated, acid/citrate/dextrose-stored red cells, in which the passive permeability of the membrane for calcium as well as for lanthanum increases considerably [90,91]. Thus, externally added lanthanum may penetrate into these ghosts and inhibit also those calcium-stimulated ATPases which are not associated with the calcium pumping [126]. Lanthanum influx, and a concomitant decrease in the ratio of lanthanum-sensitive calcium transport and lanthanum-sensitive ATP splitting was demonstrated in acid/citrate/dextrose-stored intact cells [90].

Experiments using inside-out red cell membrane vesicles may help in solving the question of the calcium-pump stoichiometry. However, the problem of relating active calcium transport to the pump-ATPase still exists in such preparations. As we have no specific inhibitor of the calcium pump, any calcium-induced ATP splitting not associated with the pump may alter the value of the calcium : ATP stoichiometry. Fig. 5 shows the relation-

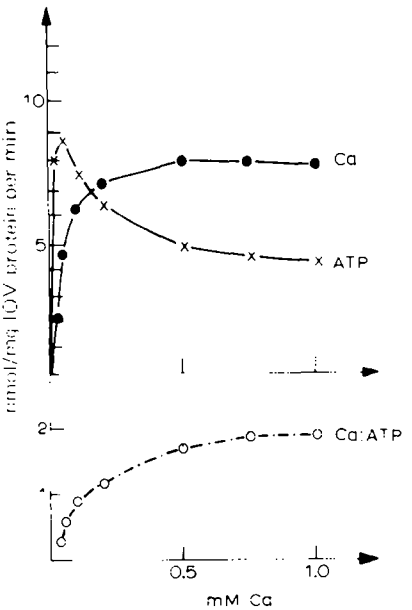


Fig. 5. Active calcium transport and ATP consumption in inside-out red cell membrane vesicles as a function of calcium concentration in the medium. Upper part: the rate of calcium uptake and the calcium-dependent ATP splitting in inside-out vesicles (IOV); lower part: the ratio of calcium transport to ATP consumption. Inside-out vesicles were prepared and calcium uptake was measured as described in Refs. 78 and 80. ATP consumption was assessed by measuring calcium-dependent inorganic phosphate liberation. Each datum point represents triplicate experiments with 5-min incubation periods. The incubation media contained 120 mM KCl, 20 mM imidazole-HCl (pH 7.4), 5 mM $MgCl_2$, 100 $\mu$M ATP, and the calcium concentration indicated ($+^{45}Ca$ tracer). Vesicle concentration = 200 $\mu$g/ml medium, temperature = 37°C [70].

ship between active calcium accumulation and calcium-dependent ATPase activity in inside-out red cell vesicles, as a function of the calcium concentration in the medium. Calcium-dependent ATPase activity is maximally activated at low calcium concentrations, whilst calcium uptake needs higher calcium concentration to become fully activated. The calcium transport : ATP consumption ratio is changed from about 0.3 : 1 to approx. 2 : 1 at increasing calcium concentrations, thus a fixed value for the stoichiometry cannot be obtained. In similar experiments, by using CaEGTA buffers, Quist and Roufogalis [69] obtained a Ca : ATP ratio of about 1.6 : 1 in inside-out vesicles, both at low and high calcium concentrations. In their experiments, a water-soluble fraction of the membrane increased calcium-stimulated ATPase activity without increasing active calcium uptake [69]. In spite of the differences in the experimental findings presented above, we may conclude that calcium-dependent ATP consumption, apparently not related to calcium pumping, can be observed in inside-out vesicles as well. Until now, no detailed study on the effects of ATP, $Mg^{2+}$, or calmodulin on the calcium-pump stoichiometry has been reported.

In order to rationalize the results presented above, two possible explanations can be offered:

(1) The Ca : ATP stoichiometry of the red cell calcium pump is 1 : 1, but in the presence of external lanthanum in intact cells, and at low calcium concentrations in inside-out vesicles the calcium transport and ATPase functions of the system dissociate. In these

cases, the calcium pump catalyzes ATP splitting without calcium translocation. Such a possibility is very much against our expectations about a pump working against thousand-fold concentration gradients, and would produce a highly unfavorable energetic balance at physiological, low cellular calcium concentrations. Therefore, this explanation, although we have no evidence against it, may be rejected on the basis of a 'sense of beauty in nature'.

(2) The Ca : ATP stoichiometry of the pump is 2 : 1, but the human red cell membrane contains calcium-stimulated ATP splitting systems which are not directly associated with calcium pumping. These systems are either 'classical' ATPases, or protein kinases + phosphatases, producing ATP splitting induced by low concentrations of cellular calcium. Such ATP-consuming reactions may be involved in the regulation of the red cell shape and plasticity, the permeability of the membrane for various compounds, or even the calcium pump. A fascinating hypothesis by Weller [127] hints at the idea that cyclic AMP-dependent protein kinase activity may regulate passive calcium movements across red cell (and also other cellular) membranes. Recently, Quist [119] suggested that $Mg^{2+}$-stimulated phosphorylation and $Ca^{2+}$-induced dephosphorylation of red cell membrane proteins may contribute to the regulation of the red cell shape and plasticity.

Many investigations have shown that in normal red cells, the work of the ($Na^+$, $K^+$) pump consumes about 20–30% of the cellular energy production [128–130]. Active calcium transport in these cells requires less than 5% of the total ATP consumption [49]. The remaining 65–75% of cellular ATP consumption is due to still unknown processes, which are calcium-dependent, membrane-bound mechanisms [131–133]. From data on biphasic ATP-dependence of ($Ca^{2+}$ + $Mg^{2+}$)-ATPase, (see also Fig. 4), Maretzki et al. [134] postulate the presence of a 'low ATP-affinity' enzyme, involved in cellular regulation but not in calcium pumping. The above data suggest that in normal red cells, intracellular regulatory processes consume much more energy than ion translocations by the pump enzymes.

## VIII. Calcium-dependent membrane phosphorylation and active calcium transport

A new approach for studying the molecular basis of the red cell calcium pump was initiated by Katz and Blostein [135] and by Knauf et al. [136], demonstrating calcium-dependent membrane phosphorylation by [$\gamma$-$^{32}$P]ATP. The authors supposed (on the analogy of the ($Na^+$, $K^+$) pump) that the phosphorylated protein is the catalytic part of the calcium pump, and isolated the phosphoprotein by SDS-polyacrylamide gel electrophoresis. The calcium-induced phosphoprotein, with an apparent molecular weight of about 140 000–150 000, was separable from the sodium-stimulated phosphoprotein (molecular weight of about 100 000), suggesting the presence of distinct and independent enzymes for the calcium pump and the ($Na^+$, $K^+$) pump, respectively (Fig. 6 and Ref. 136).

It has been convincingly demonstrated that the 140 000–150 000 dalton phosphoprotein is produced in seconds at 0°C, by micromolar concentrations of calcium, shows rapid turnover upon the addition of excess non-radioactive ATP, and its formation correlates with ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity in the membrane [137,138]. The calcium-dependent phosphoprotein is stable in acidic buffers while sensitive to hydroxylamine treatment, thus it contains an acyl-phosphate bond, as is generally characteristic for membrane ATPases (see Ref. 139). In purified ($Ca^{2+}$ + $Mg^{2+}$)-ATPase preparations, the calcium-induced, hydroxylamine-sensitive phosphorylation of the 140 000–150 000 dalton pro-

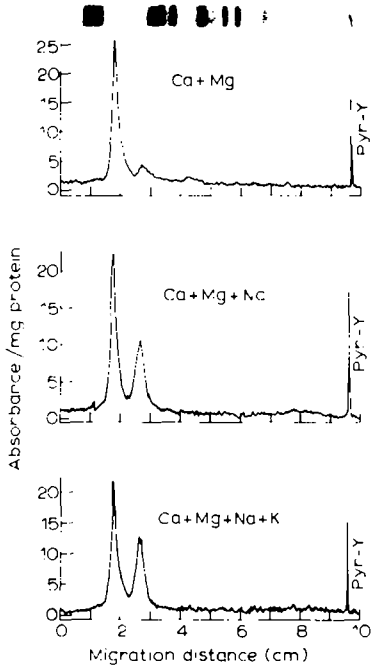


Fig. 6. Phosphorylation of fragmented red cell membranes by [$\gamma$-$^{32}$P]ATP. Calcium + magnesium produces membrane phosphorylation in a 150 000 dalton protein fraction. Sodium-dependent phosphorylation results in the labeling of an independent protein (103 000 daltons). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. At the top is a photograph of a disc gel stained with Coomassie brilliant blue to show the electrophoretic pattern of ghost proteins. The three graphs represent densitometric scans of autoradiograms of $^{32}$P. Labeling conditions: 2 $\mu$M ATP, 12 $\mu$M $MgCl_2$, 10 mM Tris-HCl, 0.5 mM $CaCl_2$. When present, the concentration of NaCl was 50 mM and KCl 10 mM. (Reproduced from results of Knauf et al. [136], by courtesy of Journal of General Physiology.)

tein was also observed [106,140], further supporting the phosphorylated-intermediate role of this membrane consituent. (It has to be mentioned that Weidekamm and Brdiczka [141] localized the calcium-dependent phosphoprotein in the 200 000 - 220 000 dalton spectrin fraction. This phosphorylation is most probably the result of some protein kinase activity [116,139].)

