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# Ca<sup>2+</sup>-DEPENDENT PHOSPHATASE AND ATPase ACTIVITIES IN EEL GILL PLASMA MEMBRANES—I. IDENTIFICATION OF Ca<sup>2+</sup>-ACTIVATED ATPase ACTIVITIES WITH NON-SPECIFIC PHOSPHATASE ACTIVITIES\*

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**Abstract**—1. The characteristics of Ca<sup>2+</sup>-activated ATPase activities previously often postulated as components for the calcium transporting system in fish gills do not fulfil the requirements of a transport Ca<sup>2+</sup>-ATPase.

2. The chelation of Ca<sup>2+</sup>- or Mg<sup>2+</sup>-ions is a prerequisite for the adenosinephosphate esters to serve as substrate for gill plasma membrane phosphatases.

3. Ca<sup>2+</sup>-activated ATP hydrolysis results from the activity of a heterogeneous pool of phosphatases located in the plasma membranes of the branchial epithelium, as is concluded from substrate specificity tests and the effects of various inhibitors on these hydrolytic activities.

4. In the present study only non-specific phosphatases could be shown.

## INTRODUCTION

Teleost fish, whether in fresh water (FW) or sea water (SW), possess efficient mechanisms to maintain blood calcium levels (Wendelaar Bonga and Van der Meij, 1980). In FW, under normal conditions but especially during periods of starvation, this control probably involves the active transfer of calcium from the ambient medium into the extracellular fluids. Indeed, it is well known that fish can extract calcium from their external environment and there is ample evidence to suggest that the major site for this uptake is associated with the branchial apparatus (Mashiko and Jozuka, 1964a; Berg, 1968). The mechanism of this transfer, however, has not been adequately studied. One of us (Fenwick, 1976, 1979), among others (Ma *et al.*, 1974; Moon, 1978; Ho and Chan, 1980; Doneen, 1981) described the presence in the gills of a Ca<sup>2+</sup>-activated adenosinetriphosphatase (Ca<sup>2+</sup>-ATPase§) which, in

ouabain-sensitive Na<sup>+</sup>/K<sup>+</sup>-ATPase, was presumed to be the enzymic basis for the active transport of calcium through the gills. This presumption was supported by various observations. Most significantly, the influx of <sup>45</sup>Ca<sup>2+</sup> in perfused, isolated, American eel gills correlated positively with Ca<sup>2+</sup>-ATPase activity (Fenwick, 1976; So and Fenwick, 1977). Additionally, the activity of this enzyme was reported to be influenced directly by hypocalcin (Ma and Copp, 1978), a hormone which can markedly influence calcium metabolism in teleosts. Further, the level of enzymic activity varied with ambient calcium concentration (Fenwick, 1978).

However, there are serious difficulties with this thesis. If active transport of calcium occurs in the gills it most likely occurs at the basal or lateral membranes where the calcium moves from an intracellular calcium concentration of about 10<sup>-7</sup> M into a body fluid calcium concentration of about 3 × 10<sup>-3</sup> M. Active transport would not be required to move the calcium from the ambient medium (10<sup>-4</sup> M) into the intracellular compartment (10<sup>-7</sup> M). However, the typical apparent affinity (K<sub>m</sub>) of the gill Ca<sup>2+</sup>-ATPase has been reported as about 0.45 mM Ca<sup>2+</sup>, a value which is not commensurate with effective functioning at the basal or lateral membrane sites. Secondly, the reported pH-optima for these activities were about 8.0 (Ma *et al.*, 1974; Fenwick, 1976) and were therefore within the favorable range for alkaline phosphatase activities. Thirdly, the Ca<sup>2+</sup>-stimulated ATP hydrolytic activity, even measured at suboptimal pH's (Fenwick, Ma), exceeded reported Na<sup>+</sup>/K<sup>+</sup>-ATPase activity to such an extent that Ca<sup>2+</sup>-transport rates would surpass expected Na<sup>+</sup>-transport rates by several orders of magnitude. By the data presented in the literature the

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§Abbreviations: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; *p*-NPP, para-nitrophenylphosphate; *p*-NP, para-nitrophenol; ATPase, adenosinetriphosphate hydrolase; *p*-NPPase, para-nitrophenylphosphatase; MS222, tricaine methanesulphonate; Tris, tris (hydroxymethyl) aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; TCA, trichloroacetic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid; EGTA, [ethylene-bis(oxyethylenenitrile)] tetraacetic acid; NTA, nitrilotriacetic acid; R24571, 1-[bis(*p*-chlorophenyl)-methyl]-3-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazoliumchloride; Me<sup>2+</sup>, Ca<sup>2+</sup> or Mg<sup>2+</sup>.



reverse is indicated (Fleming, 1973). Additionally, there has been no unqualified evidence published to indicate that ATP is the preferred substrate for the presumptive  $\text{Ca}^{2+}$ -ATPase. However, in higher vertebrates this  $\text{Ca}^{2+}$ -ATPase has been tentatively identified as an alkaline phosphatase-like activity, instead of an ATPase with characteristics of an ion transporting enzyme. In 1970, Haussler *et al.* (1970) were the first to report that alkaline phosphatase,  $\text{Ca}^{2+}$ -ATPase and much of the  $\text{Mg}^{2+}$ -ATPase activities of chick brush border membranes are properties of the same enzyme protein. More support for this idea was given by Oku and Wasserman (1978), who studied these activities in chick intestinal brush borders, and by Hanna *et al.* (1978, 1979) in their studies on these activities in rat enterocyte plasma membranes.

