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Reconstitution of the Ca^{2+}-Transport System of Human Erythrocytes

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The (Ca^{2+} + Mg^{2+})-dependent ATPase of human erythrocyte 'ghosts' was solubilized and reconstituted to form membranous vesicles capable of energized Ca^{2+} accumulation. The erythrocyte 'ghosts' for this purpose were prepared by using iso-osmotic freeze-haemolysis in the presence of Tween 20 and proteinase inhibitors to stabilize the preparation. The reconstitution procedure is similar to that developed by Meissner & Fleischer [(1974) *J. Biol. Chem.* **249**, 302–309] for skeletal-muscle sarcoplasmic-reticulum in that: (1) deoxycholate is used for the solubilization of the membrane; (2) controlled dialysis at near room temperature, rather than 0°C, is required in order to obtain a functional preparation capable of Ca^{2+} accumulation; and (3) membrane vesicles can be reassembled with protein/lipid ratio (approx. 60% protein and 40% lipid) similar to that of the original membrane.

Human erythrocytes maintain a low intracellular Ca^{2+} concentration of approx. 0.1 μM, compared with about 1 mM in blood plasma (Harrison & Long, 1968). Maintenance of this steep gradient across the membrane is due to the low passive permeability of Ca^{2+} across the plasma membrane and an outwardly directed Ca^{2+}-pumping system (Schatzmann, 1975). Erythrocyte membranes have two (Ca^{2+} + Mg^{2+})-dependent ATPase activities (ATP phosphohydrolase, EC 3.6.1.3), which differ with respect to their Ca^{2+} and substrate affinities (Horten *et al.*, 1970; Bader, 1971; Wolf, 1972). A low-affinity enzyme is believed to be involved in regulation of the shape and deformability of the erythrocyte membrane (LaCelle & Kirkpatrick, 1975), whereas the high-affinity (Ca^{2+} + Mg^{2+})-dependent ATPase is believed to be responsible for the active Ca^{2+} transport across the plasma membrane (Schatzmann, 1973). More recently, Quist & Roufogalis (1977) have suggested that the active Ca^{2+} transport is performed by the low-affinity (Ca^{2+} + Mg^{2+})-dependent ATPase.

In order to study Ca^{2+} transport distinct from other processes occurring in the entire 'ghost' membrane, it is desirable to isolate the high-affinity (Ca^{2+} + Mg^{2+})-dependent ATPase and to reconstitute the enzyme into membrane vesicles. Furthermore, reconstitution experiments can be used to study the molecular organization (Fleischer *et al.*, 1979) and the regulation of the Ca^{2+}-transport system. In the present study, we report the reconstitution of solubilized human erythrocyte membranes to form functional membrane vesicles capable of energized Ca^{2+} transport.

Abbreviations used: Tos-Lys-CH_2Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Mops, 4-morpholinepropanesulphonic acid; KIU, kallikrein-inhibitor unit (Trautschold *et al.*, 1967).

Materials and Methods

The ionophore A-23187 was supplied by Calbiochem–Behring Corp., La Jolla, CA 92037, U.S.A. Trasylol (registered trademark of Bayer A.G.) was obtained from Bayer A.G., Leverkusen, Germany. Sephadex G-25 (coarse grade) was purchased from Pharmacia, Uppsala, Sweden; di-isopropyl phosphorofluoridate, Tos-Lys-CH_2Cl and dithiothreitol were from Serva, Heidelberg, Germany; dialysis tubing with a 12000-dalton cut-off was from A. H. Thomas Co., Philadelphia, PA, U.S.A.; Insta-Gel scintillation fluid was from Packard Instrument International, Zürich, Switzerland; and $^{45}CaCl_2$ was from Amersham Buchler, Braunschweig-Wenden, Germany. All other reagents were obtained from E. Merck, Darmstadt, Germany.

Dialysis tubing was treated before use as described previously (Meissner & Fleischer, 1974). Deoxycholate was recrystallized as described by Meissner *et al.* (1973). Phosphatidylcholine was prepared from egg phospholipids (Singleton *et al.*, 1965) and the cytoplasmic activator (calmodulin) was prepared as described by Jarrett & Penniston (1978).

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Assays

Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as a standard. For lipid determinations, total phosphorus was measured as an estimate of lipid phosphorus by using a modification (Rouser & Fleischer, 1967) of the method of Chen *et al.* (1956).