The experiments on calcium-dependent membrane phosphorylation provided valuable data for the partial reactions of the red cell calcium pump. It was first shown by Rega and Garrahan [138], and confirmed by Schatzmann and Bürgin [55] and Szász et al. [142], that the formation of the phosphorylated intermediate of the pump does not require the presence of $Mg^{2+}$. Thus, the substrate for this reaction is free ATP, rather than an MgATP complex. The $K_{ATP,free}$ value in the calcium-dependent phosphorylation is about 1 $\mu$M [55], in good agreement with the data for $K_{ATP,free}$ in ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity [85] and in active calcium uptake in inside-out vesicles [78]. Calcium-dependent formation of the phosphoenzyme is accelerated by $Mg^{2+}$ [55,138,143], possibly by the action of magnesium on other partial reactions of the pump system (see below). Addition of calmodulin increases the steady-state level of the phosphoenzyme when formed in the presence of calcium and in the absence of magnesium [108,144,145], and further accelerates the rate of phosphoenzyme formation in the presence of calcium + magnesium [108].

Addition of EDTA or EGTA to the medium evokes the rapid dephosphorylation of the calcium pump intermediate (produced in the presence of calcium + magnesium), and excess non-radioactive ATP further accelerates this process [137,138]. If, however, membrane phosphorylation is produced by calcium in the absence of magnesium, dephosphorylation is slow in the presence of EDTA, EGTA or non-radioactive ATP, but is significantly accelerated by the addition of magnesium to the ATP-containing 'chasing' solutions [55,138,142]. These experiments indicate the basic role of magnesium in the dephosphorylation of the calcium-transport enzyme, and reveal that magnesium binding during phosphorylation is sufficient to allow the rapid hydrolysis of the pump intermediate. From the latter finding, Garrahan and Rega [143] postulated a magnesium-dependent conformation change of the phosphoenzyme into a form which can rapidly react with water to release $P_i$. The rate of dephosphorylation of the calcium pump intermediate is increased by $Na^+$ and $K^+$ in the presence of $Mg^{2+}$, whilst monovalent cations have no effect on the formation of the phosphoenzyme [144]. It is worth mentioning that the phosphorylated intermediate can be 'back-reacted' with ADP, i.e., the labelled phosphate from the enzyme can be transferred to ATP, in the absence of magnesium [55].

Lanthanum accelerates the phosphorylation of the calcium-pump intermediate in fragmented membranes or in inside-out vesicles, both in the presence or absence of calcium [55,142]. However, the phosphoprotein formed in the presence of lanthanum cannot be dephosphorylated by excess ATP + $Mg^{2+}$ because the phosphorylated intermediate is stabilized against hydrolysis [142]. The labeling of the calcium-pump phosphoenzyme by internal [$\gamma$-$^{32}$P]ATP in calcium-loaded intact red cells is significantly increased by the addition of external lanthanum [142], thus the inhibition of the calcium pump is caused by the 'fixation' of the transport enzyme in a phosphorylated state.

It has also to be emphasized that we do not have a specific inhibitor of the calcium pump and, if magnesium is present, and the ATP concentration is elevated, and a total membrane phosphorylation is measured, protein kinase activity significantly influences the results. When Cha and Lee [146] investigated red cell membrane phosphorylation by [$\gamma$-$^{32}$P]ATP in the presence of 3 mM $Mg^{2+}$, at 37°C, by using 1–60 min incubation periods, the most pronounced labeling occurred in the absence of calcium, and addition of calcium induced membrane dephosphorylation (similar findings were recently reported by Quist [119] and by Enyedi et al. [144]). The conclusion of Cha and Lee [146], namely that in the calcium-pump cycle, $Mg^{2+}$ is required for the phosphorylation and $Ca^{2+}$ for the dephosphorylation of the enzyme, is unlikely to be correct in the light of the results presented above.

## IX. Molecular structure of the red cell calcium pump – reconstitution experiments

The molecular basis of the red cell calcium pump is probably a membrane-spanning protein molecule, or a group of such proteins. The catalytic unit of the pump is a 140 000–150 000 dalton, strongly membrane-bound protein, phosphorylated by [$\gamma$-$^{32}$P]-ATP in the presence of calcium. However, according to our present notions on membrane structure and function, in the 'two-dimensional solvent' of the lipid bilayer, membrane proteins interact with the surrounding lipid molecules, which in turn stabilize and regulate the protein functions (see Ref. 147). This is also true for the red cell calcium pump. It has been demonstrated that ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity is lost after phospholipase treatment of isolated red cell membranes [61,148–150]. Roelofsen and Schatzmann [149] showed that phospholipase digestion of the external leaflet of the lipid bilayer in

intact red cells does not affect membrane ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity, and the degree of enzyme inactivation is directly proportional to the degradation of the glycerophospholipids in the inner membrane leaflet. By using purified phospholipases, it was shown that it is not the barrier function of the lipid layer which is necessary to obtain ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity, but rather a specific, protein-lipid association is required [149, 150]. (The latter two studies are somewhat at variance in judging the effectiveness of various lipids and detergents in reactivating ($Ca^{2+}$ + $Mg^{2+}$)-ATPase in phospholipase-digested membranes.)

The use of the non-ionic detergents, Triton X-100 and Tween 20, enabled Wolf and his coworkers [106,151,152] to isolate and purify the red cell ($Ca^{2+}$+ $Mg^{2+}$)-ATPase. They succeeded in purifying the enzyme by gel chromatography when the integral membrane proteins were protected against denaturation by mixed micelles of the detergents plus phosphatidylcholine. The preparation contained three different protein subunits with molecular weights of 145 000 ($\alpha$ subunit), 115 000 ($\beta$ subunit) and 105 000 ($\gamma$ subunit). The 145 000 dalton protein could be phosphorylated by [$\gamma$-$^{32}$P]ATP, similar to the membrane-bound enzyme. The kinetic properties of the purified ($Ca^{2+}$ + $Mg^{2+}$)-ATPase were similar to those in fragmented membranes: activation by low concentrations of calcium and ATP, stimulation by the monovalent cations, $K^+$, $Na^+$ and $NH_4^+$, and inhibition by SH-reagents and other membrane-active drugs, were reported.

According to a recent publication by Niggli et al. [140], the Triton X-100-solubilized ($Ca^{2+}$ + $Mg^{2+}$)-ATPase from red cell membrane could be further purified by using a calmodulin-affinity column in the presence of phosphatidylserine. In the presence of calcium, the ATPase remained bound to the affinity column, and, after washing off the contaminating proteins, the enzyme could be eluted by the addition of EDTA. In SDS gel

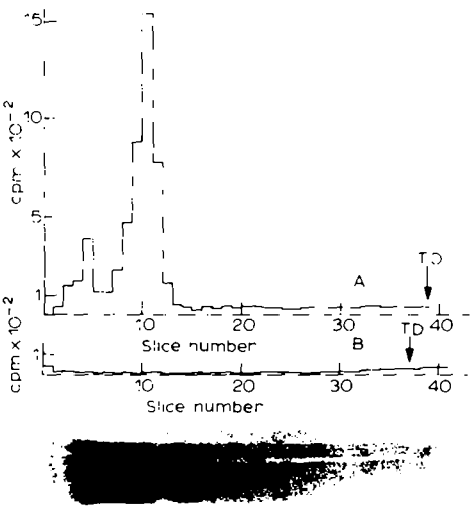


Fig. 7. Demonstration of the phosphorylated intermediate of the isolated ($Ca^{2+}$ + $Mg^{2+}$)-ATPase by labeling with [$\gamma$-$^{32}$P]ATP. (A) Distribution of $^{32}$P radioactivity in a 5% SDS-polyacrylamide gel after formation of the phosphorylated intermediate by 2 $\mu$M ATP, in the presence of 50 $\mu$M $CaCl_2$ + 12 $\mu$M $MgCl_2$. (B) Similar experiment to A, but phosphorylation in the presence of 500 $\mu$M EGTA + 12 $\mu$M $MgCl_2$. A photograph of the Coomassie blue-stained gel is shown below the diagrams. (Reproduced from results of Niggli et al. [140], by courtesy of Journal of Biological Chemistry.)

electrophoresis, only one major band with a molecular weight of 125 000 was detected, and this protein could be phosphorylated upon incubation with [$\gamma$-$^{32}$P]ATP, in the presence of calcium (Fig. 7). A minor protein fraction (205 000 daltons) was also present in the purified preparation, which possibly is a dimer of the transport enzyme.

