Doctoral Thesis

Further characterization and partial purification of the calcium, magnesium-stimulated adenosine triphosphatase of human erythrocyte membranes

Author(s):
Ronner, Peter

Publication Date:
1978

Permanent Link:
https://doi.org/10.3929/ethz-a-000132818

Rights / License:
In Copyright - Non-Commercial Use Permitted
FURTHER CHARACTERIZATION AND PARTIAL PURIFICATION OF THE CALCIUM, MAGNESIUM-STIMULATED ADENOSINE TRIPHOSPHATASE OF HUMAN ERYTHROCYTE MEMBRANES.

A DISSERTATION SUBMITTED TO THE SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH FOR THE DEGREE OF DOCTOR OF NATURAL SCIENCES

PRESENTED BY
PETER RONNER
DIPL. NATW. ETH
BORN 17th DECEMBER, 1951
CITIZEN OF SCHUEBELBACH / SZ

ACCEPTED ON THE RECOMMENDATION OF
PROF. E. CARAFOLI, REFEREE
PROF. H.J. SCHATZMANN, CO-REFEREE

1978
For my Parents,
and in memory of
my brother René.
"We can start working with time if you wish, till you can fly the past and the future. And then you will be ready to begin the most difficult, the most powerful, the most fun of all. You will be ready to begin to fly up and know the meaning of kindness and of love."

(Richard Bach, Jonathan Livingstone Seagull)
Everybody from our laboratory I would like to thank for the friendship, and all the good days we spent together. Coming to work was not only an obligation, but also a desire to meet good friends.

I want to thank Ernesto Carafoli for continuous, most helpful, moral support on this project. More than myself he always believed in its success.

The Blutspendezentrum Limmattal and their donors I would like to thank very much for perfect supply in "hepatitis"-red blood cells.

Thanks go also to Paolo Gazzotti and to Scott Peterson for very stimulating, temporary collaboration.

Financial support in the form of fellowships by the "Stipendienfonds der Basler Chemischen Industrie zur Unterstützung von Doktoranden auf dem Gebiet der Chemie", and by the ETH is greatly acknowledged.
# Table of Contents

1. Introduction  
2. Abbreviations  
3. Materials and Methods  
   3.1. General Techniques  
   3.2. Lipid Requirement of the Ca,Mg-ATPase  
   3.3. Protease Treatment of Intact Erythrocytes  
   3.4. Solubilization of the Ca,Mg-ATPase  
   3.5. Purification of the Solubilized Ca,Mg-ATPase by Gel Chromatography on Sepharose 6B  
   3.6. Preparative Isoelectric Focusing of a Partially Purified Preparation of the Ca,Mg-ATPase  
   3.7. Investigation of the Substrate Site of the Ca,Mg-ATPase using Analogues of ATP  
4. Results  
   4.1. General  
   4.2. The Lipid Requirement of the Ca,Mg-ATPase  
   4.3. Protease Treatment of Intact Erythrocytes  
   4.4. Solubilization of the Ca,Mg-ATPase  
   4.5. Purification of the Solubilized Ca,Mg-ATPase by Gel Chromatography on Sepharose 6B  
   4.6. Preparative Isoelectric Focusing of a Partially Purified Preparation of the Ca,Mg-ATPase
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7. Investigation of the Substrate Site of the Ca,Mg-ATPase using Analogues of ATP</td>
<td>69</td>
</tr>
<tr>
<td>5. Discussion</td>
<td>82</td>
</tr>
<tr>
<td>5.1. General</td>
<td>82</td>
</tr>
<tr>
<td>5.2. The Lipid Requirement of the Ca,Mg-ATPase</td>
<td>84</td>
</tr>
<tr>
<td>5.3. Protease Treatment of Intact Erythrocytes</td>
<td>89</td>
</tr>
<tr>
<td>5.4. Solubilization of the Ca,Mg-ATPase</td>
<td>91</td>
</tr>
<tr>
<td>5.5. Purification of the Solubilized Ca,Mg-ATPase by Gel Chromatography on Sepharose 6B</td>
<td>93</td>
</tr>
<tr>
<td>5.6. Preparative Isoelectric Focusing of a Partially Purified Preparation of the Ca,Mg-ATPase</td>
<td>96</td>
</tr>
<tr>
<td>5.7. Investigation of the Substrate Site of the Ca,Mg-ATPase using Analogues of ATP</td>
<td>97</td>
</tr>
<tr>
<td>6. Summary</td>
<td>109</td>
</tr>
<tr>
<td>7. References</td>
<td>112</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Human erythrocytes contain only about $10^{-7}$ M calcium (1), whereas the surrounding plasma has a calcium concentration of about 2.5 mM. Calcium is considered as an important general regulator of cell functioning (2). However, in the case of erythrocytes, no fluctuations of the intracellular calcium concentration are known, and the reason for the low calcium content remains somewhat unclear. Apparently, the K$^+$-permeability (K$^+$-loss) is increased (3-5), and the cells tend to sickle (i.e. formation of echinocytes; 6), when the intracellular calcium concentration raises above normal.

Human erythrocytes have a very low passive permeability for calcium (10-60 mmoles/h x 1 cells; 5,7-9). This leak is easily overcome by a so-called (Ca$^{2+}$ + Mg$^{2+}$)-ATPase that transports calcium, using ATP as an energy source and magnesium as a necessary cofactor. The maximal pump rate of this carrier exceeds the passive calcium leak by a factor of at least 100, the rate being 5,000 - 30,000 mmoles/h x 1 cells (1,10,11). The identity of the Ca$^{2+}$-carrier and the membrane-bound (Ca,Mg)-stimulated ATPase, as first pointed out by Schatzmann and Vincenzi (7,11), is now generally accepted, and the evidence has been reviewed by Schatzmann (1). This (Ca$^{2+}$ + Mg$^{2+}$)-ATPase has been shown to be functionally and immunologically different from the (Na$^+$ + K$^+$)-ATPase of erythrocytes (12).

The (Ca$^{2+}$ + Mg$^{2+}$)-ATPase shows a dissociation constant for calcium of about $10^{-6}$ M (10,11,13-15), and thus it would cause the intracellular calcium concentration to decrease to about $10^{-7}$ M (1), due to the large ratio of the pump
flux to the passive leak. $K_m$ values for ATP of $1 - 60 \times 10^{-6} M$ have been reported (16,17,141, this work).

Strontium is transported as efficiently as calcium (7). Magnesium, however, is not transported (7). Pb$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Mn$^{2+}$ may activate the ATPase activity to a maximal of 50% of its maximal value for calcium (14).

The (Ca$^{2+} +$ Mg$^{2+}$)-ATPase may be inhibited to 50% by 23 $\mu$M mersalyl (7), 10 $\mu$M ruthenium red (19), 10-60 $\mu$M lanthanum (20,21), or by analogues of ATP (this work). Phosphate deriving from the splitting of ATP is liberated on the inside of the erythrocytes (1,22). A stoichiometry of 1 Ca ion transported per ATP split was found by several authors (1,23,24), whereas a stoichiometry of 2 was reported for that part of the calcium transport and the ATPase activity, which may be inhibited by lanthanum (21,25,26).

From labelling studies with radioactive ATP, and using polyacrylamide gel electrophoresis of solubilized erythrocyte membranes, a molecular weight of about 140,000 was determined (27-29,34, this work).

Although several erythrocyte diseases (sickle cell anemia, hereditary spherocytosis, etc.) show formation of echinocytic structures, similar to those obtained after artificially increased intracellular calcium concentration, so far no disease has been positively identified as a defect of the (Ca$^{2+} +$ Mg$^{2+}$)-ATPase (30).

The long-term strategy, along which the work presented here has been carried out, was to compare the calcium transporting systems of several membranes, like the sarcoplasmic reticulum, the mitochondria, the erythrocytes etc. Of these systems, the (Ca$^{2+} +$ Mg$^{2+}$)-ATPase from the sarcoplasmic reticulum is known best, and shows similarities with that of erythrocytes. Its kinetics are well examined (31,32), and even a protein fragment with calcium ionophoric activity
has been isolated from the ATPase molecule (32). On the other hand, erythrocytes contain a (Na\(^+\) + K\(^+\))-ATPase that exchanges Na\(^+\) and K\(^+\). Although kinetically also well examined, this enzyme has not been isolated yet from erythrocytes, since it is present in their membrane only in a very low amount (33).

Since the possibilities of a structural analysis of the erythrocyte (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase in the intact membrane environment are restricted — the enzyme makes up 0.02\% of the total membrane protein (27,34) — the purpose of this work was to purify this enzyme as far as possible.

Many transporting, integral membrane proteins with enzymatic activity are known to require a phospholipid or a similar environment to maintain an active conformation (35,36). This is valid also for the erythrocytes (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase, as will be shown in this work. Thus to solubilize it in an active form, a detergent had to be found that could liberate the carrier enzyme from the membrane, while still leaving a sufficient lipid annulus around it.

On the other hand, several reports indicate that even proteins denatured in detergents like SDS, may still be reactivated (37). This may be very useful in purifying membrane proteins, since any of the known purification procedures might in principle be applied and include a final "reactivation" step. However, no safe method of "reactivating" SDS-treated enzymes exists today, and it is not known to what extent an isolation method may denature a membrane enzyme that has to be purified. In addition, procedures involving solubilization of membrane proteins in denaturing solvents make any purification at least a two step experiment, consisting of the actual purification step(s), and of the final reactivation step. In the case of a negative result it is therefore difficult to decide, which one of the two steps is responsible for the lack of success.
In the work presented here, the approach has been chosen to try to maintain the enzyme always "active". This procedure provides several advantages: (i) the enzyme is never inactivated, thus it most probably always retains its natural properties, (ii) each step may easily be followed by assays of enzyme activity, eliminating the need for a "label" to follow the enzyme throughout the purification process.

In the past, many highly effective purification procedures have been elaborated in enzyme biochemistry. Unfortunately, they all apply only to soluble, not membrane-bound proteins and enzymes, or to inactivated proteins. Until today only very few purification procedures are available that can be used in membrane biochemistry, i.e. especially selective solubilization of contaminant proteins, or of the desired enzyme, affinity chromatography in the presence of detergents and phospholipids. Since most of these procedures only allow a very limited degree of purification, most of the isolation work so far performed on membrane proteins has made use of naturally "enriched" membranes, that is, of membranes that contain the protein to be purified in high amounts. As examples, one may quote the \((Na^+ + K^+)\) ATPase of rabbit kidney, pig brain, and the rectal salt gland of Squalus acanthias, which was purified only 10 to 60 times with a yield of 2% to 50% (38-41). This enzyme could be obtained in virtually pure form, when isolated from naturally enriched membranes (specific activity up to 1,500 \(\mu\)moles/h \(\times\) mg protein; 38).

This thesis describes a specific phospholipid requirement of the \((Ca^{2+} + Mg^{2+})\) ATPase of human erythrocytes, improved procedures for solubilization and stabilization of the enzyme, its gel chromatography, and an approach to purification by preparative isoelectric focusing. Results on the effects of degradation of the main contaminating protein, the anion carrier, on these purification proce-
Analogues of the substrate, ATP, were tested and are discussed in connexion with substrate affinity chromatography.
2. Abbreviations

Ca,Mg-ATPase EC. 3.6.1.3., also called "Ca$^{2+}$-stimulated, Mg$^{2+}$-dependent ATPase", or "high-affinity Ca$^{2+}$-ATPase"

ATP adenosine triphosphate

PEP phosphoenolpyruvate

HEPES N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid

Tris Tris(hydroxymethyl)aminomethane

EDTA ethylenediaminetetraacetic acid

EGTA [ethylenebis(oxoethylenenitrilo)]tetraacetic acid

SDS sodium dodecyl sulfate

TEMED N,N,N',N'-tetramethylethylenediamine

BSA bovine serum albumin

Sph sphingomyelin

PC phosphatidyl choline

PE phosphatidyl ethanolamine

PS phosphatidyl serine

LPC lyso-phosphatidyl choline

DFP diisopropylfluorophosphate

TLCK N$^o$-tosyl-L-lysyl-chloromethanehydrochloride

g gravity force on earth

rpm revolutions per minute

$V_o$ void volume

$V_t$ total volume of the gel bed

$K_{av}$ partition coefficient between the liquid phase and the gel phase

$K_m$ Michaelis constant for the substrate

$K_i$ inhibitor constant

$K_d$ dissociation constant

gel bands numbered according to Steck (67)
3. **Materials and Methods**

3.1. **General techniques**

**Preparation of red blood cell membranes**

One- to three-day-old red blood cells were obtained from a local blood bank from healthy donors that had once had hepatitis. The cells were supplied in CPD-blood bank buffer (15.6 mM citric acid, 89.4 mM sodium citrate, 16.1 mM sodium dihydrogen phosphate, 128.8 mM D-glucose). The cells were washed twice in 5 volumes of 130 mM KCl, 20 mM Tris-Cl, pH 7.4 (at 4°C), and the white cells were well removed. (The washings were done in a Sorvall HS-4 rotor during 10 min at 5,500 rpm = 5,800 x g.) Packed, washed red blood cells (200 g) were lysed in 600 ml of bidistilled water at 0°C for 3 min. 200 ml of 520 mM KCl, 80 mM Tris-Cl, pH 7.4 (at 4°C) were then added to restore isotonicity. The membranes were sedimented within 10 min at 18,000 x g in a Sorvall GSA rotor at 11,500 rpm. Round bottom flasks were used. The lysing procedure was repeated about 10 times, until the membranes looked white. The final sediments were collected in 130 mM KCl, 20 mM Tris-Cl, pH 7.4 (at 4°C), and made up to a total volume of 80 to 100 ml. 5 ml portions were frozen in liquid nitrogen and stored at -80°C.

**Assay of the Ca,Mg-ATPase activity by a coupled enzyme assay (42):**

The assay mixture contained 120 mM KCl, 30 mM HEPES, 0.5 mM MgCl₂, 0.05 mM CaCl₂, 0.5 mM ATP, pH 7.4 (at 37°C), and 0.2 mM NADH, 0.5 mM PEP, 1 IU (at 25°C) of pyruvate kinase
per ml (from rabbit muscle; Boehringer, Mannheim), and 1 IU (at 25°C) of lactate dehydrogenase per ml (from hog muscle; Boehringer), and was preincubated at 37°C for 4 min. The reaction was started by adding the Ca,Mg-ATPase. The absorbance difference between 366 nm and 550 nm (as measured in a double-beam spectrophotometer thermostated at 37°C) was recorded for 4 min. EGTA was then added to a final concentration of 1 mM and the recording was continued for 4 more min. The Ca,Mg-ATPase activity was calculated from the difference of the two slopes, assuming an absorbance of 3.3/µmole of NADH x ml. The reaction was linear over a period of at least 15 min.

Protein determinations

Whenever necessary, protein solutions were dialysed before the assay, or the protein was precipitated according to the method of Bensadoun (43), using deoxycholate and perchloric acid. The protein content was then determined by the method of Lowry et al. (44).

SDS-discontinuous polyacrylamide gel electrophoresis

8% separating and 4% stacking gels of pH 8.8 and 6.8 respectively were prepared as described by Laemmli (45), and used at a volume ratio of 5 to 1. The samples, the electrode buffer, and the sample buffer were as (45), except that the sample buffer contained 4 M urea (final concentration) instead of glycerol.

The gels were run at 1 mA/gel tube (0.6 x 9 cm) for 10 min, at 2 mA/gel tube for one hour, and (meanwhile the sample had entered the separating gel) for the rest of the time at 3 mA/gel tube. The temperature was 10-15°C.

The gels were stained in 0.1% Coomassie Brilliant Blue R250, 50% (v/v) methanol, 7% (v/v) acetic acid, 43% (v/v) water for 4-10 hours at room temperature, and destained in a Bio
Rad type diffusion destainer, containing 20% (v/v) methanol, 10% (v/v) acetic acid, and 70% (v/v) water.
The gels were scanned at 560 nm in a Gilford 2400 spectrophotometer using a slit of 0.10 x 2.36 mm, and a scanning speed of 1 cm/min. Photographs were taken with a yellow filter and light from a fluorescent tube.
The same procedure for gel electrophoresis with both tubes and slabs was used.
The samples were dialysed against bidistilled water at 4°C, and concentrated by freeze-drying, whenever necessary.
3.2. LIPID REQUIREMENT OF THE CA,Mg-ATPASE

Preparation of phospholipase A₂ from Naja naja

"Pure snake venom toxin" (100 mg) from the Miami Serpentarium (USA) was dissolved in 10 ml of 20 mM potassium acetate buffer, pH 5.0, heated for 5 min in a boiling water bath, and cooled on ice. The heat-denatured proteins sedimented at 100,000 x g after 30 min. The supernatant was diluted five times with 50 mM HEPES, pH 7.4. In some experiments, the heat-treated preparation was further purified on Sephadex G-100 as described by Cremona and Kearney (46), and similar results were obtained. (Since this work has been done, Deems and Dennis (47) have described a more efficient purification procedure than the one of (46).)

The phospholipase A₂ activity was assayed using the procedure of Nieuwenhuizen et al. (48), but it was measured at 37°C.