(Ca^{2+} + Mg^{2+})-dependent ATPase activity was determined at 30°C as described previously (Arnold *et al.*, 1976), except in the measurements for the efficiency of the reconstituted Ca^{2+}-pump system, in which case the ATPase activity was measured at 20°C and in the same medium as used for Ca^{2+}-uptake measurements (Meissner & Fleischer, 1971, 1974).

Energized Ca^{2+} accumulation was measured in the presence and absence of oxalate by using $^{45}Ca^{2+}$ and Millipore filtration (Meissner *et al.*, 1973). Ca^{2+} accumulation in the presence of oxalate, referred to as Ca^{2+} loading (Meissner & Fleischer, 1974), was measured at 20°C in a medium containing 0.2–0.5 mg of vesicle protein/ml, 100 mM-KCl, 5.25 mM-$MgCl_2$, 5 mM-ATP, 100 μM-$CaCl_2$, 10 mM-Mops (pH 7.0), 10 mM-dithiothreitol, 5 mM-oxalate and 10 μg of cytoplasmic activator protein/ml. The reaction was terminated by filtration through a type GS 0.22 μm-pore-size Millipore filter. Ca^{2+} loading was calculated from the decrease of radioactivity in the filtrate. The radioactivity in the filtrate of samples containing all components except protein did not decrease significantly, i.e. about 0.5%, compared with identical samples that were not filtered, indicating that significant Ca^{2+} precipitation had not occurred. The filtrate of the mixture containing all components except protein served for determination of the zero-time concentration of $^{45}Ca^{2+}$. The filtrates (200 μl portion of each) were counted for radioactivity in 5 ml of Insta-Gel scintillation fluid.

Preparation of erythrocyte 'ghost' membranes

Centrifugations were performed at 2°C unless stated otherwise; centrifugation forces are expressed as $g_{av.}$ and the centrifugation time includes the time to reach speed. Fresh human erythrocytes (group O, Rh^+) were washed and centrifuged four times at 2°C in a Spinco type 19 rotor at 4000 g for 20 min. The medium contained 5 mM-KCl, 125 mM-NaCl, 4.3 mM-$MgCl_2$, 2 mM-Na_2HPO_4, 2 mg of glucose/ml and 10 mM-Tris/10 mM-maleate (pH 7.4).

The washed packed erythrocytes (1000 ml) were mixed with an equal volume of buffer containing 320 mM-KCl, 10 mM-$MgCl_2$, 1 mM-$CaCl_2$, 20 mM-ascorbic acid, 0.5 mM-di-isopropyl phosphorofluoridate, 500 KIU of Trasylol/ml, 20 mM-Mops (pH 7.4) and various amounts of Tween 20 for different membrane preparations. The erythrocytes were haemolysed by freezing this mixture at −80°C, followed by thawing of the haemolysate in a water bath at 15°C.

The haemolysate was centrifuged at 37000 g and 2°C for 60 min. The pellet of packed 'ghost' membranes was washed five times in a buffer (approx. 5 litres final volume) containing 200 mM-KCl, 5 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, 10 mM-Mops (pH 7.0), 10 mM-ascorbic acid, 0.5 mM-di-isopropyl phosphorofluoridate and 100 KIU of Trasylol/ml. It was centrifuged twice for 60 min at 2°C and 37000 g and three times for 45 min at 2°C and 100000 g. After each wash and centrifugation, the supernatant was removed by aspiration and the small button of unlysed cells was mechanically separated from the 'ghost' membranes and discarded. The resulting pellet of packed 'ghost' membranes was suspended in a buffer to give a final protein concentration of approx. 20 mg/ml. The buffer contained 200 mM-KCl, 5 mM-$MgCl_2$, 0.5 mM-di-isopropyl phosphorofluoridate, 250 KIU of Trasylol/ml and 500 mM-sucrose. The 'ghost' suspension was stored in a liquid-N_2 refrigerator until use.

Solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase

Solubilization of the enzyme from the erythrocyte 'ghost' membranes was done by using potassium deoxycholate at controlled detergent/protein ratios at 0°C in a medium consisting of 500 mM-KCl, 2 mM-$MgCl_2$, 1 mM-$CaCl_2$. 10 mM-dithiothreitol, 1 mM-Tos-Lys-CH_2Cl, 10 mM-di-isopropyl phosphorofluoridate, 100 KIU of Trasylol/ml and 10 mM-Mops (pH 7.0). The final protein concentration in the solubilizing medium was approx. 10 mg/ml. The mixture was left on ice for about 10 min before the separation of the solubilized material from the insoluble membrane fragments by centrifugation at 140000 g for 60 min.