The first attempts at reconstitution of the red cell calcium pump by incorporating (partially) purified ($Ca^{2+} + Mg^{2+}$)-ATPase into artificial lipid vesicles were recently reported by Gietzen et al. [153] and by Haaker and Racker [112]. The former group used the method of cholate dialysis, while the latter applied a freeze-thaw sonication method for the incorporation of the pump molecules into liposomes. In both studies, the ($Ca^{2+} + Mg^{2+}$)-ATPase was purified by using the method of Wolf et al. [106], from human and porcine erythrocytes, respectively. An ($ATP + Mg^{2+}$)-dependent calcium uptake into the vesicles was demonstrated by both groups. Active calcium transport in the freeze-thaw reconstituted vesicles was significantly stimulated by the addition of calmodulin [112]. Promising preliminary results were reported by Niggli (personal communication) in incorporating highly purified red cell calcium-pump protein into liposomes.

## X. Regulation of the red cell calcium pump

The most recent, and probably the most fascinating, area in the investigation of the red cell calcium pump is its cellular regulation. In normal red cells, active calcium extrusion is operating only at about 0.1–0.5% of its full capacity, since low passive permeability of the membrane prevents any significant calcium entry. However, in an 'emergency' situation, a 200–1000-fold increase in the calcium-pumping rate occurs, compensating even a greatly increased calcium influx. The main effector of this response is certainly the increased cellular calcium concentration, but the regulation of the pump activity seems to involve a rather complex cellular apparatus.

### *XA. Regulation by intracellular soluble proteins*

In 1973, Bond and Clough [154] first observed that membrane-free red cell hemolysate significantly stimulates ($Ca^{2+} + Mg^{2+}$)-ATPase in isolated red cell membrane fragments. They also showed that the stimulating factor is a non-dialysable, trypsin-sensitive protein, and that it is not hemoglobin. In 1976, Luthra et al. [155,156] reported the partial purification of the ($Ca^{2+} + Mg^{2+}$)-ATPase activator protein by ion-exchange chromatography. They also demonstrated the presence of this protein activator in the red cells of several mammalian species, without species specificity [156]. Parallel with these studies, experiments in various laboratories unequivocally showed that calcium-dependent reversible binding of cytoplasmic proteins to the red cell membrane basically determines the kinetics of ($Ca^{2+} + Mg^{2+}$)-ATPase activity [61–66,99,100].

The direct involvement of cytoplasmic proteins in regulating active calcium transport was first indicated by a short communication of Macintyre and Green [75], reporting the stimulation of active calcium uptake in inside-out red cell membrane vesicles by the membrane-free hemolysate. In 1978, the observations of Macintyre and Green were reinforced and extended in three different laboratories (Refs. 76–78; see Fig. 2).

A fascinating development in 1977–79 was the purification of the cytoplasmic activator protein and the recognition of its similarity (or identity) to calcium-dependent regulatory proteins in other tissues. In the early 1970's, calcium-dependent forms of brain adenylate cyclase and phosphodiesterase were characterized, and a modulator protein,

transferring calcium-sensitivity to these enzymes, was isolated (for reviews see Refs. 157 and 158). It was first shown by Gopinath and Vincenzi [159] and Jarrett and Penniston [160] that a purified brain phosphodiesterase and adenylate cyclase activator mimics red cell cytoplasmic proteins in stimulating ($Ca^{2+}$ + $Mg^{2+}$)-ATPase in isolated red cell membranes. Hinds et al. [161] and Larsen and Vincenzi [162] demonstrated that the above brain activator protein (termed calmodulin by Cheung et al. [157] in 1978) stimulates calcium uptake in inside-out red cell membrane vesicles.

From the rapidly growing number of publications concerning the functions and structure of calmodulin (according to a recent I.S.I. 'citation-cluster' investigation [163] this topic is among the fastest growing fields of basic science) the following conclusions can be outlined at present:

Calmodulin is a low (16 500) molecular weight, acidic ($pH_i$ = 4.2 – 4.3), relatively heat-stable protein with an identical or almost identical structure, and with ubiquitous occurrence in eukaryotic cells [158]. It is a divalent cation-binding protein with four binding sites: three of them with high affinity for calcium ($K_d$ of about 0.2 $\mu$M) but low affinity for $Mg^{2+}$ ($K_d$ = 140 $\mu$M), and one site with similar affinities for both ions ($K_{d,Ca}$ = 1 $\mu$M, $K_{d,Mg}$ = 20 $\mu$M [164]). Thus, the functional intracellular form of calmodulin is possibly a 3 calcium : 1 magnesium state. Calmodulin action can be selectively inhibited by its reversible, calcium-dependent binding to some neuroleptic drugs, especially to phenothiazines [165,166]. By means of chemical modifications and biophysical methods, it was shown that binding of calcium increases the helical content of calmodulin, and makes its tertiary structure more compact [158]. Calcium-dependent changes in the microenvironment of tyrosyl residue 138 on the protein were reported [167].

The role of calmodulin has been demonstrated in calcium-induced insulin release [168–170] and intestinal secretion [171], in synaptic transmission [172,173], in the regulation of active calcium transport in sarcoplasmic reticulum [174] and in synaptosomes [175,176]. Involvement of calmodulin action is also postulated in the regulation of cell multiplication [6], and fertilization of oocytes by spermatozoa [177]. The enzymes known to be regulated by calmodulin include phosphodiesterase and adenylate cyclase in various tissues, myosin light-chain kinase in muscle and non-muscle cells, phosphorylase $B$ kinase, and several other protein kinases (see Refs. 157 and 158). Thus, calmodulin possesses the ability to modulate a large number of cellular functions, possibly most of those regulated by $Ca^{2+}$. In accordance with this general physiological role, the structure and function of this protein have been highly conserved during evolution. These data confirm our belief that Nature is close-fisted when using different tools, although she is luxurious in the effects produced.

In human red cells, calmodulin affects ($Ca^{2+}$ + $Mg^{2+}$)-ATPase activity and active calcium transport possibly through a calcium-dependent direct binding to the transport enzyme [178–183]. Calmodulin binding decreases the apparent calcium affinity of the system and increases the maximum transport (ATPase) rate [73–80]. Calmodulin has no apparent effect on the ATP and $Mg^{2+}$ affinity of the pump [78,179], although it may alter the regulatory role of high ATP concentrations [108]. Calmodulin binding to the pump protects the system from inactivation during storage [182], while in certain membrane preparations the loss of activation by calmodulin is observed [69]. According to our recent experiments [184], mild trypsin digestion of inside-out red cell membrane vesicles mimics the action of calmodulin-stimulation of active calcium uptake leading to decrease in $K_{Ca}$ at unchanged $K_{ATP}$. Calmodulin has no further stimulatory action in these inside-out vesicles. These experiments indicate that a subunit of the transport

enzyme is easily digested at the internal membrane surface, and possibly the functioning of this calmodulin-binding, regulatory subunit determines the calcium affinity of the pump. The possible mode of calmodulin action on the reaction mechanism of active calcium transport in the red cell membrane is discussed in Section XI.

In 1977, Wang and Desai [185] isolated and characterized a calmodulin-binding 'inhibitory' protein, with no detectable enzyme activity. Larsen et al. [186] showed that this protein prevented calmodulin action on the calcium pump in inside-out red cell membrane vesicles. By now, several types of calmodulin-binding proteins have been isolated (see Ref. 187), possibly with functions modifying calcium + calmodulin effects on various target enzymes. According to the experiments of Au [188] and Sarkadi et al. [80], concentrated, heat-labile protein fractions of the red cell cytoplasm prevent calmodulin activation of the red cell membrane ($Ca^{2+} + Mg^{2+}$)-ATPase and calcium transport, respectively. In the original intracellular environment, these inhibitors may overrule the effect of calmodulin [80], and the actual regulatory balance is possibly a complex function of $Ca^{2+}$, $Mg^{2+}$, and ATP concentrations. The low calcium affinity of the calcium pump and its relatively low maximum transport rate in intact red cells as compared to those in resealed ghosts or in calmodulin-supplemented inside-out vesicles (see Refs. 67 and 80) indicate that calmodulin effects in the red cells may be largely supressed.