Phospholipase A₂ treatment of erythrocyte membranes

Ghost protein (15 mg) was incubated for 10 min at 37°C in a solution containing 120 mM KCl, 1% w/v BSA, 0.5 mM CaCl₂, 30 mM HEPES, pH 7.4, and various amounts of phospholipase A₂ from Naja naja (final volume, 12 ml). The reaction was stopped in ice and 35 ml of an ice-cold solution containing 130 mM KCl, 0.5% BSA, 20 mM HEPES, pH 7.4, was added. The membranes were then sedimented by centrifugation in a Sorvall SS-34 rotor for 15 min at 17,500 rpm (37,000 x g). Three more washings were carried out in the same way, but BSA was omitted in the last washing. Samples of the preparation containing about 150 μg of protein and suitable for one Ca,Mg-ATPase assay according to procedure I (this chapter) were stored in test tubes at -20°C.
Preparation of phospholipid microdispersions

PS, PC, phosphatidyl inositol, and lysolecithin were grade I, cardiolipin and phosphatidic acid were grade II, and all were obtained from Lipid Products (South Nutfield, Surrey, UK). PE was isolated from beef heart mitochondria. The phospholipids were microdispersed in 50 mM HEPES, pH 7.4, by sonication at 0°C under a stream of nitrogen and centrifuged for 30 min at 80,000 x g to remove platinum dust and large aggregates of phospholipids. The microdispersions were always kept under nitrogen and stored at 10°C. Care was taken to use the solutions before aggregation of the vesicles occurred. Lipid phosphate was measured according to a modification of the method of Chen et al. (49). Oleic acid (purum, >96%) was obtained from Fluka and was neutralized with KOH before use.

Sidedness assays

Membrane sidedness assays were carried out by measuring the accessibilities of acetylcholinesterase and NADH cytochrome c oxidoreductase (50).

Assay of the Ca,Mg-ATPase

Procedure I: About 150 µg of membrane protein, together with the desired phospholipid, were made up to a total volume of 200 µl with 50 mM HEPES, pH 7.4, and preincubated for 10 min at 25°C. The mixture was then added to a cuvette containing 800 µl of (final concentration) 120 mM KCl, 40 mM HEPES, pH 7.4, 5 mM MgCl₂, 0.05 mM CaCl₂, 0.5 mM ATP, and 0.2 mM NADH, 0.5 mM PEP, 1 IU of pyruvate kinase per ml, 1 IU of lactate dehydrogenase per ml at 37°C. Activity measurements and calculations were then carried out as described before (3.1.).

Procedure II: The reaction mixture contained 120 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂ (or 0.6 mM EGTA), 30 mM HEPES, pH
7.4, and 300 μg of membrane protein to a final volume of 1 ml. The mixture was preincubated for 3 min at 37°C and the reaction was started by adding 1 mM ATP. After 30 min of incubation at 37°C, the reaction was stopped with 1.5 ml of 3.3% SDS in 1.3 N H₂SO₄. Then 0.25 ml of 2.5% ammonium molybdate, 2 ml of water, and 0.2 ml of the Fiske and Subbarow reducing agent (BDH chemicals) were added. The optical density was read at 700 nm, 15 min after the addition of the reducing agent.
Figure 1  The structure of phospholipids and other compounds used in the studies of the lipid requirement of the Ca,Mg-ATPase.

sphingomyelin

phosphatidylcholine

phosphatidylethanolamine

phosphatidylserine

cardiolipin

R,R_1,R_2,R_3,R_4 = alkyl residues
Figure 1 (continued)

Lysoglycerophospholipids

Oleic acid

Triton X-100

Cholic acid
3.3 Protease Treatment of Intact Erythrocytes

Pronase, grade B, 45,000 P.U.K./g was obtained from Cal-biochem, α-chymotrypsin, 3 times crystallized from 4 times crystallized chymotrypsinogen from bovine pancreas, 40,000 to 50,000 U/g, was from Sigma.

The pronase treatment was carried out as described by Knauf et al. (51). Briefly, red cells were washed 3 times, incubated at 37°C for 35 min in 150 mM NaCl, 5 mM CaCl₂, 15 mM Tris-Cl, pH 7.4 (at 37°C) at a hematocrit of 50% with and without (= control) the addition of 18 P.U.K. pronase/g of packed erythrocytes. The cells were then chilled on ice, centrifuged, washed 6 times, and stored overnight at 4°C. The other day the membranes were prepared as described before (3.1.).

The chymotrypsin treatment followed exactly the procedure given by Drickamer (52). It includes inhibition of chymotrypsin at the end of the incubation by phenylmethylsulfonylfluoride (Sigma, = PMSF), centrifugation, and 3 subsequent washings. The membranes were prepared as given above.

Formation of a [γ^32P]-phosphoprotein: The incubation medium contained 130 mM KCl, 20 mM Tris-Cl, pH 7.4 (at 18°C), 0.2 mg membrane protein/ml, 2 nmoles/ml (2 μM) of [γ^32P]-ATP (specific activity 10 μCi/nmole), 12.5 μM MgCl₂, and 100 μM EGTA, or 50 μM CaCl₂. Controls were run for Ca-stimulation (EGTA), and protease treatment (control membranes).

The reaction (at 0°C) was started by the addition of the radioactive ATP, and stopped after 15 sec by the addition of 1 volume of ice-cold 10% trichloroacetic acid, 0.1 mM

* 1 P.U.K. is defined as the amount of enzyme that splits the equivalent to 1 mmole of tyrosine in a Folin protein assay, when incubated at 40°C with caseine as a substrate (53).
ATP, 0.8 mM phosphoric acid. After centrifugation for 5 min at 1,300 x g at 4°C the pellet was first washed with 2 volumes of 5% trichloroacetic acid, 0.1 mM ATP, 1 mM phosphoric acid, then with 2 volumes of bidistilled water, and was resuspended for gel electrophoresis to 1 volume of buffer containing 100 mM sodium phosphate pH 6.0, 1% SDS, bromphenolblue as a tracking dye, and 10% glycerol.

SDS-polyacrylamide gel electrophoresis of [γ³²P]-labelled membranes: 5% polyacrylamide gels of pH 6.0 were made from the following solution: 4.87% acrylamide, 0.13% bisacrylamide, 96 mM sodium phosphate pH 6.0, 0.1% SDS, 0.05% v/v TEMED, and 0.025% w/v ammoniumpersulfate. The electrode buffer contained 100 mM sodium phosphate pH 6.0 and 0.1% SDS. The gels (0.6 x 4.5 cm) were run at 15°C for 2 hours at 2 mA/gel tube and 3 more hours at 3 to 4 mA/gel tube. The gels were then frozen on dry ice, and pairs of 1 mm slices were incubated overnight with shaking in 0.7 ml of 0.05% SDS at 40°C. After addition of 7 ml Instagel (Packard, Downers Grove, Ill. USA) the vials were counted with the windows set as follows: channel A: 1.2 - 60 keV, channel B: 25 - 1,700 keV. The external standard channel ratio did not change by more than 1% in the individual gels (range 0.25 - 0.30), and the counting efficiency in channel B was found to be more than 90% for unquenched samples.
3.4. Solubilization of the Ca,Mg-ATPase

Triton X-100 was obtained from Packard, asolectin (95%, purified) from Associated Concentrates (Woodside, N.Y., USA), and PC used for solubilization and gel chromatography was grade II from Lipid Products (South Nutfield, Surrey, UK).

Procedure A

200 - 300 mg of erythrocyte membrane protein in 10 mM KCl, 10 mM Tris-Cl, 0.05 mM ATP, 0.2 mM MgCl2, 0.05 mM CaCl2, pH 7.6 (at 5°C), (final concentration 3-8 mg protein/ml) were solubilized with 0.8 - 1.2 mg Triton X-100 per mg of membrane protein at 5°C for 10 min, and were then centrifuged at 100,000 x g (Beckman TI 50 rotor at 40,000 rpm in a Sorvall OTD ultracentrifuge) for 30 - 60 min at 0°C. (The results of a 30 and a 60 min centrifugation did not differ.)

To the supernatant about 1 mg PC was added per ml (1 mg PC/mg protein). This solution was concentrated to about 10 ml in an Amicon (Lexington, USA) ultrafiltration chamber with a XM-100A filter (exclusion limit 100,000 MW). (Concentrating up to 10 times, and thereby not changing the ratio of detergent to protein, did not affect the activity of the Ca,Mg-ATPase.) The final concentrate was then made 10% w/v in glycerol (≥97%, Fluka).

The Ca,Mg-ATPase activities were determined immediately after a step had been carried out.

Procedure B

Solubilization experiments according to the procedure of Dieckvoss et al. (54,55) were carried out in the same way, except that the membranes were preincubated for 1 hour at
and that the following buffer composition was used: 150 mM KCl, 30 mM Tris-Cl, 10 mM diisopropylfluorophosphate (DFP), 50 mM cysteine, 0.1 mM MgCl$_2$, 0.05 mM CaCl$_2$, 0.6 mg PC/ml (sonicated), pH 7.3 at 0°C.
3.5. Purification of the Solubilized Ca,Mg-ATPase by Gel Chromatography on Sepharose 6B

Sepharose 6B was from Pharmacia. A Pharmacia SR 25/100 chromatographic column with flow adaptors was used (column dimensions: 2.565 x 70 - 90 cm, void volume $V_o = 110 - 130$ ml, total volume $V_t = 370 - 450$ ml). The flow rate (27.5 ml/h) was held constant with a Perpex peristaltic pump (Meyer AG, Lucerne, Switzerland), and the transmittance at 280 nm was followed by a LKB Uvicord. Five fractions of 5.7 ml each were automatically collected per hour. The chromatography was carried out in a thermostated cold-room at 4°C.

Two different buffer systems were used for the chromatography on Sepharose 6B.

Buffer system I was derived from Dieckvoss et al. (54) and contained 200 mM KCl, 0.1 mM MgCl$_2$, 0.05 mM CaCl$_2$, 10 mM cysteine (Sigma), 0.1 mM diisopropylfluorophosphate (DFP), 0.05% w/v PC (Lipid Products, grade II; dissolved in Tween 20 by stirring overnight), 0.67% Tween 20 (Merck), 30 mM Tris-Cl, pH 7.4 at 4°C. The complete buffer was stable for only 2 to 3 days, then precipitation occurred.

Therefore, and for better enzyme stability buffer system II was used: 10 mM KCl, 10 mM Tris-Cl, 0.05% w/v PC, 0.67% w/v Tween 20, 10% glycerol, pH 7.4 at 4°C.

(The concentrations and the ratio of PC and Tween 20 given, proved to be better suited for both good enzyme stability and good chromatographic separation, than a few other ratios tested. Also, Tween 20 was superior to Triton X-100.)
The assays for the Ca,Mg-ATPase activity were carried out within 5 hours of elution of the ATPase peak from the Sepharose column. Triton X-100 was assayed according to Garewal (56).

The active fractions (i.e. those with an activity of ≥1/3 of the peak fraction) were pooled and concentrated by ultrafiltration using an Amicon PM 10 filter (exclusion limit 10,000 MW).

The samples applied to the Sepharose 6B column were the preparations of solubilized and concentrated Ca,Mg-ATPase described in chapters 3.4., and 4.4.
3.6. Preparative Isoelectric Focusing of a Partially Purified Preparation of the Ca,Mg-ATPase

The ampholines of the pH ranges 3.5 - 5, 3.5 - 10, and 5 - 8 were obtained as 40% w/v solutions from LKB (Stockholm, Sweden).

The column for isoelectric focusing consisted of parts of a Liebig condenser, as manufactured by Sovirel (Levallois-Perret, France). In detail, a central tube of 1.8 x 39.7 cm took up the solution to be focused, and was cooled by a surrounding water jacket. Two layers of dialysis tubing separated the focusing solution from the electrode solution on the lower end of the central tube, but still passed the current. (A 8% polyacrylamide gel plug of 2 cm in height instead of the dialysis tubing proved unsatisfactory, since it often leaked.) Two opposite teflon tubings ended right above the dialysis membrane and served for charging and discharging the column. The upper electrode solution was directly layered on top of the focusing solution. Platinum wires served as electrodes. The focusing equipment was placed in a cold-room of 4°C, and the column was thermostated at 2°C.

The column was loaded with the gradients of ampholine, sucrose, and enzyme specified in the results section (4.6.), and by filling from the bottom, using a two-step procedure with a 3-channel Perpex peristaltic pump for gradient formation (57). The pump flux for loading and unloading the column was 72.7 ml/h. The total volume of the gradient was 80-90 ml. The electrode solutions were as follows: anode (positive, on top): 15-20 ml of 100 mM sulfuric acid, cathode (negative, on the bottom): 200 ml of 60 mM sodium hydroxide.
The focusing time at 400-550 Volts (direct current) was 20-32 hours. When discharging the column, the transmittance at 280 nm of the solution was recorded with an LKB Uvicord, and the pH was continuously recorded as the potential of a small Beckman pH combination electrode (bulb diameter 5 mm) with the solution dripping along its bulb into the tubes of an automatic fraction collector. The pH-measurements were controlled for each individual fraction by the usual procedure, and they agreed with the continuous measurements within 0.1 pH-units. The sucrose concentration was followed with an Abbé refractometer. The pH of each fraction (20-30 per column) was adjusted to pH 7.0 - 7.6 in a thermostated vessel at 20°C and using KOH or HCl for neutralization. The Ca,Mg-ATPase activity was then measured within 5 hours from discharging the column.

For gel electrophoresis and protein determinations (3.1.) the fractions had to be extensively dialysed against bidistilled water at 4°C, and freeze-dried.
3.7. Investigation of the Substrate Site of the Ca,Mg-ATPase using Analogues of ATP

The structures of the ATP analogues used in this work are given in Fig. 2. Concerning conformational, thermodynamic, and geometric data refer to Tables 8-10 in chapter 5.7.

The sodium salts of AMP-PNP (5'-adenylylimidodiphosphate) and rroATP (2,2'[1-(9-adenyl)-1'-(triphosphoryl-oxymethyl)]-dihydroxy-diethylether) were generous gifts of Dr. K.S. Boos (Department of Clinical Biochemistry, Technical University of Hannover, Hannover, Germany). rroATP was prepared according to (58). The sodium salt of BrATP (8-bromo-adenosine-5'-O-triphosphate) and the lithium salt of AMP-PCP (adenylyl[β,γ-methylene]diphosphonate) were from Boehringer (Mannheim, Germany). 10 mg of the tetra-lithium salt of AMP-PCP were converted to their H⁺-form by passage through a 0.9 x 4 cm column of Amberlite IR-120 (a strongly acidic ion exchanger) and neutralized with KOH.

The concentrations of all adenine nucleotides used were assayed spectrophotometrically, and calculated from a molar absorption coefficient of \( \varepsilon = 14,800 \, \text{M}^{-1}\text{cm}^{-1} \) at their respective \( \lambda_{\text{max}} \) values (ATP, rroATP, AMP-PNP, AMP-PCP at 258 nm; BrATP at 263 nm).

The ATPase assays with inhibitor present were done with the coupled enzyme assay described in chapter 3.1. The \( V_{\text{max}} \) of the assay reaction in the presence of ATP analogues was always at least 10 times that of the Ca,Mg-ATPase. With the exception of BrADP (for details refer to the results section, 4.7.), none of the ADP forms of the analogues was converted by pyruvate kinase to any measurable
extent (compare also to (60)). In some control experiments, phosphate was determined by the method of Fiske and Subbarow (61). For kinetic and graphical analysis all straight lines were calculated by the method of the least squares of the deviations. The standard errors of the kinetic constants were calculated from the standard errors of these regression lines. The lines were not normalized to an average $V_{\text{max}}$. 
Figure 2 The structure of ATP, and of some of its analogues

ATP  adenosine-5'-O-triphosphate
rroATP  2,2'[1-(9-adenyl)-1'-(triphosphoryl oxymethyl)]-dihydroxydiethylether
AMP-PNP  5'-adenylylimidodiphosphate
AMP-PCP  adenylyl(βγ-methylene)diphosphonate
BrATP  8-bromoadenosine-5'-O-triphosphate

Magnesium complexes with the α- and the β-phosphate group, with N(7), and probably coordinates with the enzyme (59).
4. Results

4.1. General

Properties of the membrane preparation

The preparation of red blood cell membranes used was virtually free of hemoglobin, and contained only marginal amounts of resealed vesicles (as determined by the accessibility of acetylcholinesterase and NADH cytochrome c oxidoreductase). The total activities of the Mg-ATPase and the Na,K-ATPase corresponded to less than 20% of the total Ca,Mg-ATPase. Under the usual assay conditions the Na,K-ATPase was inactive. The $K_{d, Ca}$ of the Ca,Mg-ATPase ranged around 1 $\mu$M; no low affinity Ca,Mg-ATPase was detectable. The $K_m$ for ATP was 4 - 60 $\mu$M. Under assay conditions (chapter 3.1.), the following yields in Ca,Mg-ATPase activity and protein were found (average of 8 preparations $\pm$ standard error):

4.1 $\pm$ 1.1 $\mu$moles ATP split/h x g packed cells, and

3.4 $\pm$ 1.0 mg protein/g packed cells. This corresponds to a specific activity of the Ca,Mg-ATPase of

1.4 $\pm$ 0.7 $\mu$moles ATP split/h x mg membrane protein.
4.2. The Lipid Requirement of the Ca,Mg-ATPase

When solubilization experiments for the Ca,Mg-ATPase of erythrocytes were carried out, the ATPase activity usually decreased dramatically as soon as solubilization occurred. Therefore it was decided to investigate, whether the Ca,Mg-ATPase is a lipid requiring enzyme. The properties of the erythrocyte membrane preparation used in these studies are given in chapter 4.1.