Recently, Ghijsen and Van Os (1979, 1982), Ghijsen *et al.* (1980, 1982), Van Os *et al.* (1980) and Van Os and Ghijsen (1981) showed in a series of studies on the mechanisms of calcium transport in the kidney cortex and gut of rats, that  $\text{Ca}^{2+}$ -activated ATPase activity is not homogeneous. To be more specific, they detected two different  $\text{Ca}^{2+}$ -stimulated ATP hydrolytic enzymic activities in the plasma membranes of enterocytes. One phosphatase was located on both the brush border and the basolateral membranes, had a predominantly low affinity site for calcium, and was inhibited by theophylline and L-phenylalanine, both of which are specific inhibitors of alkaline phosphatase. The other phosphatase, which was located exclusively in the basolateral membranes, had high affinity for calcium, was not inhibited by alkaline phosphatase inhibitors, but was specifically inhibited by the calmodulin antagonist chlorpromazine. Additionally, they showed that the rate of accumulation of calcium by sealed vesicles of basolateral membranes was an exponent of the activity of high calcium affinity  $\text{Ca}^{2+}$ -ATPase and not of the low calcium affinity  $\text{Ca}^{2+}$ -ATPase. As a result of these studies they concluded that the low affinity phosphatase was an alkaline phosphatase-like enzyme and that the high affinity phosphatase was the true  $\text{Ca}^{2+}$ -ATPase with characteristics of an ion transporting enzyme. This is supported by the observation that 1,25-dihydroxy vitamin  $\text{D}_3$  treated rachitic rats show concurrent increases in intestinal calcium absorption, high-affinity  $\text{Ca}^{2+}$ -ATP activity and ATP dependent  $\text{Ca}^{2+}$ -transport in the basolateral membranes.

Because of the difficulties listed earlier, and the recent evidence for the existence of at least two  $\text{Ca}^{2+}$  dependent phosphatases, we decided to re-evaluate characteristics of the putative teleost gill  $\text{Ca}^{2+}$ -ATPase using more rigorous and inclusive criteria to investigate whether the low affinity non-specific phosphatase activity or the high affinity  $\text{Ca}^{2+}$ -ATPase activity is the most likely candidate for the energy generating source of the calcium pump in eel gills. To this end we improved the previously reported procedure (Fenwick, 1976) for the isolation of gill plasma membranes. Additionally, we prevented the ATPase activity which might have resulted from mitochondrial contamination by adding as a routine specific mitochondrial ATPase inhibitors to the assay media.

## MATERIALS AND METHODS

Yellow female eels, *Anguilla rostrata* LeSueur, were used. The fish used for enzyme characterization studies had an average body weight of 1.7 kg and were obtained in the spring of 1982 from a commercial fish dealer in Quebec City, Quebec, Canada. The eels were held in running dechlorinated Ottawa city tapwater (0.45 mM  $\text{Ca}^{2+}$ , 12°C) under 16 hr of light alternating with 8 hr of darkness. During the experiments the fish were not fed. The experiments were carried out in the summer of 1982.

### Isolation of plasma membranes

Animals were quickly anaesthetized in a Tris-buffered (pH 7.4) MS-222 solution (6 g/l). The bulbus arteriosus was cannulated and the branchial apparatus was perfused with ice cold isotonic saline containing heparin (20 U/ml) to remove the blood cells from the gills. Additionally, 0.2 mM phenylmethylsulphonylfluoride (PMSF), a protease inhibitor, was added to the perfusion fluid to increase enzyme recovery. The branchial epithelium was scraped off onto an ice-cold glass plate with a glass microscope slide. The subsequent rapid (3 hr) preparative isolation procedure was carried out at 0–4°C. The collected material ( $\pm 2.5$  g/kg eel) was disrupted gently with a douncer fitted with a loose pestle (20 strokes) in 15 ml of a hypotonic buffer (pH 8.0) containing (mM): NaCl (25), PMSF (0.2) and Hepes/Tris (1). The homogenate was diluted with the same buffer to 75 ml and centrifuged 15 min at 550 g. The pellet, containing nuclei and cellular debris, was discarded. The supernatant ( $\text{H}_0$ ) was centrifuged for 30 min at 27K rpm (Beckmann SW<sub>28</sub> rotor) and yielded a pellet containing the membrane fraction ( $\text{P}_0$ ). The pellet was resuspended with a douncer (100 strokes) in 15 ml of an isotonic buffer containing (mM): sucrose (250), NaCl (12.5),  $\text{H}_2\text{EDTA}$  (0.5), Hepes/Tris (5, pH:7.5); osmolarity: 300 mOsm/l.  $\text{H}_2\text{EDTA}$  was added to ensure a leaky vesicle preparation. The suspension was diluted with the same buffer to 30 ml and centrifuged differentially: 1 Kg. 10 min, 10 Kg. 10 min, 30 Kg. 30 min. The final pellet ( $\text{P}_3$ ) was used as the eel gill plasma membrane fraction. This pellet was resuspended with a douncer (100 strokes) in the basic assay buffer (mM): Tris-HCl (20), NaCl (100), pH, 7.4. To prevent cryodamage of membrane proteins, portions as required for the assays were quickly frozen in liquid nitrogen and stored at –90°C until use.

### Assays and assay media

**Protein.** Membrane protein was estimated with a commercial reagent kit (BioRad) using bovine serum albumin as reference.

