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THE RELATIONSHIP BETWEEN Mg+Ca-ATPase AND ACTIVE CALCIUM TRANSPORT IN RESEALED HUMAN ERYTHROCYTE GHOSTS

by

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ABSTRACT

Human red blood cell ghosts were prepared by a modification of the procedure of stepwise hemolysis (57). EDTA (1.0 mM) was included in the washing procedure to remove endogenous ATP and divalent cations. Ghosts resealed with appropriate amounts of ATP, calcium and magnesium were found to have Mg+Ca-ATPase activity and linearity was maintained up to thirty minutes. Active calcium transport could be studied in these ghosts by measuring the change in the cellular concentration of calcium over time by atomic absorption spectrophotometry.

Variation in the concentration of calcium in the loading medium resulted in an activation of Mg+Ca-ATPase and two peaks were evident on the activation curve. The high and low affinity Mg+Ca-ATPase were maximally stimulated at 0.25 and 5.0 mM calcium in the loading medium, respectively.

The velocity of calcium transport was also found to be dependent on the concentration of calcium in the loading medium

and was activated over the concentration range of 0.1 to 5.0 mM calcium. A change in the concentration of cellular calcium was not evident in the absence of added ATP. In contrast to the activation of Mg+Ca-ATPase two peaks were not obtained, and the activation curve had a sigmoidal appearance.

Comparison of the calcium activation curves of Mg+Ca-ATPase and calcium transport revealed a similarity in the shape and position of the low affinity part of the Mg+Ca-ATPase and calcium transport activation curves. A stoichiometry of two (Ca:ATP) was obtained in the low affinity activity range.

Ruthenium red (0.05 to 0.4 mM) selectively inhibited the low affinity Mg+Ca-ATPase and inhibited calcium transport over the same concentration range to a similar degree. Both low affinity Mg+Ca-ATPase and calcium transport were inhibited by external ruthenium red with an I_{50} of 0.2 mM.

Propranolol, quinidine and quinine (10^{-5} to 10^{-3}M) were found to be ineffective in stimulating or inhibiting Mg+Ca-ATPase when added to the internal and external aspects of the ghosts.

Manganese, added to the loading medium over a wide concentration range, was unable to substitute for calcium in activating Mg+Ca-ATPase.

External divalent cations calcium and magnesium further increased Mg+Ca-ATPase activities when added to the external medium. Maximal stimulation occurred at a concentration of approximately 3.0 mM and calcium was almost twice as effective as magnesium.

Signatures of Examiners

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DEDICATION

To Laurie and Julian

INTRODUCTION

Human erythrocytes maintain a low intracellular concentration of calcium by means of an active calcium transport system requiring ATP (1). Schatzmann proposed that active calcium transport was associated with Mg+Ca-ATPase, analogous to the Na, K pump system (11). Some similarities between the properties of Mg+Ca-ATPase in red blood cell membrane fragments and calcium transport in resealed ghosts have been reported as indirect evidence supporting this hypothesis (12,26,30). An association between these systems was also demonstrated when both activities were studied simultaneously in resealed human erythrocyte ghosts loaded with a single concentration (1mM) of calcium (30). However, with the finding of more than one Mg+Ca-ATPase in red blood cell membrane fragments (32,33,34) it became apparent that further direct evidence showing an association between Mg+Ca-ATPase was required. Therefore, in the present thesis the association of Mg+Ca-ATPase activity and active calcium transport has been reinvestigated in the same preparation of resealed ghosts over a wide range of calcium concentrations.

Ruthenium red which has been reported to selectively inhibit Mg+Ca-ATPase activity in red blood cell membrane fragments (40) was tested on resealed ghosts to determine whether this dye would also inhibit calcium transport. Ruthenium red was also

used as a tool for investigating the association between the calcium transport system and a Mg+Ca-ATPase.

It was considered that this transport system may be a important site of drug action, particularly for drugs involved in the mobilization of calcium, because of the multitude of physiological effects regulated by internal calcium. Drugs of interest which were studied include quinine, quinidine, propranolol and tetracaine. The red blood cell ghost was used as a model membrane system in this study since red blood cells can be obtained in large quantities and ghosts free of the intracellular contents can be prepared by the method of reversal of hemolysis. The red blood cell has previously proven to be invaluable for studying the mechanism of cation transport and asymmetric ouabain inhibition of Na,K-ATPase. Many similarities have been shown between mechanisms operating on this system and those on the plasma membrane of other tissues.

LITERATURE REVIEW

Homeostatic Mechanisms Controlling The Intracellular Concentration Of Calcium

The homeostatic mechanisms by which cells maintain a low intracellular concentration of calcium has been of interest to physiologists for the past seventy years. In mammalian cells, the intracellular concentration of calcium has been estimated to be between 10^{-7}M and 10^{-5}M in contrast to a calcium concentration of 10^{-3}M in the extracellular fluid (1,2). Maintenance of a low intracellular concentration of calcium is of vital importance to the survival of the cell as an increase over the normal level has a profound effect on cellular and enzyme functions. For instance, in human erythrocytes intracellular concentrations of calcium greater than 10^{-4}M inhibits Na,K-ATPase which controls the distribution of sodium and potassium across the plasma membrane (3,4). A number of other enzymes inhibited by calcium are shown in Table I. It has also been accepted that intracellular calcium is a coupling factor in excitation-contraction coupling in muscle cells (6). In human red blood cells, intracellular calcium has been shown to regulate the passive permeability of the plasma membrane to Na^+ and K^+ (7,8), the volume of the cell (9) and membrane deformability (10).

Table I. Ion Antagonisms (5)

Enzyme	Activating Ions	Inhibiting Ions
Methionine adenosyl-transferase	Mg or Mn + (K, NH_4,Rb)	Ca or Zn
Pantothenate synthetase	Mg or Mn	Ca or Zn
Pyruvate kinase	Mg + (K,Rb,Cs)	Ca and Na or Li
5'-nucleotidase	Mg	Ca
Argininosuccinate synthetase	Mg	Ca or Mn
Glutamine synthetase	Mg or Mn	Ca
Riboflavin kinase	Mg,Zn,Co or Mn	Ca
Inorganic pyrophosphatase	Mg	Ca or Zn
Phosphopyruvate hydratase	Mg,Zn,Mn or Cd	Ca or Sr
Myosin ATPase	Ca or (K or NH_4)	Mg
Glycyl-leucine dipeptidase	Zn or Mn	Ca

A number of well-established homeostatic mechanisms by which cells control the distribution of calcium across the plasma membrane have been summarized as follows:

1. Passive permeability; Plasma membranes in the resting state are relatively impermeable to calcium under physiological conditions. However a slow exchange of calcium across the plasma membrane is evident (12,13). The distribution of calcium across the plasma membrane is also not according to the equilibrium potential as calculated by the Nernst Equation (2). In red blood cells the relatively impermeable nature of the plasma membrane does not account for the low intracellular concentration of calcium since calcium is taken up in cells depleted of

ATP or stored in the cold (7). This observation indicates that the distribution of calcium is maintained only in a metabolically active cell.

2. Active calcium transport; In human erythrocytes (12,13), He La cells (44) and L cells (45), calcium has been shown to be transported across the plasma membrane against an electrochemical potential by a mechanism requiring ATP.

3. Na-Ca Coupling; In nerve (14,15) and cardiac muscle (47), one intracellular calcium ion is exchanged for two to three sodium ions by a system not coupled to a chemical reaction.

4. Intracellular organelles;

(a) Sarcoplasmic reticulum - The sarcoplasmic reticulum is a network of tubules, vesicles, and cysternae surrounding the myofibrils (9). This structure rapidly accumulates calcium by an active transport system closely associated with a magnesium dependent ATPase (54). These structures are extremely important for relaxing the contracture of skeletal and cardiac muscle cells.

(b) Mitochondria - Mitochondria actively accumulate calcium by an active calcium transport system requiring ATP. Mitochondria may be important in maintaining a low intracellular concentration of calcium in nerve or in muscle having a poorly developed sarcoplasmic reticulum (16,17,64,69).

5. Intracellular binding and chelation; Calcium is also bound to anionic sites (such as on proteins) and chelated to molecules such as carboxylic acids and nucleotides. The relative importance of calcium binding to these sites is not known.

Active Transport In Human Red Blood Cells

Red blood cells have proved to be a useful model system for studying active transport of cations and solutes. These cells are available in large quantities in a homogeneous form and the plasma membranes can be isolated by relatively gentle means for enzyme studies. Perhaps for this reason the coupling of a transport process to a chemical reaction was first identified to occur across the plasma membrane of the red blood cell.

Wilbrandt in 1939 (19) was the first to demonstrate that the distribution of sodium and potassium was related to glycolysis. Inhibition of glycolysis by iodoacetate and sodium fluoride led to a loss of intracellular potassium and a change in the osmotic resistance of the cell. This study stimulated other workers to speculate that ATP was directly involved in the distribution of potassium, as ATP is synthesized solely by glycolysis in the human red blood cell. The importance of ATP in maintaining the intracellular levels of potassium was verified in experiments with reconstituted or resealed ghosts (22). Red blood cells may be partially hemolyzed by exposure to hypotonic solutions, usually with ionic strengths of not less than 0.02 M (20,21). The cells become leaky due to osmotic shock and ATP and other ions may be introduced into the cell. With the addition of a concentrated salt solution to this

hemolysate, the isotonicity can be restored and the restoration of low cation permeability thus obtained is adequate for studying active cation movements. Gardos (22) introduced ATP into resealed ghosts and demonstrated an accumulation of potassium by a system which required ATP. Schatzmann (23) demonstrated that this ATP dependent system was inhibited by ouabain. A link between potassium transport and the membrane bound enzyme Na,K-ATPase was recognized when Na, K-ATPase was found in crab nerve by Skou (24). Since then, Na,K-ATPase has been found in the plasma membrane of most cells, including the human erythrocyte (3). In red blood cells Na,K-ATPase and the active transport of sodium and potassium were inhibited by approximately 10^{-5}gm ml^{-1} of oubain (23). The stoichiometry and other properties of Na, K-ATPase system have now been well reviewed (24).

On the other hand, the finding of an energy dependent calcium transport system has only recently been demonstrated in red blood cells and the studies have been relatively few. Historically, Dunham and Glynn (3) in 1961 were the first to report the existence of a ouabain insensitive calcium activated ATPase in red blood cell membrane fragments (RBCMF). This enzyme which was found in these studies to have a specific activity two to three times as great as Na,K-ATPase was also found to be magnesium dependent. This enzyme will be referred to henceforth as Mg+Ca-ATPase. Schatzmann (11) reasoned that Mg+Ca-ATPase may be associated with a transport system which maintains a low concentration of intracellular calcium in red blood cells, analogous to the Na,K-ATPase system. Resealed ghosts loaded with

ATP, magnesium and calcium were found to rapidly lose cellular calcium when incubated at 37°C (Fig. 1,Panel A) (12). In ghosts loaded with 1.0 or 2.0 mM calcium, 80% of the cellular calcium was lost within ten minutes. In agreement with the hypothesis that Mg+Ca-ATPase was linked to this transport process, a concomittant increase in the concentration of inorganic phosphate (Fig. 1,Panal C) due to ATP hydrolysis was evident during the course of the calcium transport. In a previous study, Schatzmann (11) found that resealed ghosts lost calcium at a slow rate in the absence of added ATP. This result was attributed to an incomplete removal of endogenous ATP during the hemolysis procedure used in the preparation of the ghosts. This explanation was shown to be valid since cells depleted of energy by means of a 17 hr incubation in glucose free medium at 37°C showed no loss of cellular calcium unless ATP was included in the loading medium (Fig. 1,Panel B).

Ghosts resealed in the presence of 0.1 mM calcium lost calcium into an external medium containing 1.0 mM calcium. Therefore the loss of cellular calcium demonstrated by Schatzmann in resealed ghosts (11) appears to be due to an active transport system since the process was found to be dependent on ATP and operated against a concentration gradient.

However, the evidence linking calcium efflux to a Mg+Ca-ATPase was not direct. In resealed ghosts only intracellular calcium stimulated ATPase activity (12). This asymmetrical stimulation of Mg+Ca-ATPase activity by internal calcium is analogous to the Na,K-ATPase transport system where only external potassium

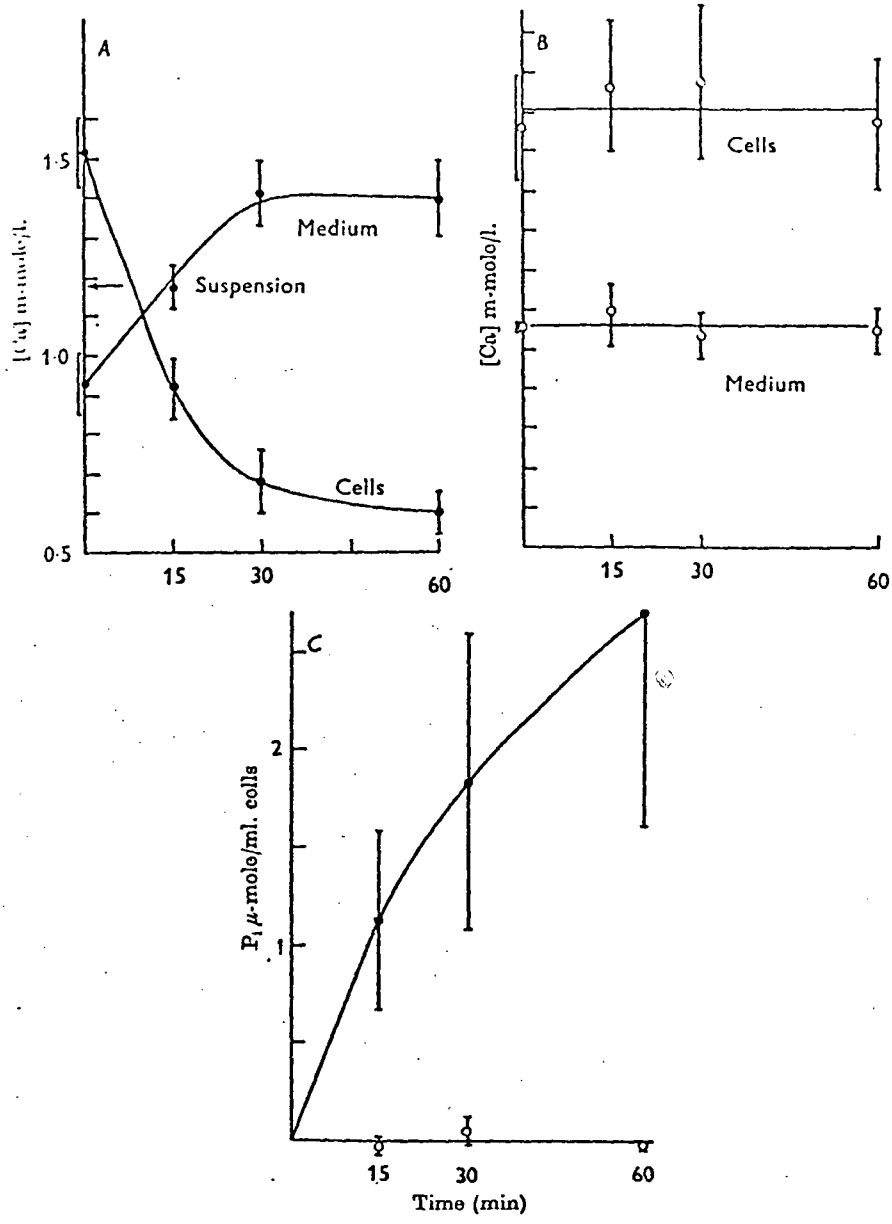


Fig. 1 Changes of Ca concentration in cells and medium and P_i release from ATP in resealed cells. Haemolysis in water containing 2 mM-Tris-ATP, 5 mM-Tris-Cl, 4 mM-$MgCl_2$ and 1 or 2 mM (in three expts. in the ATP free sample) $CaCl_2$. Reversal of haemolysis in presence of KCl. Previous to haemolysis starvation during 17 hr at 37° C in glucose-free solution (130 mM-Na, 5 mM-K, 20 mM-Tris, 155 mM-Cl). Medium: 130 mM-Na, 5 mM-K, 20 mM-Tris, 1 mM-Ca, 157 mM-Cl, 10^{-4} g/ml. ouabain. Temp. 37° C. Haematocrit: ATP sample 0·249, ATP-free sample 0·255. ● with ATP in the cells, ○ without ATP. Arrow in panel *A*: mean concentration measured in whole suspension. Four experiments, vertical bars 2 × S.E. of mean (12).

and internal sodium will stimulate Na,K-ATPase (65). Schatzmann also felt that the Mg+Ca-ATPase present in red blood cells was sensitive enough to be part of a calcium transport system which maintains a low intracellular concentration of calcium, which in the case of the human red blood cell has been estimated to be less than 5 X 10^{-5}M. In RBCMF Mg+Ca-ATPase was found to be optimally stimulated at 10^{-4}M calcium and had a threshold at 10^{-7}M.

Other evidence associating Mg+Ca-ATPase with active calcium transport was as follows:

(a) Ionic Requirements; Shin and Lee (26) were able to show that active calcium transport was dependent on intracellular magnesium. Since all the endogenous magnesium was not removed during the preparation of their ghosts, (ethylene diamine)-tetraacetic acid (EDTA) (a divalent metal ion chelating agent), was used to chelate the endogenous magnesium. Calcium transport was abolished in ghosts resealed in 1.0 mM EDTA, 2.0 mM $CaCl_2$, and 2.0 mM ATP.

(b) It has also been shown that strontium can substitute for calcium in activating Mg+Ca-ATPase in RBCMF (31) and that strontium is transported out of resealed ghosts by a mechanism requiring ATP (27).

(c) In resealed ghosts, active calcium transport does not depend on alkali metal ions for either stimulation of the velocity of calcium efflux or for a Na-Ca exchange reaction. In ghosts resealed with choline or in Tris buffer, rather than sodium or potassium, the velocity of calcium transport was not changed (26,12).

(d) Concomitant inward transport of magnesium was not observed in cells extruding calcium, nor did the addition of magnesium to the external medium stimulate the ATPase activity of resealed ghosts (79). Therefore the possibility that calcium transport is coupled to the inward movement of magnesium seems unlikely.

(e) Lastly, hydrogen ion does not seem to be coupled to calcium transport since the pH of a non-buffered medium did not change during active calcium transport (26).

2. Nucleotide specificity; Studies undertaken to determine the nucleotide requirement for calcium transport in resealed ghosts have been inconclusive. Lee and Shin reported that uridine triphosphate (UTP) and cytidine triphosphate (CTP) were as effective as ATP in supporting calcium transport in resealed ghosts (26). Guanosine triphosphate (GTP) was found to be less effective and inosine triphosphate (ITP) the poorest substrate. In another study, GTP and ITP were reported to be as effective as ATP (27). These facts do not support the contention that Mg+Ca-ATPase and calcium transport are closely associated since Mg+Ca-ATPase in RBCMF is specific for ATP (29). However, the conflicting reports on nucleotide specificity in resealed ghosts may be a result of incomplete removal of nucleoside diphosphokinase which can catalyze the synthesis of ATP by transfer of the phosphate from nucleoside triphosphates to ADP during preparation of the ghosts (35).