*XB. Regulation of the calcium pump by $Mg^{2+}$, ATP, cyclic AMP and monovalent cations*

As previously discussed, cellular magnesium is required for active calcium transport, and a maximum activity of calcium pumping is observed at millimolar magnesium concentrations. Although human red cells contain 2.5–3.0 mM magnesium, most of this divalent cation is chelated by ATP and 2,3-diphosphoglycerate, and the free $Mg^{2+}$ concentration is only about 10% of the total [189]. Therefore, actual changes in the intracellular ATP and $Mg^{2+}$ concentrations may influence the characteristics of calcium pumping. A complex effect of $Mg^{2+}$ and ATP on the kinetics of ($Ca^{2+} + Mg^{2+}$)-ATPase was suggested by Katz et al. [190]. The possible regulatory role of high cellular ATP concentrations on the calcium pump has already been discussed in subsection IVB, and Section V (see also Fig. 4).

Cyclic AMP is postulated as a general second messenger in cellular action of hormones and other stimuli, but human red cells have no significant adenylate cyclase activity to catalyze the formation of cyclic AMP [191]. At the same time, cyclic AMP-dependent membrane phosphorylation by protein kinases was convincingly demonstrated in human red cells [114–118,139], and uptake of cyclic AMP from blood plasma may be sufficient to stimulate this activity [192]. A recent report by Varghese and Cunningham [193] indicated that micromolar concentrations of cyclic AMP inhibit ($Ca^{2+} + Mg^{2+}$)-ATPase in red cell membrane fragments. The authors suggest that active calcium transport in red cells is modulated by means of a mechanism involving cyclic AMP and a membrane-associated protein kinase. In inside-out red cell membrane vesicles, cyclic AMP (also in the form of dibutyryl cyclic AMP) has no effect on active calcium transport (Sarkadi, B., unpublished data), and a possible explanation for this contradiction may be the loss of some peripheral membrane proteins during inside-out vesicle preparation.

Experiments carried out on fragmented red cell membranes, as well as on inside-out vesicles indicate that both $Na^+$ and $K^+$ in the cell interior have stimulatory actions on calcium pumping in erythrocytes (see subsection IVC). Although alterations in the $Na^+$ and $K^+$ concentrations in the physiological (or even in a pathological) range seem to be insuf-

ficient to evoke major alterations in red cell calcium pumping, the monovalent cation dependence of the plasma membrane calcium transport may be important in other tissues or in red cells of different species.

## XI. Model for the reaction mechanism of the red cell calcium pump

In this section, a putative model for the reaction mechanim of the calcium pump, and for the sites of regulatory actions is presented. The multi-step scheme in Fig. 8 summarizes earlier reports on the characteristics and regulation of the red cell calcium transport but, of course, is influenced by the author's opinion in selecting the relevant observations for constructing a self-consistent model.

The basic assumptions in the model are as follows: The calcium transport enzyme (E) in the red cell membrane at the internal membrane surface first reacts with two $Ca^{2+}$ and then the enzyme-calcium complex ($ECa_2$) is phosphorylated by cellular ATP ($EPCa_2$). The energy for calcium translocation is provided by this phosphorylation. Following calcium translocation, the affinity of the enzyme for calcium decreases by several orders of magnitude, and $Ca^{2+}$ is released into the extracellular solution. Magnesium-dependent dephosphorylation of the enzyme occurs at the internal membrane surface, and the system is ready for a new transport cycle. In the model, active calcium transport is not connected to the co- or counter-transport of monovalent or divalent cations (electroneutrality during calcium extrusion is probable ensured by chloride efflux). The stoichiometry of the calcium transport and ATP splitting in the model is 2 : 1. It has to be emphasized that the 'intramembrane positions' of the enzyme or the EP complex on the reaction scheme do not represent any specific molecular movements but merely illustrate the reaction steps.

Examining the steps of the reaction scheme, steps 1 and 2 represent the binding of $Ca^{2+}$ to the transport enzyme. Experiments demonstrating the calcium-induced formation of the phosphorylated intermediate of the calcium pump from ATP indicate that EP formation is evoked by calcium binding. The conclusion that calcium-binding sites on the

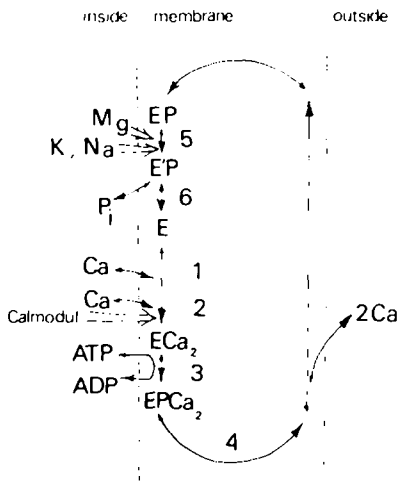


Fig. 8. Reaction scheme for active calcium transport in human red cell membrane. For details see the text.

enzyme exist in the absence of ATP is supported by experiments on *N*-ethylmaleimide-enzyme interactions [124]. According to transport experiments on resealed ghosts [54] and on inside-out membrane vesicles [79], $K_{Ca,free}$ when measured in CaEGTA buffers is between 0.5 and 5 $\mu$M. This value probably reflects the high calcium affinity of one of the binding sites on the enzyme. Cooperativity in the calcium activation of the pump, indicating the presence of more than one binding site for calcium, has been observed by some investigators [7,62,63,106]. Calculations of Ferreira and Lew [58], based on experiments with intact red cells in the presence of ionophore A23187, suggested that the calcium pump has two binding sites for calcium with similar dissociation constants (about 1 $\mu$M). In these experiments, a 'low-affinity' calcium binding may have been converted to a 'high-affinity' one by ionophore A23187 (see Refs. 63 and 112). The existence of a low-affinity binding site on the pump for calcium was shown in intact red cells, resealed ghosts and inside-out vesicles [7,18,49,54,78,79,81]. The variability in the actual $K_{Ca}$ values is most probably due to the association or dissociation of calmodulin to the pump molecule (see below).

Step 3 in the reaction mechanism is the phosphorylation of the enzyme-calcium complex by ATP. The substrate in this reaction is probably free ATP, and the value of $K_{ATP,free}$ is about 1–2 $\mu$M [55,78,85,138,142].

Step 4 represents calcium translocation and the release of $Ca^{2+}$ into the extracellular solution. The change in the affinity for calcium during this reaction amounts to several orders of magnitude. Still, in experiments where steep, reversely directed calcium concentration gradients were produced, the reversal of the calcium pump could be demonstrated. In resealed ghosts, Rossi et al. [194] observed external calcium-dependent $^{32}P_i$ incorporation into ATP, while Wüthrich et al. [195] showed picomolar amounts of net ATP synthesis by running the calcium pump backwards in inside-out membrane vesicles. It is possibly step 4 in which external lanthanum is bound to the transport enzyme, blocking any further turnover by stabilizing the system in a phosphorylated form [55, 142]. We do not think that the calcium pump is working as a mobile carrier for calcium, but external lanthanum blocking of the pump in its phosphorylated form shows the spatial accessibility of cation-binding sites at the external membrane surface during or after calcium release.

In the normal transport cycle, the EP complex is dephosphorylated at the internal membrane surface in the presence of $Mg^{2+}$. As the binding of magnesium to the enzyme during phosphorylation is sufficient to produce rapid dephosphorylation [138], a magnesium-dependent change of the EP to an E'P form is postulated (steps 5 and 6). E'P is then easily hydrolyzed to produce inorganic phosphate in the cell interior.

Based on direct measurements for the partial reactions of the pump cycle, i.e., for the formation and decomposition of the EP complex, we may work out a rough estimation of the relative rates of the individual reactions. In the absence of magnesium, calcium-induced EP formation has a $T_{1/2}$ value of 40–60 s at 0°C (Sarkadi, B. and Enyedi, Á., unpublished results) while the addition of magnesium reduces this $T_{1/2}$ value to less than 5 s [55,108,138,143] (all the data presented were obtained at 0°C). In some reports [55,138,143], an increased steady-state level of EP was noted in the presence of magnesium. However, due to the slow rate of EP formation in the absence of $Mg^{2+}$, a real steady state may not have been reached in these experiments (20–30 s incubation periods), and an underestimation of the calcium-induced EP level possibly occurred. The $T_{1/2}$ value for the dephosphorylation of the EP complex is 40–60 s in the absence of magnesium, and the decomposition of the phosphoenzyme is incomplete under these conditions [55,137,

138]. Magnesium reduces the $T_{1/2}$ value for dephosphorylation to 4–5 s and produces a complete decomposition of EP [137,138].

The above data can be fitted into a kinetic model in which the rate-limiting step in the pump cycle, in the absence of magnesium, is the transformation of EP to E'P, while in the presence of magnesium the slowest reaction (depending on the actual conditions) is either the formation of $ECa_2$, or the calcium-translocation step (see Refs. 55 and 138). In this model, the magnesium acceleration of EP formation is caused by an increase in the free enzyme concentration.