Concentration of the solubilized protein

Concentration was achieved by mixing 1 g of dry Sephadex G-25 (coarse grade) per 2.15 ml of the supernatant at 0°C. The moist gel was transferred into a fritted glass filter and the protein was washed off the gel by repeated addition of 1–2 ml portions of a solution (elution buffer) containing 500 mM-KCl, 1 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, 10 mM-dithiothreitol, 10 mM-Mops (pH 7.0) and various amounts of phosphatidylcholine, followed by centrifugation at 300 g for 8 min. The solubilized material was concentrated from approx. 3–4 mg of protein/ml to give a final protein concentration of approx. 10 mg/ml.

Dialysis

Dialysis was carried out at 16–18°C for 18 h at a buffer/sample ratio of at least 1000:1. The dialysis

buffer consisted of 400mM-KCl, 1.5mM-$MgCl_2$, 0.1mM-$CaCl_2$, 1mM-EDTA, 10mM-cysteine, 250mM-sucrose and 7.5mM-Mops (pH 7.0).

Results

The solubilization of the erythrocyte membrane and its reconstitution to form membranous vesicles capable of energized Ca^{2+} accumulation is outlined in Scheme 1.

The solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase was achieved by using deoxycholate. Erythrocyte membranes were prepared by isoosmotic freeze-haemolysis in the presence of various amounts of Tween 20 at pH 7.4 as described in the Materials and Methods section. The maximum amount of (Ca^{2+} + Mg^{2+})-dependent ATPase that can be solubilized with deoxycholate varies markedly for the 'ghost' preparations, which differ with respect to the Tween 20 concentration used during haemolysis (Fig. 1). Not more than 12% of the initial ATPase activity can be solubilized if membranes prepared without Tween 20 are treated with deoxycholate. As more Tween 20 is used for erythrocyte 'ghost' preparation, the maximum value of solubilized ATPase activity is increased. Furthermore, the maximum value of solubilized ATPase activity is shifted to lower deoxycholate/protein ratios for erythrocyte 'ghost' preparations prepared with increasing Tween 20 concentrations. Tween 20 seems to stabilize the (Ca^{2+} + Mg^{2+})-dependent ATPase and enhances solubilization with a lower concentration of deoxycholate. Corresponding to the increasing maximum values of solubilized ATPase activity obtained with erythrocyte 'ghosts' prepared with higher Tween 20 concentrations, the stability with time is also increased (results not shown). However, the high concentrations of Tween 20 used in the haemolysis step have an adverse effect in the subsequent reconstitution procedure. That is, the yield of sedimentable reconstituted ATPase activity (pellet 2) declines (Table 1) with increased concentration of Tween 20 used in preparation of the 'ghosts'. The residual Tween 20 present in the solubilized material cannot readily be removed by dialysis, thus keeping part of the ATPase soluble. A compromise concentration of 3 mg of Tween/ml was therefore used in the haemolysis step.

The amount of deoxycholate used for the solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase was optimized for each preparation by varying the weight ratio of deoxycholate to protein (see Fig. 2). The optimal ratio varies somewhat and is in the range of 0.05–0.07 for different 'ghost' membrane preparations prepared with 3mg of Tween 20/ml. Under optimal conditions approx. 60% of the initial ATPase activity is solubilized and about 30% remains membrane-bound. A higher deoxycholate concentration does not increase the yield of enzymically active solubilized (Ca^{2+} + Mg^{2+})-dependent ATPase. At a weight ratio of

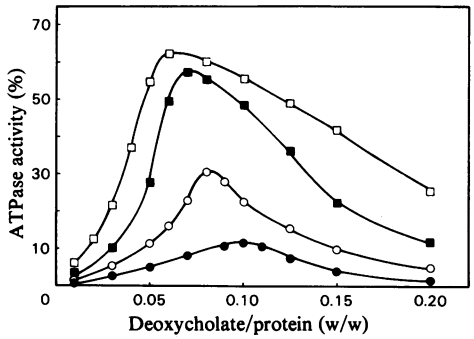


Fig. 1. *Stabilization and solubilization of the ($Ca^{2+}Mg^{2+}$)-dependent ATPase as a function of the concentrations of Tween 20 used in the preparation of erythrocyte 'ghosts'*

The erythrocyte 'ghost' preparations differ with respect to the final Tween 20 concentration used during the haemolysis step: ●, no Tween 20; ○, 1.5 mg of Tween 20/ml; ■, 3 mg of Tween 20/ml; □, 6 mg of Tween 20/ml. The actual Tween 20 concentrations of the erythrocyte 'ghost' preparations are much lower, since haemolysis is followed by five washes of the membranes. Solubilization was achieved with deoxycholate as described in the Materials and Methods section. The curves represent the solubilized (Ca^{2+} + Mg^{2+})-dependent ATPase activity (supernatant 1) as a function of the deoxycholate/protein ratio. The ATPase activity is expressed as a percentage of the activity of untreated 'ghost' membranes.