Calcium efflux is very dependent on the intracellular concentration of ATP (26). In cells pretreated with iodoacetate,

no significant transport of calcium was observed in the absence of ATP (Fig. 2). However, there was about 63% transport in ghosts not pretreated with iodoacetate in the absence of added ATP. It appears that ghosts contain approximately 0.3 mM of endogenous ATP, since approximately the same degree (58%) of calcium was transported in iodacetate treated cells when 0.3 mM ATP was added. In ghosts loaded with 1.0 mM ATP a more complete and faster removal of cellular calcium occurred.

3. Temperature dependence; Calcium transport is very sensitive to changes in temperature, as would be expected of a system coupled to a chemical reaction. High Q_{10} values of 3.5 (12) and 3.16 (26) have been reported for calcium efflux from resealed ghosts.

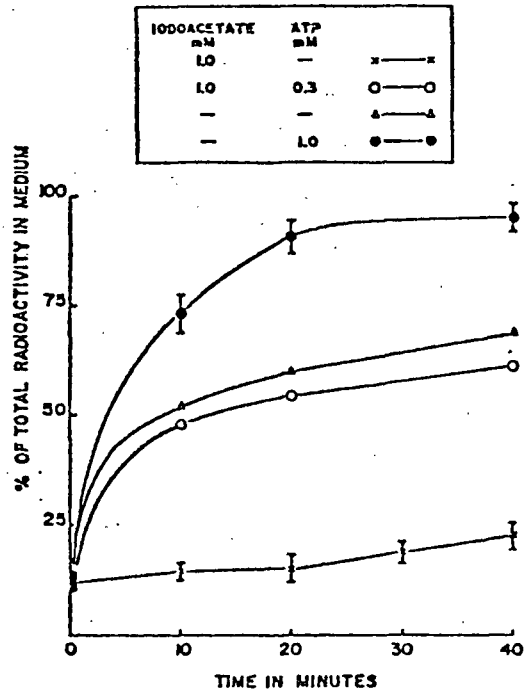


FIGURE 2. Effect of preincubation with iodoacetate. None + ATP (1 mM), the initial preincubation with iodoacetate was omitted. Other conditions, standard. None – ATP, no preincubation with iodoacetate and no ATP in lysing solution. Other conditions, standard. Iodoacetate – ATP, standard conditions except omission of ATP from solution. Iodoacetate + ATP (0.3 mM), standard conditions, except 0.3 mM ATP in lysing solution. Each point represents average of four experiments. Bars indicate standard error (SE)(26).

4. The effect of intracellular calcium on the velocity of calcium efflux; Romero and Whittam demonstrated that the velocity of calcium efflux was dependent on the intracellular concentration of calcium in red blood cells (7). Cells were loaded to give a range of internal calcium from 0.4 to 2.7 μmoles calcium per ml of cells, by incubating ATP depleted cells for various time intervals in a Ringer solution containing 10 mM calcium at 37°C. The cells were enriched with ATP by incubation with adenine and inosine and the rate of calcium loss was determined (Fig. 3). The rate of extrusion increased with increasing calcium concentration, reaching a plateau of about 2.6 μmole calcium per ml X hr. The internal calcium concentration giving half maximal activation was about 0.9 μmole per ml cells.

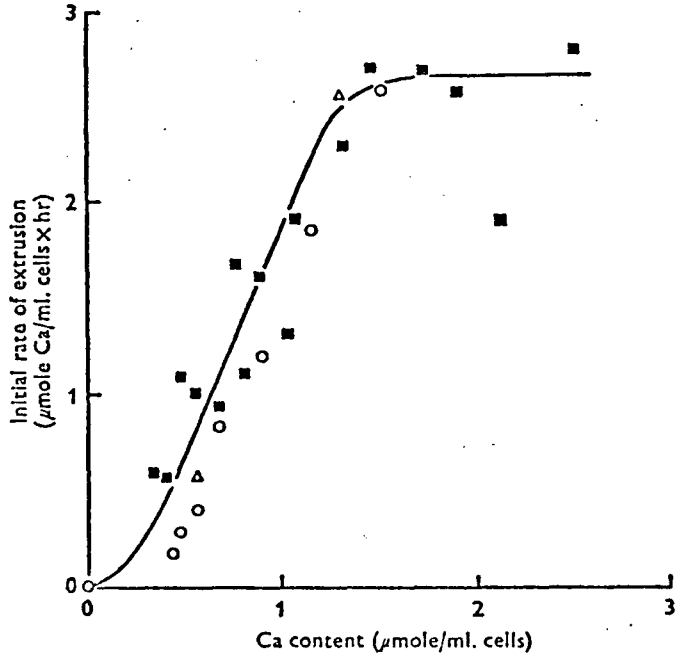


Fig. 3. The stimulation of calcium transport by internal calcium. Cells washed free of glucose were loaded with calcium by incubation at 37° C for periods up to 3 hr in Ringer solution containing (mM): NaCl, 150; $CaCl_2$, 10; Tris-HCl, 20, pH 7·6. Adenine (5 mM) and inosine (10 mM) were added, the incubation was continued and samples were taken at 10 min intervals. The rate of calcium extrusion (μmole calcium/ml. cells x hr) was calculated from the change in 10 min, and has been plotted against the mean cell calcium concentration during this time. Results are shown from three experiments (7).

Mg+Ca-ATPase Activity In Human Red Blood Cell Membrane Fragments

Since the discovery of an ouabain insensitive calcium stimulated ATPase in red blood cell membrane fragments (RBCMF), investigators have attempted to characterize this enzyme. The physiological role of Mg+Ca-ATPase however has not been completely agreed upon. As previously mentioned it was suggested that Mg+Ca-ATPase was associated with active calcium transport (12,26), whereas other investigators have felt that this enzyme may be responsible for regulating the cell volume (31). The Mg+Ca-ATPase reported by these latter workers is thought to be associated with a myofibrillar protein called spectrin (55).

Recently data has been obtained which may reconcile this difference in opinion of the functional role of Mg+Ca-ATPase. Careful studies have revealed that more than one Mg+Ca-ATPase is present in RBCMF, as differentiated by their affinities for calcium (30,32,33,34). Figure 4 illustrates the activation of Mg+Ca-ATPase in RBCMF found by Horton et al.(32). Activation by calcium appears at 10^{-8}M calcium and increases to 3.0 10^{-7}M calcium, at which point the rate of increase slows and a plateau is observed up to 3 X 10^{-5}M. The activity then rapidly increases to an optimum at 3 X 10^{-4}M calcium. The enzyme, maximally stimulated at 3 X 10^{-5}M calcium is referred to as the high affinity Mg+Ca-ATPase due to its higher affinity for calcium. The enzyme maximally stimulated at 3 X 10^{-4}M calcium is referred to as the low affinity Mg+Ca-ATPase. Other values reported in the literature indicate that the high affinity Mg+Ca-ATPase is optimally

stimulated from 10 to 50 μM calcium and the low affinity Mg+Ca-ATPase anywhere from 0.1 to 0.5 mM calcium (33,34,78,43,32). The wide variation of the reported values is attributed to the different methods used for the isolation of the RBCMF. Generally the high affinity Mg+Ca-ATPase has a specific activity of 0.3 to 0.5 μmoles Pi $mg^{-1}hr^{-1}$ compared to a specific activity of 0.6 to 0.88 μmoles Pi $mg^{-1}hr^{-1}$ for the low affinity Mg+Ca-ATPase. The presence of two Mg+Ca-ATPases has not been shown previously in whole red blood cells or in resealed ghosts. The finding of two Mg+Ca-ATPases in RBCMF further complicates studies attempting to relate active calcium transport and Mg+Ca-ATPase in resealed ghosts. Few kinetic studies have been done on these enzyme systems. The properties which have been determined are as follows:

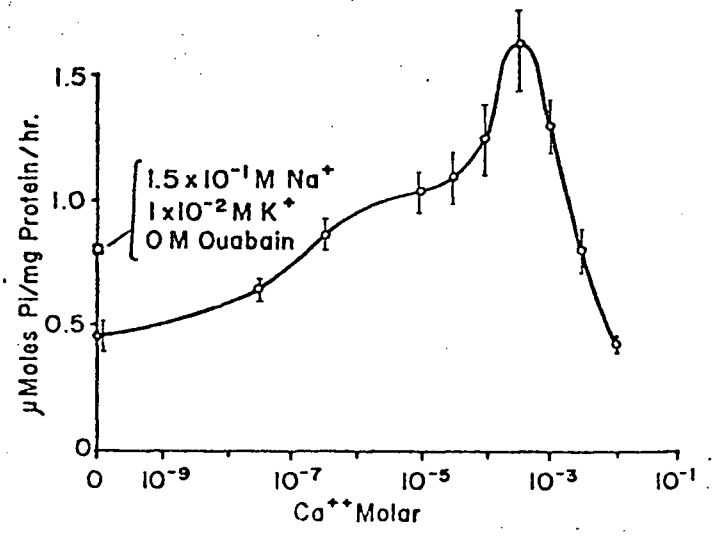


Fig.4 (Ca^{++})-dependent ATPase activity in human red cells.Each point represents the mean of four experiments with the standard error of the mean(32).

1. Ion requirements; Both high and low affinity Mg+Ca-ATPases require magnesium. Optimal concentrations of magnesium for these enzymes have been estimated to be between 3 to 8 mM(33).

2. Mg-ATP was shown kinetically to be the substrate for high affinity Mg+Ca-ATPase (33). Calcium acts allosterically as a positive autosteric effector (33). Whether or not Mg-ATP is the substrate for low affinity Mg+Ca-ATPase has not been determined.

3. Effects of other cations on Mg+Ca-ATPase activity; Both strontium and barium have been shown to be activators of Mg+Ca-ATPase (31). Strontium activates more strongly than calcium, but has a lower affinity. Careful kinetic studies were not done to determine which Mg+Ca-ATPase was activated by barium or strontium, but judging from the low affinity of these cations, the low affinity Mg+Ca-ATPase is activated at least. This finding is of special interest since strontium is also actively transported out of resealed ghosts, presumably by the same system as calcium (27).

There is evidence that Mg+Ca-ATPase in RBCMF is stimulated by univalent cations in the order of $K^+ > Na^+ > Rb^+$ (30,36). Potassium activates Mg+Ca-ATPase activity approximately 100% by a mechanism which is non-competitive with calcium (Fig. 5). The presence of both magnesium and calcium is required for this activation. Cesium and lithium were reported to have no stimulating effect, indicating that the activation by potassium, sodium and rubidium may not be due just to a change in ionic

strength (36). However, in a study done by Schatzmann and Rossi(30) lithium was shown to be about one-third as effective as potassium in activating Mg+Ca-ATPase. Sodium and potassium appear to act at the same site since no additional activation occurs when these ions are added together (36).

Activation by Na^+ or K^+ of Mg+Ca-ATPase occurs over a wide range of calcium concentrations (Fig. 6). Activation is still apparent at 1 mM, indicating that low affinity Mg+Ca-ATPase activity is activated by these cations. Schatzmann et al. (30) arbitrarily called the activity in the absence of added alkali metal cations alkali-cation-independent Mg+Ca-ATPase activity and in the presence of alkali metal cations, alkali-cation-dependent Mg+Ca-ATPase activity. Since alkali metal ions do not have any effect on active calcium transport in resealed ghosts, Schatzmann et al. (30) proposed that the extra activity seen in the presence of alkali metal cations was due to an uncoupling action of calcium on the Na,K-ATPase responsible for the transport of sodium and potassium. This hypothesis however, seems very unlikely. Bond and Green (36) felt that this effect may be a trivial property of RBCMF. For instance, these ions may expose latent Mg+Ca-ATPase activity by a membrane effect exposing the catalytic site of the enzyme. A similar effect is observed in RBCMF by freeze-thawing or detergents (34).

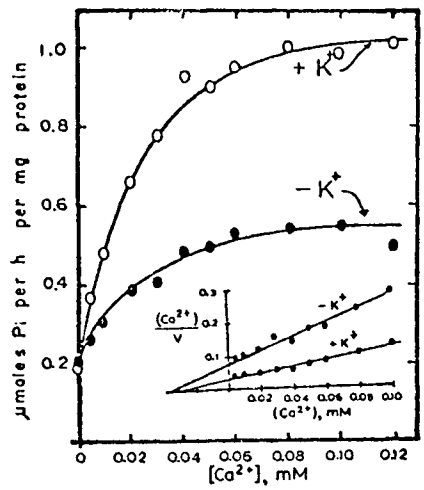


Fig. 5 ATPase activity as a function of Ca^{2+} concentration in the presence and absence of 80 mM K^+. Ordinate: total ATPase activity. The Ca^{2+} concentration given on the abscissa is the concentration added, without correction for binding by ATP or by membranes. The zero Ca^{2+} points may be slightly high, since EGTA (ethyleneglycol-bis(β-aminoethyl ester)-N,N'-tetraacetic acid) was not added to chelate possible traces of endogenous Ca^{2+}. The inset shows a Woolf plot of the data, from which K_m values were obtained. Other conditions are given in METHODS (36).

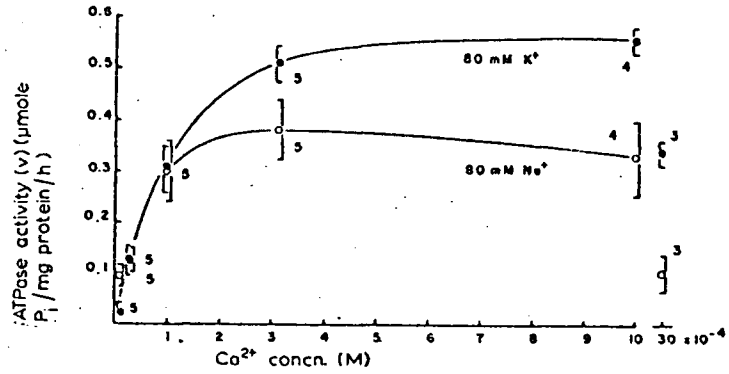


Fig.6 Effect of Na+ and K^+ on ATPase activity in hemoglobin free red blood cell membranes prepared by freezing and thawing (30).

Inhibitors Effecting Mg+Ca-ATPase And Active Ca^{2+} Transport In Red Blood Cells

In recent years numerous investigators have sought a specific inhibitor of Mg+Ca-ATPase, as ouabain has been invaluable in elucidating the mechanism of Na,K-ATPase. A specific inhibitor of Mg+Ca-ATPase and active calcium transport would be useful in providing direct evidence that calcium transport and Mg+Ca-ATPase are intimately associated. A number of drugs have been tested on this system. Among those without any effect on Mg+Ca-ATPase or calcium transport were oligomycin and ouabain (1,11) which are specific Na,K-ATPase inhibitors (37). Caffeine (1mg/ml) had no effect on active calcium transport in resealed ghosts (12).

A number of agents have been shown to be non-specific inhibitors of Mg+Ca-ATPase. Mersalyl (5.0×10^{-4}M)(11) and ethacrynic acid (10^{-4}M) (23) both inhibit ATP dependent calcium transport in resealed ghosts. However, these drugs have also been shown to equally inhibit Mg+Ca-ATPase and Na,K-ATPase activity in RBCMF in a concentration range of 10^{-6} to 10^{-3}M (5). Ethacrynic acid and mersalyl are thought to inhibit enzyme activity by binding to protein sulfhydryl groups (5). The lanthanides have been found to be useful agents for studying calcium interactions since they possess a similar radii to calcium but have much higher affinities for anionic sites due to a higher charge density (56). Holminium and praesodynium inhibit Mg+Ca-ATPase activity in RBCMF and active calcium transport in resealed ghosts, with an I_{50} of approximately 10^{-4}M (39).

However, these ions are non-specific as Na,K-ATPase and Mg-ATPase activities were also inhibited. Lanthanum has been useful for establishing the mechanism for calcium transport in mitochondria (70).

Watson and Vincenzi (40) recently reported that the inorganic dye ruthenium red selectively inhibits Mg+Ca-ATPase activity in RBCMF. Ruthenium red has previously been shown to react with carboxyl and sulphate groups on mucopolysaccharides (41). Table II illustrates the effect of ruthenium red on the ATPases of RBCMF. Mg-ATPase and Na,K-ATPase were not significantly inhibited by ruthenium red. However, both the alkali dependent and the alkali independent fractions of Mg+Ca-ATPase were almost completely inhibited by 6×10^{-5} M ruthenium red.

Table II

Effects Of Ruthenium Red On ATPases Of Erythrocyte Membranes (40)

(Values are expressed as μmoles of inorganic phosphate liberated per mg protein in 1 h.)

ATPases	ATPase activity		
	Ruthenium red concentration (M):		
	0	$6 \cdot 10^{-6}$	$6 \cdot 10^{-5}$
Mg+Ca-ATPase	0.88	0.55	0.09
alkali-cation-dependent	0.32	0.22	0.04
alkali-cation-independent	0.56	0.33	0.05
Na,K-ATPase	0.28	0.26	0.25
Mg-ATPase	0.11	0.11	0.10

This study did not distinguish between inhibition of high or low affinity Mg+Ca-ATPase as 0.12mM calcium was used in all studies. Considering the high concentration of calcium used, the low affinity Mg+Ca-ATPase, at least, was inhibited. The mechanism of action of this inhibitor also remains undetermined as the concentration of ATP, calcium and magnesium was not altered in its presence. The site of action of this drug also remains unknown, as the studies were done on RBCMF. However, ruthenium red shows potential as a tool for studying active calcium transport, since this drug has been shown to block active calcium transport in mitochondria (42).

Bader (43) recently reported that Mn^{2+} specifically activated low affinity Mg+Ca-ATPase, without affecting the high affinity Mg+Ca-ATPase in RBCMF. Bader also reported that low affinity Mg+Ca-ATPase activity was selectively inhibited by 10^{-3}M methylhydroxylamine and 5.0×10^{-5}M Cu^{2+} (EGTA: Cu^{2+} at a ratio of 10:1). This was the first report of selective inhibition of a Mg+Ca-ATPase. However, Cu^{2+} is a sulfhydryl group reagent, which renders membranes permeable to cations. Therefore this inhibitor would not be useful to study active Ca^{2+} transport in resealed ghosts or in whole red blood cells.

Active Calcium Transport In Other Tissues

Besides human erythrocytes, cultured He La cells (44) and L cells (45), ATP dependent calcium transport across the plasma membrane of other tissues has not been demonstrated. However,

a calcium or magnesium stimulated ATPase has been located in the sarcolemma of skeletal muscle (46), cardiac muscle and in guinea pig ileum (48). Hurwitz (48) suggests that ATP dependent active calcium transport may be important in lowering the intracellular concentration of calcium in guinea pig ileum, due to the poorly developed sarcoplasmic reticulum in this tissue. His hypothesis is supported by the fact that the ileum relies heavily on extracellular calcium for contractile activity. There is some evidence that calcium extrusion from squid axon may be ATP dependent (17) but the Na-Ca exchange mechanism may be more important across this plasma membrane.