As to the sites of regulatory effects on the calcium pump, $K^+$, $Na^+$, and ATP were shown to stimulate dephosphorylation of the EP complex [143,144]. Calmodulin decreases $K_{Ca}$ of the calcium pump, and increases the level of EP complex formed in the absence of magnesium [108,144,145]. In the presence of calcium + magnesium, calmodulin does not affect the steady-state level of EP but accelerates the rate of EP formation [108]. These data suggest that the predominant effect of calmodulin is to increase the rate of reaction(s) 1 and/or 2, and thus stimulate calcium pumping through the acceleration of calcium-dependent phosphoenzyme formation. Recent experiments by Rega and Garrahan [145] indicate that calmodulin also stimulates EP dephosphorylation, when measured in the absence of magnesium.

Reliable calculations for the overall turnover rate of the red cell calcium pump are hindered by our not knowing the exact number of the pump molecules in the erythrocytes. This value is probably between 700 [138] and 4500 [183] pump molecules/red cell, thus the turnover rate of the system is between 1000 and 2000 ions/pump site per min at 37°C.

## XII. Comparison of the red cell calcium pump with other active calcium transport systems

It is hoped that the calcium pump in red cells provides a model for investigating the characteristics of active calcium transport systems in other cellular membranes. As far as can be concluded from studies on the plasma membranes of dissected or cultured animal cells, (ATP + $Mg^{2+}$)-dependent calcium transport is a general way for calcium extrusion from the cell interior. The presence of such a calcium extrusion has been reported in many tissues, including liver [196], smooth muscle [197] and heart sarcolemma [198], kidney [199,200], Ehrlich ascites tumor cells [201], platelets [202,203] and neutrophil granulocytes [204]. In various excitable, and also in non-excitable, cells, calcium extrusion occurs by an $Na^+$-$Ca^{2+}$ exchange system, working at the expense of the $Na^+$ gradient established by the ($Na^+$, $K^+$) pump. However, ATP-dependent calcium extrusion seems to be present also in these cellular membranes (see Refs. 1, 2, 198, 205–207). A recent report by Gmaj et al. [200] suggests that in the basolateral membrane of kidney cells, $Na^+$-$Ca^{2+}$ exchange is responsible for the bulk flow of calcium across the epithelium, while (ATP + $Mg^{2+}$)-dependent calcium extrusion maintains the low cytoplasmic calcium concentration. DiPolo et al. [207] showed that in squid axons, vanadate inhibits ATP-dependent calcium extrusion without affecting $Na^+$-$Ca^{2+}$ exchange. It is worth emphasizing that, in human red cells, no trace of an $Na^+$-$Ca^{2+}$ exchange could be observed, while in canine red cells, volume regulation depends upon a sodium-calcium counterflow extruding sodium at the expense of calcium influx, and upon a simultaneous active calcium extrusion [208,209].

Table I shows some comparative data for the active calcium transport in the red cell

TABLE I

CHARACTERISTICS OF ACTIVE CALCIUM TRANSPORT IN HUMAN RED CELLS, SARCOPLASMIC RETICULUM (SR) VESICLES, AND MITOCHONDRIA

nTP, nucleotide triphosphate; pNPP, *p*-nitrophenyl phosphate; acetyl-P, acetylphosphate.

| Parameter | Human red cells | SR vesicles | Mitochondria |
|---|---|---|---|
| $K_{Ca,free}$ (EGTA buffers) | 0.5–5 μM | 0.3–0.5 μM | 1–5 μM |
| $V$ (nmolCa/mg protein min; at 37°C) | 10–25 | 1500–2000 | 100–200 |
| Metal ion specificity | Ca > Sr >> Ba, Mn | Ca > Sr >> Ba, Mn | Ca > Sr >> Ba, Mn |
| Energy source | ATP (+nTP) | ATP, nTP, pNPP, acetyl-P | ATP or substrate oxidation |
| $Mg^{2+}$ requirement | + | + | + (if ATP is used?) |
| Stoichiometry | 2 Ca : 1 ATP (1 Ca : 1 ATP?) | 2 Ca : 1 ATP | 2 Ca : 1 ATP 2 Ca : 1 energy conserving site |
| Most effective inhibitors | La, ruthenium red | ruthenium red, La | ruthenium red, La |
| Phosphorylated intermediate | acylphosphate 120000–150000 dalton protein | acylphosphate 100000–130000 dalton protein | none (or not shown) |
| Calmodulin regulation | + | + | not shown |
| Cyclic AMP regulation | ?+ | + | not shown |
| References | this review and 7,8,221 | 222–225 | 226,227 |

membrane and for that in sarcoplasmic reticulum and mitochondria. As indicated, the $K_{Ca,free}$ values, obtained in CaEGTA buffers, are fairly similar in the three transport systems, although the maximum transport rate is about 100 times greater in the sarcoplasmic reticulum, and about 10 times greater in the mitochondria than in human red cells. This difference is understandable if we consider that in the sarcoplasmic reticulum, 60–75% of the membrane proteins belong to the calcium pump – this system is specifically devised for the rapid elimination of calcium from the muscle cytoplasm during relaxation. Although the method of energy 'input' is somewhat different in the three pump systems, the values for stoichiometry are similar, and inhibitory effects of lanthanum and ruthenium red are also observed. Both in red cells and sarcoplasmic reticulum, calcium is actively transported away from the side of the membrane where ATP is hydrolyzed. A possible counter-transport of magnesium in sarcoplasmic reticulum during calcium uptake has been indicated [210]. An important difference between calcium transport in human red cells and sarcoplasmic reticulum is that the latter system is easily capable of synthesizing ATP during a backward calcium movement, while to make the red cell calcium pump run backward is much more difficult. Active calcium transport in red cells as well as in sarco-

plasmic reticulum is regulated by calmodulin, although in the latter system the calmodulin effect is presumed to be transferred by protein kinase actions [211]. There are also data available for the cyclic AMP-dependent, protein kinase-catalyzed regulation of active calcium transport in sarcoplasmic reticulum [212,212]. $Na^+$ and $K^+$, just as in the case of the red cell calcium pump, stimulate active calcium transport in sarcoplasmic reticulum vesicles [214,215]. Immunological cross-reaction between purified red cell $(Ca^{2+} + Mg^{2+})$-ATPase and antibody against rabbit skeletal muscle sarcoplasmic reticulum ATPase was reported by Wolf [152], supporting the structural similarity of the two enzymes.

Investigations of the ATP-fuelled calcium pumping in synaptic vesicles [216,217] and in smooth endoplasmic reticulum membranes [218] give hope for a better understanding of intracellular regulation of cytoplasmic calcium concentration. It has to be mentioned that bacteria also have a calcium pump which extrudes calcium from the cell interior (see Ref. 219). In most bacteria (e.g., in *E. coli*), the energy for calcium transport can be derived either from the electron transport chain by substrate oxidation, or from the hydrolysis of ATP. However, in *Streptococcus faecalis*, the calcium pump is fuelled only by ATP, in the presence of $Mg^{2+}$ [220]. Analysis of the similarities and differences in the various forms of membrane calcium transport may certainly increase our understanding of these processes of vital importance.

## XIII. Summary and conclusions

Local changes in intracellular calcium concentration regulate numerous basic functions in the living cells. This regulation is based upon the unequal calcium distribution among the extracellular fluid, the cytoplasm, and some intracellular organelles. The ultimate way to assure the physiological low cytoplasmic calcium concentration is the active extrusion of calcium by plasma membrane pumps.

Active calcium extrusion in human red cells is of vital importance as an increase in cellular calcium is harmful to most red cell functions. The calcium pump is a membrane-bound system, catalyzing the uphill extrusion of calcium by using the energy of cellular ATP. According to a reaction scheme (Section XI), intracellular binding of calcium to the pump evokes an ATP-dependent phosphorylation of the transport enzyme, and then a transmembrane calcium movement. For the dephosphorylation of the enzyme, i.e., for the continuous work of the pump, the presence of cellular magnesium is required. No co- or counter-transport of cations is connected to active calcium extrusion. The stoichiometry of the pump is either 1 Ca : 1 ATP, or more probably, 2 Ca : 1 ATP. The molecular basis of the red cell calcium pump is a membrane-spanning protein with a molecular weight of 125 000–150 000.

Calcium extrusion from human red cells is effectively stimulated by small increases in the cellular calcium concentration. The pump is also influenced by changes in cellular $Mg^{2+}$, ATP, or monovalent cation concentrations and by the action of cytoplasmic regulatory protein(s). The protein activator of the red cell calcium pump has a molecular structure similar or identical to the calcium-dependent regulatory protein, calmodulin, found in various tissues of various animal species. In red cells, calmodulin increases the maximum transport rate of the calcium pump and increases its calcium affinity as well.