**$\text{Na}^+/\text{K}^+$ -ATPase.**  $\text{Na}^+/\text{K}^+$ -ATPase was assayed by the method of Bonting and Caravaggio (1963). The incubation was started by the addition of a 20- $\mu\text{l}$  sample of the membrane preparation, containing 10–20  $\mu\text{g}$  protein, to 400  $\mu\text{l}$  medium. The ouabain-sensitive,  $\text{K}^+$ -dependent activity was calculated as the difference in activities measured in two media. Medium A, yielding total ATPase activity, consisted of (mM): NaCl (100),  $\text{MgCl}_2$  (5),  $\text{H}_2\text{EDTA}$  (0.1),  $\text{Na}_2\text{ATP}$  (3), KCl (12.5) and imidazole (30); pH 7.4. Medium E, yielding the ouabain-insensitive,  $\text{K}^+$ -independent activity, consisted of medium A without KCl but with added ouabain (1.0 mM). The incubation was performed at 37°C for 10–30 min. The reaction was stopped on ice and by adding 100  $\mu\text{l}$  ice-cold TCA (40%). To determine ATP hydrolysis, the liberated  $\text{P}_i$  was estimated as described by Fenwick (1976) with a Technicon auto-analyzer.

### Alkaline phosphatase (pH 10.4)

Membrane alkaline phosphatase was estimated with a commercial reagent kit (Sigma) for the assay of serum or plasma alkaline phosphatase: *p*-nitrophenylphosphatase



Table 1. Recovery and purification of eel gill plasma membranes (P<sub>3</sub>-fractions)

	Na <sup>+</sup> /K <sup>+</sup> -ATPase	Alkaline phosphatase	Succinate dehydrogenase	Protein
Recovery (%)	13.1±2.33 (6)	1.8 ± 0.76 (6)	3.39±0.39 (7)	2.6±0.57 (7)
Purification	61.9±5.76	0.91 ± 0.21	1.62±0.36	—

Recoveries were expressed as percentages of the total enzyme activities in the initial homogenate (H<sub>0</sub>). The purification factors represent the ratios of the specific activities in the plasma membrane fraction (P<sub>3</sub>) and the initial homogenate (H<sub>0</sub>). Mean values (±SEM) are given, with the number of observations in parentheses.

(*p*-NPPase) activity was assayed at pH 10.4 in a glycine buffer in the presence of 5 mM Mg<sup>2+</sup>. Eel gut alkaline phosphatase (Sigma, type XIX) served as a reference. Activities were expressed in units eel gut alkaline phosphatase equivalents on basis of *p*-NP release. After the reaction was stopped with 1 N NaOH, *p*-NP was measured at 420 nm.

#### Succinate dehydrogenase

Succinate dehydrogenase (SDH) activity was estimated in a medium (pH 8.0) containing (mM): K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> (50), Na<sub>2</sub>-succinate (50), sucrose (25), and 1 mg/ml 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazoliumhydrochloride (*p*-INT) according to Pennington (1961). The reaction was started by the addition of samples varying from 20–100 μl to 1 ml assay medium and incubations were carried out at room temperature until appropriate pink coloration. SDH activities were determined on the same day as isolation. The reaction was stopped by the addition of 100 μl TCA (40%) and extraction was done with 4 ml ethylacetate. The activity was determined in the organic phase by measuring the absorbance at 490 nm after overnight phase separation at 4°C. Activities were expressed as A<sub>490</sub>/hr mg protein.

#### Ca<sup>2+</sup>-stimulated phosphatases

The basic medium for Ca<sup>2+</sup>-stimulated phosphatase activities (pH 7.4) consisted of (mM): NaCl (100), ouabain (0.1), NaN<sub>3</sub> (5), oligomycin B (5 μg/ml), Tris-HCl (20); disodium salts of ATP, ADP, AMP and *p*-NPP were used as substrates. Ca<sup>2+</sup> ~ or Mg<sup>2+</sup> ~ substrate complexes were prepared by adding equimolar concentrations of the chloride salts of either ion, assuming a 1 : 1 ratio for Me<sup>2+</sup> ~ chelation by adenosinephosphate-esters (Sapper *et al.*, 1980). Incubations were performed on 20-μl samples in a total volume of 500 μl for 30 min at 25 or 37°C. Substrate hydrolysis was estimated as described for the Na<sup>+</sup>/K<sup>+</sup>-ATPase assay.

In all assays blanks were prepared by adding membrane samples after the reaction was stopped.

Lipophilic inhibitors (chlorpromazine, phenothiazine, R24571) were dissolved in ethanol (100%) and brought to the required concentration in the assay medium (ethanol concentrations did not exceed 0.1%). Membrane samples were preincubated with the desired final concentration of these inhibitors for 15 min at 37°C as suggested for R24571 by Gietzen *et al.* (1981). Ethanol treated samples served as controls. All assays were performed in plastic tubes.

Water soluble inhibitors (theophylline, 1-phenylalanine, cysteine) were directly dissolved in the assay medium.

#### Reagents

All reagents used were of the highest purity commercially available. Ultrapure water was used in all assays. R24571 was purchased from Janssen Pharmaceutica, Beerse, Belgium. All other chemicals were obtained from Sigma (St. Louis, MO).

#### Statistics and calculations

Values are expressed as mean values±SEM. Statistical

analysis of the data was carried out applying Student's *t*-test. Significance was accepted with *P*<0.05 when *n*<4, or with *P*<0.02 when *n*>4 (*α*=5%). Apparent *K<sub>m</sub>*-values and *V<sub>max</sub>*-values were calculated by means of Lineweaver-Burk transformation of the Michaelis-Menten equation. Linear regression analysis was based on the least square method.