Effect of Degradation of Phospholipids on the Ca,Mg-ATPase

When the Ca,Mg-ATPase activity of intact membranes was measured in the presence of increasing amounts of phospholipase A2, no inhibition was observed, unless BSA was present in the incubation medium (Fig. 3). Since BSA removes the hydrolysis products (i.e., fatty acids and lysocompounds), the experiment shows that these products (or at least one of them) maintain the enzyme activity.

Effect of Removal of Phospholipids on the Ca,Mg-ATPase

In the experiment described in Fig. 4, erythrocyte membranes were partially depleted of phospholipids and washed with BSA to remove fatty acids and lysocompounds. The incubation and washing procedure caused some loss of activity, which never exceeded 20%. In the experiment described, the phospholipase activity was less than optimal, since the calcium concentration in the medium was kept low (0.5 mM) to minimize resealing of the membranes, which would then still contain phospholipase after the washing. In fact, at the end of the phospholipase incubation, about 15% of the membranes were resealed and right-side out, about 10% were
**Figure 3** Effect of the partial degradation of phospholipids on the Ca,Mg-ATPase activity of erythrocyte ghosts.

The reaction mixture contained 120 mM KCl, 30 mM HEPES, pH 7.4, 5 mM MgCl₂, 0.1 mM CaCl₂ (or 0.6 mM EGTA), 300 µg ghost protein, and purified heat-treated phospholipase A₂ at the concentration shown in the figure. When present, bovine serum albumin (fatty acid free) was 0.3%. The reaction was continued for 30 min in the presence of 1 mM ATP. (O) no BSA; (●) with BSA.
Figure 4 Decrease of the Ca,Mg-ATPase activity due to removal of the phospholipids from the membrane. Membranes were incubated for 10 min at 37°C in a solution containing 120 mM KCl, 1% bovine serum albumin, 0.5 mM CaCl₂, 30 mM HEPES, pH 7.4, and up to 8 IU of phospholipase A₂. Membranes were subsequently washed three times with 130 mM KCl, 0.5% bovine serum albumin, 20 mM HEPES, pH 7.4, and once without bovine serum albumin. Activity and phospholipid degradation are expressed as a percentage of the values found in the intact ghosts. Symbols reflect different experiments.
resealed and inside-out, and about 75% appeared to be unsealed. (It must be pointed out, however, that the results with the marker NADH-cytochrome c oxidoreductase should be interpreted with some care, because the assay shows some latency in unsealed ghosts and because saponin, which is used in the assay, causes a mild inactivation of the enzyme (50).)

In the experiments described in Fig. 4, erythrocyte membranes were partially depleted of phospholipids as described under Materials and Methods (3.2.). Almost complete loss of the activity was observed, when about 50% of the phospholipids were still present in the membrane. Sphingomyelin, which represents up to 25% of the total phospholipids, is not degraded by phospholipase A₂, and PC is hydrolyzed very slowly (62). Accordingly, membranes treated with 9 IU phospholipase A₂/mg of membrane protein contained, after the incubation and the washings, all of the sphingomyelin, 55% of the original PC, and 30 and 15% of the original PE and PS, respectively.

Reactivation of the Ca,Mg-ATPase by Detergents, Lysocompounds and Fatty Acids

In a first series of reactivation experiments, the influence of detergents on the Ca,Mg-ATPase of phospholipase A₂-treated membranes was assayed. As shown in Fig. 5, cholate, at a concentration that was not inhibiting the enzyme in the intact membranes, had no effect on the activity. This observation indicates that the low activity in phospholipase-treated membranes was not due to the limited accessibility of the enzyme. On the contrary, the addition of Triton X-100 increased the Ca,Mg-ATPase activity. This phenomenon evidently represents a reactivation effect and has a maximum at about 0.3 μmol of Triton X-100 per mg of protein.
Figure 5  Effect of detergents on the Ca,Mg-ATPase activity of phospholipid-depleted ghosts.

Partially lipid-depleted ghosts were obtained by treatment of intact ghosts with purified phospholipase A$_2$ (0.7 IU/mg of protein) for 15 min at 32°C in the presence of 0.8 mM CaCl$_2$; the washing of the vesicles was performed as described under 3.2. The Ca,Mg-ATPase activity was measured as described in Figure 3, using 300 µg of lipid-depleted ghosts. Bovine serum albumin was not present in the medium. (O) Triton X-100; (●) K-cholate.

In the experiment shown in Fig. 6, various concentrations of lysolecithin and oleate were added to membranes partially depleted of phospholipids. Lysolecithin did not induce any appreciable reactivation of the Ca,Mg-ATPase activity, whereas oleate restored the activity completely, when added at a concentration of 1 µmol/mg of protein. These results
Figure 6 Effect of oleic acid and lysolecithin on the Ca,Mg-ATPase activity of phospholipid-depleted ghosts.

Partially phospholipid-depleted ghosts were prepared and assayed as described in Fig. 5 in the presence of different concentrations of lysolecithin (●) and K-oleate (O).

indicate clearly that the ATPase activity in membranes treated with phospholipase, and still containing the products of phospholipid degradation (Fig. 3), is maintained by fatty acids rather than by lysocompounds. This is at variance with the findings recently published by Coleman and Bramley (63), and Schatzmann and Roelofsen (64,65).
By degrading the phospholipids with phospholipase C, and being thus unable to wash out the diglycerides formed, they could reactivate the Ca,Mg-ATPase to a maximal value of 60% of its control (65) by addition of lysolecithin at a concentration of about 1 μmole / mg of protein.

**Reactivation by Phospholipids**

To determine whether or not phospholipase A2 remained bound to the membranes after the phospholipid depletion, the following experiments were carried out. Membranes treated with 9 IU phospholipase A2 / mg of protein were incubated with an excess of [14C]-PC as in the preincubation step for the Ca,Mg-ATPase assay, but at 37°C, thus accelerating the hydrolysis about tenfold. The lipids were extracted and fractionated, and their radioactivity was measured. The amount of fatty acids formed was 0.065 ± 0.025 (SD of five experiments) μmol / mg of protein, which is not sufficient to reactivate the enzyme.

Furthermore, addition of 0.4% BSA to the reactivated enzyme in the ATPase assay abolished the reactivation by oleic acid almost completely, whereas the reactivation by phospholipids decreased by only 10% or less.

Figure 7 shows an experiment in which the various phospholipids normally present in erythrocyte membranes were used to reactivate the partially phospholipid-depleted Ca,Mg-ATPase. Clearly, in this experiment, PS gave the best reactivation. Using membrane preparations which were less depleted of phospholipids, the specificity was less pronounced. Other negatively charged phospholipids, like cardiolipin, phosphatidic acid, and phosphatidylinositol, gave good reactivations of the enzyme as well (not shown).

The concentrations of phospholipids necessary for optimal reactivation (1 to 2 μmol / mg of protein) are not very different from those of the total phospholipids in
Figure 7  Reactivation of the Ca,Mg-ATPase in partially phospholipid-depleted ghosts by PS, PE, and PC. Sixty-five percent of the membrane phospholipids were removed by phospholipase A₂ treatment as described in Fig. 4. Preincubation of the membranes with microdispersions of phospholipids was carried out for 10 min at 25°C. Ca,Mg-ATPase activity was then measured using procedure I (3.2.) for 5 min at 37°C. The control ghosts incubated without phospholipase had an activity of 0.52 µmol of ATP split / h x mg of protein, without the addition of phospholipids. Symbols represent reactivation by PS (●), PE (○), and PC (▲).

(µmol phospholipid added / mg protein)

(µmol phospholipid added / mg protein)

(Ca²⁺+Mg²⁺)-ATPase activity (µmoles/h x mg protein)

(µmol phospholipid added / mg protein)
the intact erythrocyte membrane (0.57 µmol / mg of protein). Cardiolipin showed optimal reactivation at an approximate concentration of 0.25 µmol / mg of protein; at higher concentrations, some inhibition was observed.
4.3. Protease Treatment of Intact Erythrocytes

Table I shows the results of membrane preparations done from pronase and chymotrypsin-treated erythrocytes. Both of them showed a 20% decreased specific Ca,Mg-ATPase activity, and a correspondingly decreased yield in total Ca,Mg-ATPase activity.

Figures 8 and 9 show superposed scans and a photograph, respectively, of SDS-polyacrylamide gels of membranes from pronase- and chymotrypsin-treated cells, respectively, together with their controls. More than 95% of band 3 were degraded by the protease treatment.

Figure 10 shows the protein and the $^{32}$P-label patterns of control and chymotrypsin-treated membranes on 5% SDS-polyacrylamide gels of pH 6.0. The $^{32}$P-peaks of the two preparations were identical within the accuracy of this method (+ 10,000 MW), and behaved uniformly upon coelectrophoresis. In case the Ca,Mg-ATPase would be degraded, one would expect either the $^{32}$P-peak to be decreased, or the 65,000 MW fragment to be labelled. The former possibility may be ruled out by the 133% yield in phosphoprotein of chymotrypsin-treated membranes as compared to the control. The latter possibility was found to apply to the phosphorylated band 3 (52,66), which, however, does not represent an acyl-phosphate of an ATPase (52,66).

The Ca,Mg-stimulated phosphoprotein, and thus most probably the Ca,Mg-ATPase (27), banded at the highest apparent molecular weights of band 3 in SDS gels (Fig. 10). Two sets of separate experiments were carried out using the same labelling technique with untreated membranes and electrophoresis on discontinuous pH 6.6, 4% / pH 8.8, 8% gels (chapter 3.1.), and standardizing internally with the known molecular weights of all prominent protein bands.
TABLE I  Effect of pronase and chymotrypsin treatment of human erythrocytes on the
(Ca$^{2+}$ + Mg$^{2+}$)-ATPase activity in membrane preparations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ca,Mg-ATPase activity, yield</th>
<th>specific Ca,Mg-ATPase activity</th>
<th>membrane protein, yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/h x g packed cells</td>
<td>µmoles/h x mg protein</td>
<td>mg protein/g of packed cells</td>
</tr>
<tr>
<td>control</td>
<td>4.3 (100%)</td>
<td>1.28 (100%)</td>
<td>3.4 (100%)</td>
</tr>
<tr>
<td>pronase-treated</td>
<td>3.0 (70%)</td>
<td>1.04 (81%)</td>
<td>2.9 (85%)</td>
</tr>
<tr>
<td>control</td>
<td>3.8 (100%)</td>
<td>1.33 (100%)</td>
<td>2.9 (100%)</td>
</tr>
<tr>
<td>chymotrypsin-treated</td>
<td>3.4 (88%)</td>
<td>1.03 (78%)</td>
<td>3.3 (114%)</td>
</tr>
</tbody>
</table>

Intact erythrocytes were treated with pronase or chymotrypsin as described in methods (3.3.), and membranes were then prepared by the standard procedure (see 3.1.).
Figure 8  Scans from a SDS-polyacrylamide gel electrophoresis of membranes from pronase-treated and untreated erythrocytes.

Intact erythrocytes (50% hematocrit) were treated with 18 P.U.K. pronase/g of packed cells for 35 min at 37°C and then washed 6 times (3.3.). The membranes of both
Legend to Fig. 8 (continued):
treated and untreated cells were then prepared by the
standard procedure (3.1.). 100 µg of membrane protein
each were subject to discontinuous gel electrophoresis
on 4% / 8% gels of pH 6.8 / 8.8, and stained with Coo-
massie Blue. The scans were done at 560 nm.
--- control, ---- pronase-treated.

(according to 67). They revealed a molecular weight of
126,000 ± 4,000 for the Ca,Mg-ATPase (band 3 shows an
average molecular weight of 95,000).
Intact erythrocytes (50% hematocrit) were treated with 90 U chymotrypsin/g of packed red cells, and incubated for 90 min at 37°C (3.3.). Then, phenylmethylsulfonyl-fluoride (=PMSF) was added to a final concentration of 0.6 mg/g packed red cells to stop proteolysis. The cells were washed 3 times and the membranes were prepared by the standard procedure (3.1.). Discontinuous gel electrophoresis with 100 µg of membrane protein each, and staining with Coomassie Blue were carried out as described in Fig. 8, but using a 3 mm slab-gel instead. Left: control; right: chymotrypsin treated.
Figure 10 SDS-PAGE of $^{32}$P-labelled membranes from chymotrypsin-treated and untreated erythrocytes.
Legend to Fig. 10

Membranes from chymotrypsin-treated and untreated erythrocytes were (32)P-labelled by incubation with [γ(32)P]-ATP in the presence of 12.5 μM MgCl$_2$, and 50 μM CaCl$_2$ (-----) or 100 μM EGTA (-----) for 15 sec at 0°C. Gel electrophoresis was carried out on 5% SDS-polyacrylamide gels of pH 6.0 at 10-15°C. The gels (0.6 x 4.5 cm) were sliced and counted for (32)P.

A: 140 μg membrane protein from untreated cells, B: from chymotrypsin treated cells, and C: coelectrophoresis of both 70 μg membrane protein from untreated and chymotrypsin-treated human erythrocytes.
4.4. Solubilization of the Ca,Mg-ATPase

Table II shows the results of solubilizing the Ca,Mg-ATPase from erythrocyte membranes by Triton X-100, using an optimized procedure, slightly modified from that of Dieckvoss et al. (54,55). Under the conditions given in Table II and in the methods part (3.4.), mainly bands 3, 4.5, and almost all of the accountable Ca,Mg-ATPase activity were solubilized (Fig. 11). Since this procedure resulted in a very labile enzyme preparation (Fig. 12), the procedure A described in chapter 3.4. was used. It omits protease inhibitors and SH-reagents, but includes the addition of phospholipids and glycerol to the supernatant.

Table III shows the results of this procedure (A) with control and chymotrypsin-treated membranes. Fig. 13 shows the scans of SDS-polyacrylamide gels of chymotrypsin-treated membranes, the solubilized and non-solubilized proteins. The results are essentially identical to those of the procedure B as modified from Dieckvoss et al., except that the stability of the Ca,Mg-ATPase was much better (Fig.12).

Using pronase-treated membranes instead of chymotrypsin-treated ones, the same results were obtained, but due to a lower (and suboptimal) Triton concentration (1.6 mg / mg of protein), a specific activity of up to 5 μmoles/h x mg protein, a purification factor of up to 5, a yield in Ca,Mg-ATPase activity in the supernatant of up to 60%, and in protein of 10%, could be achieved.

It should be stressed, that the concentration of Triton X-100 needed for solubilization of the Ca,Mg-ATPase from pronase- or chymotrypsin-treated membranes was always significantly higher than for untreated membranes (Fig. 14, the curve for the control is taken from S.W. Peterson (68), by his kind permission).
TABLE II  Solubilization of erythrocyte membranes with Triton X-100, using a procedure slightly modified from that of Dieckvoss et al. (54,55); (procedure B).

<table>
<thead>
<tr>
<th>Sample</th>
<th>specific Ca,Mg-ATPase activity (μmoles/h x mg)</th>
<th>purification factor</th>
<th>yield in Ca,Mg-ATPase activity (%)</th>
<th>yield in protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>thawed membranes</td>
<td>1.56 ± 0.41 (6)</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>preincubated membr.</td>
<td>1.20 ± 0.39 (4)</td>
<td>0.85 ± 0.13 (4)</td>
<td>74 ± 10 (4)</td>
<td>100</td>
</tr>
<tr>
<td>solubilized membr.</td>
<td>0.88 ± 0.28 (4)</td>
<td>0.88 ± 0.54 (4)</td>
<td>56 ± 11 (4)</td>
<td>100</td>
</tr>
<tr>
<td>pellet (100,000 x g)</td>
<td>0.03 ± 0.06 (4)</td>
<td>-</td>
<td>1 ± 3 (4)</td>
<td>71 ± 16 (4)</td>
</tr>
<tr>
<td>supernatant</td>
<td>3.20 ± 1.32 (5)</td>
<td>2.12 ± 0.64 (5)</td>
<td>42 ± 5 (5)</td>
<td>23 ± 5 (5)</td>
</tr>
<tr>
<td>concentrated spnt</td>
<td>3.38 ± 1.37 (5)</td>
<td>2.31 ± 0.66 (5)</td>
<td>38 ± 5 (6)</td>
<td>18 ± 4 (5)</td>
</tr>
<tr>
<td>amicon filtrate</td>
<td>0.00 ± 0.00 (3)</td>
<td>-</td>
<td>0 ± 0 (3)</td>
<td>6 ± 4 (3)</td>
</tr>
</tbody>
</table>

Erythrocyte membranes were solubilized by 0.8 ± 0.1 (6) mg Triton X-100 / mg membrane protein (the optimal amount) at a concentration of 4.1 ± 0.8 (6) mg protein / ml. The preincubation was 60 min at 0°C. Medium (slightly modified from 54,55): 150 mM KCl, 30 mM Tris-Cl, 10 mM diisopropylfluorophosphatase (DFP), 50 mM cysteine, 0.1 mM MgCl₂, 0.05 mM CaCl₂, 0.6 mg PC (sonicated) / ml, pH 7.3 at 0°C.
Figure 11  SDS-polyacrylamide gel electrophoretic protein patterns of membranes from untreated erythrocytes, of the pellet and the supernatant after selective solubilization by Triton X-100.
Legend to Fig. 11:

After a 60 min preincubation (at 0°C) in the presence of 10 mM diisopropylfluorophosphate, the Ca,Mg-ATPase from human erythrocytes was selectively solubilized by procedure B (see 3.4.), using 0.8 mg Triton X-100/mg of membrane protein and in the following medium: 150 mM KCl, 30 mM Tris-Cl, 0.1 mM MgCl₂, 0.05 mM CaCl₂, 10 mM diisopropylfluorophosphate, 50 mM cysteine, 0.6 mg PC (sonicated) / ml, pH 7.3 at 0°C. The solubilization was carried out at 5°C for 10 min. The centrifugation lasted 30 min at 100,000 x g at 0°C. The protein pattern was analysed by SDS-polyacrylamide gel electrophoresis on discontinuous 4%/8%, pH 6.8/8.8 gels. TD = tracking dye. Bands are numbered according to Steck (67).
A: membranes, B: pellet, C: supernatant (solubilized proteins).