Active calcium transport in human red cells is a useful model for investigating the characteristics of ATP-fuelled pumps in the plasma membranes. The red cell calcium pump has basic features similar to active calcium transport in the plasma membrane of different eukaryotic cells, in sarcoplasmic reticulum, or in bacterial membranes.

## Acknowledgements

The author wishes to thank Drs. G. Gárdos, Ilma Szász and H.J. Schatmann for their valuable advice in preparing the manuscript, and Mrs. M. Sarkadi for her technical assistance in the experiments presented.

## References

1 Baker, P.F. (1972) Progr. Biophys. Mol. Biol. 24, 177–223
2 Baker, P.F. (1976) Symp. Soc. Exp. Biol. 30, 67–88
3 Ebashi, S. (1976) Annu. Rev. Physiol. 38, 293–314
4 Rasmussen, H. and Goodman, D.B.P. (1977) Physiol. Rev. 57, 421–509
5 Case, R.M. (1980) Cell Calcium 1, 1–5
6 Whitfield, J.F., Boynton, A.L., Macmanus, J.P., Sikorska, M. and Tsang, B.K. (1979) Mol. Cell. Biochem. 27, 155–179
7 Schatzmann, H.J. (1975) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 6, pp. 125–168, Academic Press, New York
8 Sarkadi, B. and Tosteson, D.C. (1979) in Membrane Transport in Biology (Giebisch, G., Tosteson, D.C. and Ussing, H.H., eds.), Vol. 2, pp. 117–160, Springer-Verlag, Berlin
9 Harrison, D.G. and Long, C. (1968) J. Physiol. 199, 367–381
10 Schatzmann, H.J. and Vincenzi, F.F. (1969) J. Physiol. 201, 369–395
11 Lichtman, M.A. and Weed, R.I. (1973) in Red Cell Shape (Bessis, M., Weed, R.I. and Leblond, P.F., eds.), pp. 79–93, Springer-Verlag, Berlin
12 Dunn, M.J. (1974) Biochim. Biophys. Acta 352, 97–116
13 Plishker, G.A. and Gitelman, H.J. (1976) J. Gen. Physiol. 68, 29–41
14 Lew, V.L. and Beaugé, L.A. (1979) in Membrane Transport in Biology (Giebisch, G., Tosteson, D.C. and Ussing, H.H., eds.), Vol. 2, pp. 81–116, Springer-Verlag, Berlin
15 Lew, V.L. and Ferreira, H.G. (1978) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 10, pp. 217–271, Academic Press, New York
16 Porzig, H. (1972) J. Membrane Biol. 8, 237–258
17 Szász, I. and Gárdos, G. (1974) FEBS Lett. 44, 213–216
18 Romero, P.J. and Whittam, R. (1971) J. Physiol. 214, 481–507
19 Garrahan, P.J. and Rega, A.F. (1967) J. Physiol. 193, 459–466
20 Gunn, R.B. and Tosteson, D.C. (1971) J. Gen. Physiol. 57, 593–609
21 Szász, I., Sarkadi, B. and Gárdos, G. (1977) J. Membrane Biol. 35, 75–95
22 Reed, P.W. (1973) Fed. Proc. 32, 635
23 Reed, P.W. (1976) J. Biol. Chem. 251, 3489–3494
24 Kirkpatrick, F.H., Hillman, D.G. and LaCelle, P.L. (1975) Experientia 31, 653–654
25 Weed, R.I., LaCelle, P.L. and Merrill, E.W. (1969) J. Clin. Invest. 48, 795–811
26 LaCelle, P.L., Kirkpatrick, F.H. and Udkow, M. (1973) in Erythrocytes, Thrombocytes, Leukocytes (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds.), pp. 49–52, Thieme, Stuttgart
27 White, J.G. (1974) Am. J. Pathol. 77, 504–514
28 Sarkadi, B., Szász, I. and Gárdos, G. (1976) J. Membrane Biol. 26, 257–270
29 Palek, J., Stewart, G. and Lionetti, F.J. (1974) Blood 44, 583–597
30 Kirkpatrick, F.H. (1976) Life Sci. 19, 1–18
31 Lorand, L., Weissmann, L.B., Epel, D.L. and Lorand, J.B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4479–4481
32 Allan, D. and Michell, R.H. (1975) Nature 258, 348–349
33 Allan, D. and Michell, R.H. (1977) Biochem. J. 166, 495–499
34 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4457–4461
35 Sheetz, M.P. and Singer, S.J. (1977) J. Cell. Biol. 73, 638–646
36 Gárdos, G. (1958) Acta Physiol. Acad. Sci. Hung. 14, 1–5
37 Gárdos, G. (1959) Acta Physiol. Acad. Sci. Hung. 15, 121–125
38 Blum, R.M. and Hoffman, J.F. (1971) J. Membrane Biol. 6, 315–328
39 Blum, R.M. and Hoffman, J.F. (1972) Biochem. Biophys. Res. Commun. 46, 1146–1152

40 Gárdos, G., Sarkadi, B. and Szász, I. (1975) FEBS (Fed. Eur. Biochem. Soc.) Proc. Meet. 35, 167–180
41 Lew, V.L. and Ferreira, H.G. (1976) Nature 263, 336–338
42 Gárdos, G., Szász, I. and Sarkadi, B. (1977) Acta Biol. Med. Ger. 36, 823–829
43 Simons, T.J.B. (1976) J. Physiol. 256, 209–225
44 Yingst, D.R. and Hoffman, J.F. (1978) Biophys. J. 23, 463–471
45 Putney, J.W. (1979) Pharmacol. Rev. 30, 209–245
46 Dunham, E.T. and Glynn, I.M. (1961) J. Physiol. 156, 274–293
47 Hoffman, J.F. (1962) Circulation 24, 1201–1213
48 Davis, P.W. and Vincenzi, F.F. (1971) Life Sci. 10, 401–406
49 Sarkadi, B., Szász, I., Gerlóczi, A. and Gárdos, G. (1977) Biochim. Biophys. Acta 464, 93–107
50 Sarkadi, B., Szebeni, J. and Gárdos, G. (1980) in Alfred Benzon Symposium No. 14, pp. 220–235, Munksgaard, Copenhagen
51 Schatzmann, H.J., (1966) Experientia 22, 364–368
52 Lee, K.S. and Shin, B.C. (1969) J. Gen. Physiol. 54, 713–729
53 Olson, E.J. and Cazort, R.J. (1969) J. Gen. Physiol. 53, 311–322
54 Schatzmann, H.J. (1973) J. Physiol. 253, 551–569
55 Schatzmann, H.J. and Bürgin, H. (1978) Ann. N.Y. Acad. Sci. 307, 125–147
56 Bürgin, H. and Schatzmann, H.J. (1979) J. Physiol. 287, 15–32
57 Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970–6977
58 Ferreira, H.G. and Lew, V.L. (1976) Nature 259, 47–49
59 Wolf, H.U. (1972) Biochem. J. 130, 311–314
60 Scharff, O. (1972) Scand. J. Clin. Lab. Invest. 6, 313–319
61 Quist, E.E. and Roufogalis, B.D. (1975) Arch. Biochem. Biophys. 168, 240–251
62 Scharff, O. (1976) Biochim. Biophys. Acta 443, 206–218
63 Scharff, O. and Foder, B. (1977) Biochim. Biophys. Acta 483, 416–424
64 Scharff, O. and Foder, B. (1978) Biochim. Biophys. Acta 509, 67–77
65 Farrance, M.L. and Vincenzi, F.F. (1977) Biochim. Biophys. Acta 471, 49–58
66 Farrance, M.L. and Vincenzi, F.F. (1977) Biochim. Biophys. Acta 471, 59–66
67 Larsen, F.L., Hinds, T.R. and Vincenzi, F.F. (1978) J. Membrane Biol. 41, 361–376
68 Quist, E.E. and Roufogalis, B.D. (1975) FEBS Lett. 50, 135–139
69 Quist, E.E. and Roufogalis, B.D. (1977) J. Supramol. Struct. 6, 375–381
70 Sarkadi, B., Szász, I., Gárdos, G. (1979) XIth Int. Congr. Biochem., Toronto, Abstr. 116
71 Steck, T.L. and Kant, J.A. (1971) Biochemistry 10, 172–180
72 Steck, T.L. (1974) in Methods in Membrane Biology (Korn, E., ed.), Vol. 2, pp. 245–281, Plenum Press, New York
73 Weiner, M.L. and Lee, K.S. (1972) J. Gen. Physiol. 59, 462–473
74 Macintyre, J.D. and Green, J.W. (1976) J. Gen. Physiol. 68, 12a P
75 Macintyre, J.D. and Green, J.W. (1977) Fed. Proc. 36, 271
76 Macintyre, J.D. and Green, J.W. (1978) Biochim. Biophys. Acta 510, 373–377
77 Hinds, T.R., Larsen, F.L. and Vincenzi, F.F. (1978) Biochem. Biophys. Res. Commun. 81, 455–461
78 Sarkadi, B., Macintyre, J.D. and Gárdos, G. (1978) FEBS Lett. 89, 78–82
79 Sarkadi, B., Schubert, A. and Gárdos, G. (1979) Experientia 55, 1045–1047
80 Sarkadi, B., Szász, I. and Gárdos, G. (1980) Biochim. Biophys. Acta 598, 326–338
81 Schatzmann, H.J. and Rossi, G.L. (1971) Biochim. Biophys. Acta 241, 379–392
82 Ferreira, H.G. and Lew, V.L. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 53–92, Academic Press, New York
83 Olson, E.J. and Cazort, R.J. (1974) J. Gen. Physiol. 63, 590–600
84 Cha, Y.N., Shin, B.C. and Lee, K.S. (1971) J. Gen. Physiol. 57, 202–215
85 Schatzmann, H.J. (1977) J. Membrane Biol. 35, 149–158
86 Muallem, S. and Karlish, S.J.D. (1979) Nature 277, 238–240
87 Olson, E.J. (1979) J. Membrane Biol. 48, 265–284
88 Wüthrich, A. and Schatzmann, H.J. (1980) Cell Calcium 1, 21–36
89 Schatzmann, H.J. and Tschabold, M. (1971) Experientia 27, 59–61
90 Szász, I., Sarkadi, B. and Gárdos, G. (1978) Br. J. Haematol. 39, 559–568
91 Szász, I., Sarkadi, B., Schubert, A. and Gárdos, G. (1978) Biochim. Biophys. Acta 512, 331–340