Table 1. *Yield of reconstituted ATPase activity (pellet 2) as a function of the Tween 20 concentration used during haemolysis*

Erythrocyte membranes were prepared by using various concentrations of Tween 20 during the haemolysis step. Solubilization and reconstitution were performed as outlined in Scheme 1. The ATPase activity of pellet 2 is related to the activity of the dialysed sample. The remainder of ATPase activity up to 100% remains soluble (supernatant 2).

Tween 20 (mg/ml)	Yield of sedimentable reconstituted ATPase activity (pellet 2) (%)
0	76
1.5	66
3	59
6	54
12.5	48
25	23
50	10
75	7.3
100	5.4

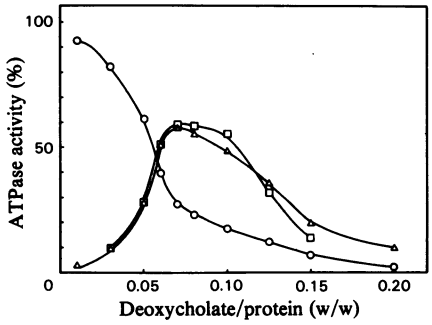


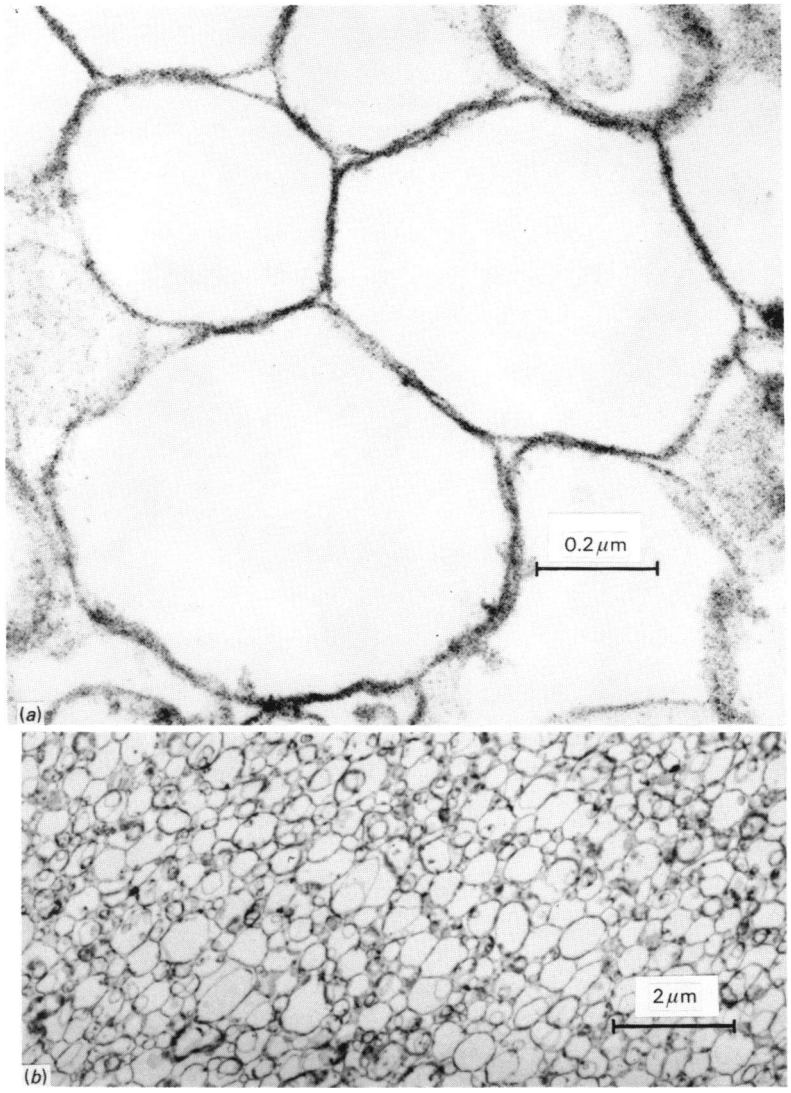
Fig. 2. *Solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase as a function of the deoxycholate/protein ratio*
Erythrocyte 'ghost' membranes prepared with 3mg of Tween 20/ml (approx. 10mg of protein/ml) were incubated with potassium deoxycholate at 0°C for 10min. To separate the insoluble matter from the solubilized material, the mixture was centrifuged at 140000$g_{av.}$ for 60min. The pellet was resuspended in the buffer used for solubilization. Part of the solubilized material was dialysed against dialysis buffer for 12h at 4°C. ATPase activity of the solubilized material (△), the solubilized material after dialysis (□) and the pellet (○). The ATPase activity is expressed as a percentage of the activity of untreated 'ghost' membranes.