## RESULTS

### Isolation procedure

Table 1 shows the percentage recovery and the purification factors for several marker enzymes in the P<sub>3</sub>-fraction. Na<sup>+</sup>/K<sup>+</sup>-ATPase was used as plasma membrane marker, while succinate dehydrogenase was taken as a marker for mitochondrial membranes. The P<sub>3</sub>-fraction contained about 13% of the initial Na<sup>+</sup>/K<sup>+</sup>-ATPase and underwent a 60-fold purification. Succinate dehydrogenase activity was purified to only a factor of about 1.6 and showed a recovery of about 3.4%. Although a high degree of purification for plasma membranes was obtained as judged by the Na<sup>+</sup>/K<sup>+</sup>-ATPase purification factor, the P<sub>3</sub>-fraction still showed mitochondrial contamination. Therefore, we included oligomycin and sodium azide in all our ATPase assays to specifically exclude mitochondrial ATPase activities. Only around 2% of the initial alkaline phosphatase (measured at pH 10.4) was recovered with a purification factor of 0.91. The use of alkaline phosphatase as plasma membrane marker was suggested by Ma *et al.* (1974) but we conclude this enzyme to be inappropriate in the present study. We prefer the use of Na<sup>+</sup>/K<sup>+</sup>-ATPase as a plasma membrane marker because of its membrane-specificity and its specific ouabain-sensitivity.

### Test for substrate accessibility of the membrane vesicles

Tween-80 had no significant effect on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of membrane vesicles of the P<sub>3</sub>-fraction (Table 2). As detergent treatment of sealed vesicles should increase Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Brotherus *et al.*, 1979) as a result of improved substrate accessibility, we concluded that the membrane vesicles of the P<sub>3</sub>-fraction were sufficiently leaky and that they did not require detergent treatment for optimal substrate- and ion-accessibility. Apparently the use of EDTA in the isolation procedure secures optimal enzyme activities in this plasma membrane vesicle preparation.

### Effects of storage on Ca<sup>2+</sup>- or Mg<sup>2+</sup>-activated ATP hydrolysis

In Fig. 1 the effects of storage at -90°C on the



Table 2. Effects of Tween-80 on the Na<sup>+</sup>/K<sup>+</sup>-ATPase activities in eel gill plasma membranes (P<sub>3</sub>-fractions)

	Na <sup>+</sup> /K <sup>+</sup> -ATPase (μmol P <sub>i</sub> /hr/mg protein) Controls	Detergent treated
Tween-80	51.25±14.1 (100%)	52.14±15.4 (103%)

Na<sup>+</sup>/K<sup>+</sup>-ATPase activities were used as an indicator of substrate accessibility of the membrane vesicle preparation. Samples were preincubated with Tween-80 (0.1% v/v) at 37°C for 10 min and subsequently incubated for 15 min at 37°C. Similar, buffer preincubated, samples served as controls. Mean values (±SEM) are given for six different samples, with the percentage activity in parentheses.

specific activities of Ca<sup>2+</sup>- or Mg<sup>2+</sup>-activated ATP hydrolysis is presented. The Ca<sup>2+</sup>-induced ATP hydrolytic activity proved to be more stable than the Mg<sup>2+</sup>-induced ATP hydrolytic activity with 61 and 37% of the original activity, respectively, being present after eight days. As a result of the difference in sensitivity towards storage at low temperature, the ratio of Mg<sup>2+</sup>-induced ATP hydrolysis to Ca<sup>2+</sup>-induced ATP hydrolysis decreased from 0.86 to 0.61 after one day and to 0.53 after 8 days (*P*<0.05). Both curves showed the characteristics of a curve composed of a fast and a slow component. A rapid decrease is observed during the first day of storage, followed by a slower decrease in activity thereafter. Comparable results were presented by Ma *et al.*