92 Nakao, T., Nagano, K., Adachi, K. and Nakao, M. (1963) Biochem. Biophys. Res. Commun. 13, 444–448
93 Wins, P. and Schoffeniels, E. (1966) Biochim. Biophys. Acta 120, 341–350
94 Rosenthal, A.S., Kregenow, F.M. and Moses, H.L. (1970) Biochim. Biophys. Acta 196, 254–262
95 Clarke, M. and Griffith, J. (1972) Fed. Proc. 31, 412
96 Hayashi, H., Jarrett, H.W. and Penniston, J.T. (1978) J. Cell. Biol. 76, 105–115
97 White, M.D. and Ralston, G.B. (1980) Biochim. Biophys. Acta 596, 472–475
98 Wolf, H.U. (1972) Biochim. Biophys. Acta 266, 361–375
99 Vincenzi, F.F. and Farrance, M.L. (1977) J. Supramol. Struct. 7, 301–306
100 Vincenzi, F.F. (1978) Ann. N.Y. Acad. Sci. 307, 229–231
101 Pfleger, H. and Wolf, H.U. (1975) Biochem. J. 147, 359–361
102 Watson, E.L., Vincenzi, F.F. and Davis, P.W. (1971) Biochim. Biophys. Acta 249, 606–610
103 Bond, G.H. and Green, J.W. (1971) Biochim. Biophys. Acta 241, 393–398
104 Szász, I. (1975) Acta Biochim. Biophys. Acad. Sci. Hung. 10, 25–30
105 Scharff, O. (1978) Biochim. Biophys. Acta 512, 309–317
106 Wolf, H.U., Dieckvoss, G. and Lichtner, R. (1977) Acta Biol. Med. Ger. 36, 847–858
107 Richards, D.E., Rega, A.F. and Garrahan, P.J. (1978) Biochim. Biophys. Acta 511, 194–201
108 Muallem, S. and Karlish, S.J.D. (1980) Biochim. Biophys. Acta 597, 631–636
109 Glynn, I.M. and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13–55
110 Taylor, D., Baker, R. and Hochstein, P. (1977) Biochem. Biophys. Res. Commun. 76, 205–211
111 Plishker, G.A. and Gitelman, H.J. (1977) J. Membrane Biol. 35, 309–318
112 Haaker, H. and Racker, E. (1979) J. Biol. Chem. 254, 6598–6602
113 Roses, A.D. and Appel, S.H. (1973) J. Biol. Chem. 248, 1408–1411
114 Avruch, J. and Fairbanks, G. (1974) Biochemistry 13, 5507–5514
115 Greenquist, A.C. and Shohet, S.B. (1975) in Erythrocyte Structure and Function (Brewer, G.J., ed.), pp. 515–531, Alan R. Liss Inc., New York
116 Hosey, M.M. and Tao, M. (1977) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 9, pp. 233–321, Academic Press, New York
117 Hosey, M.M. and Tao, M. (1977) Biochim. Biophys. Acta 482, 348–357
118 Fairbanks, G. and Avruch, J. (1974) Biochemistry 13, 5514–5521
119 Quist, E.E. (1980) Biochem. Biophys. Res. Commun. 92, 631–637
120 Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959
121 Pouchan, M.I., Garrahan, P.J. and Rega, A.F. (1969) Biochim. Biophys. Acta 173, 151–154
122 Garrahan, P.J., Pouchan, M.I. and Rega, A.F. (1970) J. Membrane Biol. 3, 26–42
123 Rega, A.F., Richards, D.E. and Garrahan, P.J. (1973) Biochem. J. 136, 185–194
124 Richards, D.E., Rega, A.F. and Garrahan, P.J. (1977) J. Membrane Biol. 35, 113–124
125 Schatzmann, H.J. and Roelofsen, B. (1977) FEBS (Fed. Eur. Biochem. Soc.) Proc. Meet. 42, 389–400
126 Sarkadi, B., Szász, I. and Gárdos, G. (1979) J. Membrane Biol. 46, 183–184
127 Weller, M. (1978) Mol. Cell. Biochem. 20, 95–102
128 Sen, A.K. and Post, R.L. (1964) J. Biol. Chem. 239, 345–352
129 Whittam, R. and Ager, M.E. (1965) Biochem. J. 97, 214–227
130 Funder, J. and Wieth, J.O. (1967) Acta Physiol. Scand. 71, 113–124
131 Brox, D., Peterman, B. and Frunder, H. (1977) Acta Biol. Med. Ger. 36, 611–619
132 Frunder, H. (1977) Acta Biol. Med. Ger. 36, 475–476
133 Till, U., Peterman, H., Wenz, J. and Frunder, H. (1977) Acta Biol. Med. Ger. 36, 597–610
134 Maretzki, D., Reimann, B., Klatt, D. and Rapoport, S. (1980) FEBS Lett. 111, 269–271
135 Katz, S. and Blostein, R. (1973) Fed. Proc. 32, 287
136 Knauf, P.A., Proverbio, F. and Hoffman, J.F. (1974) J. Gen. Physiol. 63, 324–336
136 Katz, S. and Blostein, R. (1975) Biochim. Biophys. Acta 389, 314–324
138 Rega, A.F. and Garrahan, P.J. (1975) J. Membrane Biol. 22, 313–327
139 Schrier, S.L. (1977) Blood 50, 227–237
140 Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958
141 Weidekamm, E. and Brdiczka, D. (1975) Biochim. Biophys. Acta 401, 51–58
142 Szász, I., Hasitz, M., Sarkadi, B. and Gárdos, G. (1978) Mol. Cell. Biochem. 22, 147–152
143 Garrahan, P.J. and Rega, A.F. (1978) Biochim. Biophys. Acta 513, 59–65
144 Enyedi, Á., Szász, I., Sarkadi, B., Bot, G. and Gárdos, G. (1980) 28th Int. Congr. Physiol. Sci., Budapest, Abstr., in the press