deoxycholate to protein of 0.07 or below, deoxycholate does not inhibt the ATPase activity, since approx. 100% activity is accountable for between supernatant and pellet fractions and dialysis of the solubilized material does not increase the ATPase activity. The deoxycholate-solubilized (Ca^{2+} + Mg^{2+})-dependent ATPase is fairly stable. At higher ratios of detergent to protein in the range 0.07–0.11 there is inhibition and/or inactivation, which is at least in part reversible; under these conditions dialysis of the solubilized samples results in an increased ATPase activity. At ratios higher than 0.12, the inhibition and/or inactivation of the enzyme is irreversible.

Thus the procedure developed for the solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase activity from the erythrocyte membrane suspension makes use of deoxycholate in the optimal range of the deoxycholate/protein ratio of 0.05–0.07 (w/w), a treatment that achieves partial solubilization of the 'ghost' membranes (Scheme 1). The insoluble residue, consisting of membranous fragments, is removed by centrifugation in a Spinco 60 Ti rotor for 60min at 0°C and 140000g to form pellet 1. Supernatant 1 contains the solubilized material in a concentration of 3–4mg of protein/ml.

Erythrocyte 'ghost' suspension
↓ Solubilization with deoxycholate at a deoxycholate/protein ratio of 0.05–0.07 (w/w), 0°C, pH 7.0
Partially solubilized 'ghosts'
↓ Centrifugation for 60 min at 0°C and 140000$g_{av.}$
↘ Pellet 1 (discarded)
Supernatant 1 = solubilized material
↓ Concentration by Sephadex G-25 (coarse grade)
↘ Sephadex gel containing most of the aqueous medium and part of the lipids, proteins and deoxycholate
Concentrate
↓ Dialysis for 18h at 17°C
↘ Deoxycholate
Dialysed sample
↓ Centrifugation for 60min at 0°C and 140000$g_{av.}$
↘ Supernatant 2 (discarded)
Pellet 2
↓ Two washes in a medium containing 200mM-KCl, 5mM-$MgCl_2$, 5mM-dithiothreitol, 250mM-sucrose and 10mM-Mops (pH 7.0). Centrifugations for 60min at 0°C and 140000$g_{av.}$
↘ Supernatant 3 (discarded)
Pellet 3 = reconstituted vesicles

Scheme 1. *Solubilization and reconstitution procedure*
For further details see the Materials and Methods section.



EXPLANATION OF PLATE 1

Electron micrographs of reconstituted vesicles

The vesicles exhibit (Ca^{2+} + Mg^{2+})-dependent ATPase and Ca^{2+}-pump activity and were prepared under standard conditions (see legend to Table 4). The vesicles were fixed in 2% (w/v) OsO_4 in 20mM-cacodylate buffer (pH 7.2) containing 120mM-NaCl and 2.4mM-$CaCl_2$. The pellet was block stained with uranyl acetate, embedded in Araldite, and sliced in thin sections. Magnifications: (*a*) ×85000, (*b*) ×8500.

The solubilized sample is concentrated by adding dry Sephadex G-25 (see the Materials and Methods section) to give a final protein concentration of approx. 10mg/ml. The resulting concentrate is slightly turbid even when the buffer used for eluting the protein from the gel contains no phosphatidylcholine. This indicates that part of the deoxycholate is removed during the concentration step, leading to the formation of some vesicles, which are incapable of energized Ca^{2+} accumulation.

The concentrate was then dialysed against dialysis buffer (see the Materials and Methods section) for 18h at 16–18°C. In the course of dialysis the sample becomes more turbid. Removal of the detergent leads to spontaneous assembly of membrane vesicles. The vesicles are recovered by centrifugation at 140000*g* for 60min, and supernatant 2 is discarded. The sediment, pellet 2, is then washed twice as indicated in Scheme 1 to remove residual deoxycholate. The wash serves also to remove loosely bound protein from the vesicles, and to separate protein-poor and protein-rich vesicles. Pellet 3, obtained from the last centrifugation, consists of functional reconstituted vesicles.