It was found that ATP and the ionic strength had no influence on the stability of the solubilized Ca,Mg-ATPase, and that 10 mM diisopropylfluorophosphate (DFP), 1 mM TLCK, 50 mM cysteine, 1 mM EDTA, removal of Triton X-100 by Bio-Beads SM-2 destabilized the enzyme. Addition of acidic phospholipids at a concentration of about 1 mg/mg of solubilized protein, and storage in a solution of 10% w/v glycerol stabilized it.
Figure 12 Stability of the Triton X-100 solubilized Ca,Mg-ATPase. Comparison of procedures A and B.

The supernatant obtained from the solubilization of human erythrocyte membranes by procedure B, as modified from that of (54,55), was stored at 4°C (•••••). The initial activity of the supernatant is taken as 100%. The protein concentration was 0.4 mg/ml. Medium: 150 mM KCl, 30 mM Tris-Cl, 10 mM diisopropylfluorophosphate, 50 mM cysteine, 0.1 mM MgCl₂, 0.05 mM CaCl₂, 0.6 mg PC (sonicated) / ml, pH 7.3 at zero degrees.

The supernatant obtained by procedure A was stabilized by the addition of 1 mg asolectin/mg solubilized protein, and by the addition of 10% w/v glycerol. The medium then contained: 10 mM KCl, 10 mM Tris-Cl, 0.05 mM ATP, 0.2 mM MgCl₂, 0.05 mM CaCl₂, 10% w/v glycerol, 3.9 mg asolectin/ml, pH 7.6 at 5°C. The protein concentration was 3.9 mg/ml. (O—O). 0.9 mg Triton X-100 were used for solubilization in both cases.
TABLE III  Solubilization of chymotrypsin-treated erythrocytes membranes with Triton X-100 according to procedure A (3.4.).

<table>
<thead>
<tr>
<th>Sample</th>
<th>specific Ca,Mg-ATPase activity (µmoles/h x mg protein)</th>
<th>purification factor</th>
<th>yield in Ca,Mg-ATPase activity (*) (%)</th>
<th>yield in protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>membranes</td>
<td>1.16 ± 0.28</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>solubilized membranes</td>
<td>0.81 ± 0.43</td>
<td>0.67 ± 0.21</td>
<td>75 ± 29 (76 ± 40)</td>
<td>100</td>
</tr>
<tr>
<td>pellet (100,000 x g, 1 h)</td>
<td>0.03 ± 0.03</td>
<td>-</td>
<td>1 + 1 (0 ± 0)</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>supernatant</td>
<td>2.3 ± 1.4</td>
<td>1.9 ± 0.7</td>
<td>62 ± 22 (60 ± 12)</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>supernatant, PC added, concentrated, glycerol added</td>
<td>1.85 ± 1.20</td>
<td>1.5 ± 0.7</td>
<td>50 ± 23 (34 ± 1)</td>
<td>27 ± 4</td>
</tr>
</tbody>
</table>

(*) The values for the control membranes are given in parentheses. All data are average values ± standard error of 2 experiments. The amount of Triton X-100 used was 1.2 mg/mg protein in control, and 2.3 mg/mg protein in chymotrypsin-treated membranes. Both concentrations were near optimal. 1 mg PC was added per mg of solubilized protein, and the concentrated supernatant was made 10% w/v in glycerol (see 3.4.).
Figure 13  SDS-polyacrylamide gel electrophoretic protein patterns of membranes from chymotrypsin-treated erythrocytes, of the pellet and the supernatant after selective solubilization by Triton X-100.
Legend to Fig. 13:

The Ca,Mg-ATPase of membranes from chymotrypsin-treated human erythrocytes was selectively solubilized by Triton X-100 by procedure A (3.4.), using 2.3 mg Triton X-100 / mg membrane protein. Medium: 10 mM KCl, 10 mM Tris-Cl, 0.05 mM ATP, 0.2 mM magnesium chloride, 0.05 mM calcium chloride, pH 7.6 at 5°C. The solubilization was carried out at 5°C for 10 min. Centrifugation was done at 100,000 x g for 30 min, at 0°C. The protein patterns of the supernatant and the pellet were analysed by SDS-polyacrylamide gel electrophoresis on discontinuous 4%, pH 6.8 / 8%, pH 8.8 gels. TD = tracking dye. Bands are numbered according to Steck (67).
A: membranes, B: pellet, C: supernatant (solubilized proteins).
Figure 14  Solubilization of the Ca,Mg-ATPase by Triton X-100, using membranes from untreated and pronase-treated human erythrocytes.

Membranes from pronase-treated (O—O) and from untreated (●—●) human erythrocytes were solubilized with the amount of Triton X-100 / mg membrane protein given in the diagrams. The solubilization was carried out at 5°C, by procedure B, as described in Fig. 11.

A shows the yield in Ca,Mg-ATPase activity in the supernatant after 1 h centrifugation at 100,000 x g, relative to the starting activity,

B shows the specific activity of the solubilized Ca,Mg-ATPase.
4.5. **Purification of the Solubilized Ca,Mg-ATPase by Gel Chromatography on Sepharose 6B**

The Ca,Mg-ATPase was selectively solubilized from human erythrocyte membranes (ghosts) with Triton X-100 by either one of the two procedures given in chapter 3.4., concentrated by ultrafiltration and applied to a Sepharose 6B column.

Fig. 15 shows a typical elution diagram of the proteins solubilized from membranes prepared from intact erythrocytes and run in buffer system I, i.e. in the presence of the protease inhibitor DFP (diisopropylfluorophosphate) and of cysteine. Below the diagram, gels of several fractions out of the region of the peak with the Ca,Mg-ATPase activity are shown. Fig. 16 shows the densitometric trace of a discontinuous, Coomassie Blue-stained SDS-gel of these pooled fractions with the Ca,Mg-ATPase activity. Obviously, the anion transport protein (i.e. 98% of the proteins of band 3) is the major contaminant of the Ca,Mg-ATPase. The Ca,Mg-ATPase is not part of the anion carrier, as was shown by the experiments on the protease treatment of intact erythrocytes.

The dominant absorbance peak mainly indicates the elution of the Triton X-100 micelles. However, the Ca,Mg-ATPase still contains tightly bound Triton X-100.

Table IV shows the results (with the average and the standard errors) obtained from several similar experiments, using either membranes from untreated or from pronase-treated erythrocytes.
Elution diagram of Triton X-100 solubilized erythrocyte membrane proteins from a Sepharose 6B column, using buffer system I.

Figure 15
Legend to Fig. 15:

The Ca,Mg-ATPase was selectively solubilized by Triton X-100 from the membranes of untreated human erythrocytes, as described in Fig. 11. The concentrated supernatant (3.4.) was applied to a Sepharose 6B column (2.565 x 70.7 cm) and eluted in the following buffer (system I; 3.5.) at a flow rate of 27.5 ml/h: 200 mM KCl, 0.1 mM magnesium chloride, 0.05 mM calcium chloride, 10 mM cysteine, 0.1 mM diisopropylfluorophosphate (DFP), 0.05% w/v PC, 0.67% w/v Tween 20, 30 mM Tris-Cl, pH 7.4 at 4°C. The Ca,Mg-ATPase activity was measured within 5 hours after elution. The Triton micelle peak was identified in separate experiments without protein present, hemoglobin (Hb) was identified spectrophotometrically. Discontinuous SDS-polyacrylamide gels were as described in Fig. 11. 20 mg of protein were applied to the column.

The Sepharose 6B column was calibrated with ferritin, catalase, aldolase, serum albumin, ovalbumin. The results agreed within 10% with the specifications of the manufacturer. The size of the eluted particles was identical within experimental error for both untreated and pronase-treated membranes. Thus, the micelle of the eluted Ca,Mg-ATPase had an apparent molecular weight of 155,000 ± 20,000, corresponding to a diameter of about 100 Å.

Fig. 17 shows an elution diagram of a Sepharose 6B column using buffer system II and the proteins solubilized from chymotrypsin-treated membranes. A slab gel given below the diagram shows the protein patterns of the eluted fractions. A small separation of the peak of the Ca,Mg-ATPase and the peak of the 65,000 MW fragment of band 3 may be seen (for the chymotrypsin-treatment refer to chapter 3.3.). Correspondingly, a faint band (indicated by the arrow) migrating slower than the remaining proteins of band 3 may be seen to culminate together with the Ca,Mg-ATPase - peak (this band roughly corresponds to the one of the 32P-phosphoprotein). Table IV shows the corresponding
Figure 16  Densitometric trace of a discontinuous SDS-polyacrylamide gel of the pooled fractions with Ca,Mg-ATPase activity of a Sepharose 6B column.

The Ca,Mg-ATPase was selectively solubilized from untreated human erythrocyte membranes by Triton X-100, and the solubilized proteins were fractionated on a Sepharose 6B column as described in Fig. 15. The polyacrylamide gel was run and stained as described in Fig. 15. The scanning was done at 560 nm, using a slit of 0.10 mm. TD = tracking dye.
TABLE IV  Purification of the Triton X-100 solubilized Ca,Mg-ATPase of human erythrocyte membranes by gel chromatography on Sepharose 6B, using buffer system I, II.

<table>
<thead>
<tr>
<th>Sample</th>
<th>specific Ca,Mg-ATPase activity (µmoles/h x mg)</th>
<th>purification factor</th>
<th>yield in Ca,Mg-ATPase activity (%)</th>
<th>yield in protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer system I:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal membranes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>applied (spnt)</td>
<td>3.25 ± 1.25 (6)</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATPase, pooled</td>
<td>14.2 ± 7.6 (6)</td>
<td>4.3 ± 1.1 (6)</td>
<td>78 ± 21 (7)</td>
<td>25 ± 10 (7)</td>
</tr>
<tr>
<td>total of column</td>
<td>-</td>
<td>-</td>
<td>119 ± 30 (7)</td>
<td>-</td>
</tr>
<tr>
<td>most active fract.</td>
<td>24.3 ± 7.3 (3)</td>
<td>7.5 ± 0.4 (3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>pronase-treated:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>applied (spnt)</td>
<td>3.66 ± 2.29 (2)</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATPase, pooled</td>
<td>6.7 ± 1.5 (2)</td>
<td>2.5 ± 1.9 (2)</td>
<td>61 ± 47 (2)</td>
<td>26 ± 1 (2)</td>
</tr>
<tr>
<td>total of column</td>
<td>-</td>
<td>-</td>
<td>101 ± 72 (2)</td>
<td>-</td>
</tr>
<tr>
<td>most active fract.</td>
<td>9.9 ± 0.6 (2)</td>
<td>3.4 ± 1.9 (2)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE IV  (Continued)

<table>
<thead>
<tr>
<th>Sample</th>
<th>specific Ca,Mg-ATPase activity (μmoles/h x mg)</th>
<th>purification factor</th>
<th>yield in Ca,Mg-ATPase activity (%)</th>
<th>yield in protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer system II:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chymotrypsin-treated membranes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>applied (spnt)</td>
<td>1.85 + 1.20 (2)</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATPase, pooled</td>
<td>2.06 + 0.59 (2)</td>
<td>2.06 + 0.51 (2)</td>
<td>71 + 23 (2)</td>
<td>42 + 13 (2)</td>
</tr>
<tr>
<td>total of column</td>
<td>-</td>
<td>-</td>
<td>75 + 32 (2)</td>
<td>-</td>
</tr>
<tr>
<td>most active fract.</td>
<td>3.09 + 1.09 (2)</td>
<td>1.88 + 0.63 (2)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The conditions for the chromatography on Sepharose 6B were those described in Figs. 15, and 17. The pronase- and the chymotrypsin-treatment were carried out as described in Figs. 8 and 9. The average values are given + their standard error, number of experiments in (()).
Figure 17: Elution diagram from a Sepharose 6B column of Triton X-100 solubilized membrane proteins from chymotrypsin-treated erythrocytes.
The Ca,Mg-ATPase was selectively solubilized by Triton X-100 from the membranes of chymotrypsin-treated human erythrocytes (see Fig. 9) by the procedure described in Fig. 13. The concentrated supernatant (3.4.) was applied to a Sepharose 6B column (2.565 x 84.2 cm) and eluted in the following buffer (system II; 3.5.) at a flow rate of 27.5 ml/h: 10 mM KCl, 10 mM Tris-Cl, 0.05% w/v PC, 0.67% w/v Tween 20, 10% w/v glycerol, pH 7.4 at 4°C. All the assays were done as described in Fig. 15. 50 mg of protein were applied to the Sepharose column.

results in a purification scheme.

The Ca,Mg-ATPase activity of both, pronase- and chymotrypsin-treated erythrocytes was found to be less active and less stable, when run over a Sepharose 6B column, as compared to the one of untreated erythrocytes. Since the membranes of protease-treated cells, when stored at 4°C, never lost more than 5% of their initial activity during 7 days, the reason for the instability is thought to be the larger amount of Triton X-100 needed for the solubilization of the enzyme from these membranes, as compared to the untreated ones (chapter 4.4.; 2.3 mg Triton X-100/mg of protein, instead of 0.8 mg Triton X-100/mg of protein).

The results in Table V suggest, that the size of the eluted particles is significantly larger in the experiments using buffer system II, than in system I. One might speculate, that in system II the enzyme is eluted as a dimer, whereas it occurs as a monomer in system I. However, more accurate and detailed results are required to clarify this point.
TABLE V  The size of the Triton X-100 solubilized Ca,Mg-ATPase from human erythrocyte membranes, as eluted in mixed micelles from the Sepharose 6B column.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_{av}$ of the Ca,Mg-ATPase micelle</th>
<th>corresponding MW of the Ca,Mg-ATPase micelle</th>
<th>$K_{av}$ Triton micelle</th>
<th>$K_{av}$ hemoglobin micelle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer system I:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal membranes</td>
<td>0.374 ± 0.018</td>
<td>155,000 ± 18,000</td>
<td>0.532 ± 0.004</td>
<td>0.686 ± 0.016</td>
</tr>
<tr>
<td>pronase-treated membranes</td>
<td>0.379 ± 0.013</td>
<td>150,000 ± 12,000</td>
<td>0.544 ± 0.000</td>
<td>-</td>
</tr>
<tr>
<td>Buffer system II:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal membranes:</td>
<td>0.283</td>
<td>276,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>chymotrypsin-treated membranes</td>
<td>0.294 ± 0.018</td>
<td>257,000 ± 30,000</td>
<td>0.505 ± 0.015</td>
<td>-</td>
</tr>
</tbody>
</table>

The conditions for the Sepharose 6B column chromatography were those described in Figs. 15 and 17 (and chapter 3.5.). $K_{av}$ is the relative elution volume $(V_e - V_o)/(V_e - V_o)$. The column was calibrated with ferritin, catalase, aldolase, serum albumin and ovalbumin (3.5.). The results are given as the average ± standard error, number of experiments in ().
4.6. Preparative Isoelectric Focusing of a Partially Purified Preparation of the Ca,Mg-ATPase

Erythrocyte membranes were solubilized with Triton X-100. The soluble proteins were concentrated (PC was added as described in 3.4.), passed through a Sepharose 6B column, and the active fractions were pooled and concentrated. (All these procedures were already described in chapters 3.4, 3.5, 4.4, and 4.5) The preparation then contained a Ca,Mg-ATPase, purified 3 - 10 times in terms of specific activity, and mainly contaminated with the anion carrier protein (i.e. "band 3"). The focusing gradients were established as described in the legend to Table VI. A typical example of the time course of the decay of the current flowing through the column is given in Fig. 18.

Fig. 19 shows the profiles of the protein content, the Ca,Mg-ATPase activity, and of the pH along the isoelectrically focused column. In this case, a preparation of Ca,Mg-ATPase from chymotrypsin-treated membranes (see 3.3, 4.3) was used. A SDS-polyacrylamide slab gel of successive fractions of the same isoelectric focusing column is shown in Fig. 20. The purification remained only marginal, in that no single band could be detected to separate from the others. However, a shift was consistently observed between the main ATPase peak and the main protein peak. Using the preparation of Ca,Mg-ATPase from chymotrypsin-treated erythrocytes, the following pH values were found for the different maxima: protein at 4.82 ± 0.30 (2), Ca,Mg-ATPase activity at 5.01 ± 0.03 (2), and specific Ca,Mg-ATPase activity at 6.05 ± 0.06 (2).
Focusing time (h)

Figure 18  A typical curve of the time course of the decay of the current flowing through the isoelectric focusing column.

The focusing column (1.8 x 31.4 cm) contained gradients of 0.6 - 3% ampholine pH 3.5-10, and of 5 - 30% sucrose. The sample (17 mg protein) was gradually concentrated towards the center of the column. Total volume of the focusing solution: 80 ml. The focusing was done at 400 V at 2°C. The resistance increased from 45 kΩ initially, to 200 kΩ at the end of the focusing.
Figure 19  Preparative isoelectric focusing on a sucrose gradient using a partially purified preparation of the Ca,Mg-ATPase from erythrocytes.