145 Rega, A.F. and Garrahan, P.J. (1980) Biochim. Biophys. Acta 596, 487–489
146 Cha, Y.N. and Lee, K.S. (1976) J. Gen. Physiol. 67, 251–261
147 Bretscher, M.S. and Raff, M.C. (1975) Nature 258, 43–49
148 Coleman, R. and Bramley, T.A. (1975) Biochim. Biophys. Acta 382, 565–575
149 Roelofsen, B. and Schatzmann, H.J. (1977) Biochim. Biophys. Acta 464, 17–36
150 Ronner, P., Gazzotti, P. and Carafoli, E. (1977) Arch. Biochem. Biophys. 179, 578–583
151 Wolf, H.U. and Gietzen, K. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1272
152 Wolf, H.U. (1978) FEBS Meet., Dresden, Abstr. No. 1061.
153 Gietzen, K., Seiler, S., Fleischer, S. and Wolf, H.U. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., ed.), pp. 519–522, Elsevier/North-Holland, Amsterdam
154 Bond, G.H. and Clough, D.L. (1973) Biochim. Biophys. Acta 323, 592–599
155 Luthra, M.G., Hildebrandt, G.R. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 164–179
156 Luthra, M.G., Hildebrandt, G.R., Kim, H.D. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 180–186
157 Cheung, W.Y., Lynch, T.J. and Wallace, R.W. (1978) in Advances in Cyclic Nucleotide Research (George, W.J. and Ignarro, L.J., eds.), Vol. 9, pp. 233–251, Raven Press, New York
158 Wolff, D.J. and Brostrom, C.O. (1979) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A., eds.), Vol. 11, pp. 28–88, Raven Press, New York
159 Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209
160 Jarrett, H.W. and Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210–1216
161 Hinds, T.R., Larsen, F.L. and Vincenzi, F.F. (1978) Biochem. Biophys. Res. Commun. 81, 455–461
162 Larsen, F.L. and Vincenzi, F.F. (1979) Science 204, 306–309
163 Small, H. and Greenlee, E. (1979) Detecting Emergent Growth Areas in Science: An Interim Report and Predictions for 1979, Institute for Scientific Information
164 Wolff, D.J., Poirier, P.G., Brostrom, C.O. and Brostrom, M.A. (1977) J. Biol. Chem. 252, 4108–4117
165 Levin, R.M. and Weiss, B. (1977) Mol. Pharmacol. 13, 690–697
166 Levin, R.M. and Weiss, B. (1978) Biochim. Biophys. Acta 540, 197–204
167 Gietzen, K., Mansard, A. and Bader, H. (1980) Biochem. Biophys. Res. Commun., in the press
168 Richman, P.G. and Klee, C.B. (1979) J. Biol. Chem. 254, 5372–5376
169 Sugden, M.C., Christie, M.R. and Ashcroft, S.J.H. (1979) FEBS Lett. 105, 95–100
170 Valverde, I., Vandermeers, A., Anjaneyulu, R. and Malaisse, W.J. (1979) Science 206, 225–227
171 Ilundain, A. and Naftalin, R.J. (1979) Nature 279, 446–448
172 DeLorenzo, R.J., Freedman, S.D., Yohe, W.B. and Maurer, S.C. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1838–1842
173 Wood, J.G., Wallace, R.W., Whitaker, J.N. and Cheung, W.Y. (1980) J. Cell Biol. 84, 66–76
174 Katz, S. and Remtulla, M.A. (1978) Biochem. Biophys. Res. Commun. 83, 1373–1379
175 Sobue, K., Ichida, S., Yoshida, H., Yamazaki, R. and Kakiuchi, S. (1979) FEBS Lett. 99, 199–202
176 Kuo, C.H., Ichida, S., Matsuda, T., Kakiuichi, S. and Yoshida, H. (1979) Life Sci. 25, 235–240
177 Jones, H.P., Bradford, M.M., McRorie, R.A. and Cornier, M.J. (1978) Biochem. Biophys. Res. Commun. 82, 1264–1272
178 Hanahan, D.J., Taverna, R.D., Flynn, D.D. and Ekholm, J.E. (1978) Biochem. Biophys. Res. Commun. 84, 1009–1015
179 Au, K.S. (1978) Int. J. Biochem. 9, 735–743
180 Au, K.S. (1979) Int. J. Biochem. 10, 637–643
181 Lynch, T.J. and Cheung, W.Y. (1979) Arch. Biochem. Biophys. 194, 165–170
182 Niggli, V., Ronner, P., Carafoli, E. and Penniston, J.T. (1979) Arch. Biochem. Biophys. 198, 124–130
183 Jarrett, H.W. and Kyte, J. (1979) J. Biol. Chem. 254, 8237–8244
184 Sarkadi, B., Enyedi, Á., Szász, I. and Gárdos, G. (1980) 28th Int. Congr. Physiol. Sci., Budapest, Abstr., in the press
185 Wang, J.H. and Desai, R. (1977) J. Biol. Chem. 252, 4175–4184
186 Larsen, F.L., Raess, B.U., Hinds, T.R. and Vincenzi, F.F. (1978) J. Supramol. Struct. 9, 269–274

187 Grand, R.J. and Perry, S.V. (1979) Biochem. J. 183, 285–295
188 Au, K.S. (1978) Int. J. Biochem. 9, 477–480
189 Flatman, P. and Lew, V.L. (1977) Nature 267, 360–362
190 Katz, S., Roufogalis, B.D., Landman, A.D. and Ho, L. (1979) J. Supramol. Struct. 10, 215–225
191 Rodan, S.B., Rodan, G.A. and Sha'afi, R.I. (1976) Biochim. Biophys. Acta 428, 509–515
192 Thomas, E.L., King, L.E. and Morrison, M. (1979) Arch. Biochem. Biophys. 196, 459–464
193 Varghese, S. and Cunningham, E.B. (1980) Biochim. Biophys. Acta 596, 468–471
194 Rossi, J.P.F.C., Garrahan, P.J. and Rega, A.F. (1978) J. Membrane Biol. 44, 37–46
195 Wüthrich, A., Schatzmann, H.J. and Romero, P. (1979) Experientia 35, 1589–1590
196 Van Rossum, G.D.V. (1970) J. Gen. Physiol. 55, 18–32
197 Casteels, R., Goffin, S., Raeymaekers, L. and Wuytack, F. (1973) J. Physiol. 231, 19P
198 Caroni, P. and Carafoli, E. (1980) Nature 283, 765–767
199 Moore, L., Fitzpatrick, D.F., Chen, T.S. and Landon, E. (1974) Biochim. Biophys. Acta 345, 405–418
200 Gmaj, P., Murer, H. and Kinne, R. (1979) Biochem. J. 178, 549–560
201 Charlton, R.R. and Wenner, C.E. (1978) Biochem. J. 170, 537–544
202 Käser-Glanzmann, R., Jakábová, M., George, J.N. and Lüscher, E.F. (1977) Biochim. Biophys. Acta 466, 429–440
203 Käser-Glanzmann, R., Jakábová, M., George, J.N. and Lüscher, E.F. (1978) Biochim. Biophys. Acta 512, 1–12
204 Jackowski, S., Petro, K. and Sha'afi, R.I. (1979) Biochim. Biophys. Acta 558, 348–352
205 DiPolo, R. (1977) J. Gen. Physiol. 69, 795–813
206 Lew, V.L. (1978) Nature 274, 421–422
207 DiPolo, R., Rojas, H.R. and Beaugé, L. (1979) Nature, 281, 228–229
208 Parker, J.C., Gitelman, H.J., Glossom, P.S. and Leonard, D.L. (1975) J. Gen. Physiol. 65, 84–96
209 Brown, A.M. (1979) Biochim. Biophys. Acta 554, 195–203
210 Kanazawa, T., Yamada, S., Yamamoto, T. and Tonomura, Y. (1971) J. Biochem. 70, 95–123
211 Hörl, W.H. and Heilmeyer, L.M.G. (1978) Biochemistry 17, 766–772
212 Schwartz, A., Entman, M.L., Kaniike, K., Lane, L.K., VanWinkle, W.B. and Bornet, E.P. (1976) Biochim. Biophys. Acta 426, 57–72
213 Tada, M., Ohmori, F., Yamada, M. and Abe, H. (1979) J. Biol. Chem. 254, 319–326
214 Jones, L.R., Besch, H.R. and Watanabe, A.M. (1977) J. Biol. Chem. 252, 3315–3323
215 Shikegawa, M. and Pearl, L.J. (1976) J. Biol. Chem. 251, 6947–6952
216 Rahamimoff, H. and Abramovitz, E. (1978) FEBS Lett. 92, 163–167
217 Papazian, D., Rahamimoff, H. and Goldin, S.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3708–3712
218 Moore, L., Chen, T.S., Knapp, H.R. and Landon, E.J. (1975) J. Biol. Chem. 250, 4562–4568
219 Simoni, R.D. and Postma, P.W. (1975) Annu. Rev. Biochem. 44, 523–554
220 Kobayashi, H., Brunt, J. and Harold, F.M. (1978) J. Biol. Chem. 253, 2085–2092
221 Roufogalis, B.D. (1979) Can. J. Physiol. Pharmacol. 57, 1331–1348
222 Hasselbach, W., Suko, J. and Stromer, M.H. (1975) Ann. N.Y. Acad. Sci. 264, 335–349
223 MacLennan, D.H. and Holland, P.C. (1975) Annu. Rev. Biophys. Bioeng. 4, 377–404
224 Hasselbach, W. (1977) Biophys. Struct. Mech. 3, 45–54
225 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 2–79
226 Bygrave, F.L. (1978) Biol. Rev. 53, 47–79
227 Carafoli, E. and Crompton, M. (1978) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 10, pp. 151–217, Academic Press, New York