The characteristics of fractions in the different stages of a typical solubilization and reconstitution study are shown in Table 2. The solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase with deoxycholate is non-selective. At the ratio of deoxycholate to protein that was found to be optimal for the solubilization of the ATPase, 30–40% of the total membrane protein is solubilized, containing 50–60% of the initial ATPase activity. In addition 70–80% of the phospholipid is solubilized from the 'ghost' membranes, as revealed by total phosphorus determination on supernatant 1. The insoluble membrane residue forming pellet 1 has a decreased lipid/protein ratio and contains about 20–30% of the initial ATPase activity. By concentrating the solubilized material (supernatant 1) the ATPase activity is decreased by 30–50% as compared with supernatant 1. The Sephadex G-25 gel retains about two-thirds of the protein and phospholipid of supernatant 1. The key step of the reconstitution process is the controlled dialysis, which yields membrane vesicles (pellet 2) with selective enrichment of (Ca^{2+} + Mg^{2+})-dependent ATPase activity. Pellet 2, obtained by centrifugation of the dialysed sample at 140000*g* for 60min, contains about 50–60% of the ATPase activity of the dialysed sample, and the remainder of this activity remains soluble (supernatant 2). Dialysis for longer periods does not significantly increase the amount of sedimentable (Ca^{2+} + Mg^{2+})-dependent ATPase. Pellet 2 is washed twice, which results in the removal of about half of the total protein and phospholipid. The specific ATPase activity in the reconstituted membrane is in the range of 250–350munits/mg of protein, which is 10–15 times higher than that in erythrocyte 'ghosts' (Table 2). The phospholipid/protein ratio of the reconstituted vesicles is approx. 24μg of phosphorus/mg of protein, only slightly increased as compared with erythrocyte 'ghost' membranes (Table 2). The reconstituted preparation containing (Ca^{2+} + Mg^{2+})-dependent ATPase of erythrocytes is capable of energized Ca^{2+} transport in the presence of oxalate, but no transport is detectable in the absence of oxalate (Table 3). This difference undoubtedly reflects the lesser sensitivity of the assay in the absence of oxalate. Electron microscopy reveals sealed membranous vesicles. The majority of the vesicles have a diameter of 200–600nm (Plate 1*b*). Most of the reconstituted vesicles are unilamellar (Plate 1*a*).

The time course of Ca^{2+} loading by reconstituted vesicles is shown in Fig. 3. Ca^{2+} accumulation is an energized process requiring ATP (Table 3). Addition of the ionophore A-23187 to the assay medium

Table 2. *Characteristics of the fractions at different stages in the course of a typical reconstitution experiment*
The individual steps are described in the Results section and outlined in Scheme 1. In this special experiment 1mg of phosphatidylcholine/ml was added to the elution buffer. The methods used are described in the Materials and Methods section. (Ca^{2+} + Mg^{2+})-dependent ATPase activity was assayed at 30°C. A unit of ATPase activity represents 1μmol of product formed/min.

Step	Total protein (mg)	Total phosphorus (mg)	Phospholipid/protein ratio (μg of P/mg of protein)	Total ATPase activity (units)	ATPase specific activity (munits/mg of protein)
'Ghosts'	950.6	17.3	18.2	24.3	25.5
Supernatant 1	379.9	13.5	35.5	12.5	32.9
Pellet 1	581.7	3.7	6.4	7.8	13.4
Concentrate	125.4	4.2	33.5	6.8	54.2
Supernatant 2	80.3	3.5	43.8	3.3	41.1
Pellet 2	20.3	0.53	26.1	4.0	197.0
Pellet 3 = reconstituted vesicles	11.2	0.27	24.1	3.4	303.6

Table 3. *Ca^{2+}-loading rate and capacity of reconstituted vesicles*

The reconstituted vesicles were prepared under the standard conditions as described in the legend of Table 4. Ca^{2+} loading of the control was assayed at 20°C in a medium containing 100mM-KCl, 5.25mM-$MgCl_2$, 5mM-ATP, 100μM-$CaCl_2$, 10mM-Mops (pH 7.0), 10mM-dithiothreitol, 5mM-oxalate and 10μg of cytoplasmic activator protein/ml. Samples containing 2μM-ionophore A-23187 were incubated for 15min at 20°C before the reaction was started. The Ca^{2+}-loading rate is calculated from the Ca^{2+} accumulation within the first 1min. Ca^{2+}-loading capacity represents the Ca^{2+} accumulation per mg of protein in a 15min period. The various assay conditions were tested with two or more different preparations. Each value is the mean of at least eight independent determinations.