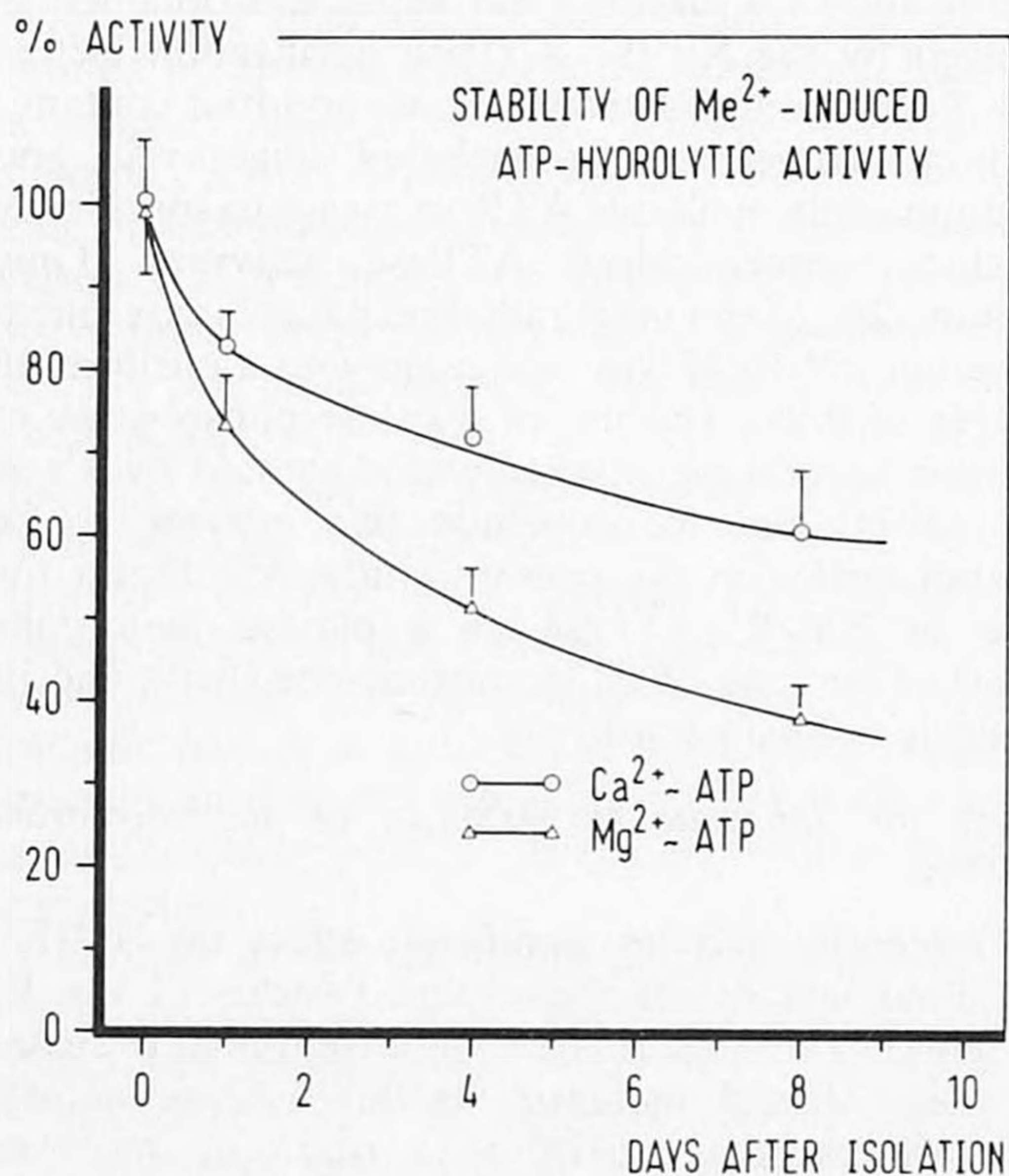


Fig. 1. Effects of storage on Me<sup>2+</sup>-induced ATP hydrolytic activities in eel gill plasma membranes. Aliquots of P<sub>3</sub>-fractions were frozen in liquid nitrogen and stored at -90°C. Me<sup>2+</sup>-induced ATP hydrolysis (at 3 mM Mg~ATP or Ca~ATP) was assayed on the day of isolation and 1, 4 and 8 days afterwards, as described in Materials and Methods. Specific activities at day 0 were designated 100% activity and amounted to 52.8±7.2 μmol P<sub>i</sub>/hr/mg protein for Ca~ATP and 45.4±8.9 μmol P<sub>i</sub>/hr/mg protein for Mg~ATP. Mean values and standard errors of the mean are given; *n*=4.