There is some evidence that the Na-Ca exchange mechanism evident in nerve, may also operate in skeletal muscle (49), cardiac muscle (50) and smooth muscle (51). The requirement of external sodium for calcium efflux appears to be very specific, since external lithium, choline or potassium cannot substitute for sodium. It has been estimated that two to three external sodium ions are exchanged for one internal calcium ion by this mechanism (17). The direction of the Na-Ca exchange mechanism can be reversed in direction in nerves bathed in solutions deficient in external sodium. Other features of the Na-Ca exchange mechanism in cyanide poisoned squid axons are shown in Table III, taken from Baker (17). Cyanide was added in this preparation to release calcium from mitochondria and thus prevent interference of calcium uptake by mitochondria when making calcium flux measurements.

Table III

Features Of The Na-Ca Exchange Mechanism In Cyanide Poisoned Squid Axons(17).

1. It is temperature sensitive and has a Q_{10} of about three.
2. It is insensitive to high concentrations of the cardiac glycoside ouabain.
3. It is inhibited by externally applied lanthanum ions (80).
4. It is very dependent on the ionic composition of the external medium. Removal of external calcium reduces the efflux to a variable extent. Baker and Crawford (81) found no effect whereas Blaustein and Hodgkin (82) found that removal of external calcium reduced the efflux to about half (ie. 2 pmoles/cm^2sec to 1 pmole/cm^2sec). In the absence of external calcium, complete replacement of sodium by lithium,potassium, choline or sugar causes a further reduction of efflux to about one-twentieth of its original value, 0.1 pmole/cm^2sec.

Nerve and muscle cells also maintain a low intracellular calcium concentration by means of the sarcoplasmic reticulum and mitochondria (17,54). Both of these structures can accumulate calcium by an active ATP dependent system. Estimates of the calcium sequestering capacity of sarcoplasmic reticulum *in vitro* suggests that this membrane system is able to lower the cytoplasmic calcium concentration below the *in vivo* level required for contraction (approx. 10^{-6}M)(54). It has also been shown that the speed of calcium accumulation by sarcoplasmic reticulum is compatible with the time of tension decay in muscle (52). Calcium is accumulated against an activity gradient by an active transport system which is coupled to a Mg+Ca-ATPase (53). This enzyme has a low Km for calcium, (approx. 10^{-7}M) and a requirement for magnesium, optimal at 5.0 mM. A number of models describing this transport system have been reported (54). Since this transport system shares a number of

properties to the Mg+Ca-ATPase in human erythrocytes, a scheme taken from Martonosi (54) showing the molecular mechanism relating ATP hydrolysis to calcium transport is outlined as follows:

1. $E + ATP \rightleftharpoons E\text{-}ATP$

2. $E\text{-}ATP + 2Ca \rightleftharpoons E\begin{matrix}Ca\\ \diagup\\ \text{---}ATP\\ \diagdown\\ Ca\end{matrix}$

Outside

3. $E\begin{matrix}Ca\\ \diagup\\ \text{---}ATP\\ \diagdown\\ Ca\end{matrix} \rightleftharpoons E\sim P\begin{matrix}Ca\\ \diagup\\ \\ \diagdown\\ Ca\end{matrix} + ADP$

---

4. $E^*\sim P\begin{matrix}Ca\\ \diagup\\ \\ \diagdown\\ Ca\end{matrix} \rightleftharpoons E^* + P_i + 2Ca$ Inside

---

5. $E^* \rightleftharpoons E$

E=enzyme, E*=the conformationally altered form of the carrier.

In this scheme ATP binds to the enzyme in the absence of calcium (step 1), which promotes the binding of calcium (step 2). The bound calcium then activates the phosphate transfer from ATP leading to the formation of a phosphoprotein intermediate (step 3). The carrier than undergoes a conformational change with the translocation of calcium from the outer to the inner surface of the sarcoplasmic reticulum. Release of calcium from this low affinity carrier (step 4), followed by hydrolysis of the phosphoprotein, completes the cycle (step 5).

METHODS AND MATERIALS

Outdated citrated human blood (O positive) was obtained from the Canadian Red Cross and stored for not more than two weeks at 5°C. Blood was used within twenty days of collection.

Preparation Of Red Blood Cell Ghosts

Red blood cell (RBC) ghosts were prepared by a modification of the procedure of stepwise hemolysis outlined by Schrier (57). Whole blood (150 ml) was suspended in one volume of 0.155 M NaCl in eight 40 ml polypropylene centrifuge tubes. The tubes were centrifuged at 4°C for ten minutes at 2,000 X g in a refrigerated centrifuge (eg. International model, B-20, 870 angle head). The supernatant and white buffy layer were removed by suction. Considerable care was taken to remove the top buffy coat as completely as possible, despite a considerable loss of red cells (approx. one-third). The red cells were resuspended with a glass stirring rod and washed again in the same tubes with ten volumes of 0.155 M NaCl with subsequent removal of the supernatant and any remaining white cells. For a summary of the preparation, loading and assay procedures to be outlined refer to Table IV.

The cells were then partially hemolyzed by suspension in ten volumes of 0.08 M NaCl containing 1.0 mM EDTA (disodium salt) and centrifuged at 8,000 X g for ten minutes. Temperature

Table IV

Preparation Of RBC Ghosts, Loading, And Assay Procedure

I Preparation:

1. Wash with 0.155M NaCl (twice)
2. 0.08M NaCl + 1.0mM EDTA
3. 0.06M NaCl + 1.0mM EDTA
4. 0.04M NaCl + 1.0mM EDTA
5. 0.015M NaCl + 5.0mM TRIS-MALEATE, pH 7.1

II Loading:

1.0ml ghosts + 3.0ml of solution containing; 4.0mM Na_2ATP, 4.0mM $MgCl_2$, 10mM TRIS, pH7.1 and various concentrations of $CaCl_2$

III Resealing:

1. Add 0.2ml of 2.876M NaCl
2. Stand at R.T., 10 min.
3. Wash with 2.0mM $MgCl_2$, 0.125M NaCl, and 20mM TRIS-MALEATE (twice)
4. Suspend to 3.0ml in assay medium (as above) + 1mM $CaCl_2$

was maintained at 2-4°C during hemolysis procedures to ensure that the ghosts did not reseal immediately after hemolysis (58). EDTA was included in the hemolysis medium to remove endogenous divalent metal cations such as calcium and magnesium. Chelating agents have also been found to be useful in facilitating the removal of soluble enzymes and hemoglobin from the cellular interior (59) without affecting resealing (58).

The ghosts were then suspended in ten volumes of 0.06 M NaCl containing 1.0 mM EDTA and were centrifuged at 13,000 X g for ten minutes at 2-4°C. At this step the cells lost a considerable amount of hemoglobin as evidenced by a very dark supernatant. A much higher centrifugal force is required at this step to spin down the red cells as the cells have become swollen and therefore have much less density than the whole RBC. The ghosts were hemolyzed in ten volumes of 0.04 M NaCl containing 1.0 mM EDTA and centrifuged at 15,000 X g for ten minutes at 2-4°C. The ghosts were finally resuspended in ten volumes of 0.05 M NaCl and 0.005 M Tris-Maleate (pH 7.1) and centrifuged at 20,000 X g for ten minutes at 2-4°C. The pellet at this stage was pink in color. The pellet was also viscous in nature and could be poured into a 40 ml polypropylene centrifuge tubes. The solid red button remaining in the bottom of the centrifuge tubes consists of cells which would not hemolyze under these conditions or type III ghosts (58) and was discarded. These ghosts were used within fifteen minutes in the loading step.

Loading Procedure

1.0 ml of ghosts (approx. 10^8 ghosts or 5.0 mg of membrane protein) was pipeted into a 15 ml polypropylene test tube (calibrated) containing 3.0 ml of solution, to give a final concentration of 4.0 mM Na_2ATP, 4.0 mM $MgCl_2$ and 10.0 mM Tris-maleate (pH 7.1).Unless otherwise stated ,the concentration of calcium was varied in this medium. When required, monovalent cations and drugs were also included. The tbes were allowed to stand five to ten minutes to allow the ions to equilibriate before resealing at 2-4 °C (12).

Resealing And Washing Procedure

Partially hemolyzed red blood cell ghosts can retain their original shape and permeability by restoration of isotonicity (20,21). The RBC ghosts were resealed by the addition of 0.2 ml of 2.876 M NaCl solution. The tubes were well shaken and placed in a water bath at room temperature for exactly ten minutes. Restoration of isotonicity and the increase in temperature both facilitate resealing (58). Ghosts not allowed to stand for as long as ten minutes at room temperature were found to take up less calcium as measured by atomic absorption spectrophotometry since they did not reseal as completely and calcium was lost during the washing. The tubes were returned to the ice bath after ten minutes.

The resealed ghosts were washed with 5.5 ml of an isotonic solution containing 125.0 mM NaCl, 2.0 mM $MgCl_2$ and 20.0 mM Tris-Maleate (pH 7.1) at 5,000 X g for ten minutes at 2-4°C. The resulting pellet forms a compact pellet at this stage. The resealed ghosts were washed once more in 10.0 ml of the above solution. The clear supernatant was aspirated off leaving approximately 2.5 ml of solution. In most experiments 0.1 ml of 30 mM $CaCl_2$ was added to the tubes to give a final concentration of 1.0 mM calcium. Required amounts of ruthenium red and other drugs were also added at this stage. The resealed ghosts were finally brought to a final volume of 3.0 ml in the previously graduated test tubes with the addition of the isotonic washing solution.

Incubation Procedures

In all experiments the reaction was started by immersing the tubes in a shaking water bath at 37°C. A short delay of three minutes in reaching the temperature of 37°C was inevitable in these experiments as was evident in the efflux measurements (Fig. 13).

Determination of ATPase Activity

ATPase activity was determined by measuring the inorganic phosphate released from ATP by a modification of the method of Fiske and SubbaRow (60,63). The procedure is as follows:

The enzyme reaction was terminated by the addition of 1.0 ml of cold silicotungstic acid (8.0%) in perchloric acid (1.2 M), usually after a thirty minute incubation period. The tubes were placed in ice, to minimize ATP hydrolysis, for at least one hour. The tubes were then centrifuged at 20,000 X g at 0-4°C in a refrigerated centrifuge (eg. International model, B-20, 874 angle head). 3.0 ml of the supernatant was transfered into a second test tube (eg. 20 X 150 mm) containing 1.4 ml distilled water and kept in ice. At zero time, 0.4 ml of molybdate reagent was added followed immediately by the addition of 0.2 ml of aminonaptholsulfonic acid reagent and stirred on a vortex for fifteen seconds. The color was allowed to develop for thirty minutes at room temperature. After thirty minutes the color was read in a spectrophotometer (eg. Coleman-Hitachi 124) at 660 nm against a distilled water reference in a 1.0 cm light path cuvet. KH_2PO_4 (analytical reagent) was used as the primary standard.

ATPase activity was found to be linear up to thirty minutes in resealed ghosts (Fig. 7). Mg+Ca-ATPase activity was determined by measuring the difference in ATPase activity in the absence and presence of calcium in the loading medium. Since the ATPase activity in the absence of added calcium can not be estimated (eg. ghosts do not reseal under this condition) the activity in the absence of added calcium was estimated by extrapolation of the calcium activation curve to zero calcium.

Na,K-ATPase activity was determined by measuring the activity

in ghosts resealed with NaCl (2.876 M) with the addition of external potassium (0-15.0 mM). Ouabain (0.1 mM) abolished the activity stimulated by external potassium.

Na,K-ATPase and Mg+Ca-ATPase activity was reported as μmoles of Pi released from ATP per mg of membrane protein per hour (μmoles P_i $mg^{-1}hr^{-1}$).

Determination Of The Velocity Of Calcium Transport

Calcium transport or efflux was measured as the change in cellular calcium with time at 37°C. The incubation was terminated by the addition of 6.0 ml of an ice cold solution containing NaCl (119 mM) and lanthanum (6.0mM). The tubes were immediately placed in a ice bath. $LaCl_3$ was included in this solution to displace loosely bound external calcium and to inhibit the calcium transport system (83). This procedure was invoked in order to obtain a more accurate determination of intracellular calcium and calcium tightly bound in the membrane. In practice, little difference was found when the ghosts were washed with ice cold NaCl solution (0.155 M) alone. The tubes were centrifuged for ten minutes at 5,000 X g at 2-4°C. The supernatant was removed by aspiration and the pellet was washed once more in 6.0 ml of the same solution.

Calcium was extracted from the pellet by a slight modification of the wet ashing procedure reported by Sparrow et al.(61). Briefly, 1.0 ml of 3.0 M trichloracetic acid: glacial acetic acid, 1:1, was added to dissolve the pellet. The pellet was

resuspended with a vortex mixer and the tubes were placed for fifteen minutes in a water bath at 70°C. After this time, 2.0 ml of distilled water was added. The tubes were mixed by vortex and were placed in the water bath for ten minutes at 70°C. They were then allowed to cool to room temperature, allowing coagulation of the protein, and made up to a final volume of 5.0 ml with 30.0 mM $LaCl_3$ (approx. 2.0 ml) in a calibrated test tube. $LaCl_3$ was added to prevent interference from phosphates and inorganic ions in preparation for atomic absorption spectrophotometry. The precipitate was removed by centrifugation at 7,500 X g for ten minutes. The concentration of calcium in the protein free supernatant was determined by measuring the absorption at the 4227 Å line from a hollow cathode calcium lamp on a Varian-Techtron atomic absorption spectrophotometer, model AA-5. A nitrous oxide-acetylene flame was used. A lamp current (hollow calcium cathode lamp) was set at 4.0 mA and the slit width at 125 μ. Standard $CaCO_3$ solutions were prepared from standard reference solutions obtained from Fisher Scientific Company. A standard curve obeying Beers law was obtained between 10-100 μM calcium.

Protein Assay

The concentration of protein was determined by the method of Lowry (62) for insoluble proteins. Bovine serum albumin (Sigma, 3X recrystallized) was used as a standard. Standard curves were linear from 50 to 250 μg of protein when read at

500 nm. 1.0 ml of the hemoglobin free ghost preparation was found to contain 4.4 mg of membrane protein.

Hemoglobin free ghosts were prepared by washing the ghosts three additional times in 0.015 M NaCl and 0.005mM Tris-Maleate (pH 7.1) for ten minutes at 20,000 X g. Temperature was controlled at 2-4°C during these washings. No measurable change in the specific activity of Mg+Ca-ATPase between hemoglobin containing and hemoglobin free ghosts was obtained.

Washing Procedure

During the course of this work, it was found that the method for washing the polypropylene test tubes was very critical for obtaining maximal ATPase activities. Therefore the following washing procedure was adopted. The test tubes were soaked overnight in tap water containing 0.5% Liquinox detergent. The tubes were rinsed with tap water and soaked in 10% HNO_3 for at least one hour in 5.0 mM EDTA (tetrasodium salt). The tubes were well rinsed with tap water and then rinsed ten times with glass distilled water and allowed to dry in air.

A loss of low affinity Mg+Ca-ATPase activity results in test tubes soaked in 0.5% Liquinox alone, presumably due to a build up of detergent in the tubes.

RESULTS AND DISCUSSION

Properties Of The Red Blood Cell Ghost Preparation

Of fundamental importance to this study was the preparation of a red blood cell ghost population which could be made 'leaky' and then resealed to regain the original cation permeability characteristics of the whole cell. It was also important that the activity of the various ATPase systems were not destroyed during the preparation. In previous studies, Schatzmann et al. (12) and others (26,27) prepared ghosts by a method involving a one step hemolysis, followed by resealing. This procedure did not allow a complete removal of endogenous enzymes which can synthesize ATP (70). Furthermore, endogenous divalent metal cations and ATP were not completely removed by this method. Therefore in order to reduce the endogenous cations and ATP concentrations in these preparations, the cells were preincubated at 37°C for 17 hr in glucose free medium (12) or were incubated in the presence of iodoacetate (26). These two methods are undesirable since some destruction of the plasma membrane must occur as evidenced by hemolysis during incubation.

In this study, therefore, a modification of the procedure of stepwise hemolysis as outlined by Schrier (57) was adopted as described in the Methods. EDTA (disodium salt) (1.0 mM)

was included in the washing solutions to insure removal of Mg^{2+}, Ca^{2+}, ATP and soluble enzymes (74). Care, however, had to be taken in using EDTA in membrane preparations, since under certain conditions EDTA solubilizes membrane bound proteins (67). Furthermore, total removal of membrane divalent cations, Mg^{2+} and Ca^{2+}, could result in a ghost population which may not reseal, as these ions are thought to be structural components of the plasma membrane (67).

Under the conditions used in this study (see Methods), the preparion remained relatively impermeable to divalent and monovalent cations and retained ATPase activity. Evidence supporting the viability of this preparation for transport studies was as follows:

1. Under the light microscope, the ghosts regained their original biconcave disc shape after resealing (magnification 500X).

2. The specific activities obtained for Na,K-ATPase and Mg+Ca-ATPase were comparable to those reported in other studies (40,65). The Na, K-ATPase and Mg+Ca-ATPase activities were shown to be asymmetrically stimulated by external potassium and internal calcium, respectively. Such characteristics can only be demonstrated in cells with an intact plasma membrane.

3. A loss of cellular calcium against a concentration gradient was demonstrated (Fig. 13), whereas no loss of cellular calcium occurs in frozen red blood cell membrane fragments used in our studies. In addition, no change in the cellular concentration of magnesium occurred in the resealed ghosts,

indicating that tne membrane is also not freely permeable to magnesium.

4.No visual loss of hemoglobin occurred during washing subsequent to resealing.

Ion Requirements For Resealing

Maizels et al.(75),in an early study ,reported that calcium was an essential requirement for maintaining red blood cell membrane permeability. Recently, however, Bramely et al.(76) claimed magnesium could substitute for calcium in resealing, though less effectively. However, in the present study, inclusion of at least 0.05 mM calcium in the loading medium was found to be an absolute requirement for resealing. This was demonstrated in an experiment where ghosts were loaded in a medium containing no added calcium, followed by the normal procedure for resealing. Subsequent suspension of these ghosts in a medium containing 1.0 mM calcium resulted in an uptake of cellular calcium approximately equivalent to the values normally obtained with 'leaky ghosts' (i.e. 0.06 μmoles $Ca^{2+}mg^{-1}$). On incubation at 37°C, loss of cellular calcium was observed. This result clearly indicates that the ghosts had not resealed in the loading medium devoid of calcium and suspension in a medium containing calcium allowed entry of calcium into the cell interior and subsequent resealing. The presence of 4.0 mM magnesium was thus not sufficient for complete resealing in these preparations. The difference between these results and

the results reported by Bramely et al. (76) may be due to the use of EDTA in our studies, which reduces endogenous calcium to very low levels.

Na,K-ATPase Activity In Resealed Red Blood Cell Ghosts

Investigation of Na,K-ATPase activity was undertaken to determine whether the integrity of this transport enzyme had been preserved. In ghosts loaded with 0.5 mM calcium and 0.1 mM EGTA, linearity in ATP splitting was observed for thirty minutes (Fig. 7). Deviation from linearity occurred after thirty minutes, presumably due to a depletion of ATP. Calcium was included in the loading medium to facilitate resealing of the ghosts, and did not appear to inhibit the Na,K-ATPase activity. Thirty minutes was therefore chosen as the incubation time in subsequent Na,K-ATPase experiments. Figure 7 shows that 0.2 mM ouabain, when applied externally, reduced total ATPase activity from 0.525 to 0.36 μmoles Pi $mg^{-1}hr^{-1}$. The difference accounts for the Na,K-ATPase present.