A preparation of Ca,Mg-ATPase (9 mg protein) selectively solubilized by Triton X-100 and chromatographed on Sepharose 6B, was isoelectrically focused on a gradient of 1.5 - 3 - 0% ampholine pH 3.5 - 5, and 0 - 0 - 3% ampholine pH 5 - 8, and of 10 - 40% sucrose. The gradient contained 0.3% Triton X-100. The sample was evenly distributed in the upper half of the gradient (left part in this diagram; compare with Table VI, Expt. B). The focusing was done at 2°C, and 550 V for 22 hours.

(●●●) pH, measured in individual fractions; (Δ—Δ) Ca,Mg-ATPase activity, measured after neutralisation; (○—○) protein content.
Figure 20  SDS-polyacrylamide slab gel electrophoresis of the fractions of an isoelectric focusing column of a partially purified preparation of the Ca,Mg-ATPase from human erythrocytes.

A preparation of partially purified Ca,Mg-ATPase (selective solubilization by Triton X-100 and chromatography on Sepharose 6B) from membranes of chymotrypsin-treated human erythrocytes was isoelectrically focused as described in Fig. 19 (this experiment is identical with that of Fig. 19). Gel electrophoresis was carried out on a discontinuous 4%, pH 6.8 / 8%, pH 8.8 3 mm slab gel. The gel was stained with Coomassie Blue. The dominant band represents the 65,000 MW fragment of band 3 (due to chymotrypsin-treatment; 4.3.).
The proteins, banding at their pI, precipitated within a few hours of focusing. Increasing the concentration of Triton X-100 above 0.3% led to increased inactivation of the Ca,Mg-ATPase. Decreasing the amount of protein to be focused caused difficulties in the accuracy of enzyme determination, and increasing the concentration of ampholines did not prove very efficient in preventing precipitation. The isoelectric focusing was thus carried out with as little protein, and as flat a gradient of ampholines, as possible. When readjusting the pH, the precipitated proteins could be resolubilized within seconds.

Table VI summarizes the results of several isoelectric focusing experiments. The overall yield in Ca,Mg-ATPase activity was only 20%. However, the focused enzyme could be partially reactivated (i.e., its activity increased up to 3 times) by addition of PS (Fig. 21). The Triton X-100 was not removed for these reactivation experiments, and might have prevented a better reactivation.

Considering complete reactivation possible, the best purification obtainable with isoelectric focusing using the method described (3.6.) would thus be about 2 times.
TABLE VI  Preparative isoelectric focusing of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase from human erythrocyte membranes, partially purified by selective solubilization, and gel filtration through Sepharose 6B.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific Ca,Mg-ATPase activity (µmoles/h x mg protein)</th>
<th>purification factor</th>
<th>yield in Ca,Mg-ATPase activity (%)</th>
<th>yield in protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal membranes, solubilized (proc. B), Sepharose (system I):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>applied sample</td>
<td>5.6 ± 1.6 (2) 1</td>
<td>(2)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>fraction of ATPase peak</td>
<td>3.2 (1) 0.5 (1)</td>
<td>(2)</td>
<td>5 ± 1 (2)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>column overall</td>
<td>-</td>
<td>-</td>
<td>19 ± 11 (2)</td>
<td>81 (1)</td>
</tr>
<tr>
<td>chymotrypsin-treated membranes, solubilized (proc. A), Sepharose (system II):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>applied sample</td>
<td>2.7 ± 0.3 (2) 1</td>
<td>(2)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>fraction of ATPase peak</td>
<td>1.6 ± 1.0 (2) 0.6 ± 0.4 (2)</td>
<td>(2)</td>
<td>5 ± 4 (2)</td>
<td>17 ± 0 (2)</td>
</tr>
<tr>
<td>column overall</td>
<td>-</td>
<td>-</td>
<td>21 ± 16 (2)</td>
<td>86 ± 9 (2)</td>
</tr>
</tbody>
</table>
Legend to Table VI

Focusing procedure in the case of normal membranes:
16-19 mg protein were applied and evenly distributed in a gradient of 0.6%-3% ampholines pH 3.5 - 10, and of 5%-30% sucrose. The concentration of Triton X-100 was 0.3%.

Focusing procedure in the case of chymotrypsin-treated membranes:
9-26 mg protein were applied. Sucrose gradient 10%-40%. 0.3% Triton X-100. Expt. A: 0.6%-3% ampholines pH 3.5 - 10. The sample was applied in a gradient towards the center. Expt. B: Gradient of 1.5%-3%-0% ampholines pH 3.5 - 5, and 0%-0%-3% ampholines pH 5-8. The sample was evenly distributed in the second half of the gradient.

For details of focusing refer to methods (3.6.). Average values are given with the standard error and the number of experiments in ( ).
Figure 21  Reactivation of a preparation of isoelectrically focused Ca,Mg-ATPase from human erythrocytes by addition of phosphatidylserine.

The Ca,Mg-ATPase in two fractions of pH 4.99 (O—O) and 4.75 (●—●) of an isoelectric focusing column (see Fig. 19) was reactivated by addition of microdispersed phosphatidylserine (PS; chapter 3.2.). The fractions stemmed from the column described in Figs. 19, and 20.
4.7. Investigation of the Substrate Site of the Ca,Mg-ATPase using Analogues of ATP

The structure of the analogues described here, was given in Fig. 2, chapter 3.7. For conformational and thermodynamic data refer to Tables VIII, IX, and X, chapter 5.7.

rroATP

rroATP has the ribose ring opened between C(2') and C(3'). As may be seen from the Lineweaver-Burk plot (Fig. 22) and from the Dixon plot (Fig. 23), rroATP inhibits the Ca,Mg-ATPase competitively with respect to ATP with an inhibitor constant ($K_i$) of only 1.4 - 1.5 mM (Fig. 22, this is a lower limit for $K_i$, not using the values of $[ATP]^{-1} = 75 \text{ mM}^{-1}$; otherwise no $K_i$ may be calculated), and 0.7 - 4.6 mM respectively (Fig. 23).

The $V_{max}$ values of the three calculated regression lines in the Lineweaver-Burk plot are identical with probabilities of 65-85% according to the student $t$-test (null hypothesis). Therefore, the statement of competitive inhibition seems to be statistically justified.

As may be seen from Table VII, rroATP is split at a rate at least 100 times slower than ATP.

AMP-PNP

AMP-PNP has its $\beta$- and $\gamma$-phosphorous linked by a -NH-group instead of the usual -O-.

Figs. 24 and 25 show a Lineweaver-Burk and a Dixon plot, respectively, of the inhibition of the Ca,Mg-ATPase by AMP-PNP. Noncompetitive inhibition with respect to ATP was observed in all experiments carried out.
The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of rroATP was measured by a coupled enzyme assay (3.1.). The assay mixture contained 120 mM KCl, 30 mM HEPES, 0.5 mM magnesium chloride, 0.05 mM calcium chloride, the amount of ATP and the analogue specified in the diagram, and 0.2 mM NADH, 0.5 mM PEP, 1 IU (at 25°C) of pyruvate kinase per ml, and 1 IU (at 25°C) of lactate dehydrogenase per ml. The assay mixture was preincubated at 37°C for 4 min and the reaction started by adding the membranes (150 µg/ml). The reaction (decrease of absorption of NADH) was followed spectrometrically for 4 min, once with calcium present, and once after addition of 1 mM EGTA. The difference was taken as the Ca,Mg-ATPase activity. Assay temperature: 37°C. 

(••••) no inhibitor present, (O--O) with 72 µM rroATP, (△--△) with 180 µM rroATP. Linear regressions gave a $K$ for ATP of $65 \pm 3$ µM (standard error), and a $K_i$ for rroATP of 1.4 mM (O), and of 1.5 mM (△).
TABLE VII  Determination of the rate of phosphate splitting by the \((\text{Ca}^{2+} + \text{Mg}^{2+})\)-ATPase from ATP and rroATP

<table>
<thead>
<tr>
<th>Compound</th>
<th>concentration range tested (mM)</th>
<th>specific Ca,Mg-ATPase activity (μmoles/h x mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (control)</td>
<td>0.5</td>
<td>1.16</td>
</tr>
<tr>
<td>rroATP</td>
<td>0.02 - 5.00 (6 samples)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The samples were incubated in the same medium as for standard assays (3.1.), but MgCl₂ was 5 mM, CaCl₂ was 1 mM and the incubation time was 20 min. Phosphate was determined by the method of Fiske and Subbarow (61).

In Fig. 24, the \(V_{\text{max}}\) values for inhibitor present were identical to the control with probabilities of 0.4%, 2.5%, and 10%, respectively, as determined by the student t-test (probabilities among each others: 8%, 25%, 55%), whereas the apparent \(K_{\text{m}}\) values were identical with a probability of 45%-95% (average 68%). Thus, noncompetitive inhibition is highly probable.

The inhibitor constant \(K_{i}\) was 230 μM in the Lineweaver-Burk, and 200 μM in the Dixon plot; it generally ranged between 150 μM and 230 μM. The Ca,Mg-ATPase therefore showed an affinity approximately 20 times lower for AMP-PNP than for ATP.

The replots of the slopes and the intercepts (taken from Fig. 24) versus the inhibitor concentration are linear (error of 1, and 3%, respectively), and show a \(K_{i}\) of 195 μM and 183 μM, respectively. The inhibition type (69)
Figure 23  Dixon plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue rroATP.

The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of rroATP was measured as described in Fig. 22. (Δ—Δ) with 13 μM ATP, (○—○) with 27 μM ATP, (●—●) with 67 μM ATP present together with the concentration of rroATP given in the diagram. The linear regression lines intersected at a K_i of 4.6 mM, 0.7 mM, and 1.3 mM, respectively.

is therefore linear noncompetitive. (Parabolic noncompetitive inhibition indicating combined action of at least 2 molecules of inhibitor on the enzyme can not be excluded
Figure 24  Lineweaver-Burk plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue AMP-PNP.

The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of AMP-PNP was measured as described in Fig. 22.

(●—●) no inhibitor present, (○—○) with 155 μM AMP-PNP, (△—△) with 246 μM AMP-PNP, (▲—▲) with 310 μM AMP-PNP present.

The linear regression gave a $K_i$ for ATP of $9.98 \pm 0.22 \mu M$, and an average $K_i$ for AMP-PNP of $233 \pm 16 \mu M$ (individual $K_i$ values: $267 \pm 27 \mu M; 231 \pm 16 \mu M; 203 \pm 38 \mu M$).

with certainty. However, the Ca,Mg-ATPase was never reported to show cooperativity with respect to ATP.)
Figure 25 Dixon plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue AMP-PNP.

The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of AMP-PNP was measured as described in Fig. 22. 

(Δ—Δ) with 6 μM ATP, (O—O) with 12 μM ATP, (•—•) with 121 μM ATP present, together with the concentration of AMP-PNP given in the diagram. 
The linear regression lines gave a Kᵢ for AMP-PNP of 196 ± 11 μM (individual values: 178 ± 10 μM, 197 ± 24 μM, 214 ± 20 μM).

AMP-PCP

AMP-PCP has its β- and γ-phosphorous linked by a -CH₂- group, instead of the usual -O-. This bond cannot be split by an ATPase (70).

Figs. 26 and 27 show a Lineweaver-Burk, and a Dixon plot, respectively, of the inhibition of the Ca,Mg-ATPase by AMP-PCP.
Figure 26  Lineweaver-Burk plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue AMP-PCP.

The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of AMP-PCP was measured as described in Fig. 22.

(○—○) no inhibitor present, (O—O) with 210 μM AMP-PCP, (△—△) with 419 μM AMP-PCP present.

The linear regression gave a $K_i$ for ATP of 4.55 ± 0.21 μM, and an average $K_i$ for AMP-PCP of 320 ± 60 μM (individual values 278 ± 13 μM, 362 ± 17 μM).
Clearly, AMP-PCP inhibited competitively. In a student t-test, the $V_{\text{max}}$ values were identical with probabilities of 80%-90%. The $K_m$ for ATP was 4.5 µM, the $K_i$ for AMP-PCP 320 µM (Fig.26), and 350 µM (Fig.27), respectively. In another membrane preparation, showing a $K_m$ for ATP of 9.2 µM, a $K_i$ of $600 \pm 300$ µM was found. The Ca,Mg-ATPase, therefore, shows an affinity for AMP-PCP about 75 times lower than for ATP.

**BrATP**

In BrATP, the C(8)-hydrogen of ATP is replaced by bromine. This causes the heterocyclic adenine ring system to be oriented syn, instead of anti, towards the ribose ring (Table IX, chapter 5.7.). Figure 28 shows a Lineweaver-Burk plot, and Fig. 29 a Dixon plot of the inhibition by BrATP of the ATP turnover of the Ca,Mg-ATPase. $K_i$ values for BrATP of $76 \pm 10$ µM, and $89 \pm 4$ µM, respectively, were calculated, whereas the $K_m$ for ATP was $4.6 \pm 0.2$ µM. Competitive inhibition by BrATP was observed with respect to ATP. (In a student t-test the $V_{\text{max}}$ values were identical with probabilities of 45%, 55%, and 85%). Other experiments with the same membrane preparation gave $K_i$ values of $116 \pm 60$ µM. Approximately, the affinity of the Ca,Mg-ATPase for BrATP is thus 20 times lower than for ATP.

In contrast to another report (71) it was found, that BrADP is phosphorylated by pyruvate kinase (from rabbit muscle), however at a rate about 220 times more slowly than ADP (Fig. 30, note the difference in scale for ADP and BrADP). In the usual assays, the ADP was therefore differentiated from BrADP (by the pyruvate kinase) by a factor of about 20.

Fig. 31 shows a Lineweaver-Burk plot for BrATP as a substrate for the Ca,Mg-ATPase. Its $V_{\text{max}}$ is about 10 times lower than that of ATP ($V_{\text{max}}$ for BrATP: 0.15 µmoles/h x mg protein, for ATP: 1.20 µmoles/h x mg protein). Its
Figure 27 Dixon plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue AMP-PCP. The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of AMP-PCP was measured as described in Fig. 22. (Δ-Δ) with 1.8 μM ATP, (▲-▲) with 2.8 μM ATP, (○-○) with 4.6 μM ATP, (●-●) with 46 μM ATP present, together with the concentration of AMP-PCP given in the diagram. The linear regression lines gave a $K_i$ for AMP-PCP of 346 ± 23 μM (the individual values range between 323 μM and 387 μM).
Figure 28 Lineweaver-Burk plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue BrATP.

The activity of the Ca,Mg-ATPase of human erythrocyte ghost membranes in the presence of BrATP was measured as described in Fig. 22.

(•—•) no inhibitor present, (O—O) 58 µM BrATP, (Δ—Δ) 290 µM BrATP. The linear regression gave a $K_m$ for ATP of 4.55 ± 0.21 µM, and an average $K_i$ for BrATP of 76 ± 10 µM.
Figure 29 Dixon plot of the inhibition of the erythrocyte Ca,Mg-ATPase by the ATP analogue BrATP.

The Ca,Mg-ATPase activity of human erythrocyte ghost membranes in the presence of BrATP was measured as described in Fig. 22. (Δ—Δ) with 2.8 μM ATP, (O—O) with 4.6 μM ATP, (●—●) with 46 μM ATP, together with the concentration of BrATP given. The linear regression lines gave an average $K_i$ for BrATP of $89 \pm 4$ μM (individual values $81 \pm 14$ μM, $89 \pm 4$ μM, and $100 \pm 23$ μM).
Figure 30  Comparison of the phosphorylation rate of ADP and BrADP by the pyruvate kinase.

The assay mixture was prepared as described in Fig. 22, but with the amount of pyruvate kinase specified in the diagram, and in the absence of erythrocyte membranes. The concentration of pyruvate kinase was the rate limiting factor in this assay reaction. The initial phosphorylation rates of ADP and BrADP differed by a factor of 220, BrADP being more slowly. ADP (o), BrADP (●).

K_m was found to be 230 µM, i.e., 50 times higher than that for ATP. It should be stressed, that these results (Fig. 31) were obtained using a 50 fold larger amount of pyruvate kinase than in the usual assay mixture. (Under these assay conditions the pyruvate kinase then
Figure 31  BrATP as a substrate of the erythrocyte membrane Ca,Mg-ATPase.

The Ca,Mg-ATPase activity of human erythrocyte ghost membranes with BrATP as the substrate was measured as described in Fig. 22, but with 50 times the usual amount of pyruvate kinase. A K_m for BrATP of 230 ± 10 μM was calculated, the V_max being 0.15 μmoles/h x mg protein, instead of 1.20 μmoles/h x mg protein in the case of ATP.

turned over the BrADP at a V_max of 0.7 μmoles/mg membrane protein x h.)
5. Discussion

5.1. General

Since the experiments on the Ca,Mg-ATPase of erythrocytes described in this work were done without considering a postulated activator protein (72,73), it is pertinent to briefly discuss this problem here.

Hanahan et al. (74) first found, that human erythrocyte membranes prepared at low ionic strength showed less Ca,Mg-ATPase activity than those prepared at physiological ionic strength. This was confirmed also in the course of the work presented here. Therefore, membranes were always prepared by a cyclic procedure of hypotonic lysis and isotonic washings (chapter 3.1.). The loss of activity in hypotonically prepared membranes might conceivably be due to the formation of small membrane vesicles, which do not sediment during centrifugation (10 to 40 min at 20,000 x g). Using such vesicles of a diameter of less than 0.2 μ and formed upon aging of erythrocytes (75), latent Ca,Mg-ATPase activity could be detected only in the presence of large amounts of Triton X-100 (>20 mg Triton/mg membrane protein; not shown).