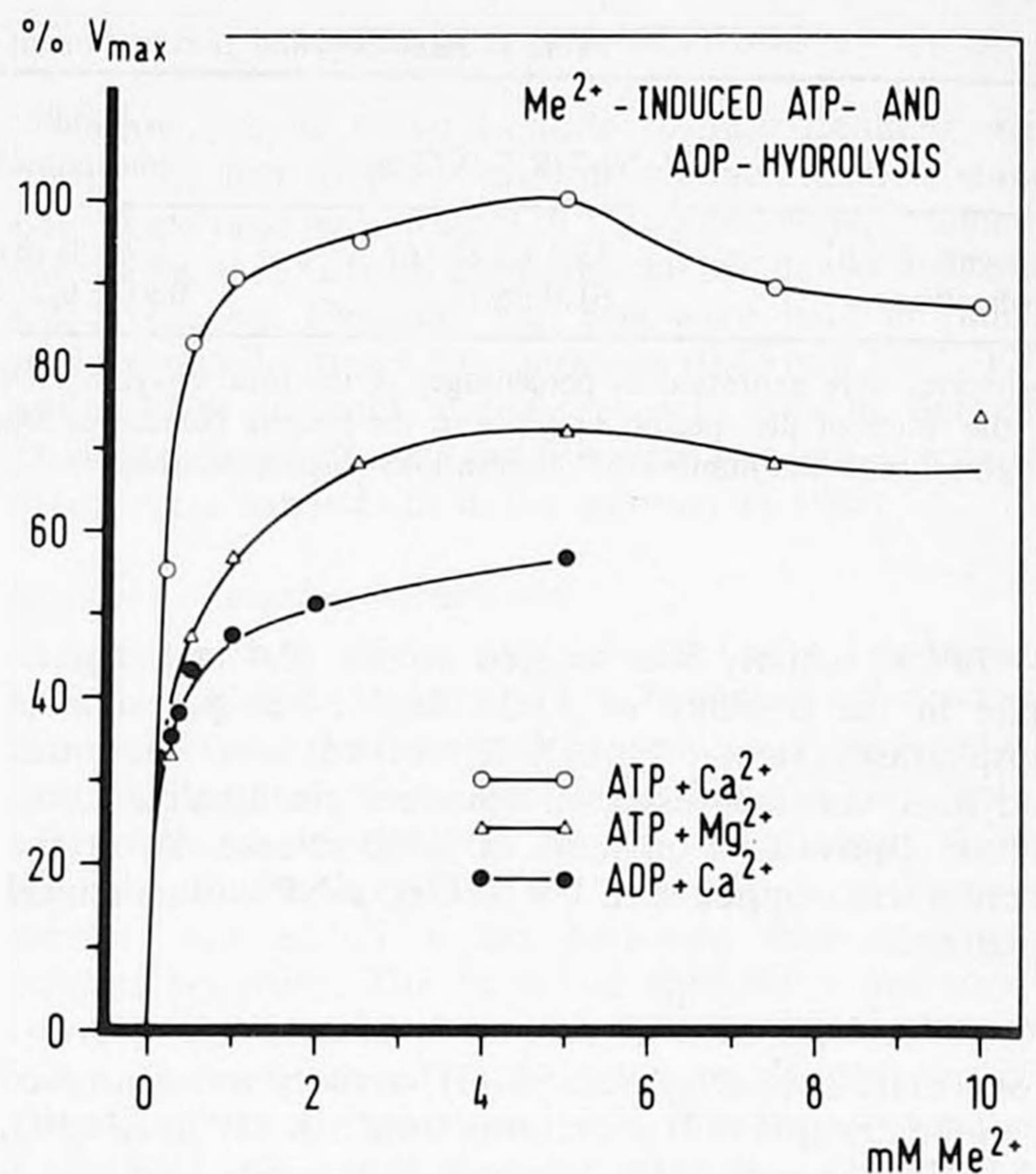


Fig. 2. Effects of different concentrations of Ca<sup>2+</sup>- or Mg<sup>2+</sup>-ions on ATP and ADP hydrolysis by eel gill plasma membranes (P<sub>3</sub>-fraction). Activities were expressed as percentages of the maximum hydrolytic activity (observed at 5 mM Ca<sup>2+</sup> and 5 mM ATP). Specific activities amounted to 60.2±2.8 μmol P<sub>i</sub>/hr/mg protein for 5 mM Ca~ATP, 34.7±8.1 μmol P<sub>i</sub>/hr/mg protein for 5 mM Ca~ADP and 43.1±3.9 μmol P<sub>i</sub>/hr/mg protein for 5 mM Mg~ATP. Me<sup>2+</sup>-concentrations that resulted in half-maximal activation of substrate hydrolysis (calculated with Lineweaver-Burk plots): 78 μM Ca<sup>2+</sup> for ATP, 51 μM Ca<sup>2+</sup> for ADP and 101 μM Mg<sup>2+</sup> for ATP. Mean values of six observations are given.

(1974) who stored their gill membrane preparations at -20°C. It is tempting to suggest that the difference between Ca<sup>2+</sup>- and Mg<sup>2+</sup>-induced ATP hydrolysis in sensitivity to storage was a result of the differential inactivation of a heterogeneous enzyme pool present in eel gill plasma membranes.

#### Activation of adenosinephosphate-ester hydrolysis by Ca<sup>2+</sup>- or Mg<sup>2+</sup>-ions

Significant hydrolysis of adenosinephosphate-esters upon incubation with eel gill plasma membranes was strictly dependent on the presence of Ca<sup>2+</sup>- or Mg<sup>2+</sup>-ions. This requirement for hydrolytic activity may involve activation of enzymes or a modification of the conformation of the substrates due to the chelation of these ions to adenosine phosphate-esters. The high concentrations of the ions required for maximum activation (5 mM Ca<sup>2+</sup>+5 mM ATP; Fig. 2) favor the latter explanation.

In a solution with a mixture of ATP, ADP and AMP and non-saturating concentrations of Ca<sup>2+</sup>- or Mg<sup>2+</sup>-ions, these ions will preferentially be chelated with the most complex ligand (ATP) because the affinity of the adenosinephosphate-esters for Ca<sup>2+</sup>- or Mg<sup>2+</sup>-ions increases with increasing numbers of phosphate groups in these esters (Table 3). At equimolar concentrations of Ca<sup>2+</sup> and ATP, enzy-



Table 3. Equilibrium constants for adenosinephosphate-esters and  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ions

Ligand	$\text{Mg}^{2+}$	$\text{Ca}^{2+}$
ATP	3.61–4.40	3.14–4.34
ADP	3.01–3.60	2.78–3.34
AMP	1.69–2.00	1.41–1.85

The combination of ligands with metal ions is given by:

$$\text{ML}_{n-1} + \text{LML}_n, \text{ where } K_n = \log(\text{ML}_n)/(\text{ML}_{n-1})(\text{L})$$

$K_1$ -values are given for media with an ionic strength of 0.1–0.2 mol/l at 20–30°C. Data were collected from: *Stability Constants*, Special publication No. 17, London, The Chemical Society, 1964.

matic breakdown of the  $\text{Ca}^{2+}\sim\text{ATP}$  complex will give  $\text{Ca}^{2+}\sim\text{ADP}$  and phosphate as reaction products. If, on the other hand, a surplus of ATP is present relative to  $\text{Ca}^{2+}$ -ions, upon enzymatic hydrolysis of  $\text{Ca}^{2+}\sim\text{ATP}$  the  $\text{Ca}^{2+}$ -ions will migrate from the reaction product  $\text{Ca}^{2+}\sim\text{ADP}$  to the free

ATP, which leads to a reaction mixture of  $\text{Ca}^{2+}\sim\text{ATP}$ , and free ATP and ADP.