In an experiment to determine whether Na,K-ATPase could be activated asymmetrically, the concentration of potassium was varied externally in ghosts resealed with NaCl (Fig. 8). Maximal stimulation of ATPase activity occured at 8.0 mM external potassium. In the presence of 0.1 mM external ouabain the ATP splitting stimulated by external potassium was abolished.

Since the total ATPase activity of the ghosts in the presence of 0.1 mM ouabain was equal to the activity in the absence

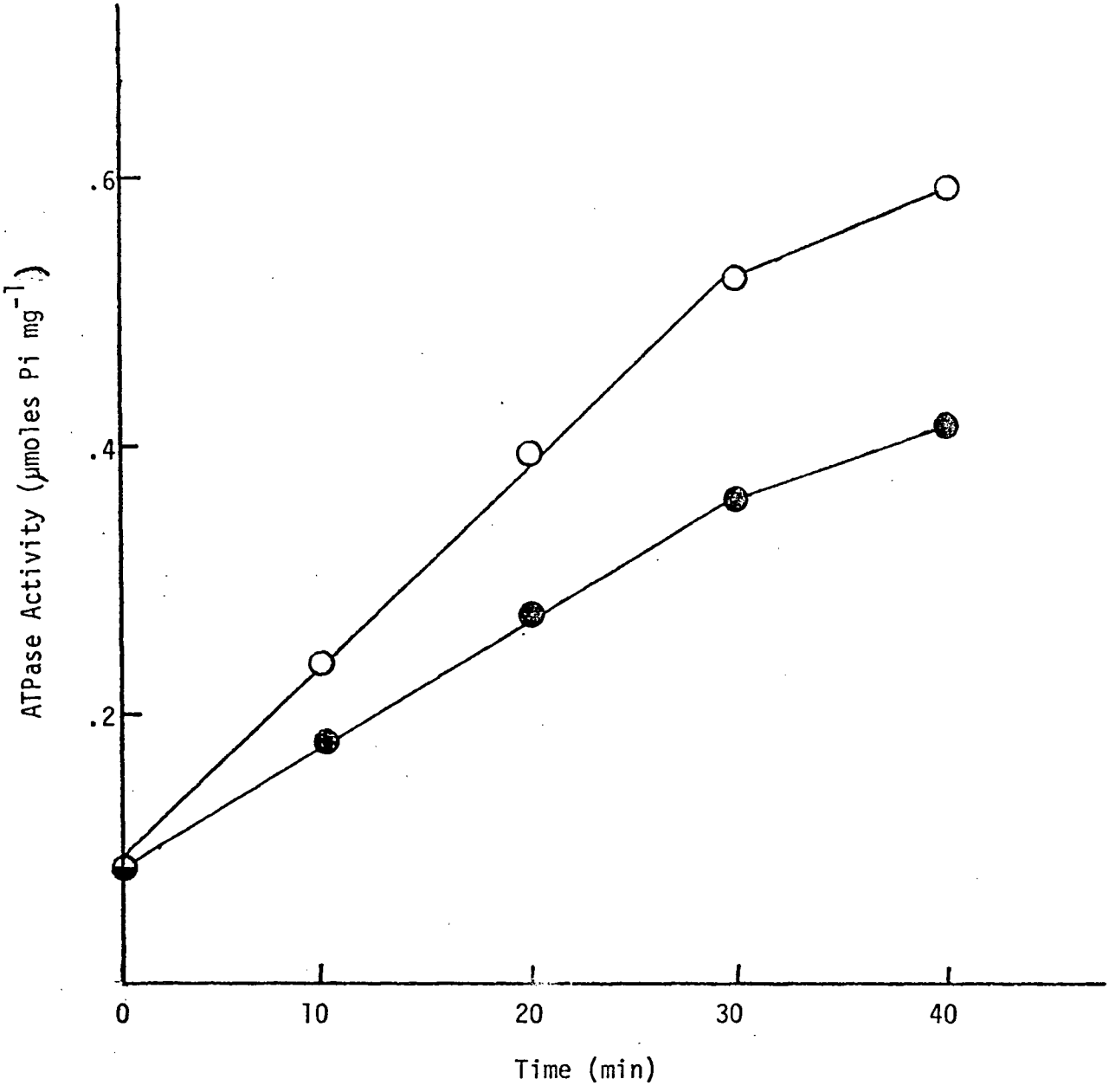


Figure 7. Time course of ATPase activity in the absence of external ouabain (○) and in the presence of 0.2 mM external ouabain (●). The concentration of calcium in the loading medium was 0.5 mM.

of added potassium, no Na, K-ATPase activity was present in ghosts resealed with NaCl, unless external potassium was added. In future determinations of Mg+Ca-ATPase activity, interference from ATP splitting due to Na,K-ATPase in cells resealed with NaCl would not be expected, since external potassium was not used.

Addition of 15.0 mM potassium in the loading medium, in the absence of external potassium, did not increase the basal rate of ATPase activity (0.3 μmoles $mg^{-1}hr^{-1}$), indicating that only external potassium activates Na, K-ATPase. Addition of 0.1 mM ouabain in the loading medium did not inhibit stimulation due to external potassium. These two findings demonstrate that both oubain and potassium have external binding sites, in agreement with results reported by previous workers (65). The result also supports the view that the cells have resealed, since internal potassium does not have access to the outside of the membrane.

In the presence of 1.0 mM external calcium (Fig. 8), the activation of Na,K-ATPase by external potassium was essentially unchanged. The specific activity of Na,K-ATPase (at 10.0 mM external potassium) was approximately 0.35 μmoles Pi $mg^{-1}hr^{-1}$ in the presence or absence of 1.0 mM external calcium. Therefore external calcium does not affect the specific activity of Na, K-ATPase.

As a comparison, the specific activity of Na,K-ATPase obtained in a preparation of frozen RBCMF by Watson et al.(40) was 0.28 μmoles Pi $mg^{-1}hr^{-1}$. The maximal value obtained in this study was 0.35 μmoles Pi $mg^{-1}hr^{-1}$.

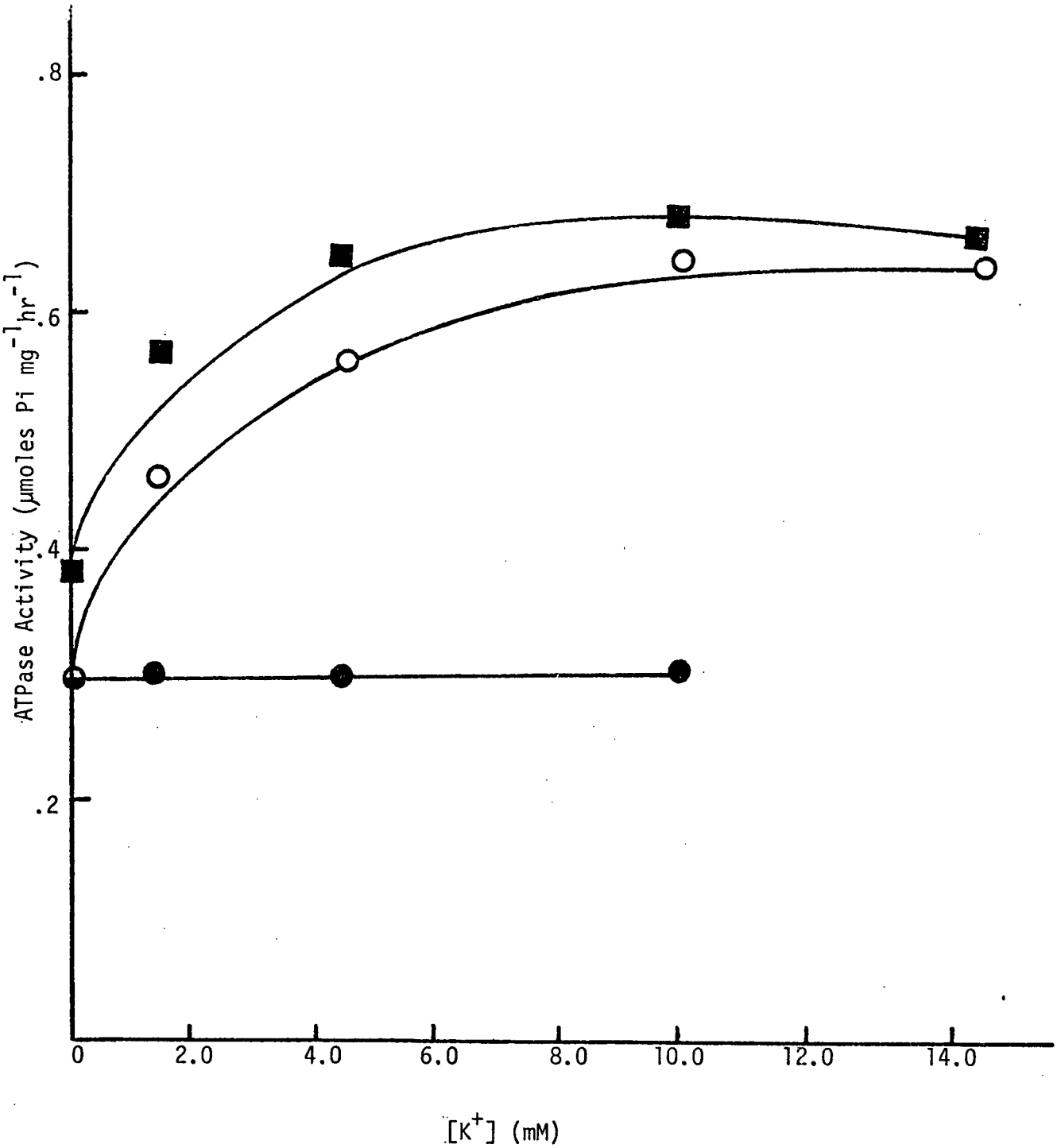


Figure 8. Activation of Na,K-ATPase activity by external potassium in the presence of 1.0 mM external calcium (■),zero external calcium (○) and 0.1 mM external ouabain (●).The concentration of calcium in the loading medium was 0.5 mM.Other conditions were standard.Each point represents the mean of two experiments.

Mg+Ca-ATPase Activity In Resealed Red Blood Cell Ghosts

Variation of the concentration of calcium in the loading medium led to a marked increase in ATPase activity (Fig. 9). Two peaks were obtained, possibly representing two calcium stimulated Mg+Ca-ATPases. The activity was corrected for Mg-ATPase and non-enzymatic hydrolysis as in the Methods. The finding of two Mg+Ca-ATPases in resealed ghosts was in agreement with earlier studies on frozen RBCMF, where the high affinity Mg+Ca-ATPase was optimally activated by approximately 10^{-5} to 10^{-6}M calcium, and a low affinity Mg+Ca-ATPase with optimal activation at approximately 10^{-5} to 10^{-4}M calcium. In resealed ghosts (Fig. 9), high affinity Mg+Ca-ATPase and low affinity Mg+Ca-ATPase were maximally stimulated by 0.25mM and 5.0mM calcium in the loading medium, respectively. The marked differences in affinity for calcium between RBCMF and resealed ghosts led to the conclusion that the concentrations of calcium in the loading medium were much higher ($\sim$ 10 fold) than the actual free intracellular concentrations of calcium at the beginning of the incubation. For instance, in ghosts loaded with 5.0mM calcium, there may not be an efficient 1:1 loading ratio between the loading medium and the cell interior (38). Furthermore, some of the loaded calcium may have been lost during the resealing procedure, which required an incubation for ten min. at room temperature (see Methods). A considerable amount of calcium may be chelated by binding sites that are not available in RBCMF, especially since such sites may

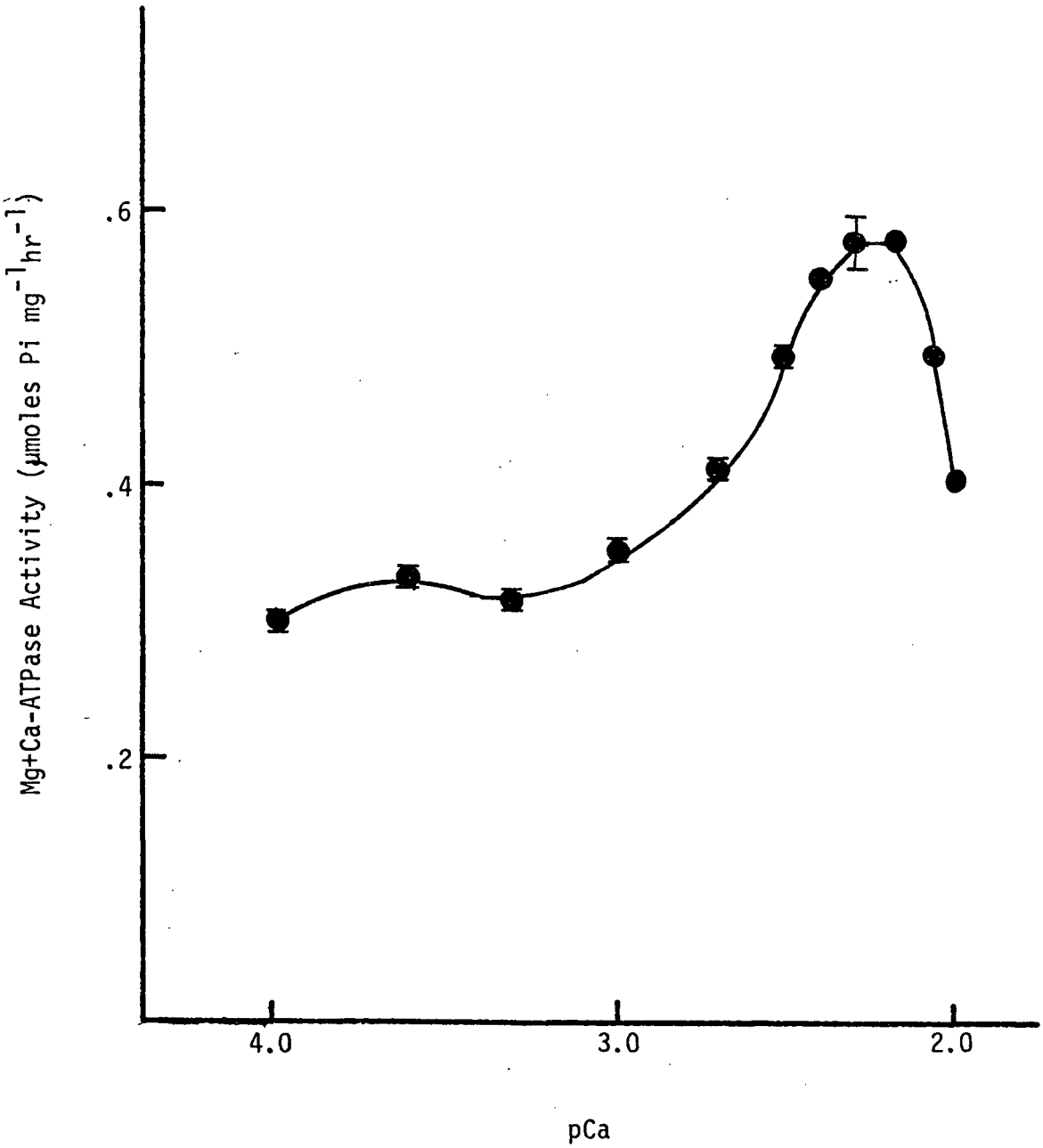


Figure 9. Effect of varying the concentration of calcium in the loading medium on Mg+Ca-ATPase activity. Each point represents the mean of three experiments (± standard errors).

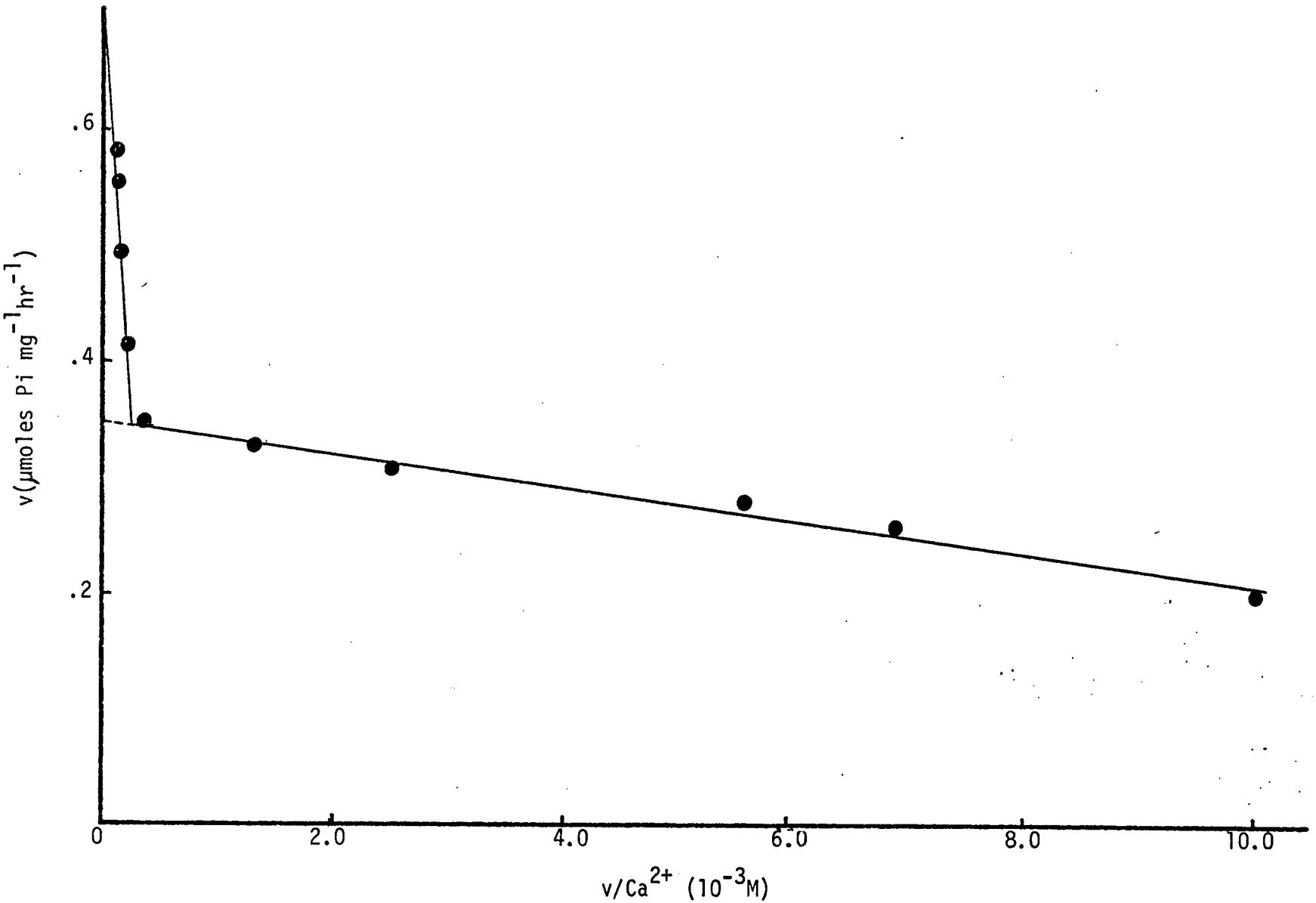
have been depleted by EDTA. Therefore the concentrations of calcium indicated on the abcissa are not true intracellular concentrations of calcium, but represent a relative intracellular concentration of calcium added in the loading medium.