Addition of the "20,000 x g supernatant" of the hemolysate of erythrocytes, although free of accessible Ca,Mg-ATPase activity, to membranes was reported to increase their Ca,Mg-ATPase activity (72). This was interpreted with the presence of an activator of the Ca,Mg-ATPase in the hemolysate. Using membranes of low specific Ca,Mg-ATPase activity the "activator protein" stimulated the activity up
to 3 - 6 times but not more (72,73,76-79). Tests for "latent" ATPase activity were not carried out in these studies. It was found that the activator protein was 100 times more abundant than the Ca,Mg-ATPase itself (77).

The activator protein was "purified" by several groups. However, no correlation could be found between a stained band and the eluate of fractions of analytical polyacrylamide gels (77), or the activator might not be of protein nature, since the absorbance at 260 nm was 50% larger than at 280 nm (78). An activation of the Ca,Mg-ATPase could also be obtained with the activator of cAMP nucleotide hydrolase (EC. 3.14.17.), Troponin C, or Parvalbumin (78,79). Recent work (80-82) claims also an effect of the activator on the calcium transport in erythrocytes.
5.2. The Lipid Requirement of the Ca,Mg-ATPase

The erythrocyte membrane consists of 50 % (w/w) protein, 10 % carbohydrates, 20 % cholesterol, and 20 % phospholipids (83). Ways and Hanahan (84) determined the following phospholipid composition, which was confirmed by others (83,85) (the predominant fatty acid composition is given in parenthesis): Sphingomyelin 24 % (24:0, 24:1), phosphatidylcholine 30 % (18:2), phosphatidyl ethanolamine 26 % (20:4, 22:5, 22:6), phosphatidyl serine 15 % (18:0, 20:4). The phospholipids are asymmetrically distributed (85-88):

![Diagram showing the distribution of phospholipids in the inner and outer leaflets of the erythrocyte membrane.]

(inner membrane leaflet) Sph (outer membrane leaflet) PC (inner membrane leaflet) PE (outer membrane leaflet) PS

(the length of the shaded bars is proportional to the amount of the respective phospholipid).

Phospholipase A (EC 3.1.1.4) from Naja naja or porcine pancreas requires Ca$^{2+}$ (89-91), and degrades all glycerophospholipids
but not sphingomyelin (83). The products, fatty acids and lysocompounds, may be removed by washing with BSA (92).

Phospholipase C (EC 3.1.4.3) from Clostridium welchii or Bacillus cereus splits the phospholipid head-group off, producing diglycerides, which aggregate to droplets of 300-1000 nm in diameter (93,94). They may be removed by lipase treatment (94), or by extraction with dry ether (92). Phospholipase C from Cl. welchii does not degrade PS, but all other glycerophospholipids and sphingomyelin; B. cereus phospholipase C has the same specificity, but also slowly degrades PS (65,91). Sphingomyelinase C does not degrade glycerophospholipids (65).

The Ca,Mg-ATPase of human erythrocyte membranes is physiologically maintained active by phospholipids, artificially also by either fatty acids, or lysocompounds, or both of them (Fig. 3). This agrees with a later finding of Roelofsen and Schatzmann (65), that total degradation of the glycerophospholipids by phospholipase A$_2$ from porcine pancreas inhibited the Ca,Mg-ATPase to 80-85%, but to 100%, when fatty acids and lysocompounds were removed by BSA (65).

Fig. 4 shows, that the ATPase activity is lost completely, when 50 % of the phospholipids are degraded. This may be due to the following: (i) sphingomyelin, which makes up 25 % of the membrane phospholipids, is not degraded by phospholipase A$_2$ from N. naja, and has no influence on the ATPase activity (64,65), (ii) PS is degraded fastest (91), and actually seems to maintain the ATPase activity (Fig. 7). Concurring results were also obtained by others: Wheeler et al. (95), using the detergent Lubrol for unspecific lipid removal from erythrocyte membranes, observed complete loss of Na,K-ATPase activity at 90-100 % removal of phospholipids. (This ATPase was reported to require PS to be active (92)). Coleman and Bramley (63) noticed complete loss of the erythrocyte Ca,Mg-ATPase activity at 40-70 % phospholipid hydrolysis using an only partially purified phospholipase C from Cl. welchii, although
this phospholipase, when pure, does not degrade PS (65,91). Roelofsen and Schatzmann (64,65) found direct proportionality between the percentage of glycerophospholipids remaining and the percentage of Ca,Mg-ATPase activity, when using pure phospholipase C from B. cereus or Cl. welchii. In conclusion, inactivation studies do not definitely exclude PS from keeping the Ca,Mg-ATPase in an active state.

Phospholipid depletion caused noncompetitive inhibition relative to ATP of a Ca,Mg-ATPase of Escherichia coli (96) or sarcoplasmic reticulum (93), and noncompetitive inhibition relative to calcium in the erythrocyte Ca,Mg-ATPase (65). This agrees with the proposal, that removal of phospholipids affects the affinity of the substrate site (Ca$^{2+}$, ATP) reversibly and decreases the maximal turnover rate of the ATPase, most probably by a gradual conformational change.

Fig. 5 shows, that Triton X-100 reactivates the lipid depleted erythrocyte Ca,Mg-ATPase, and that this effect is most probably not due to increased substrate permeability, since cholate exhibited no effect. This parallels a report by The and Hasselbach (98) on a preparation of sarcoplasmic Ca,Mg-ATPase, delipidated by the action of phospholipase A$_2$ (N. naja) and BSA. This ATPase could be reactivated by addition of (a suboptimal amount of) 1.5 μmoles Triton X-100/mg protein.

The possibility, that Triton X-100 surrounds the Ca,Mg-ATPase and that it reactivates specifically because of its hydrophobic part, is improbable for the following reasons: (i) other lipids like LPC, PC, PE, etc. fail to reactivate; (ii) the reactivation by Triton X-100 was much less pronounced in membranes degraded more extensively. The explanation could thus be, that Triton reactivates, because it allows undegraded lipids to reassemble to a more favorable configuration around the ATPase.

The results in Fig. 3 demand, that either fatty acids or lysoglycerophospholipids, or both of them, protect the
Ca,Mg-ATPase activity and Fig. 6 suggests, that only fatty acids do so. Meissner and Fleischer (97) reported, that oleic acid reactivated the phospholipid depleted sarcoplasmic Ca,Mg-ATPase at 0.4 μmoles/ mg protein, and The and Hasselbach (98,99) found an optimal amount of 0.7 - 1.06 μmoles/ mg protein for the same system. They determined an optimal chain length of C_{16} to C_{18}, having the double bond around C(9). Using a partially purified, solubilized preparation of erythrocyte Ca,Mg-ATPase for reconstitution, Peterson et al. (100) recently confirmed the reactivating effect by oleate.

After lipid depletion by phospholipase C, and without removal of the diglycerides formed, all researchers observed an inhibition of the erythrocyte Ca,Mg-ATPase after addition of fatty acids (63-65), and an activation by lysolecithin (63-65). In the preparation described here lysolecithin had no reactivating effect. However, also Meissner and Fleischer (97) and The and Hasselbach (99) could reactivate a preparation of phospholipase A treated sarcoplasmic Ca,Mg-ATPase with lysolecithin.

Among the phospholipids used for reactivation of the erythrocyte Ca,Mg-ATPase, those negatively charged were found to be most efficient. This was also true for phospholipids which are not normally found in erythrocyte membranes (cardiolipin, phosphatidyl inositol, and phosphatidic acid, which is present only in a small amount). Among the phospholipids normally found in the erythrocyte membrane, PS was clearly the best in reactivation. That PS naturally keeps the Ca,Mg-ATPase active is further supported by the following: (i) The outer phospholipid leaflet of the membrane may be digested completely without loss of enzyme activity (65). PS is conferred almost exclusively to the inner side of the membrane (85). (ii) The degradation of PS somewhat parallels the decrease in ATPase activity (discussion above; 63-65,101). (iii) Treatment of membranes with phospholipase C from Cl. welchii, that de-
grades all phospholipids except PS, inactivates the ATPase to only 64 % (65). This inhibition is probably due to the decreased ratio of lipid to protein. (iv) PS was found to reactivate best also in membranes consisting of sphingomyelin, diglycerides and small amounts of residual glycerophospholipids (64,65). (v) Also the Na,K-ATPase of erythrocytes exhibits specificity for PS (92).

Degradation of sphingomyelin (65) or addition of sphingomyelin to glycerophospholipids depleted membranes (65) did not affect Ca,Mg-ATPase activity. Splitting off the polar head group of phospholipids leads to inactivation of the ATPase (63-65,101,102). This does not necessarily mean, that only the polar head groups keep the enzyme active, since also the diglycerides formed separate into small droplets (93,94). Generally, these diglycerides complicate the interpretation of reactivation studies (63-65).

Marinetti (103) concluded from crosslinking experiments of PE and PS with dinitrodifluorobenzene, that one third of the total PS is tightly clustered around the proteins, whereas the other two thirds occurs as di-,tri-, or tetramers, surrounded by other phospholipids.

Better understanding of the interaction of the Ca,Mg-ATPase with phospholipids will be possible once the enzyme is available in pure form.
5.3. PROTEASE TREATMENT OF INTACT ERYTHROCYTES

It was first reported by Knauf et al. (27), that in erythrocyte membranes a phosphoprotein formed upon Ca,Mg-stimulation, presumably the Ca,Mg-ATPase, resisted to almost complete proteolysis of the membrane proteins facing the plasma side, i.e. essentially bands 3, PAS 1, and PAS 3 (104). The results presented here confirmed, that the Ca,Mg-ATPase activity is only slightly decreased (20 %, Table 1), the amount of phosphorylated protein roughly similar to the control (30 % increased), and that the molecular weight changes by less than 10 000, upon pronase or chymotrypsin treatment. Thus, the Ca,Mg-ATPase presumably spans the membrane to enable calcium transport and its release into the hydrophilic phase, but does not protrude into the plasma space with any part cleavable by chymotrypsin or pronase. This picture still agrees with results obtained by Roelofsen and Schatzmann (64,65) showing that degradation of the phospholipids of the outer leaflet of the membrane does not decrease Ca,Mg-ATPase activity although degradation of the inner leaflet does so.

Bender et al. (105) showed, that glucose transport of erythrocytes decreased by <20 % during extensive treatment with pronase. It is generally accepted, that pronase or chymotrypsin treatment of erythrocytes fragments band 3 to a 65,000 MW protein remaining membrane bound (52,66,105,106), spanning the membrane (52,66,106), leaving the NH₂-terminal intact (52,66), and a 30,000 MW protein, representing the COOH-terminal, containing some 20 % carbohydrates (104) and being highly hydrophobic (66).

A molecular weight of 126,000 as found in this work is in good agreement with data from Knauf et al. (142,000 - 150,000, 27), Wolf (105,000 - 145,000, 28), and Drickamer (135,000 - 170,000, 34).
The question, whether the Ca,Mg-ATPase "belongs" to band 3 is still unanswered. Usually, the term "band 3" is only an operational designation for one or several proteins to migrate on SDS-polyacrylamide gels in a broad diffuse zone of an average molecular weight of 95,000. The reason for the diffuse zone is generally thought to be heterogenous glycosylation of the dominant protein (107), i.e. the anion carrier. Actually, the individual protein molecules of band 3 were found to band always at the same $r_f$-value within the diffuse zone (107). The glyceraldehyde-3-phosphate dehydrogenase is thought to be part of the anion carrier too. Also, PAS-1 bands in the band 3 region (104), as well as the acetylcholinesterase (104), and the Na,K-ATPase (5). The heterogeneity in proteins of band 3 is further supported by the impossibility to degrade band 3 completely by pronase- or chymotrypsin-treatment. This indicates the presence of resistent protein peaks (51). Apart from the data of Knauf et al. (27), there is no reason to exclude the Ca,Mg-ATPase from band 3, when one understands band 3 as an operational gelelectrophoretic designation of a family of proteins.
5.4. Solubilization of the Ca,Mg-ATPase

Wolf and Gietzen (108) first published, that the erythrocyte Ca,Mg-ATPase may be selectively solubilized by Triton X-100. However, they specified only the concentration of detergent, neglecting the protein concentration and the ratio of detergent to protein needed for solubilization. This omission makes the reproducibility of the work (28,54,55,109,110) difficult. Since the critical micellar concentration of Triton X-100 is low (0.016% w/v, 111), and protein concentrations of more than 1 mg/ml are used during solubilization, the ratio of Triton X-100 to protein, rather than the absolute concentration of Triton, becomes the important parameter in solubilization. This is further supported by results presented here (3.4.), showing that the Triton X-100 solubilized Ca,Mg-ATPase may be concentrated up to 10 times without any loss of activity, although the concentration of Triton increases to 5%. Furthermore, the preparations of Wolf and coworkers (28,54,55,109,110) lost activity completely within 2 days (Fig. 12).

The method described here, allows to extract the Ca,Mg-ATPase either selectively together with band 3 at a low yield, or with up to 60% yield (Table III), thereby solubilizing also bands 1,2, and 4.5 (Fig. 11). This solubilized Ca,Mg-ATPase, stored at 4°C, still retained 30% of its initial activity during 7 days. This stability may compare to the preparation by Dixon and Hokin of Lubrol extracted Na,K-ATPase from Electrophorus electricus, which retained 60% of its activity, when stored at 0°C for 6 days (41). The stabilization by phospholipids may be explained by the phospholipid requirement of the Ca,Mg-ATPase (4.2.). The nature of stabilization by polyols like glyc-
erol, although also true for the Ca,Mg-ATPase of sarcoplasmic reticulum and the F$_1$-ATPase of mitochondria, is still unknown. However, protection of the substrate site on the enzyme is likely.

As Fig. 14 shows, membranes of pronase- or chymotrypsin-treated erythrocytes release the Ca,Mg-ATPase only at a 2- to 3-fold higher concentration of Triton X-100 than membranes of untreated cells. One may assume, that the fragment split from band 3 is very hydrophilic, due to its high carbohydrate content of about 20% by weight (104). Thus, the residual 65,000 fragment of band 3 would be more hydrophobic and need more detergent for solubilization than the intact protein. However, recalculation of Drickamers data (66) on the band 3 fragments shows the 65,000 MW fragment to be more hydrophilic than the original 95,000 MW protein.

Triton X-100 seems to be one of only a few detergents that solubilize the Ca,Mg-ATPase in an active form. Wolf (112) reported Tween 20 to be partially effective, whereas deoxycholate, widely used for sarcoplasmic reticulum Ca,Mg-ATPase, inactivated the erythrocyte Ca,Mg-ATPase by more than 95% as soon as solubilization occurred (this work, not shown; and 112).

(The "soluble Ca,Mg-ATPase" preparations reported by Quist and Roufogalis (113) and by Weidekamm and Brdiczka (114), obtained by incubation of intact membranes at low ionic strength are not considered to represent the solubilized Ca-transporting ATPase described here. This is further supported by Kirkpatrick's work (115).)
5.5. Purification of the Solubilized Ca,Mg-ATPase by Gel Chromatography on Sepharose 6B

Gel filtration on Sepharose 6B proved to be a partially useful tool in purifying the Triton X-100 solubilized Ca,Mg-ATPase of erythrocyte membranes (Table 4). Almost no inactivation occurred and complete recovery of the Ca,Mg-ATPase activity was achieved, but the purification factor amounted to only about 5. Since during gel chromatography the proteins stay in micelles of detergent and phospholipids, they become more uniform in apparent size, which is thought to be the reason for the low separating effect of the Sepharose 6B column. Even degradation of band 3, the major contaminant, to a fragment of 65,000 MW by protease treatment did not allow separation from the Ca,Mg-ATPase of MW about 130,000.

It should be stressed, that although a large Triton X-100 micelle peak eluted from the Sepharose column, when run in Tween 20, a large amount of Triton remained still bound to the Ca,Mg-ATPase and its contaminating proteins, which could be eluted from these proteins, when adsorbed to hydroxylapatite (not shown).

The points made by Wolf et al. (28) about the purification of the solubilized enzyme could be confirmed in this work (not shown), i.e. loss of activity, when the enzyme is subjected to chromatography with or without detergent and in the absence of phospholipids, and the ineffectiveness of divalent metal ions to prevent this.

Wolf et al. (28) have used a technique almost identical to the one described here to purify this enzyme on crosslinked Sepharose CL-6B. (Actually, the method described here has been taken and modified from theirs.) They usually started with a solubilized enzyme much more contaminated with spec-
trin than the preparation used here. Their separation pattern is very similar to the one presented here. However, their $V_t$ (total volume) of the Sepharose CL-6B column seems to be considerably overestimated. Recalculation of their data, using hemoglobin as the reference peak, shows their Ca,Mg-ATPase activity to elute at a $K_{av}$ of about 0.285, which happens to be identical to the peak position found in this work with buffer system II. As was suggested in the results section (4.5.), the position of this peak might suggest the enzyme to be eluted as a dimer, which is further supported by the fact, that Wolf and coworkers' gels (28) show, that a considerable part of band 3 was not solubilized, but aggregated, and thus eluted in the void volume. Therefore, the enzyme was most probably not dissociated, due to the low amount of detergent present. There is some kinetic support for the assumption of an enzyme dimer from the reports by Ferreira and Lew (10), And Galo et al. (116), who found a Hill coefficient of 2 for the Ca,Mg-ATPase.