	Ca^{2+} loading	
Conditions	Rate (nmol of Ca^{2+}/min per mg of protein)	Capacity (nmol of Ca^{2+}/mg of protein)
Control	20	105
−Oxalate	0	0
−ATP	0	0
+Ionophore A-23187	3	3.5
−Additional cytoplasmic activator	17.5	97

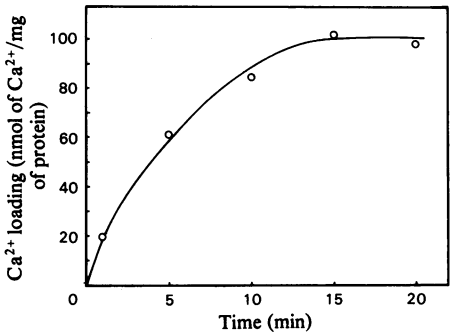


Fig. 3. *Time course of Ca^{2+} loading by reconstituted vesicles*

Reconstituted membranes were prepared from 'ghosts' as described in the Results section (Scheme 1). Ca^{2+} accumulation was measured at 20°C in the presence of oxalate by using the Millipore filtration technique (see the Materials and Methods section). Each time point is the mean of 12 determinations.

significantly decreases the Ca^{2+}-loading rate and capacity (Table 3). Calmodulin, the cytoplasmic activator, was added (10μg/ml of assay medium), to ensure full saturation of the Ca^{2+}-pump system with activator. Near-optimum Ca^{2+}-loading capacity was obtained, as well as Ca^{2+}-loading rates (Table 3) and (Ca^{2+} + Mg^{2+})-dependent ATPase activity (results not shown), even without added activator.

The characteristics of the reconstituted Ca^{2+}-pump system are highly dependent on the conditions used for reconstitution (Table 4). Dialysis carried out at low temperatures (0–4°C) yields vesicles with very low transport ability. Use of dialysis buffer without sucrose gives vesicles that are less effective in transporting Ca^{2+} than those prepared under standard conditions. The quality of the vesicles is further dependent on the phosphatidylcholine concentration in the elution buffer that is used for washing the protein from the Sephadex G-25 gel during the concentration step. A phosphatidylcholine concentration of 2mg/ml yields vesicles with a coupling efficiency of 0.30 and a Ca^{2+}-loading rate of 31nmol of Ca^{2+}/min per mg of protein.

Discussion

The solubilization of the (Ca^{2+} + Mg^{2+})-dependent ATPase from erythrocyte membranes and its reconstitution to form unilamellar membrane vesicles capable of Ca^{2+} accumulation has been achieved. The Ca^{2+} accumulation is an energy-requiring transmembrane process, since no accumulation occurs in the absence of ATP and the ionophore A-23187 abolishes transport (cf. Scarpa & Inesi, 1972).

The Ca^{2+} pump is a minor constituent (<0.6%) of the erythrocyte membrane (Wolf *et al.*, 1977), whereas it is the major constituent (>70% of the protein) in the skeletal-muscle sarcoplasmic-reticulum membrane (Meissner, 1975). A major difference is that the pump from the erythrocyte membrane is regulated by a cytoplasmic factor, calmodulin, which combines with the pump when the cytoplasmic Ca^{2+} concentration rises (Luthra *et al.*, 1976; Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1978). The nature of the regulation of the Ca^{2+} pump by, and its interaction with, calmodulin represents an added dimension in the investigation of the Ca^{2+} pump of the erythrocyte membrane which can be studied in reconstituted membranes.

Biological membranes consist mainly of protein and phospholipid. For most membranes, the protein content is equal to or greater than the lipid content.