An Eadie plot (77) of the calcium activation data, revealed two straight lines (Fig. 10). The Vmax and the calcium dissociation constant for the low affinity Mg+Ca-ATPase were calculated to be 0.690 μmoles Pi $mg^{-1}hr^{-1}$ and 1.23 mM respectively. A Vmax and Ka of 0.340 μmoles Pi $mg^{-1}hr^{-1}$ and 1.26 X 10^{-5}M were obtained for the high affinity Mg+Ca-ATPase. Values reported by Bader (78) for the specific activities of Mg+Ca-ATPases in RBCMP were 0.5 and 0.8 μmoles Pi $mg^{-1}hr^{-1}$ for the high and low affinity Mg+Ca-ATPase, respectively. The slightly lower values reported here for the resealed ghosts may be due to assaying at slightly less than optimal conditions. There are preliminary indications that an increase in the concentration of magnesium in the loading medium will further enhance Mg+Ca-ATPase activity in this preparation.

The Effect Of External Divalent Cations On ATPase Activity

Previously, Schatzmann and Vincenzi (12) reported that only intracellular calcium stimulated ATPase activity in resealed ghosts, while external calcium had no significant effect. However, external divalent cations were found to significantly stimulate ATPase in this ghost preparation (Fig. 11). The ordinate represents the increase in ATPase activities over

Figure 10. Eadie plot of calcium activation of Mg+Ca-ATPases in ghosts.


.6
.4
.2
v(μmoles Pi mg^{-1}hr^{-1})
0
2.0
4.0
6.0
8.0
10.0
v/Ca^{2+} (10^{-3}M)

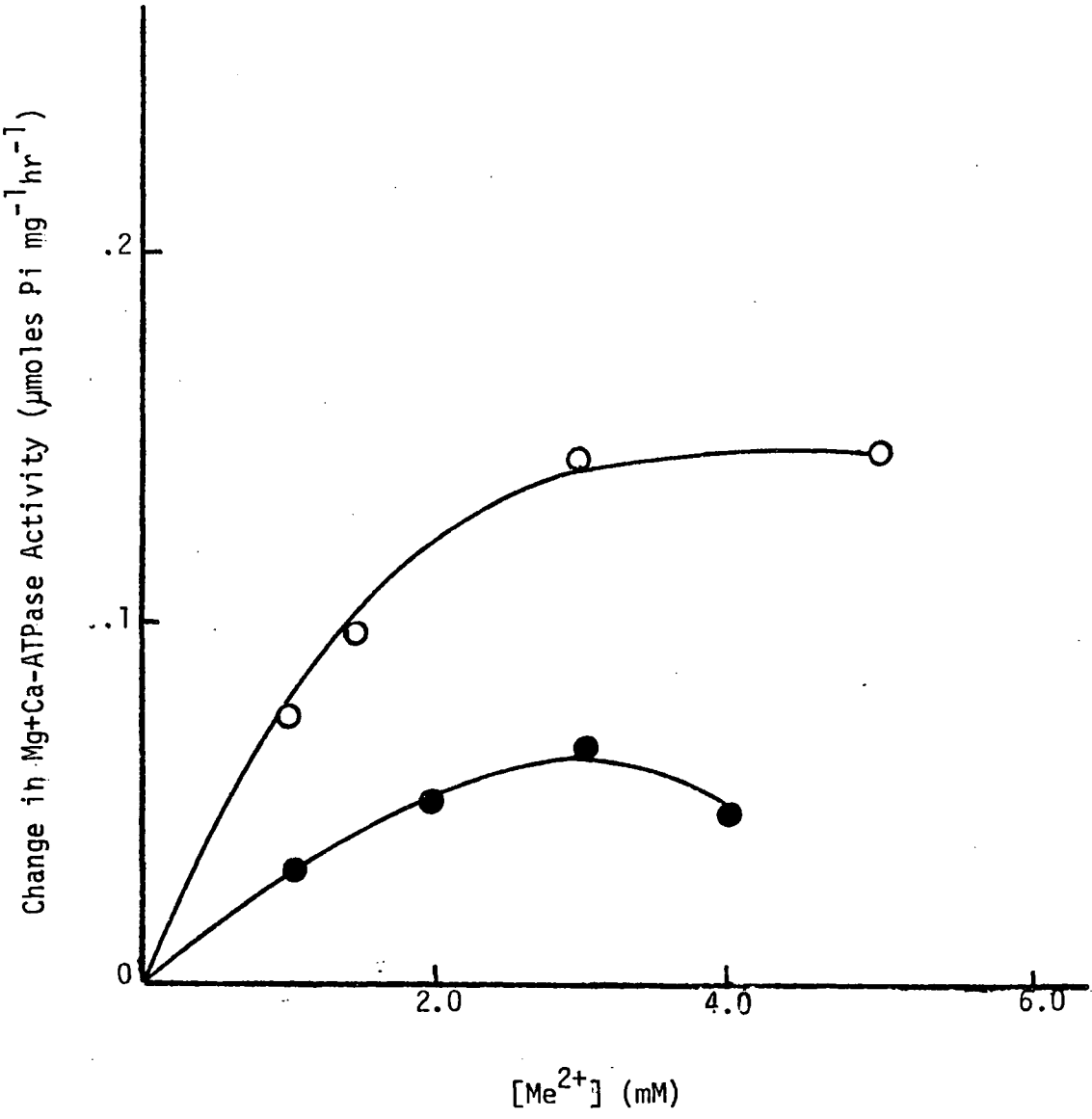


Figure 11. The effect of external divalent cations on ATPase activity. The ghosts were loaded with 0.5 mM Calcium and 0.4 mM EGTA. Other conditions were standard. Ca (○), Mg (●).

basal levels obtained in the absence of added external divalent cations. The maximal increase in ATPase activity occured at 3.0 mM external calcium, with an increase over basal activity of 0.140 μmoles Pi $mg^{-1}hr^{-1}$. External magnesium was only about one-half as effective as external calcium in increasing ATPase activity, with a maximal increase of 0.06 μmoles Pi mg^{-1} hr^{-1}.

Since increasing the external potassium concentration (in the presence of 0.1 mM ouabain) did not affect Mg+Ca-ATPase activity, stimulation of ATPase activity appears to require divalent metal cations.

To determine if magnesium and calcium were entering the cell or were stimulating an external ATPase such as Na,K-ATPase or Mg+Ca-ATPase, the concentration of calcium was varied in the loading medium of resealed ghosts suspended in external medium containing zero calcium and 1.0 mM calcium (Fig. 12). Since there is no apparent shift in the position of the calcium activation curve in the presence of 1.0 mM calcium, external calcium does not have access to the cellular interior. The stimulation must therefore be due to calcium acting on the external surface of the membrane.

Since external calcium did not affect the Na,K-ATPase activity as shown previously (Fig. 8) the extra ATPase activity seen in the presence of 1.0 mM external calcium is not due to stimulation of Na,K-ATPase at an external binding site. The possibility that some ghosts were present which did not reseal and were being activated is unlikely because of the high

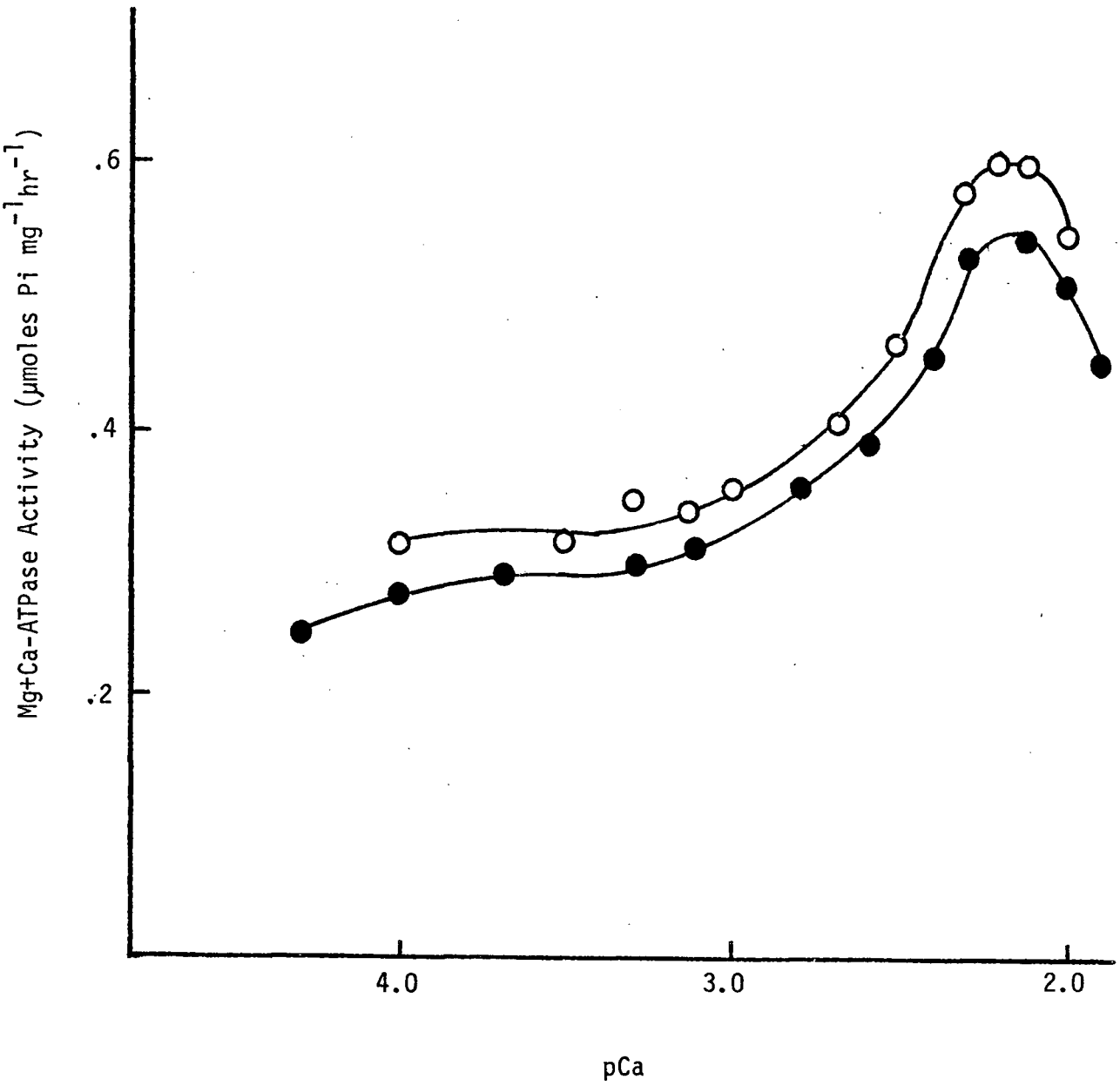


Figure 12. Effect of varying the concentration of calcium in the loading medium on Mg+Ca-ATPase activity in the absence of external calcium (●) and in the presence of 1.0 mM external calcium (○).0.4 mM EGTA was included in the loading medium.Other conditions were standard.Each point represents the mean of at least two experiments.

concentrations of divalent cations required for this activation, relative to the concentrations required to stimulate membrane fragments.

It was speculated that calcium and magnesium may be replacing endogenous magnesium and calcium removed from the membrane by EDTA. The binding of calcium or magnesium may restore the conformation Mg+Ca-ATPase, resulting in an increased activity..

The Effect Of Internal Calcium On Calcium Efflux

Qualitative studies indicating that the velocity of calcium efflux is dependent on the internal concentration of calcium have been previously reported in resealed ghosts (30) and in whole red blood cells (7). Schatzmann and Rossi (30), however, were unable to demonstrate a correlation between activation of Mg+Ca-ATPase and the velocity of calcium efflux in their preparations of resealed ghosts.

In this study ghosts were loaded with different concentrations of calcium and resealed as in the Methods. The loss of cellular calcium was determined as a function of time at 37°C. (Fig. 13). In ghosts loaded over the concentration range of 0.1 to 5.0 mM calcium, a rapid loss of cellular calcium was apparent. In most of the ghosts an equilibrium level of cellular calcium was reached within ten minutes. The concentration of calcium in the ghosts at time zero was directly proportional

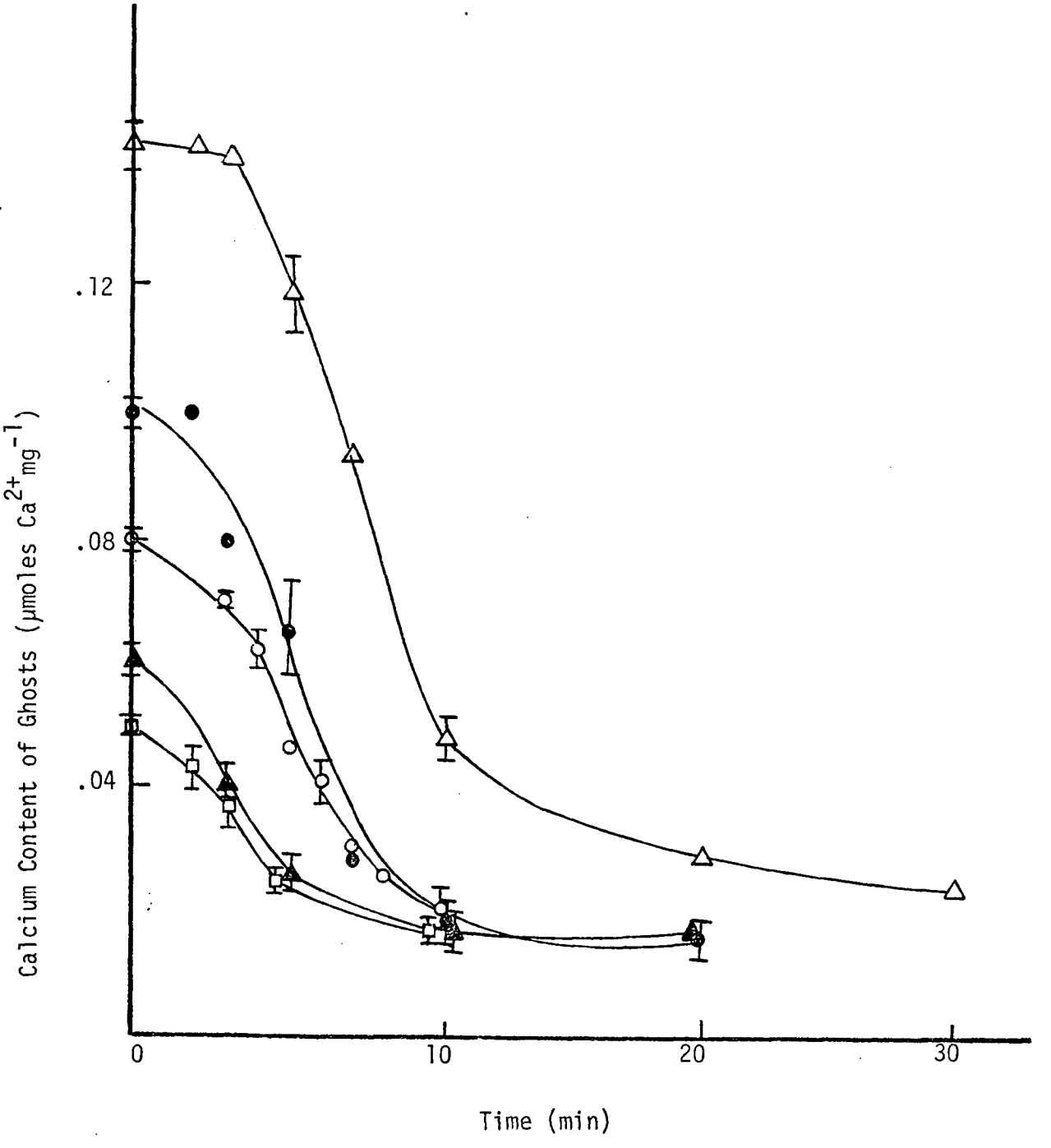


Figure 13. Changes in the concentration of cellular calcium with time. Each point represents the mean of two to three experiments (± standard errors), except where no bars are shown. The concentration of calcium in the loading medium was 0.5 mM (□), 1.0 mM (▲), 2.0 mM (○), 3.0 mM (●) and 5.0 mM (△).

to the concentration of calcium in the loading medium. This is shown in figure 14 where a plot of μmoles Ca^{2+} mg^{-1} against the concentration of calcium in the loading medium yielded a straight line.

Evidence was obtained showing that the loss of cellular calcium was due to active calcium transport. For instance, if ATP was not included in the loading medium, a loss of cellular calcium over time did not occur, indicating that the calcium loss was an ATP dependent process. This finding also shows that endogenous ATP was removed during the preparation of the ghosts. Furthermore, a loss of cellular calcium occurred in ghosts loaded with less than 1.0 mM calcium, showing that intracellular calcium was transported against an electrochemical potential.