The yield in ATPase activity of the Sepharose 6B column (Table 4) agrees well with the report of Wolf et al. (28). However, the protein estimates differ by a factor of almost 30, since in the case of Wolf et al. the procedure of Lowry was used (44), without properly correcting for interfering substances. Wolf et al. (28) claim to have obtained the pure Ca,Mg-ATPase after Sepharose CL-6B gel chromatography, and they show a gel with 3 so-called subunits of this ATPase: $\alpha$ of MW 145,000, $\beta$ of MW 115,000, and $\gamma$ of MW 105,000. "Subunit $\alpha$" agrees well with the known molecular weight of the Ca,Mg-ATPase (27,34, this work), and could be labelled as a $^{32}$P-phosphoprotein. Also, this band commonly occurs in membranes, and has a molecular weight of about 150,000, but is of yet unknown nature. The Ca,Mg-ATPase makes up some 0.02% of the total membrane protein (27,43), and may thus be seen as only 3%
of the total protein in the reported stage of purification (assuming Wolf et al.'s protein determination to be correct). "Subunits $\beta$" and "$\gamma$" represent classical bands remaining intact after partial proteolysis of band 3, which, in their hands, most probably occurred between chromatography and gel electrophoresis.

In conclusion, Wolf et al. (28) did not prepare a pure Ca,Mg-ATPase, but instead, highly purified band 3 together with the ATPase, which makes up only a very minor part of the total protein. Further points in favour of this conclusion are: (i) The ATPase is not eluted as a complex of these subunits, since the size of the eluted particle corresponds to only one "subunit" (155,000 "MW"). (ii) The amino acid composition of Wolf and coworkers' "Ca,Mg-ATPase" (54) agrees within 20% with the one published by Furthmayr et al. (117) on the pure band 3, with the only exception of lysine. (iii) As reported in this work (chapter 4.3.), the "pure Ca,Mg-ATPase" according to Wolf, i.e. band 3, may be degraded to a 65,000 MW fragment affecting neither the Ca,Mg-ATPase activity and the yield in phosphoprotein formation, nor the position of the phosphorylated band on SDS-polyacrylamide gels.
5.6. PREPARATIVE ISOELECTRIC FOCUSING OF A PARTIALLY PURIFIED PREPARATION OF THE CA,MG-ATPASE

Isoelectric focusing proved to be rather ineffective in separating the solubilized and Sepharose 6B purified Ca,Mg-ATPase from contaminating proteins, i.e. mainly the anion carrier protein, or its 65,000 MW fragment. Apparently, these proteins have a very similar pI (4.8 to 5.0). Similarly, Das et al. (118) found most of the erythrocyte membrane proteins to band between pH 3.8 and 4.8 in isoelectric focusing on polyacrylamide gels. Shami et al. (119), using a system similar to the one described here, but with 1% Triton X-100 and 1.5% ampholines, found band 3 as a precipitate at pH 4.2. Peter- son et al. (100), using an analytical isoelectric focusing system, located the Ca,Mg-ATPase between pH 4 and pH 7, but did not achieve any purification. It should be stressed, that only PC - which is electroneutral over a wide pH range - was added to the solubilized Ca,Mg-ATPase, because the negatively charged phospholipids would move into the acidic electrode buffer, together with the ATPase.

The problem of the precipitation of the proteins during focusing (120) might conceivably be circumvented by using the method of preparative flat-bed isoelectric focusing on Sephadex (121). The results presented here confirm, that isoelectric focusing is a technique applicable in a very early stage of purification (121), rather than to a partially purified protein.
5.7. Investigation of the Substrate Site of the Ca,Mg-ATPase

Using Analogues of ATP

Tables 8 to 10 provide detailed information about the properties of the ATP analogues used in this work. It should be kept in mind, that most ATP or ADP requiring, enzyme-catalyzed processes were found to consist of 2 steps: a binding step and a subsequent reaction step (122,123).

\textbf{rro ATP}

Because of its open ribose ring rroATP has a much higher conformational mobility than ATP (124):

\begin{center}
\includegraphics[width=0.3\textwidth]{rroatp.png}
\end{center}

This additional mobility does no longer allow the formation of a stable complex with Mg\textsuperscript{2+} (Table 8). The exocyclic group orientation and the heterocycle / ribose orientation are no longer restricted (Table 9). It was suggested (58), that the large change in enthropy needed for binding and fixation of rroATP might be the reason for its partial ineffectiveness as a substrate, since rroATP is structurally very similar to ATP.

In rat liver mitochondria rroATP (or rroADP) showed a binding constant of about 90 \( \mu \text{M} \) towards the adenine-nucleotide carrier \( (K_m \text{ ATP} = 41 \mu \text{M}) \), but was not transported across the membrane (58). rroADP and rroATP were competitive inhibitors of the ADP/ATP translocator.

Spinach chloroplasts bound rroADP to their \( \text{CF}_1 \)-ATPase, but
<table>
<thead>
<tr>
<th>Compound</th>
<th>Bond length 1)</th>
<th>Bond angle 1)</th>
<th>metal binding constants 2)</th>
<th>second pK$_a$ 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1.61</td>
<td>130</td>
<td>4500</td>
<td>1800</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>1.68</td>
<td>127</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>AMP-PCP</td>
<td>1.79</td>
<td>117</td>
<td>12900</td>
<td>4800</td>
</tr>
<tr>
<td>rroATP</td>
<td>1.61</td>
<td>130</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>BrATP</td>
<td>1.61</td>
<td>130</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

1) Determined for sodium pyrophosphate and its analogs
2) Measured in 100 mM KCl, 25 mM Tris-Cl, pH 7.4 at 25°C
3) Refer to (70)

* Each value is larger than the corresponding one for AMP-PCP.
** Each value is smaller than the corresponding one for ATP.
TABLE IX  Conformation of ATP and of some of its analogues (140).

<table>
<thead>
<tr>
<th>Compound</th>
<th>heterocycle to ribose ring orientation</th>
<th>pseudorotation of the ribose ring</th>
<th>exocyclic group orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>anti &gt; syn</td>
<td>53% S, 47% N</td>
<td>gg &gt; (tg, gt)</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>anti &gt; syn</td>
<td>nd.</td>
<td>nd.</td>
</tr>
<tr>
<td>AMP-PCP</td>
<td>anti &gt; syn</td>
<td>nd.</td>
<td>nd.</td>
</tr>
<tr>
<td>rroATP</td>
<td>no preferred orientation</td>
<td>not existing (open ring)</td>
<td>no rotational restrictions</td>
</tr>
<tr>
<td>BrATP</td>
<td>syn</td>
<td>nd.</td>
<td>tg ~ gt &gt; gg</td>
</tr>
</tbody>
</table>

Concerning symbols for conformational descriptions refer to Table 10. nd. = not determined.
TABLE X Parameters for the description of the conformation of ATP, and of its analogues (123,140).

1) pseudorotation = puckering of the ribose ring:

The terms "endo" and "exo" refer to the orientation of a C-atom towards, or away from N(9).

N-type: C(3') endo, C(2') exo

activation energy at room temperature: 20 kJ/mol for ATP, = 10^9 rotations/sec.

S-type: C(3') exo, C(2') endo

2) exocyclic group orientation C(4') - C(5'); Newman projections:

3 possible rotamers:

gauche-gauche: gauche-trans: trans-gauche:
TABLE X  (continued)

3) syn-anti equilibrium of the heterocycle / ribose ring orientation:

syn:

\[
\begin{align*}
\text{NH}_2 & \quad \text{NH}_2 \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{OH} & \quad \text{OH} \quad \text{OH} \\
\end{align*}
\]

\[\phi = 150 \pm 45 ^\circ\]

anti:

\[
\begin{align*}
\text{NH}_2 & \quad \text{NH}_2 \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{OH} & \quad \text{OH} \quad \text{OH} \\
\end{align*}
\]

\[\phi = -30 \pm 45 ^\circ\]

activation energy at room temperature: 26 kJ/mol for ATP, \(= 10^8\) rotations/sec
they could not phosphorylate it (125). The overall process of phosphorylation requires $\text{Mg}^{2+}$; probably MgADP is the actual substrate (126). The lack of a stable Mg-complex of rroADP might therefore be the reason for the lack of phosphorylation of rroADP, since in addition the binding process was largely independent of $\text{Mg}^{2+}$ (127).

The results presented here show very weak binding of rroATP to the enzyme ($K_a \sim 1$ to 2 mM), no splitting of rroATP by the ATPase, and most probably competitive inhibition with respect to ATP. The high value for $K_a$ suggests a specific requirement of the ATPase for the conformation of the ATP molecule in solution, i.e. one or more of the following: (i) the exocyclic group orientation $C(4') - C(5')$, (ii) the anti orientation of the heterocycle towards the ribose ring, or (iii) the structure of the ribose. On the other hand, it cannot be excluded that ATP is bound as its $\text{Mg}^{2+}$-complex (involving the $\alpha$- and the $\beta$-phosphate group, and the fourth orbital of N(7)), and rroATP would then fail because of its unstable $\text{Mg}^{2+}$-complex.

**AMP-PNP**

The P-NH-P bond length and angle of AMP-PNP as well as its conformation (Table 9), are close to the ones of ATP (Table 8). However, the affinity of AMP-PNP to $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$ is at least 3 times higher than the one of ATP (Table 10). Because of its less electronegative nitrogen, AMP-PNP has a $pK_a$ for the second proton of the $\gamma$-phosphate group of only 7.8 (Table 8). AMP-PNP is thought to resist cleavage by ATPases (70), which was confirmed recently in the case of the Na,K-ATPase from renal medulla (128). Various reports about the mitochondrial ATPase complex (18, 60, 129, 130) suggest, that AMP-PNP inhibits its hydrolytic site ($K_m[\text{ATP}] \sim 5 \times 10^{-4} \text{M}$) competitively with a $K_i$ of 0.1 - 1.3 $\mu$M. AMP-PNP is thereby not split and the oxidative phosphorylation site for ADP remains unaffected. Using the calcium modulated heavy meromyosin ATPase as a model, Yount
et al. (131) found competitive inhibition by AMP-PNP and a $K_i$ of 3.1 $\mu$M ($K_m[ATP]=1.2$ $\mu$M). (However, a Lineweaver–Burk plot for $1/[ATP]$, as constructed from the data presented in the Dixon plot, shows bent curves).

In the experiments presented here, an inhibition constant for AMP-PNP towards the red blood cell Ca,Mg-ATPase of 200 $\mu$M at a $K_m[ATP]$ of about 10 $\mu$M was found. In comparison to the other systems discussed above (where $K_i - K_m$), this suggests a more specific structural requirement of the Ca,Mg-ATPase in the region of the terminal phosphate groups than usual. (This may concern the complete dissociation of the protons of the $\gamma$-phosphate group, the P-N-P bond length or angle, or the stability of the AMP-PNP-Mg complex). The type of noncompetitive inhibition (i.e. the affinity of the Ca,Mg-ATPase to ATP remains constant, but its maximal velocity in presence of inhibitors is decreased even at infinite substrate concentration) is unexpected. There are essentially two explanations for this behaviour: (i) the AMP-PNP binds at a site different from the ATP binding site, but still affects the binding or the catalytic site, (ii) AMP-PNP binds to the binding site and forms a stable complex, which can not be readily dissociated by high concentrations of ATP. Explanation (i) may be rejected, because it seems unlikely from the results presented here, that AMP-PNP should bind to a different site than rroATP, AMP-PCP, BrATP, and ATP taking into account its rather similar structure. Explanation (ii) seems more logical. In all likelihood, AMP-PNP is not so reactive, that the binding site should be blocked by a covalently bound complex of Enzyme-AMP-PNP. Three reports, however, suggest that the Enzyme-AMP-PNP complex dissociates only in the range of minutes (18, 60, 130). Recalculation of the results of Philo and Schwyn (130) on the soluble beef heart mitochondrial ATPase shows also, that the dissociation of the E-AMP-PNP complex (at 50 $\mu$M AMP-PNP, 30°C, and pH 7.6) is about 200 times slower than its formation (this ratio increases proportionally with increa-
sing concentration of AMP-PNP). The decay rate of the ATP complex is several orders of magnitudes faster (122). Thus, the inhibition of the Ca,Mg-ATPase by AMP-PNP is most probably competitive in nature, but in the assay described appeared to be noncompetitive, because of the slow dissociation rate of the Enzyme-AMP-PNP complex. Kinetically, this view agrees with the present concepts in enzymology (132, 133).

**AMP-PCP**

AMP-PCP is not split by ATPases (70,134). It binds Mg$^{2+}$ and Ca$^{2+}$ with an affinity 3 times higher than ATP (Table 8), and its γ-phosphate group shows a dissociation constant pK$_a$ for the second proton of only 8.5 (Table 8). The geometry of the terminal phosphate groups of AMP-PCP differs substantially from both ATP and AMP-PNP. The P-C bond is 10% longer than the regular P-O bond, and the P-C-P bond angle is much more acute (117°) than the corresponding one in ATP (130°). This is most probably the reason for the lower affinity of AMP-PCP to the ATPase (K$_m$ = 320 - 250 μM) as compared to AMP-PNP (K$_m$ 200 - 230 μM). The competitive type inhibition was expected.

Yount et al. (131) found a K$_i$ of 200 μM at a K$_m$ of 1.2 μM in the case of the Ca-modulated heavy meromyosin ATPase, and observed competitive inhibition. Hegyvary and Post (134) determined for the Na,K-ATPase of guinea pig kidney cortex in flow dialysis experiments a K$_i$ of 10 μM and a K$_m$ ATP of 0.2 μM.

**BrATP**

Due to the large bromine atom in position C(8), BrATP has its adenine ring system oriented strictly syn instead of anti (Table 9). This causes a lowered affinity to Mg$^{2+}$ and Ca$^{2+}$ (Table 8) and a different exocyclic group orientation (Table 9). Since in ATP the activation energy for the anti to syn conformational change is only 26 kJ/mole (at 25°C, Table 10, 123), ATP may be bound by the enzyme in either position. Only preliminary results have been published on this point.
so far (71,135).
In the experiments presented here (Fig. 28,29), a $K_i$ for BrADP to the erythrocyte Ca,Mg-ATPase of 75 to 90 $\mu$M was found. The inhibitor was competitive to ATP. BrATP was split by the ATPase at a 10 times lower rate than ATP. The $K_m$ for BrATP did not differ significantly from its $K_i$. The results do not allow to decide, whether the low affinity of BrATP, decreased 20-fold relative to ATP, is due to its inversely oriented heterocycle or its lack of Mg$^{2+}$ complexation. The orientation of the exocyclic group plays probably a minor role, since its rotational barrier is low compared to the one of the heterocycle.

Nucleoside diphosphate kinase (E.C. 2.7.4.6) isolated from beef liver (71) or measured in situ in mitochondria from rat liver (135) showed an identical $K_m$ for BrATP and ATP. This enzyme therefore does not require a specific orientation of the heterocycle. The mitochondrial adenine nucleotide carrier bound BrATP similar to ATP with a $K_i$ of about 50 $\mu$M. However, it was not translocated (136). Another ATP analogue with a substituted C(8), 8-azido-ATP, showed a $K_m$ of 100 $\mu$M with the Na,K-ATPase of human erythrocytes (137).

Table 11 shows a summary of results obtained with several ATP analogues for a few selected systems.

The mitochondrial ADP/ATP carrier apparently requires for nucleotide binding at least 3 negative charges on the phosphate chain and is highly specific for the N(1) - C(6) region of the adenine ring. In addition, to induce transport, the sugar moiety has to be closely related to ribose. In fact, the only analogue known to be transported is 9-(3'-deoxyribofuranosyl)-adenine-5'-triphosphate (3'dATP) or the respective diphosphate compound (123). An analogous requirement for the (3')-hydroxyl group however, was found for the leucocyte adenosine carrier (139).

The Na,K-ATPase was found to be primarily specific in the region of the C$_5$ amino group of the adenine ring (as the ADP/
### TABLE XI  Inhibition of some ATP-dependent enzymes by analogues of ATP.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Mitochondrial ADP/ATP carrier</th>
<th>Leucocyte Na,K-adenosine ATPase, various sources</th>
<th>Ca,Mg-ATPase erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-P-NH-P-β (AMP-PNP)</td>
<td>3 (70)</td>
<td></td>
<td>20 (*)</td>
</tr>
<tr>
<td>γ-P-CH$_2$-P-β (AMP-PCP)</td>
<td></td>
<td>50 (134)</td>
<td>75 (*)</td>
</tr>
<tr>
<td>2′dATP</td>
<td>2 (58)</td>
<td></td>
<td>25 (*)</td>
</tr>
<tr>
<td>BrATP</td>
<td>1 (136)</td>
<td></td>
<td>20 (*)</td>
</tr>
<tr>
<td>GTP</td>
<td></td>
<td>3 (139)</td>
<td>800 (138) (20) (102)</td>
</tr>
<tr>
<td>ITP</td>
<td></td>
<td>2 (139)</td>
<td>1200 (138) (12) (102)</td>
</tr>
<tr>
<td>Analogue</td>
<td>Mitochondrial adenosine ATPase, ADP/ATP carrier</td>
<td>Leucocyte Na, K- adenosine ATPase, ADP/ATP carrier</td>
<td>Various sources erythrocytes</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>CTP</td>
<td><img src="image" alt="CTP Structure" /></td>
<td>5 (139)</td>
<td>350 (134, 9(102) 138)</td>
</tr>
</tbody>
</table>

All values tabulated equal $K_i$(analogue)/$K_m$(ATP), except values in (), which represent $V$(ATP)/$V$(analogue), when the concentration of the analogue is 1 mM. The second part of the columns with the figures in () gives the literature references. (*) = measured and given in this work.
ATP carrier). The triphosphate chain and the ribose moiety ranged second in importance (134,138).
In the case of the Ca,Mg-ATPase of erythrocytes it seems best to assume analogous requirements (compare also to 16), since the results presented do not allow further description.