Figure 2 shows the effects of different concentrations of  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ions on ATP or ADP hydrolysis at fixed concentrations of ATP or ADP (5 mM). Maximum activities consistently occurred at equimolar concentrations of divalent ion and ATP or ADP. The  $V_{\max}$ -value for  $\text{Mg}^{2+}$ -activation of ATP hydrolysis was 71.5% that of the  $V_{\max}$ -value found for  $\text{Ca}^{2+}$ -activation of ATP hydrolysis. The  $V_{\max}$ -values for  $\text{Ca}^{2+}$ -activation of ADP hydrolysis came to 57% of the  $V_{\max}$ -values for  $\text{Ca}^{2+}$ -activation of ATP hydrolysis. The apparent  $K_m$ -values for  $\text{Mg}^{2+}$ - or  $\text{Ca}^{2+}$ -induced substrate hydrolysis were  $78\ \mu\text{M}$   $\text{Ca}^{2+}$  for ATP,  $51\ \mu\text{M}$   $\text{Ca}^{2+}$  for ADP and  $101\ \mu\text{M}$   $\text{Mg}^{2+}$  for ATP. ATP-hydrolysis was increasingly diminished at  $\text{Mg}^{2+}$ - or  $\text{Ca}^{2+}$ -concentrations exceeding the ATP concentrations (2.5 or 5.0 mM).

In Figs 3(a) and (b) and Table 4 the results of substrate-specificity determinations are shown. Ap-

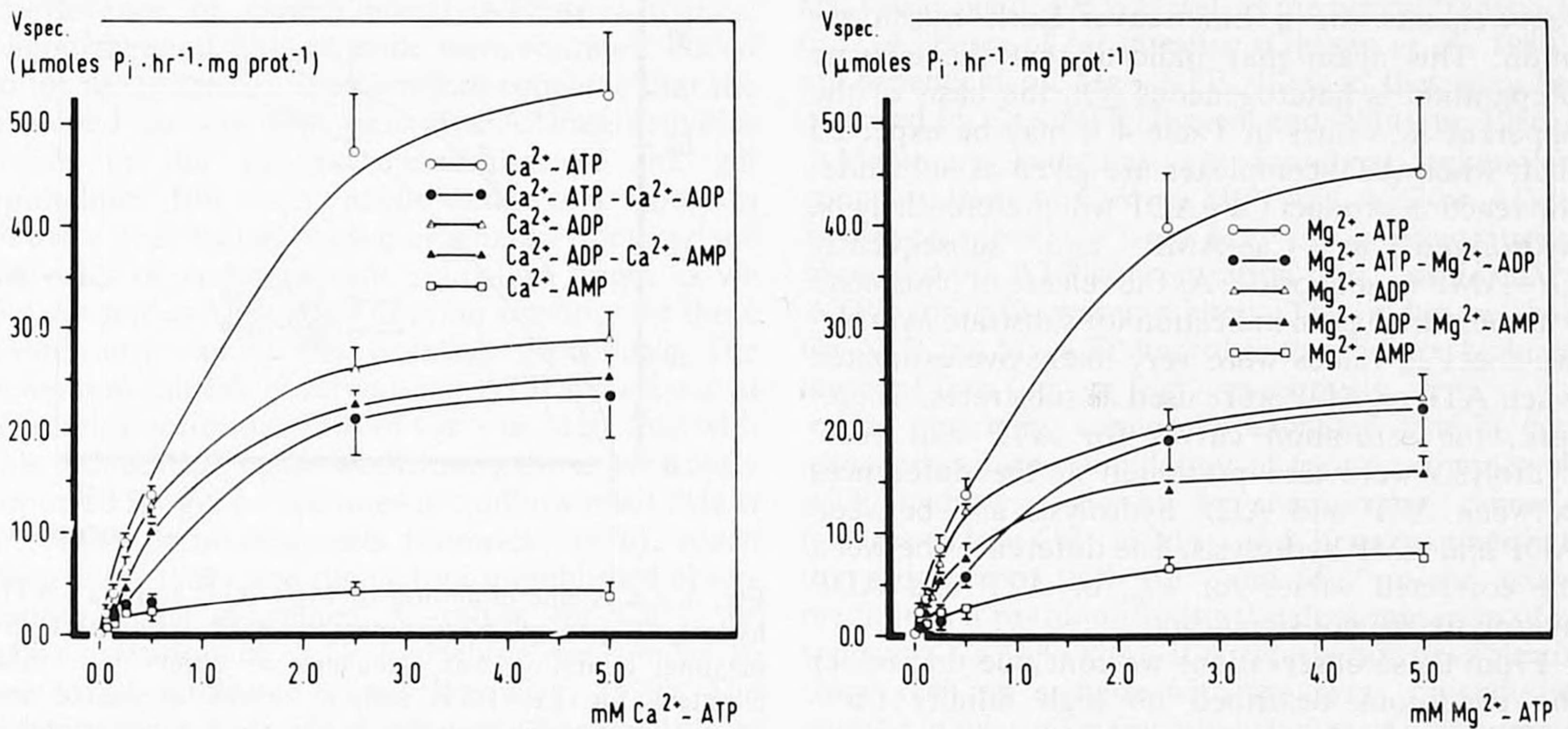


Fig. 3. Eel gill phosphatase substrate specificity tests. Aliquots of  $\text{P}_3$ -fractions were incubated with equimolar concentrations of adenosine-phosphate esters and either  $\text{Ca}^{2+}$  (left) or  $\text{Mg}^{2+}$  (right). Four plasma membrane preparations were tested with six different substrates each. Additionally, curves are shown that represent the difference between the specific activities measured with ATP and ADP, and the difference between the specific activities measured with ADP and AMP. Half-maximal activation concentrations and maximum velocities (calculated with Lineweaver–Burk plots) are given in Table 4. Mean values and standard errors of the mean are given;  $n = 4$ .