Table 4. *Effect of preparation conditions on the properties of the reconstituted vesicles*

Reconstitution was achieved by removal of the detergent by using Sephadex G-25 followed by dialysis. Standard conditions for the reconstitution process are defined as follows: elution buffer contains 1 mg of exogenous phosphatidylcholine; dialysis at 16–18°C for 18h against dialysis buffer. ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity and Ca^{2+} loading were assayed at 20°C in a medium as described in the Materials and Methods section. The Ca^{2+}-loading rate refers to the Ca^{2+} accumulation per mg of protein in the first 1 min. Ca^{2+}-loading capacity indicates the amount of Ca^{2+} accumulation per mg of protein in a 15 min period. The efficiency of the reconstituted Ca^{2+}-pump system is defined as mol of Ca^{2+} transported/mol of ATP hydrolysed, and was calculated from the rate of Ca^{2+} loading and ATPase activity during the first 1 min. Three different preparations of reconstituted vesicles were done under standard conditions or with a elution buffer containing 2 mg of phosphatidylcholine/ml. The preparations under the other conditions were single experiments. Each value represents the mean of four or more independent determinations.

Variation of conditions	ATPase activity (munits/mg of protein)	Ca^{2+} loading: Rate (nmol of Ca^{2+}/min per mg of protein)	Ca^{2+} loading: Capacity nmol of Ca^{2+}/mg of protein)	Efficiency Ca^{2+}/ATP
Standard	95	20	105	0.21
Dialysis at 0–4°C	190	<2	<2	<0.011
Dialysis buffer without sucrose	124	4	25	0.032
Elution buffer with no phosphatidylcholine	112	14	39	0.12
Elution buffer with 2 mg of phosphatidylcholine/ml	104	31	61	0.30

For example, the membranes of both the erythrocyte and sarcoplasmic reticulum from skeletal muscle consist of approx. 60% protein and 40% lipid (Meissner & Fleischer, 1971). The procedure described by Meissner & Fleischer (1974) was the first in which functional membrane vesicles were reconstituted with lipid content similar to that of the original membrane. Such reconstituted membranes are suited for biophysical studies to characterize membrane structure, including motional parameters (e.g. the motion of the polar head groups or the hydrophobic portions of the phospholipids), so that membrane structure can be correlated with membrane function (Fleischer *et al.*, 1979). Preparations consisting of a large excess of phospholipid (Racker, 1972) reflect mainly the characteristics of the lipid alone and are unsuitable for the study of membrane structure and the nature of lipid–protein interaction.

The solubilization of the ($Ca^{2+} + Mg^{2+}$)-dependent ATPase from the erythrocyte membrane with Triton X-100 (Wolf & Gietzen, 1974) and its purification by mixed-micelle-gel chromatography (Wolf *et al.*, 1977) has already been reported, but our attempts to reconstitute membrane vesicles without large excess of lipid, and capable of energized Ca^{2+} accumulation, have been largely unsuccessful, as were those of Peterson *et al.* (1978). The reconstitution of the Ca^{2+}-transport protein of the erythrocyte membrane is difficult, since it appears to be labile when solubilized with Triton X-100 (Peterson *et al.*, 1978).

The successful reconstitution reported here involved two key steps: (1) the preparation of erythrocyte 'ghost' membranes by an iso-osmotic freeze-haemolysis procedure, by using Tween 20 and proteinase inhibitors at pH 7.4 to stabilize the preparation; and (2) the use of deoxycholate for solubilization, which could readily be removed by controlled dialysis. The solubilization and reconstitution procedure results in a 10-fold enrichment in ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity. The reconstitution procedure is similar to that developed by Meissner & Fleischer (1974) for reconstitution of sarcoplasmic reticulum from rabbit skeletal muscle in that: (1) deoxycholate is the detergent used; (2) dialysis must be carried out at near room temperature rather than in the cold (0–4°C), in order to reconstitute membrane vesicles capable of energized Ca^{2+} accumulation; and (3) membrane vesicles of high protein content can be reconstituted with lipid/protein ratio similar to that of the original membrane, i.e. 60% protein and 40% phospholipid.

The results presented here do not totally exclude the suggestion of Quist & Roufogalis (1977) that the active Ca^{2+} transport is performed by the low-affinity ($Ca^{2+} + Mg^{2+}$)-dependent ATPase, since the preparation of reconstituted vesicles is just about 10% pure with regard to the high-affinity ($Ca^{2+} + Mg^{2+}$)-dependent ATPase. However, from unpublished work (K. Gietzen, S. Fleischer & H. U. Wolf) we can conclude that the high-affinity system indeed is responsible for the Ca^{2+} transport across the erythrocyte membrane as postulated by Schatzmann (1973).

It is now desirable to purify the Ca^{2+} pump from the erythrocyte membrane so that it can be reconstituted to form functional membrane vesicles suitable for the study of membrane structure and the

regulation of the Ca^{2+} pumping. The present study shows the feasibility of the reconstitution approach for this purpose.

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