In conclusion, an affinity chromatography on ATP-Sepharose 4B with the ATP linked at its C(8) position over a spacer to the Sepharose 4B seems to be a promising tool in further purifying the Ca,Mg-ATPase of human erythrocytes.
6. Abstract

The Ca,Mg-ATPase of human erythrocyte membranes was found to have a specific lipid requirement for phosphatidylserine. The Ca,Mg-ATPase was selectively solubilized by Triton X-100, and its stability could be considerably increased by addition of phospholipids, and of glycerol. Chromatography of the solubilized Ca,Mg-ATPase on Sepharose 6B yielded a preparation contaminated mainly with the erythrocytes anion carrier. Preparative isoelectric focusing on a sucrose gradient, of this Sepharose-purified Ca,Mg-ATPase did not allow separation of the ATPase from the anion carrier. Pronase- or chymotrypsin-treatment of intact erythrocytes degraded the anion carrier to a 66,000 MW fragment. However, this fragment could not be separated from the Ca,Mg-ATPase by chromatography on Sepharose 6B, or by preparative isoelectric focusing, either. The effect of some analogues of ATP on the Ca,Mg-ATPase was tested in intact membranes. It is concluded, that ATP substituted at its C(8) position and linked to Sepharose 4B, is a promising tool in further purifying the Ca,Mg-ATPase by affinity chromatography.
ZUSAMMENFASSUNG


Ein reproduzierbares Verfahren zur Solubilisierung der Ca,Mg-ATPase und zur Chromatografie in gemischten Detergens / Phospholipid - Mizellen auf Sepharose 6B wurde aus demjenigen von Dieckvoss et al. (Poster No. P 316, FEBS Symposium on the Biochemistry of Membrane Transport, Zurich, 1976) entwickelt.

Die solubilisierte Ca,Mg-ATPase konnte durch Zugabe von Phospholipiden und Glyzerin stabilisiert werden. Der Erythrozyten-Anionencarrier stellte die hauptsächliche Proteinverunreinigung der selektiv solubilisierten und auf Sepharose chromatografierten Ca,Mg-ATPase dar. Der Abbau dieses Proteins zu einem 65 000 MG Fragment durch Pronase- oder Chymotrypsin-Behandlung intakter Erythrozyten erlaubte ebenfalls keine Trennung von der Ca,Mg-ATPase (MG ca. 125 000).

Präparative isoelektrische Fokussierung in Triton X-100 auf einem Saccharose-Gradienten führte weder zur Trennung der Ca,Mg-ATPase vom Anionencarrier, noch von dessen 65 000 MG Fragment.

Analoge des ATP mit einem geöffneten Ribose-Ring, mit β,γ-Phosphatgruppen, die durch eine Amino- oder Methylen-
7. References

   Active calcium transport and Ca\(^{2+}\)-activated ATPase in human red cells.

   Regulation of intracellular calcium.

   Variable Ca sensitivity of a K-selective channel in intact red cell membranes.

4. T.J.B. Simons, J. Physiol. (Lond.) 256, 227-244 (1976)  
   Ca-dependent K-exchange in human red blood cell ghosts.

   The influx of calcium into human erythrocytes during cold storage. Influences of extracellular pH, intracellular ATP and efflux of univalent cations.


   Calcium movements across the membrane of human red cells.

   Ca-uptake by ATP-depleted red cells from different species with and without associated increase in K-permeability.

   On the ATP dependence of Ca-induced increase in K permeability observed in human red cells.

    Use of ionophore A 23187 to measure cytoplasmic Ca buffering and activation of the Ca pump by internal Ca.

    Dependence on calcium concentration and stoichiometry of the Ca pump in human red cells.
The effects of an antiserum to Na,K-ATPase on the ion-transporting and hydrolytic activities of the enzyme.

Ca transport in human erythrocytes. Separation and reconstitution of high and low affinity (Mg+Ca)-ATPase activities in membranes prepared at low ionic strength.

Activation of membrane-bound high-affinity calcium ion-sensitive adenosine triphosphatase of human erythrocytes by bivalent metal ions.

Ca activation of membrane-bound Ca,Mg-ATPase from human red blood cells prepared in the presence or absence of Ca.

Studies on a Ca-dependent ATPase of human erythrocyte membranes. Effects of Ca and H.

Calcium ion-dependent p-nitrophenyl phosphate phosphatase activity and calcium ion-dependent adenosine triphosphatase activity from human erythrocyte membranes.

Action of the adenosine triphosphate analog adenylylimidodiphosphate in mitochondria.

Ca-activated membrane ATPase: selective inhibition by ruthenium red.

Active calcium ion uptake by inside-out and right-side out vesicles of red blood cell membranes.

Determination of the stoichiometry of the calcium pump in human erythrocytes using lanthanum as a selective inhibitor.
   Investigation of the accompaniment of calcium during active calcium transport from human erythrocyte ghosts.

   Endocytosis in resealed human erythrocyte ghosts: abnormalities in sickle cell anemia.


   Transport parameters and stoichiometry of active calcium ion extrusion in intact human red cells.

   Association of (Ca+Mg)-ATPase activity with ATP-dependent Ca uptake in vesicles prepared from human erythrocytes.

   Electrophoretic separation of different phosphoproteins associated with Ca-ATPase and Na,K-ATPase in human red cell ghosts.

   Purification and properties of high-affinity Ca-ATPase of human erythrocyte membranes.

   Ca-stimulated membrane phosphorylation and ATPase activity of the human erythrocyte.

   Genetic abnormalities of cation transport in the human erythrocyte.

   Biochemical and clinical aspects of sarcoplasmic reticulum function.
The calcium transport ATPase of sarcoplasmic reticulum.

Comparison of sources of a phosphorylated intermediate in transport ATPase.

The red cell membrane contains three different adenosine triphosphatases.

Role of phospholipids in transport and enzymic reactions.

Lipid requirement of membrane-bound enzymes.

Reversible denaturation of enzymes by sodium dodecyl sulfate.

Purification and properties of the (sodium + potassium)-activated adenosine triphosphatase and reconstitution of sodium transport.

Purification and properties of Na,K-ATPase from pig brain.

Purification of (Na+K)-ATPase: active site determinations and criteria of purity.

Studies on the characterization of the sodium-potassium transport adenosine triphosphatase.

On the mechanism of Na- and K-stimulated hydrolysis of adenosine triphosphate.
   Assay of proteins in the presence of interfering materials.

   Protein measurement with the Folin phenol reagent.

   Cleavage of structural proteins during the assembly of the head of bacteriophage T4.

46. T. Cremona, E.B. Kearney, J. Biol. Chem. 239, 2328-2334 (1964)
   Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase.

   Characterization and physical properties of the major form of phospholipase A2 from cobra venom (Naja naja naja) that has a molecular weight of 11,000.

   Phospholipase A2 (phosphatide acylhydrolase, EC 3.1.1.4) from porcine pancreas.

   Microdetermination of phosphorous.

   Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes.

   Chemical characterization and pronase susceptibility of the Na+K pump-associated phosphoprotein of human red blood cells.

52. L.K. Drickamer, J. Biol. Chem. 251, 5115-5123 (1976)
   Fragmentation of the 95,000 dalton transmembrane polypeptide in human erythrocyte membranes.

   A proteolytic enzyme of streptomyces griseus. Purification of a protease of streptomyces griseus.
Purification of the high-affinity Ca-ATPase of human 
erthrocyte membranes by application of mixed micelles.

55. G. Dieckvoss, H.U. Wolf, Abstract and Poster No. P316, 
FEBS Symp. Zurich 1976 on the Biochemistry of Membrane 
Transport, (1976) 
Purification of the high-affinity Ca\(^{2+}\)-ATPase of human 
erthrocyte membranes.

A procedure for the estimation of microgram quantities 
of Triton X-100.

57. J.S. Fawcett, in "Methodological Developments in Bio¬
chemistry", ed. E. Reid, vol. 2 "Preparative Techni¬
ques", pp. 61-80 (1973) 
Isoelectric focusing.

58. K.S. Boos, E. Schlimme, D. Bojanovski, W. Lamprecht, 
Properties of the ribose-ring-opened adenine nucleo¬
tide, 2,2'\[1'-\text{(9-adenyl)}-l'-(tri-,diphosphoryl-oxymethyl)]
-dihydroxydiethylether in mitochondrial adenine-nucleo¬
tide translocation.

59. M. Cohn, T.R. Hughes, J. Biol. Chem. 235, 3250-3253 
(1960) 
Phosphorous magnetic resonance spectra of adenosine 
di- and triphosphate.

60. H.S. Penefsky, J. Biol. Chem. 249, 3579-3585 (1974) 
Differential effects of adenylylimidodiphosphate on 
adenosine triphosphate synthesis and the partial re¬
actions of oxidative phosphorylation.

(1925) 
The colorimetric determination of phosphorous.

ward, L.L.M. vanDeenen, Biochim. Biophys. Acta 241, 
925-929 (1971) 
Action of pure phospholipase A\(_2\) and phospholipase C 
on human erythrocytes and ghosts.

63. R. Coleman, T.A. Bramley, Biochim. Biophys. Acta 382, 
565-575 (1975) 
Hydrolysis of erythrocyte membrane phospholipids by a 
preparation of phospholipase C from Clostridium welchii. 
Deactivation of (Ca+Mg)-ATPase and its reactivation by 
added lipids.
Some aspects of the Ca-pump in human red blood cells.

The lipid requirement of the (Ca+Mg)-ATPase in the human erythrocyte membrane, as studied by various highly purified phospholipases.

Fragmentation of the band 3 polypeptide from human erythrocyte membranes.

The organization of proteins in the human red blood cell membrane.

68. S.W. Peterson, present address: Department of Bacteriology, University of California, Los Angeles, USA.
personal communication.

Steady state kinetics.

ATP analogs.

Chromatographische Untersuchung des Substratverhaltens von 8-Brom-adenosin-5'-O-triphosphat gegenüber Nucleosiddiphosphatkinase.

A soluble protein activator of Mg,Ca-ATPase in human red cell membranes.

Studies on an activator of the (Ca+Mg)-ATPase of human erythrocyte membranes.

74. D.J. Hanahan, J. Eckholm, G. Hildenbrandt, Biochem. 12, 1374-1387 (1973)
Biochemical variability of human erythrocyte membrane preparations, as demonstrated by sodium-potassium-magnesium and calcium adenosine triphosphatase activities.
Release of spectrin-free vesicles from human erythrocytes during ATP depletion.

Observations on the (Ca+Mg)-ATPase activator found in various mammalian erythrocytes.

Purification of an activator of human erythrocyte membrane (Ca+Mg)-ATPase.

Partial purification of the (Ca+Mg)-ATPase activator from human erythrocytes: its similarity to the activator of 3':5'-cyclic nucleotide phosphodiesterase.

Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca+Mg)-ATPase.

Further studies on the red cell calcium pump using inside-out vesicles as a model.

Plasma membrane Ca transport: stimulation by a protein activator from red blood cells or bovine brain.

Modulator binding protein inhibits activation of red blood cell membrane (Ca+Mg)-ATPase.

Localization of red cell membrane constituents.

84. P. Ways, D.J. Hanahan, J. Lipid Res. 5, 318-328 (1964)
Characterization and quantification of red cell lipids in normal man.

The asymmetric distribution of phospholipids in the human red cell membrane.
Evidence for an asymmetric distribution of phospholipids in the human erythrocyte membrane.

Phospholipase A₂ as a probe of phospholipid distribution in erythrocyte membranes. Factors influencing the apparent specificity of the reaction.

Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases.

Haemolysis of intact human erythrocytes by purified cobra venom phospholipase A₂ in the presence of albumin and Ca.

The effect of fatty acids and of albumin on the action of a purified phospholipase A₂ from cobra venom on synthetic lecithins.

The use of phospholipases in the determination of asymmetric phospholipid distribution in membranes.


A structural study of the modification of erythrocyte ghosts by phospholipase C.

Lytic and non-lytic degradation of phospholipids in mammalian erythrocytes by pure phospholipases.
Lipid requirement of the membrane sodium-plus-potassium ion-dependent adenosine triphosphatase system.

Phospholipid requirements of ATPase of Escherichia coli.

The role of phospholipid in Ca-stimulated ATPase activity of sarcoplasmic reticulum.

Unsaturated fatty acids as reactivators of the calcium dependent ATPase of delipidated sarcoplasmic membranes.

Properties of the sarcoplasmic ATPase reconstituted by oleate and lysolecithin after lipid depletion.

Partial purification and reconstitution of the Ca,Mg-ATPase of erythrocyte membranes.

ATPase and phosphatase activities from human red cell membranes: II. The effects of phospholipases on Ca-dependent enzymic activities.

Active uptake of Ca and Ca-activated Mg-ATPase in red cell membrane fragments.

Arrangement of phosphatidylserine and phosphatidylethanolamine in the erythrocyte membrane.

The red cell membrane.
Proteins of the human erythrocyte membrane as modified by pronase.


Isolation and characterization of band 3, the predominant polypeptide of the human erythrocyte membrane.

The solubilization of high-affinity Ca-ATPase of human erythrocyte membranes.

Partial purification of soluble high-affinity Ca-ATPase of human erythrocyte membranes.

Phosphorylation of partially purified high-affinity Ca-ATPase of human erythrocyte membranes.

Solubilization of membranes by detergents.

Purification of the Ca-dependent ATPase of human erythrocyte membranes.

Calcium transport in human erythrocytes. Separation and reconstitution of high and low Ca affinity (Mg+Ca)-ATPase activities in membranes prepared at low ionic strength.

Extraction and localization of a (Ca+Mg)-stimulated ATPase in human erythrocyte spectrin.
Calcium and magnesium ATPases of the spectrin fraction of human erythrocytes.

Kinetic changes of the erythrocyte (Ca+Mg)-adenosine-triphosphatase of rats fed different fat-supplemented diets.

Isolation of the major intrinsic transmembrane protein of the human erythrocyte membrane.


Rapid quantitative separation of the major glycoproteins (PAS 1,2 and 3) from other human red cell membrane proteins in a non-denaturing medium by affinity chromatography.

120. A. Winter, C. Karlsson, LKB Application Note No. 219 (1976)
Preparative electrofocusing in density gradients.

121. A. Winter, H. Perlmutter, H. Davies, LKB Application Note No. 198 (1975)
Preparative flat-bed electrofocusing in a granulated gel with the LKB 2117 Multiphor.

Kinetic analysis of ATPase mechanisms.

123. K.S. Boos, Dissertation, Technical University of Hannover, (1977)
Bindungs- und Transportverhalten Pentose-modifizierter Adeninnucleotide gegenüber dem mitochondrialen Adeninnucleotid-Carrier.
Substrate properties of yeast tRNA(Phe) oxidized and reduced at the 3'-terminal ribose.

Properties of ribose modified ADP analogues in photophosphorylation of spinach chloroplasts.

Inhibition of photophosphorylation by ATP and the role of magnesium in photophosphorylation.

Effects of external factors on photophosphorylation and exchange of CF₁-bound adenine nucleotides.

Active transport of sodium and potassium ions by the sodium and potassium ion-activated adenosine triphosphatase from renal medulla.

Structure, function and regulation of the mitochondrial adenosine triphosphatase complex of rat liver.

Inhibition of the soluble ATPase from mitochondria by adenylylimidodiphosphate.

Interaction of P-N-P and P-C-P analogs of adenosine triphosphate with heavy meromyosin, myosin, and actomyosin.


134. C. Hegyvary, R.L. Post, J. Biol. Chem. 246, 5234-5240 (1971)
Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase.
Enzymatic properties of the inner and outer membranes of rat liver mitochondria.

Activity of 8-C-Bromoderivatives of ATP and ADP in the mitochondrial adenine nucleotide translocation system.

Interactions of a photo-affinity ATP analog with cation-stimulated adenosine triphosphatases of human red cell membranes.

On the specificity of the ATP-binding site of (Na+K)-activated ATPase from brain microsomes.

Membrane transport of nucleosides in rabbit polymorphnuclear leucocytes.

Investigations of mitochondrial adenine nucleotide translocation by means of nucleotide analogs.

Role of magnesium in the (Ca+Mg)-stimulated membrane ATPase of human red blood cells.

Studies on the active transport of calcium in human red cells.
Curriculum Vitae

I was born in St. Gallen on Dec 17, 1951, grew up in Rorschacherberg and attended there the usual schools.

1966 - 1970 Gymnasium in St. Gallen
1970 Matriculation
1970 - 1974 Studies in general chemistry, and finally biochemistry at the Division of Natural Sciences at the ETH in Zurich.
1974 Diploma (Dipl. Natw.) and diploma work at the Institute for Biochemistry II, Prof. G. Semenza, on the "Solubilization of sucrase from small-intestinal brush border membranes".
1974 - 1978 Work on this thesis at the Institute for Biochemistry III under supervision by Prof. E. Carafoli. Teaching and supervising in various laboratory courses, diploma works, and in 3 "FEBS Advanced Courses" on "Membrane Biochemistry: Transport and Bioenergetics", held in Zurich.