As the concentration of calcium in the loading medium was increased from 0.1 mM to 5.0 mM, there is an increase in the steepness of the slopes of the curves, indicating that the velocity of transport is increasing as the concentration of calcium is increased in the loading medium. The efflux curves obtained conform to a first order process, in that for each curve the velocity of efflux decreases exponentionally with time. Thus a plot of log calcium content from the data in figure 13 against time yielded straight lines. From these straight lines extrapolated points for the first ten minutes were obtained. Initial velocities could then be accurately determined by replotting the data on a plot of concentration of calcium against time. The first three minutes were not used in the calculations of initial velocities since this time

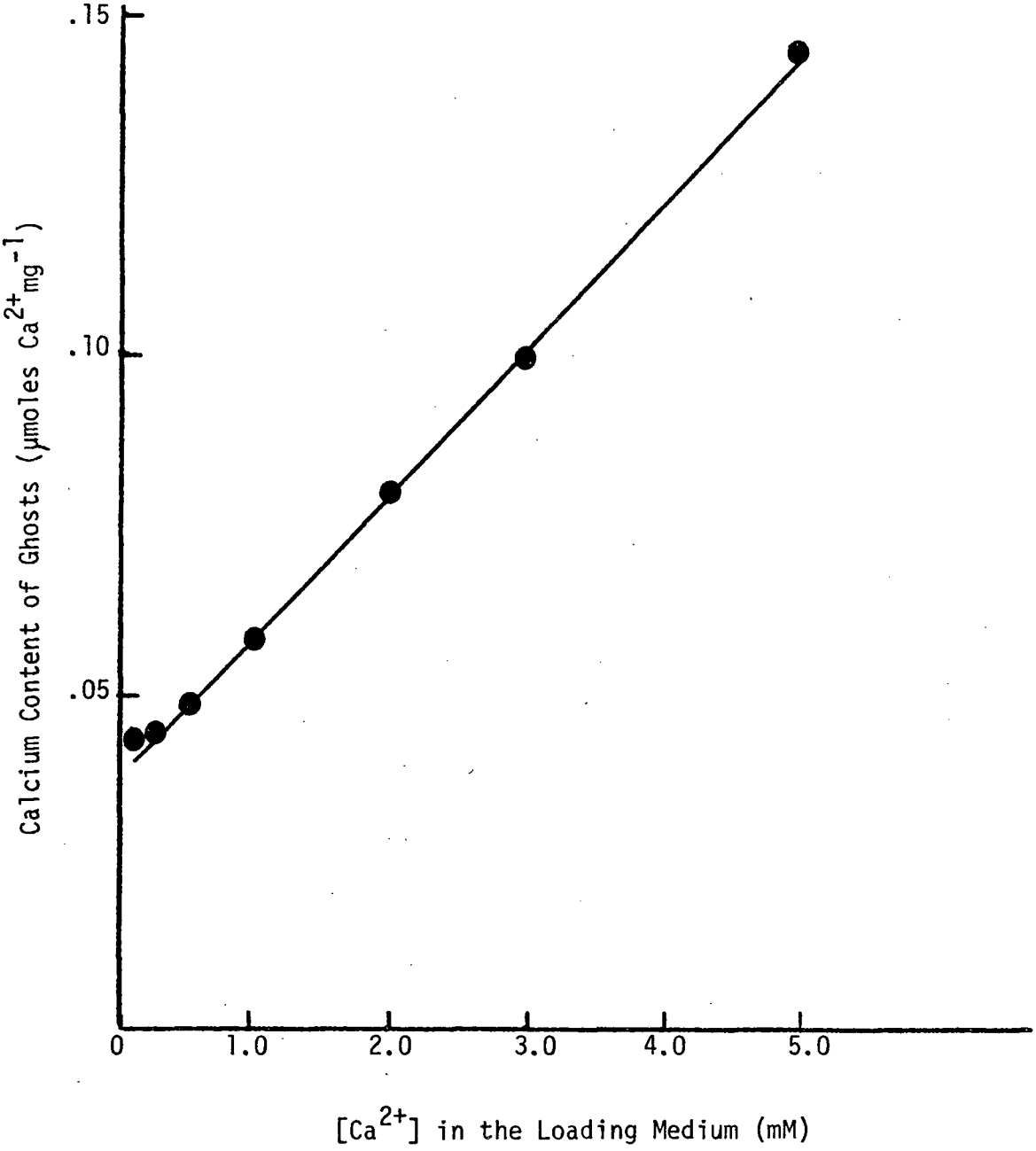


Figure 14. Relationship between cellular calcium and calcium in the loading medium. Each point represents the mean of three experimental determinations (± standard errors).

represents the time required for the tubes to warm up from 0°C to 37°C (see Fig. 13).

A plot of the initial velocities of calcium efflux against the concentration of calcium in the loading medium yielded a sigmoidal curve (Fig. 15, open circles). Increasing the concentration of calcium from 0.1 mM to 1.0 mM did not result in a large increase in the velocity of calcium efflux. A more marked stimulation of calcium efflux occurred in ghosts loaded with concentrations of calcium greater than 1.0 mM. In contrast to the activation of Mg+Ca-ATPase activity two peaks were not obtained suggesting that there is only one calcium transport system present. Figure 15 indicates that calcium stimulation of both ATPase activity and calcium efflux are parallel in the calcium concentration range of 1.0 to 5.0 mM, where the low affinity Mg+Ca-ATPase is thought to operate. A comparison of the curves in this region indicates a stoichiometry of two calcium ions transported per molecule of ATP hydrolyzed. At calcium concentrations of less that 1.0 mM, where the high affinity Mg+Ca-ATPase is thought to operate, the stoichiometry measured appears to be less than one, and the shapes of the calcium efflux and the ATPase calcium activation curves do not coincide. This suggests that at low calcium concentrations there is an ATPase splitting (high affinity Mg+Ca-ATPase) which is not coupled to calcium transport.

The stoichiometry of two (Ca:ATP) contrasts to the previously reported value of 0.77 (12), but agrees with the stoichiometry found for calcium transport in muscle sarcoplasmic

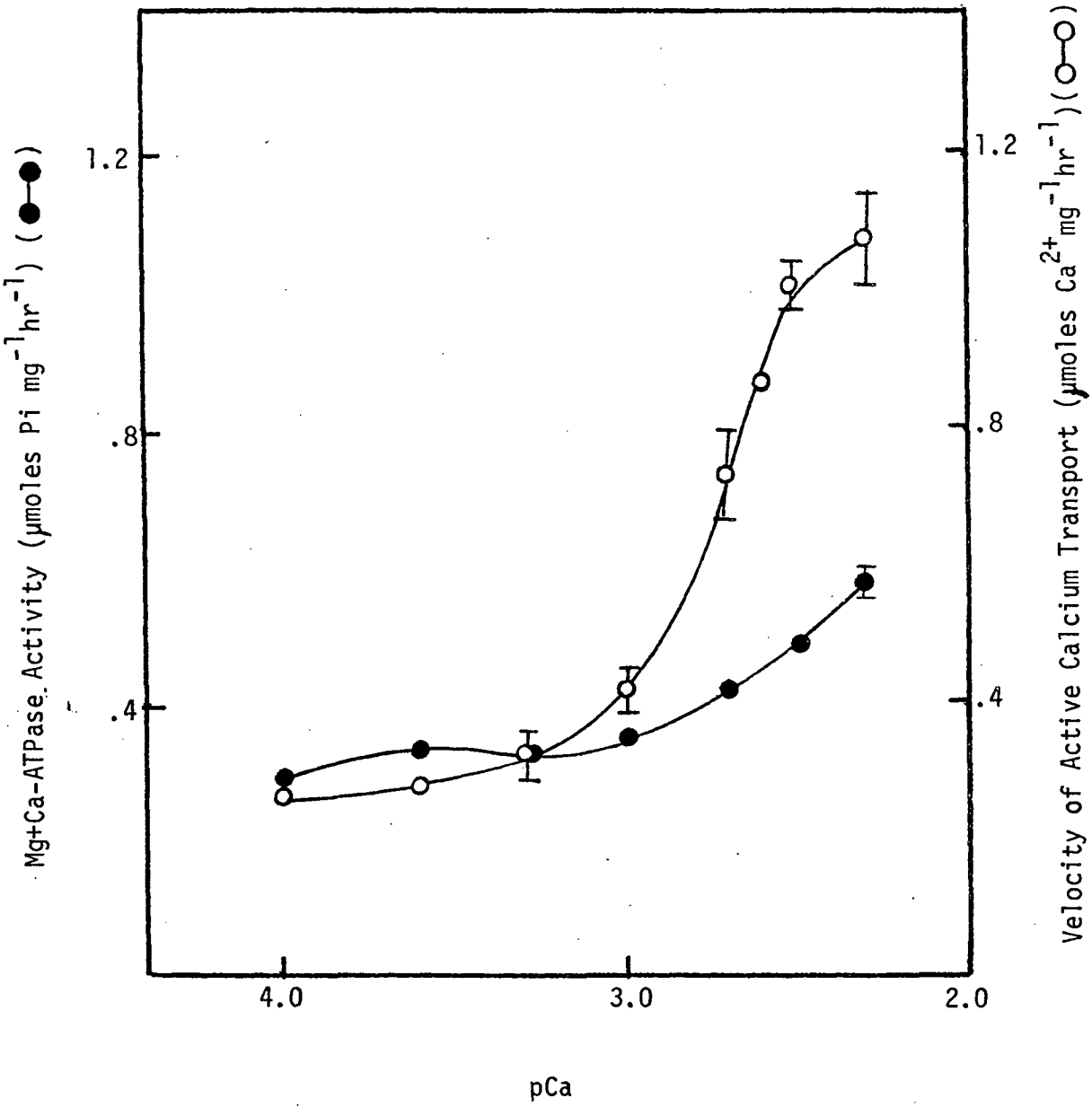


Figure 15. Comparison of the velocity of active calcium transport and Mg+Ca-ATPase activity as a function of calcium in the loading mediumThe Mg+Ca-ATPase data represents the mean of three experiments and the standard errors are smaller than the circles drawn.The calcium transport data represent the mean of three determinations (± standard errors),except where no bars are shown,where points represent the mean of duplicate determinations.

reticulum (54). It seems more reasonable that the calcium pump in red blood cells would also have a high stoichiometry, since less energy would have to be expended in extruding a given amount of intracellular calcium.

The efflux data was plotted according to Hill (68) in order to investigate the meaning of the sigmoidal shape of the calcium efflux curve (Fig. 16). At least two straight lines were obtained with Hill coefficients (n) of approximately 0.39 for concentrations of calcium less than 1.0 mM and 1.0 for ghosts loaded with greater than 1.0 mM calcium. This change in n values may mean that the calcium transport system has a cooperative component. Binding of one molecule of calcium may increase the affinity of the transport system for the binding of another molecule of calcium, at a threshold value of approximately 1.0 mM calcium in the loading medium. Changes in n values often reflect a change in the conformation of spacial arrangement of enzyme subunits. A subunit structure for Mg+Ca-ATPase in red blood cells has not been reported. However, Kyte (71) recently reported that the Na,K-pump in the plasma membrane of renal cortex appears to have a subunit structure. An alternative explanation is that the break in the slope of figure 16 may be due to the presence of two calcium activated calcium transport systems. The results presented above do not rule out this possibility. However, results obtained using ruthenium red (to be discussed later) make this explanation less likely.

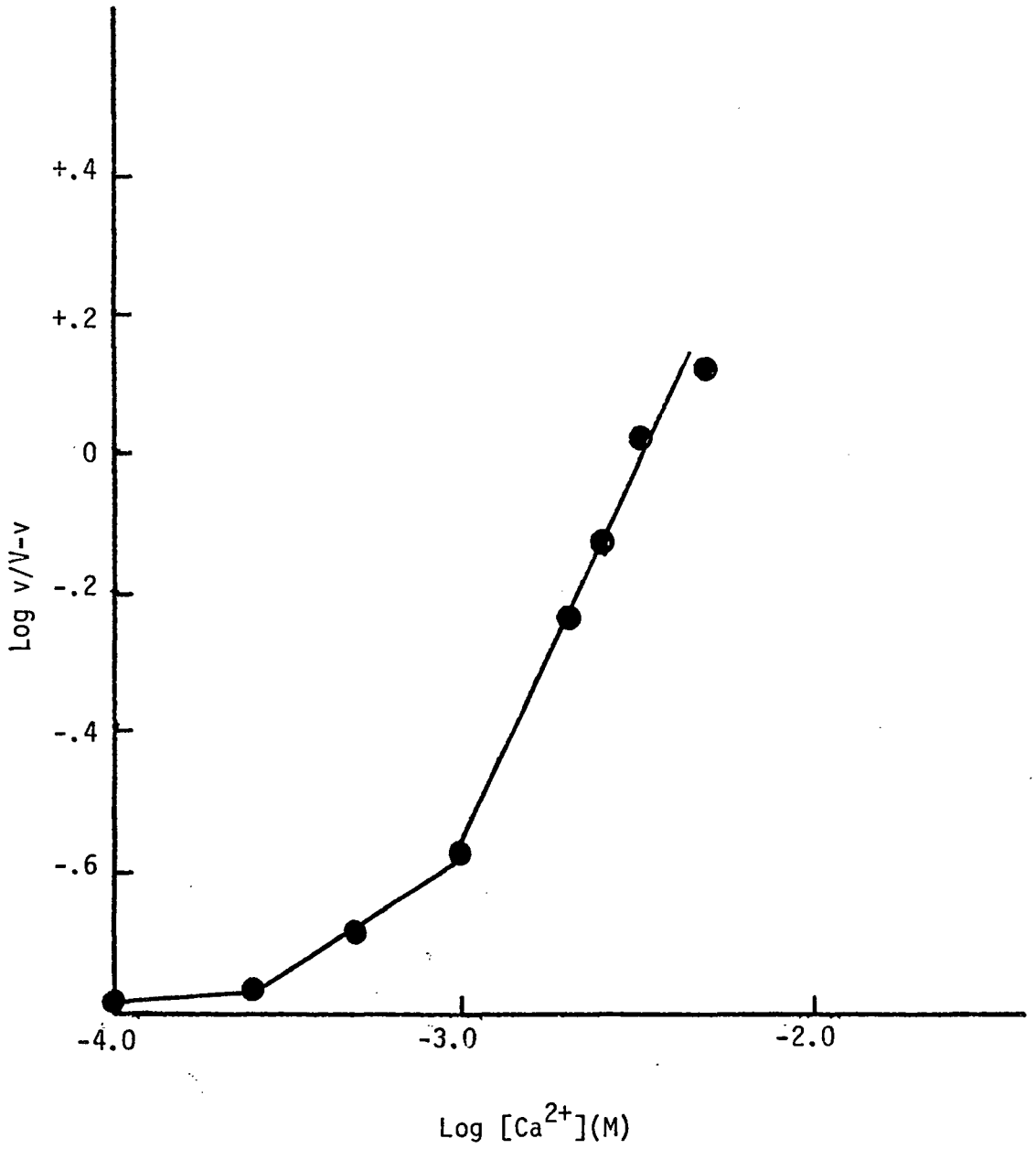


Figure 16. Hill plot of calcium activation of calcium efflux in ghosts.v is the velocity of calcium efflux and V is the maximum velocity of this efflux, as determined from a Lineweaver-Burk plot.

The Effect Of Ruthenium Red On Mg+Ca-ATPase Activities

In the previous section, a similarity between the shape of the calcium activation curves for the low affinity Mg+Ca-ATPase and the calcium transport system was noted. This association was further investigated using ruthenium red as a tool. Watson et al. (40) reported that ruthenium red, an inorganic dye used in staining mucopolysaccharides, selectively inhibited Mg+Ca-ATPase activity in RBCMF. No distinction, however, was made between inhibition of high or low affinity Mg+Ca-ATPase activity or whether calcium transport was also inhibited.

In this study ruthenium red, added externally, was found to inhibit Mg+Ca-ATPase in a dose dependent manner in ghosts loaded with 3.0 and 5.0 mM calcium (Fig. 17). In the ghosts loaded with 3.0 and 5.0 mM calcium, the inhibition curves were found to be parallel. Since the I_{50} value of ruthenium red at both concentrations of internal calcium was 0.2 mM, the mechanism of inhibition would not be expected to be competitive with respect to calcium. This finding is not surprising in view of the facts which suggest that ruthenium red inhibits by binding to anionic sites on the outside of membranes. Calcium has previously been shown here and elsewhere (12) to activate Mg+Ca-ATPase on the internal surface of the membrane. Evidence supporting an external binding site for ruthenium red are as follows:

(1) A preincubation time was not required. Ghosts preincubated in 0.2 mM ruthenium red for 0, 10, 20 and 30 minutes at

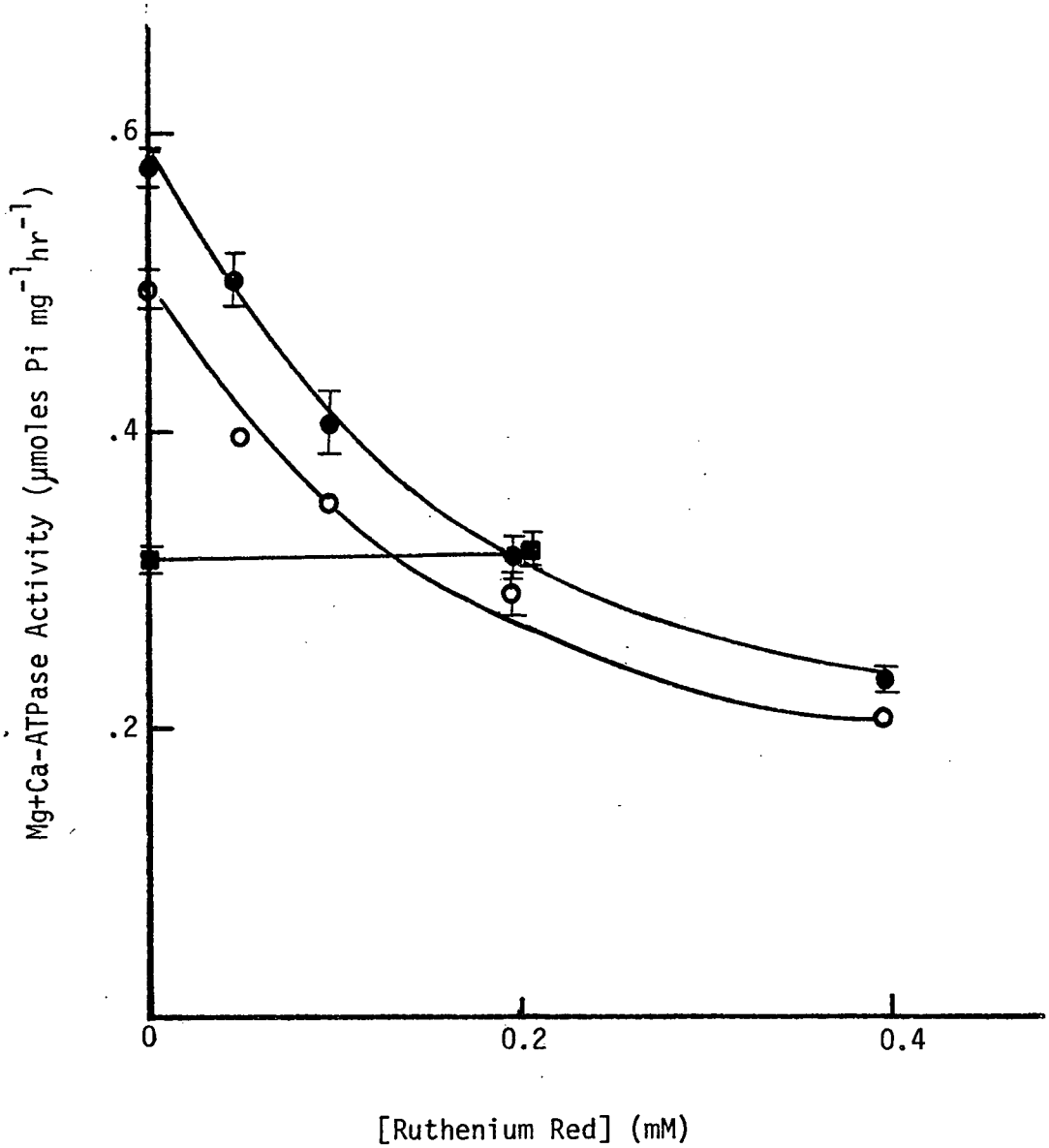


Figure 17. Effect of ruthenium red in the external medium on Mg+Ca-ATPase activity.The concentration of ruthenium red was varied in ghosts loaded with o.5 mM calcium (■),3.0 mM calcium (○) and 5.0 mM calcium (●).

2-4°C. inhibited low affinity Mg+Ca-ATPase by 50%. If ruthenium red was penetrating into the interior of the ghosts, and then inhibiting Mg+Ca-ATPase, greater inhibition would be expected for ghosts preincubated for longer periods of time.

(2) Ruthenium red does not penetrate through the plasma membrane of intact cells as evidenced from electron microscopy (72).

(3) Penetration of ruthenium red through the plasma membrane of resealed ghosts would be very slow since ruthenium red possesses a hexavalent positive charge (40).

Ruthenium red, therefore, probably exerts its inhibition by binding to mucopolysaccharides on the outside surface of the ghost. Binding at these sites may induce an unfavourable conformational perturbation of the membrane bound Mg+Ca-ATPase, leading to a reduction in the catalytic efficiency of this enzyme. Binding to an allosteric site would also favour a non-competitive type of inhibition by ruthenium red.

In ghosts resealed with 0.5 mM calcium, 0.2 mM external ruthenium red did not affect Mg+Ca-ATPase activity (Fig. 17). In order to explain this analomous result, the concentration of calcium was varied in the loading medium, the ghosts were resealed and suspended in a medium containing 0.2mM ruthenium red (Fig.18). Ruthenium red had essentially no effect on Mg+Ca-ATPase activity at concentrations of calcium lower than 1.0mM, where the high affinity Mg+Ca-ATPase is considered to operate. However, the low affinity Mg+Ca-ATPase was markedly inhibited by ruthenium red. The % inhibition was dependent on

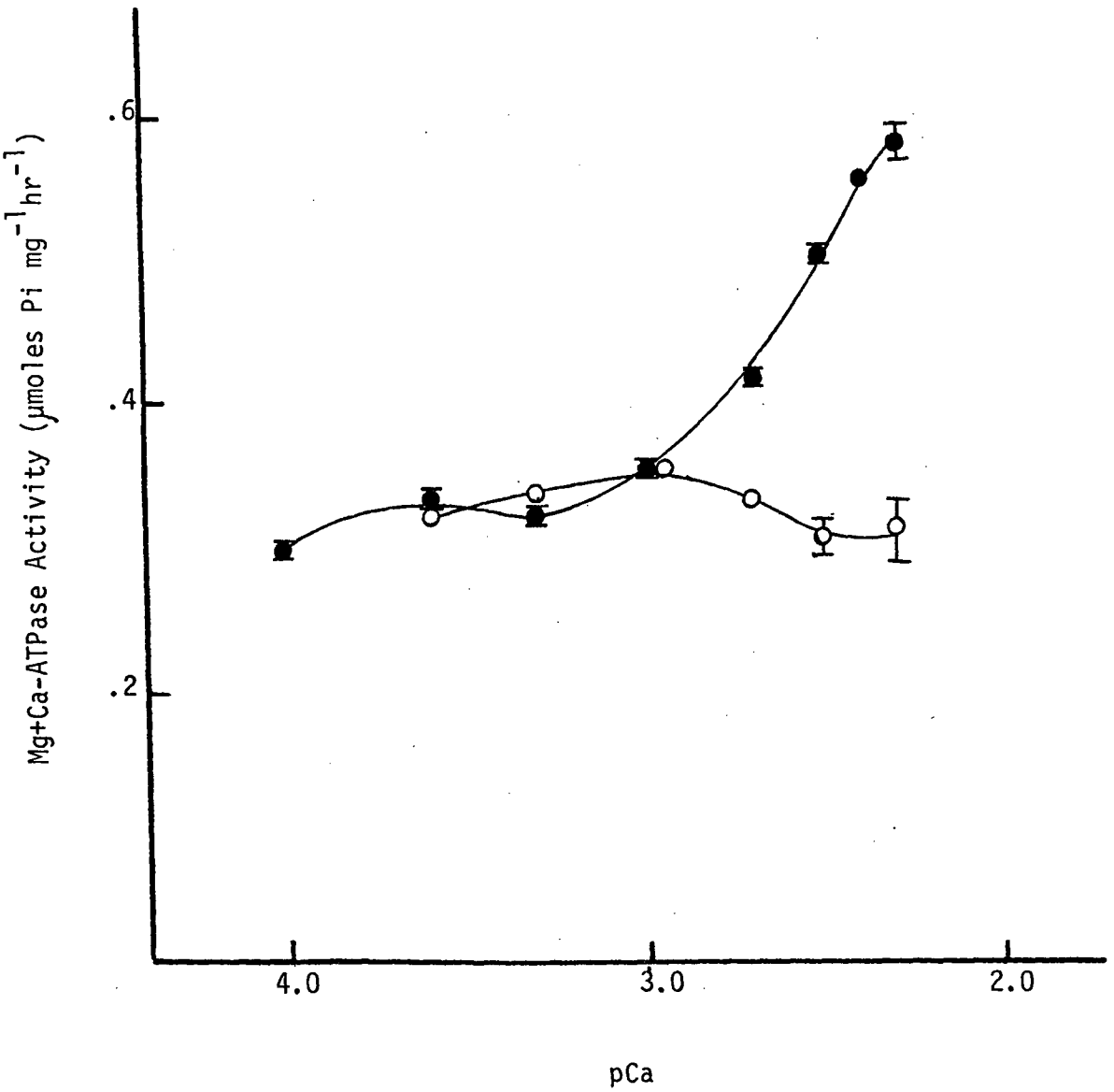


Figure 18. The effect of ruthenium red on the activation of Mg+Ca-ATPase. The calcium concentration in the loading medium was varied in the absence of ruthenium red (●) and in the presence of 0.2 mM ruthenium red (○)in the external medium.Where bars are shown the data represent the mean of three experiments (± standard errors).Other points represent the mean of duplicate determinations.

the concentration of calcium in the loading medium. For example, ghosts loaded with 2.0 mM calcium were inhibited by 27% whereas ghosts loaded with 3.0 mM calcium were inhibited by 42% (see Fig. 21).

The Effect Of Ruthenium Red On Calcium Transport

Since ruthenium red was shown to selectively inhibit low affinity Mg+Ca-ATPase, this dye showed promise as a tool for investigating whether or not a Mg+Ca-ATPase is associated with calcium transport. In ghosts loaded with 3.0 mM calcium, external ruthenium red inhibited the velocity of active calcium transport in a dose dependent manner (Fig. 19). The I_{50} value was estimated to be 0.2 mM, which is the same value estimated for the inhibition of the low affinity Mg+Ca-ATPase (Fig. 17). Thus both the active calcium transport system and low affinity Mg+Ca-ATPase have a similar affinity for ruthenium red.

The effect of 0.2 mM external ruthenium red on the activation of active calcium transport by internal calcium was also determined (Fig. 20). The pattern of ruthenium red inhibition of calcium transport was essentially the same as that on low affinity Mg+Ca-ATPase. The % inhibition by ruthenium red increased with an increase in the concentration of calcium from 1.0 mM to 3.0 mM and then leveled off (Fig. 21). Thus the degree of inhibition by 0.2 mM ruthenium red depends on the concentration of calcium in the loading medium (see Fig. 20). There are

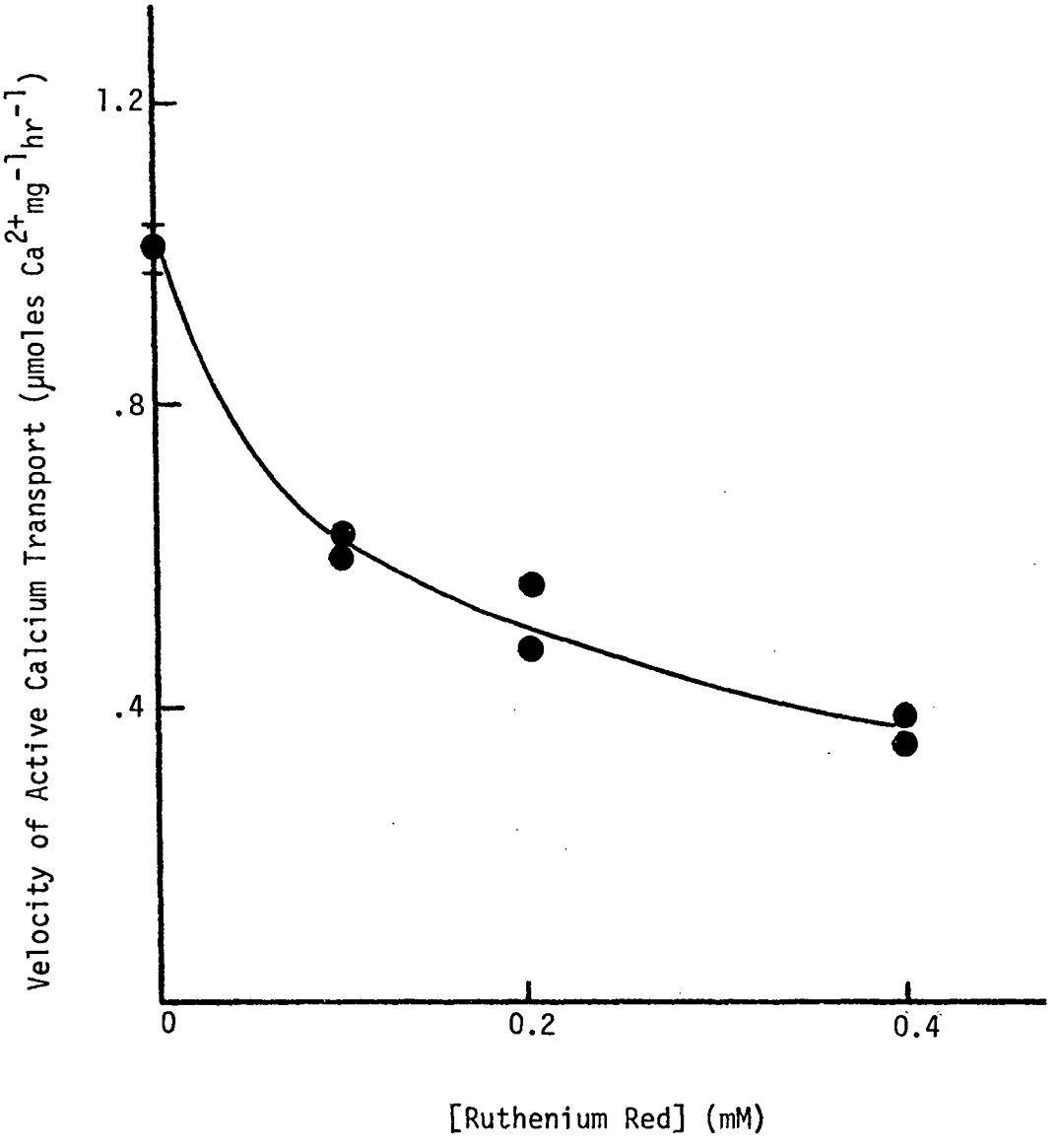


Figure 19. Effect of ruthenium red in the external medium on the velocity of active calcium transport in ghosts loaded with 3.0 mM calcium.

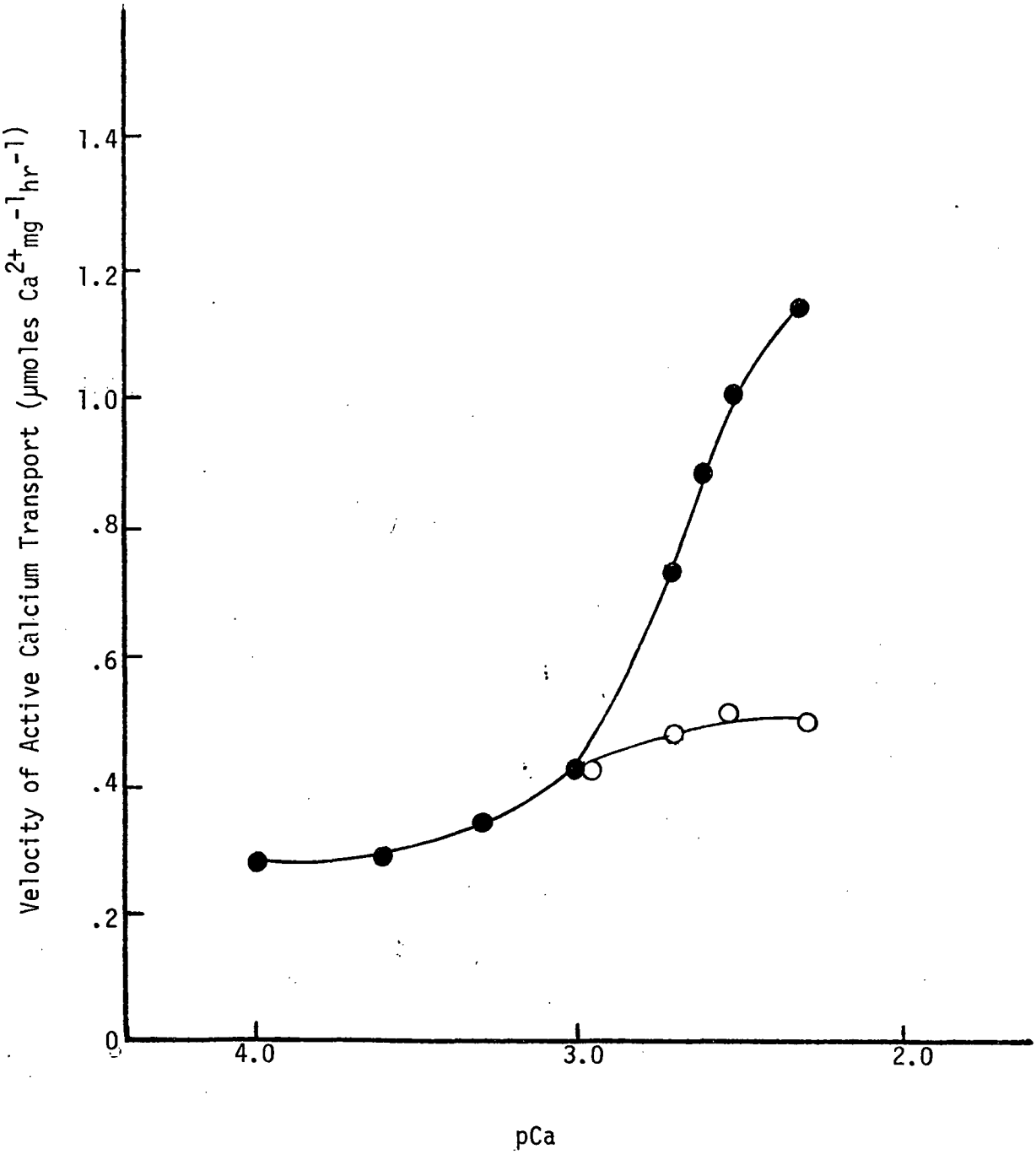


Figure 20. The effect of ruthenium red on the velocity of active calcium transport. The calcium concentration in the loading medium was varied in the absence of ruthenium red (●) and in the presence of 0.2 mM ruthenium red (○). The data indicated by the open circles represent the mean of two determinations.

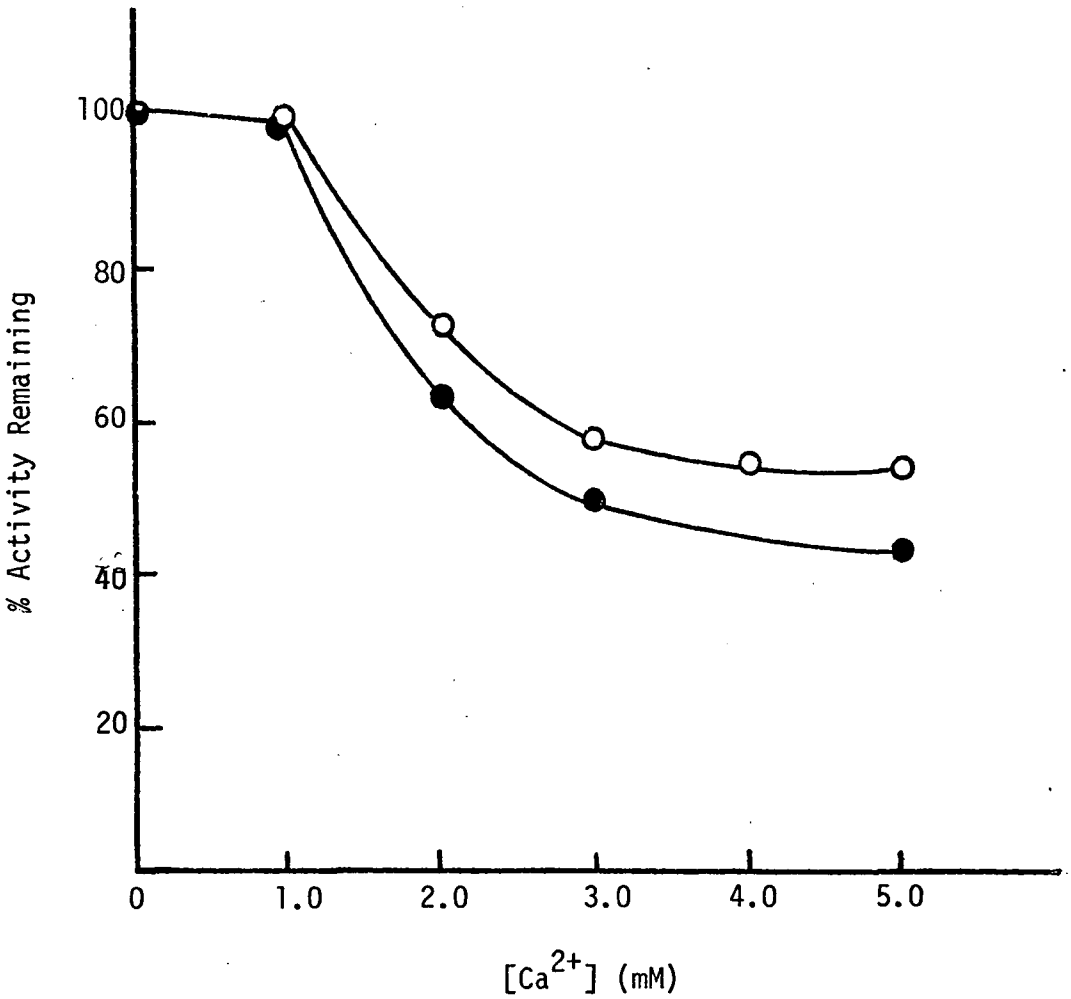


Figure 21. The effect of ruthenium red (0.2 mM) in the external medium on Mg+Ca-ATPase activity (○) and active calcium transport (●) as a function of the concentration of calcium in the loading medium.

two possible explanations for this behaviour. It is possible that there are more binding sites available for ruthenium red at higher concentrations of internal calcium, since the volume of the red cell is regulated by the internal concentration of calcium (10). A more likely explanation becomes apparent upon reexamination of figure 16. It was previously noted that there was a change in the Hill coefficients from .39 to 1.0 as the concentration of calcium was varied in the loading medium. It was suggested that this change may be due to a change in the subunit structure of the enzyme. If low affinity Mg+Ca-ATPase and the calcium transport system are indeed associated, the same explanation would hold here too. Thus the binding of ruthenium red to external mucopolysaccharides could prevent the ability of calcium to affect an interaction of the transport enzyme subunits or an increase in cooperativity. Ruthenium red could thus bind to and stabilize the less active form of the transport system so that only a certain maximal velocity of calcium efflux and Mg+Ca-ATPase activity would be obtainable. Support of this hypothesis awaits isolation of the low affinity Mg+Ca-ATPase and identification of a subunit structure.

Preliminary experiments also indicate that inhibition by ruthenium red may be temperature dependent. For instance, 0.4 mM external ruthenium red increased the lag time for calcium efflux in ghosts loaded with 3.0 mM calcium from three to four minutes. Furthermore, membranes must be preincubated at 2 to 4°C. in order for inhibition to be obtained. The possibility thus exists that ruthenium red can only bind to one

form of the transport enzyme. This form may be the more efficient transport form, induced by high concentrations of intracellular calcium and possibly by low temperatures.

The Effect Of Drugs (Quinine, Quinidine, and Propranolol) On Mg+Ca-ATPase

Quinidine has been shown to inhibit the binding of calcium in isolated sarcoplasmic reticulum (79). The possibility was considered that this drug may have effects on other systems controlling the distribution of calcium, such as active calcium transport across the plasma membrane. The human erythrocyte was used as a model system to determine whether quinidine or quinine affected low affinity Mg+Ca-ATPase in resealed ghosts or RBCMF. Both quinidine and quinine were found to be ineffective over a concentration range of 10^{-5}M to 10^{-3}M in both preparations. Interference by these drugs on the inorganic phosphate assay (63) at concentrations greater than 10^{-3}M was observed.

Propranolol was also without effect on Mg+Ca-ATPase activity in resealed ghosts in the concentration range of 10^{-5} to 10^{-3}M.

The effect of these drugs on active calcium transport was not determined.

The Effect Of Manganese On Mg+Ca-ATPase Activity

Bader (43) recently reported that manganese can substitute for calcium in activating low affinity Mg+Ca-ATPase in RBCMF. Manganese was reported to activate low affinity Mg+Ca-ATPase with the same potency as calcium and to have no effect on high affinity Mg+Ca-ATPase. Therefore manganese was considered as a potential tool for differentiating enzyme function.

The concentration of manganese was varied in the loading medium in the presence or absence of 1.0 mM external calcium (Fig. 22). Calcium was not included in the loading medium in these experiments. Inhibition of ATPase activity was observed at concentrations of manganese less than 1.0 mM. The activity returned to the control level at 5.0 mM manganese. The concentrations of manganese was also varied in the absence of 1.0 mM external calcium to ensure that the slight activation occurring between 3.0 and 5.0 mM manganese was due to manganese and not external calcium leaking into the ghosts. Since their was no appreciable change in the two results, manganese may be able to substitue for calcium in resealing the ghosts, however, its effect on activating Mg+Ca-ATPase seems negligible.

Manganese did not activate calcium activated ATPase in RBCMF. It is possible that contamination of the manganese with calcium could account for the difference in results obtained here and those reported by Bader (43).

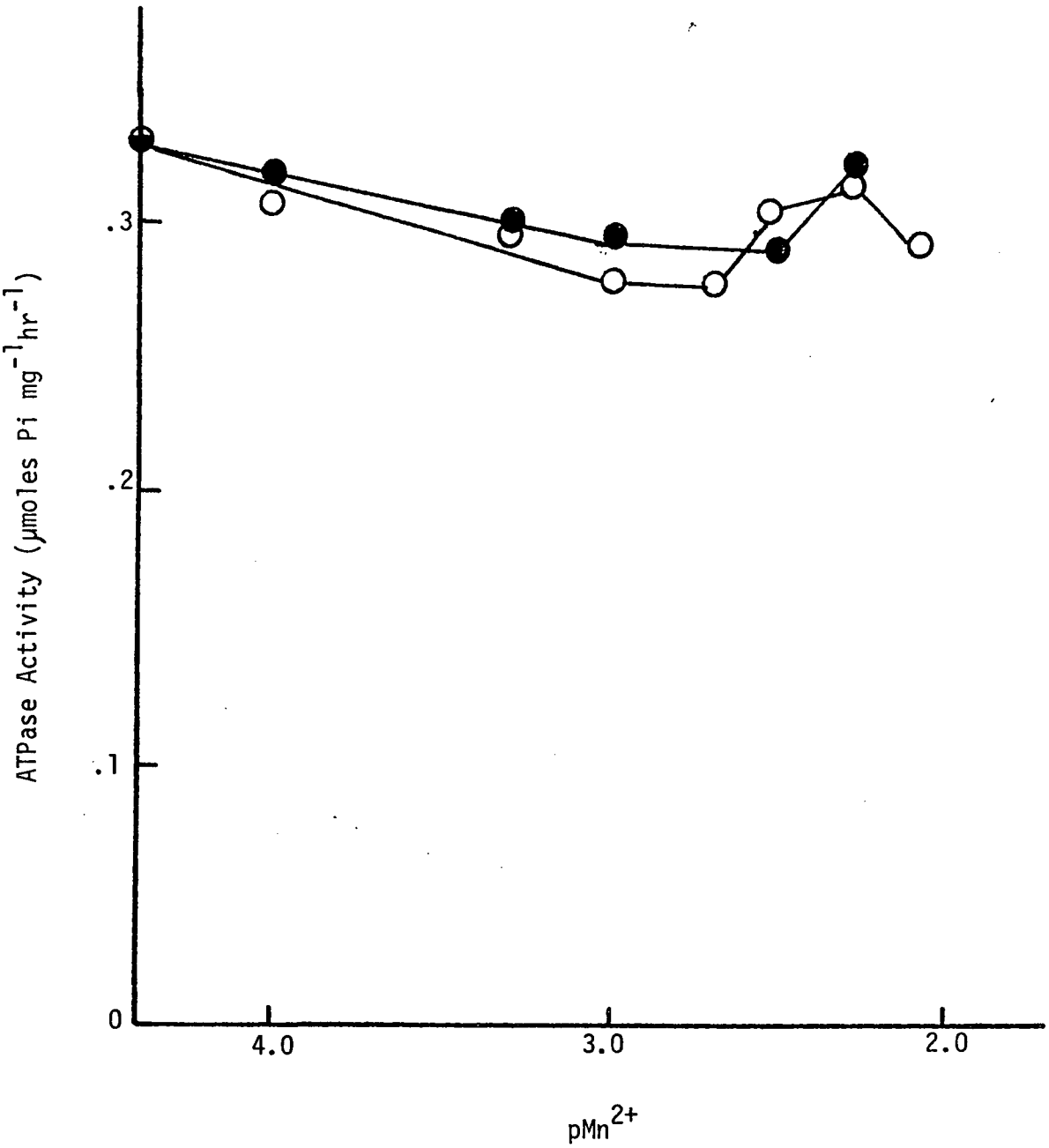


Figure 22. Effect of varying the concentration of manganese in the loading medium on ATPases activity in the absence of external calcium (○) and in the presence of 1.0 mM external calcium(●).Each point represents a single experiment.

CONCLUSIONS

A suitable preparation of resealed erythrocyte ghosts was developed in this study. The use of 1.0 mM EDTA in the washing procedure resulted in a ghost population essentially free of endogenous ATP and divalent cations. This method of preparation has advantages over previous methods used for preparing ATP free ghosts since long preincubation periods in metabolically depleted media or iodoacetate treatment of the whole cells prior to preparation of the ghosts is not required. Ghosts prepared by this method required the addition of calcium ($\geq$ .05 mM) to the loading medium in order to reseal. Magnesium was unable to substitute for calcium in this respect. The use of EDTA did not result in removal or denaturation of membrane ATPase(s) since specific activities comparable to those reported in the literature were obtained for both Na,K-ATPase and Mg+Ca-ATPase. Further evidence was given indicating that the ghosts had resealed (p 35) and that Na,K-ATPase and Mg+Ca-ATPase could be asymmetrically stimulated by cations.

In contrast to a report by Schatzmann and Vincenzi (12) it was found that external divalent cations (Mg and Ca) further increased Mg+Ca-ATPase activity in this ghost preparation. The mechanism of action was attributed to the replacement of membrane divalent cations removed by EDTA (not used by Schatzmann and Vincenzi). Presumably calcium or magnesium are required to maintain the most favourable conformation of this enzyme. It was of interest that the specific activity of Na,K-ATPase was

not increased by these external divalent metal cations. This study was the first to show the presence of a high and low affinity Mg+Ca-ATPase in resealed human erythrocyte ghosts. Previous studies had shown a high and low affinity Mg+Ca-ATPase in RBCMF. However, the concentrations of calcium in the loading medium were more than ten times higher than the concentrations of calcium required to achieve optimal activation in RBCMF. It was suggested that the concentrations of calcium in the loading medium do not represent the true intracellular concentration of free calcium, for reasons outlined on p 42 and reference 30.

The present studies provide strong evidence that only the hydrolysis of ATP due to low affinity Mg+Ca-ATPase is coupled to calcium transport. A stoichiometry of two was found in contrast to a previous estimate by Schatzmann and Vincenzi (12), who reported a stoichiometry of 0.77. However, their study (12) was done with only one internal calcium concentration (1.0 mM). In addition, the present study has shown that a stoichiometry of less than one is obtained at low concentrations of calcium where the high affinity Mg+Ca-ATPase is also functioning. The results further indicate that the operation of the calcium pump is a cooperative process; the pump becoming more efficient as the concentration of calcium is increased. This mechanism may be very important to the survival of the cell in situations in which the internal calcium concentration becomes high. Ruthenium red may prove to be a useful tool for investigating the cooperative aspects of the mechanism of calcium

transport, since this dye inhibits the more efficient form of the transport system. The finding that the inhibition by ruthenium red increased with increasing concentrations of calcium in resealed ghosts was not found in RBCMF where ruthenium red inhibited equally at all concentrations of calcium (76). This finding implies that in RBCMF only one state of the enzyme can exist, in contrast to the enzyme in the resealed ghost.

The finding that low affinity Mg+Ca-ATPase may be associated with calcium transport was at first somewhat surprising since one might expect that the Mg+Ca-ATPase with the highest affinity for calcium might be best suited for maintaining a low intracellular calcium. However the low affinity Mg+Ca-ATPase possesses a higher specific activity than the high affinity Mg+Ca-ATPase and may possess a threshold sufficiently low to maintain a low intracellular concentration of calcium. In conditions of low pH and low oxygen tension which can occur in the spleen (84,85), extracellular calcium can enter the red blood cell. In such cases the low affinity Mg+Ca-ATPase may be well adapted to handling high internal calcium conditions.

The function of the high affinity Mg+Ca-ATPase was not determined in this study, but may be associated with the actin-like protein (spectrin) isolated from the inner aspect of the RBC (55).

Quinine, quinidine and propranolol were found to have no effect on Mg+Ca-ATPase in resealed ghosts when added either externally or internally. These drugs are thought to be membrane

stabilizing agents and it was anticipated that Mg+Ca-ATPase activity might be blocked if these drugs act by expansion of the membrane and by producing unfavourable conformational perturbations in the membrane microenvironment.

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APPENDIX

Table A

Effect of internal calcium on the velocity of calcium efflux.

-log [Ca] in the loading medium (M)	μmoles Ca mg^{-1} hr^{-1} mean	± SE	n
4.0	.279		1
3.6	.291	.042	2
3.3	.337	.027	3
3.0	.427	.031	3
2.7	.742	.072	3
2.5	1.035	.021	3
2.3	1.15	.076	3

Table B

Effect of internal calcium on the velocity of Mg+Ca-ATPase.

-log [Ca] in the loading medium (M)	μmoles Pi mg^{-1} hr^{-1} mean	± SE	n
4.0	.296	.005	3
3.6	.333	.007	3
3.3	.316	.006	3
3.0	.350	.007	3
2.7	.411	.004	3
2.52	.4926	.020	4
2.39	.5525		1
2.3	.577	.014	3

Table C

Effect of external ruthenium red on the velocity of calcium transport.

[Ca] in the loading medium (mM)	[Ruthenium Red] (mM)	μmoles $Ca^{2+} mg^{-1} hr^{-1}$
3.0	0	1.074, 1.012, 1.02
3.0	.1	.610, .635
3.0	.2	.475, .565
3.0	.4	.341, .385

Table D

Changes in the calcium concentration of resealed ghosts with time.

[Ca] in the loading medium (mM)	Time (min.)	μmoles $Ca^{2+} mg^{-1}$ mean	± SE	n
0.1	0	.443		1
0.1	5	.223		1
0.1	10	.176		1
0.1	20	.202		1
0.25	2	.455	.001	2
0.25	4	.330		1
0.25	6	.261		1
0.25	8	.197	.004	2

Table D (cont'd)

[Ca] in the loading medium (mM)	Time (min.)	µmoles Ca^{2+} mg^{-1} mean	± SE	n
0.5	0	.474	.006	4
0.5	2	.432	.045	2
0.5	3	.366	.025	2
0.5	4	.364		
0.5	5	.252	.002	2
0.5	6	.258	.042	2
0.5	7	.184		
0.5	8	.188		
0.5	10	.152	.016	3
0.5	20	.136		1
1.0	0	.585	.027	4
1.0	2	.398		1
1.0	3	.398	.022	2
1.0	5	.269	.026	3
1.0	6	.239		1
1.0	7	.193		1
1.0	10	.173	.002	2
1.0	20	.215	.011	2
2.0	0	.807	.007	3
2.0	3	.6965	.014	2
2.0	4	.621	.039	2
2.0	5	.454		1

Table D (cont'd)

[Ca] in the loading medium (mM)	Time (min.)	µmoles $Ca^{2+}mg^{-1}$ mean ± SE		n
2.0	6	.412	.039	3
2.0	7	.307		1
2.0	8	.273		1
2.0	10	.229	.003	3
3.0	0	1.017	.009	3
3.0	2	.98		1
3.0	3	.797		1
3.0	5	.624	.075	3
3.0	7	.284		1
3.0	10	.202	.025	3
3.0	20	.612	.007	3
5.0	0	1.425	.036	3
5.0	2	1.44		
5.0	3	1.445		
5.0	5	1.19	.089	3
5.0	7	0.930		1
5.0	10	.489	.034	2
5.0	20	.296		1
5.0	30	.250		

Table E

Effect of ruthenium red (0.2 mM) on activation of Mg+Ca-ATPase in the loading medium.

[Ca] in the loading medium (mM)	[Ruthenium Red] (mM)	μmoles Pi $mg^{-1}hr^{-1}$ mean ± SE	n
0.25	0.2	.301 .023	3
.5	0.2	.321 .010	3
1.0	0.2	.356 .012	3
3.0	1.0	.301 .009	3
5.0	1.0	.3076 .025	3

Table F

Effect of varying the concentration of ruthenium red on Mg+Ca-ATPase activity.

[Ca] in the loading medium (mM)	[Ruthenium Red] (mM)	μmoles Pi $mg^{-1}hr^{-1}$ mean ± SE	n
0.5	0	.3166 .006	3
0.5	.2	.321 .011	3
3.0	0	.4926 .020	3
3.0	.05	.391	1
3.0	.10	.352	1

Table F (cont'd)

[Ca] in the loading medium (mM)	[Ruthenium Red] (mM)	µmoles Pi $mg^{-1}hr^{-1}$ mean ± SE		n
3.0	.20	.301	.009	3
3.0	.40	.210		1
5.0	0	.577	.014	3
5.0	.05	.501	.026	3
5.0	.1	.406	.023	3
5.0	.2	.307	.025	3
5.0	.4	.232	.011	3

Table G

Effect of 0.2 mM external ruthenium red on the activation of calcium transport by internal calcium.

[Calcium] in the loading medium (1.0 mM)	[Ruthenium Red] (mM)	µmoles $Ca^{2+}mg^{-1}hr^{-1}$
1.0	0.2	.425, .440
2.0	0.2	.479, .466
3.0	0.2	.475, .565
5.0	0.2	.430, .540

Table H

Effect of varying the concentration of calcium in the loading medium in the presence and absence of external calcium.

- all conditions are standard except 0.4 mM EGTA was included in the loading medium.

-log [Ca] in the loading medium (M)	[Ca] in the external medium (mM)	μmoles Pi $mg^{-1}hr^{-1}$
4.0	1.0	.310,.318
3.52	1.0	.313,.325
3.30	1.0	.360,.342
3.125	1.0	.340,.346
3.0	1.0	.349,.367
2.699	1.0	.400,.406
2.523	1.0	.462,.473
2.30	1.0	.586,.574
2.125	1.0	.594,.606
2.0	1.0	.491,.499
2.22	1.0	.495,.504
4.30	0	.254,.248
4.0	0	.272,.282
3.699	0	.290,.298
3.30	0	.295,.304
3.125	0	.310,.318
2.823	0	.358,.364
2.602	0	.384,.396
2.426	0	.423,.480

Table H (cont'd)

-log [Ca] in the loading medium (M)	[Ca] in the external medium (mM)	μmoles Pi $mg^{-1}hr^{-1}$
2.30	0	.490, .460
2.20	0	.50, .516
2.125	0	.541, .553
2.0	0	.498, .518
1.9	0	.440, .468

Table I

Effect of manganese on Mg+Ca-ATPase activity in resealed ghosts.

-log $[Mn]^{++}$ in the loading medium	$[Ca]^{++}$ in the external medium	μmoles Pi $mg^{-1}hr^{-1}$
4.0	1.0	.318
3.3	1.0	.302
3.0	1.0	.296
3.52	1.0	.288
3.30	1.0	.324
4.0	0	.306
3.3	0	.296
3.0	0	.278
3.7	0	.281
3.52	0	.302
3.30	0	.314
3.126	0	.290

Table J

Effect of ouabain on ATPase activity on ghosts resealed with KCl (2.876 M)
- Ca in the loading medium was 0.5 mM.

Time (min)	[Ouabain] in the external medium (mM)	μmole Pi $mg^{-1}hr^{-1}$
0	0	.156
10	0	.203
30	0	.535
40	0	.605
0	0.2	.156
10	0.2	.225
20	0.2	.306
30	0.2	.392
40	0.2	.447

Table K

Activation of Na,K-ATPase by external potassium in resealed ghosts.
- Ca^{2+} in the loading medium was 0.5 mM and calcium was not present in the loading medium when indicated.

$[K^+]$ in the external medium (mM)	[Ouabain] in the external medium	$[Ca^{2+}]$ in the external medium	μmoles Pi $mg^{-1}hr^{-1}$
0	0.1	0	.302
1.5	0.1	0	.300
4.5	0.1	0	.298
10.0	0.1	0	.300
0	0	0	.303,.297,.337
1.5	0	0	.447,.460
4.5	0	0	.550,.558
10.0	0	0	.638,.646
15.0	0	0	.636,.645
0	0	1.0	.365,.373
1.5	0	1.0	.562,.570
4.5	0	1.0	.643,.656
10.0	0	1.0	.676,.682
15.0	0	1.0	.648,.660

Table L

Effect of external calcium on ATPase activity.
-the ghosts were loaded with 0.5 mM calcium and .4 mM EGTA. Other conditions were standard.

$[Ca^{2+}]$ in the external medium (mM)	μmoles Pi $mg^{-1}hr^{-1}$
1.0	.073,.070
1.5	.098,.104
2.0	.118,.130
3.0	.340,.335
5.0	.146,.140

Table M

Effect of external magnesium on ATPase activity.
-conditions same as Table L.

$[Mg^{2+}]$ in the external medium	μmoles Pi $mg^{-1}hr^{-1}$
1.0	.02,.03
2.0	.044,.050
3.0	.062,.055
4.0	.048,.052