Table 4. Apparent  $K_m$  values and  $V_{\max}$  values for  $\text{Ca}^{2+}\sim$  and  $\text{Mg}^{2+}\sim$  substrate complexes in eel gill plasma membranes

Substrate	Apparent $K_m$	$V_{\max}$ Calculated	Observed
$\text{Ca}\sim\text{ATP}$	$0.83 \pm 0.13$	$38.16 \pm 4.67$	$52.78 \pm 7.24$ (100%)
$\text{Ca}\sim\text{ADP}$	$0.53 \pm 0.13$	$23.95 \pm 5.43$	$28.93 \pm 2.63$ (55%)
$\text{Ca}\sim\text{AMP}$	$0.10 \pm 0.04$	$3.43 \pm 0.53$	$3.69 \pm 0.30$ (7%)
$\text{Ca}\sim p\text{-NPP}$	0.14	2.76	$2.76 \pm 0.12$ (5%)
$\text{Mg}\sim\text{ATP}$	$0.52 \pm 0.10$	$29.03 \pm 7.43$	$45.42 \pm 8.85$ (100%)
$\text{Mg}\sim\text{ADP}$	$0.38 \pm 0.12$	$20.12 \pm 3.55$	$23.39 \pm 2.33$ (51%)
$\text{Mg}\sim\text{AMP}$	$0.20 \pm 0.07$	$5.34 \pm 1.07$	$7.35 \pm 0.86$ (16%)
$\text{Mg}\sim p\text{-NPP}$	0.14	3.03	$3.03 \pm 0.64$ (7%)

$K_m$ -values (mM) and  $V_{\max}$ -values ( $\mu\text{mol Pi/hr/mg protein}$ ) were calculated for individual samples by means of Lineweaver–Burk plots. Calculated  $V_{\max}$ -values were compared with observed  $V_{\max}$ -values. Mean values are given ( $\pm$  SEM) for four different samples. Significance of differences is given in the text.



parent  $K_m$ -values and  $V_{max}$ -values were calculated on the basis of incubations of plasma membranes with varying concentrations of equimolar amounts of phosphate-esters (ATP, ADP, AMP or *p*-NPP) and divalent ions ( $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ).  $V_{max}$ -values for ADP as compared to ATP (=100%) are 55 and 51% for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  respectively. The  $V_{max}$ -values for Mg~AMP were twice as high as observed for Ca~AMP or *p*-NPP in combination with either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ .

Apparent  $K_m$ -values decreased with decreasing numbers of phosphate groups in the substrate, i.e. the apparent affinities for the simplest substrates (AMP and *p*-NPP) were found to be the highest. No difference was found in apparent affinity between AMP or *p*-NPP in combination with either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . The apparent affinity for Mg~ATP and Mg~ADP was significantly higher than for Ca~ATP or Ca~ADP.

For the ATP complexes as substrates the calculated and observed  $V_{max}$ -values differed significantly. This suggested that the results did not fulfil the requirements for a Lineweaver-Burk transformation. This again may indicate that the enzyme preparation is heterogeneous. On the basis of the apparent  $K_m$ -values in Table 4 it may be expected that, when ATP-complexes are given as substrate, the reaction product Ca~ADP will preferentially be hydrolyzed to Ca~AMP and subsequently Ca~AMP to adenosine. As the release of phosphate was measured as an indication for substrate hydrolysis, the  $V_{max}$ -values were very likely overestimated when ATP or ADP were used as substrates. Therefore, the saturation curves for ATP and ADP hydrolysis were also presented as the differences between ATP and ADP hydrolysis and between ADP and AMP hydrolysis. The differences between the corrected values for  $V_{max}$  of ATP and ADP hydrolysis are not significant.

From these observations we conclude that under the conditions described no high affinity  $\text{Ca}^{2+}$ -ATPase can be demonstrated. Instead the observed hydrolytic activities represent non-specific phosphatase activities. We therefore examined the effects of several inhibitors on Ca~ATP and Mg~ATP hydrolysis in another set of experiments.

#### *Effects of inhibitors on Ca~ATP and Mg~ATP hydrolysis (Table 5)*

Under our assay conditions, up to 10 mM L-phenylalanine (a known inhibitor of gut alkaline phosphatase) had no inhibitory effect on either Ca~ATP or Mg~ATP hydrolysis. Conversely, theophylline, a more potent inhibitor of alkaline phosphatase (Ghijzen *et al.*, 1980), inhibited significantly more Ca~ATP hydrolysis than Mg~ATP hydrolysis (23.4 and 14.6% respectively). Both activities were inhibited by up to 80% with L-cysteine (10 mM). However, the calculated 50% inhibition occurred at 2.27 mM cysteine for Ca~ATP hydrolysis and 6.08 mM cysteine for Mg~ATP hydrolysis (Fig. 4).

R24571, chlorpromazin and phenothiazin have been reported to inhibit calcium transport related high-affinity  $\text{Ca}^{2+}$ -ATPase activities by competition with calmodulin at very low inhibitor concentrations

Table 5. Effects of various inhibitors on Ca~ATP and Mg~ATP hydrolysis in eel gill plasma membranes

Inhibitor	Maximum inhibition (%)	
	Ca~ATP (3 mM)	Mg~ATP (3 mM)
L-Phenylalanine (10.0)	0.0	0.0
Theophylline (1.25)	23.4	14.6
Cysteine (10)	77.2	76.7
R24571 (0.01)	5.1	14.1
Chlorpromazine (0.1)	6.1	9.0
Phenothiazine (0.001)	6.0	10.8

Inhibition (%) at the most effective inhibitor concentration (mM) is given for the average of six individual samples tested with and without inhibitors.

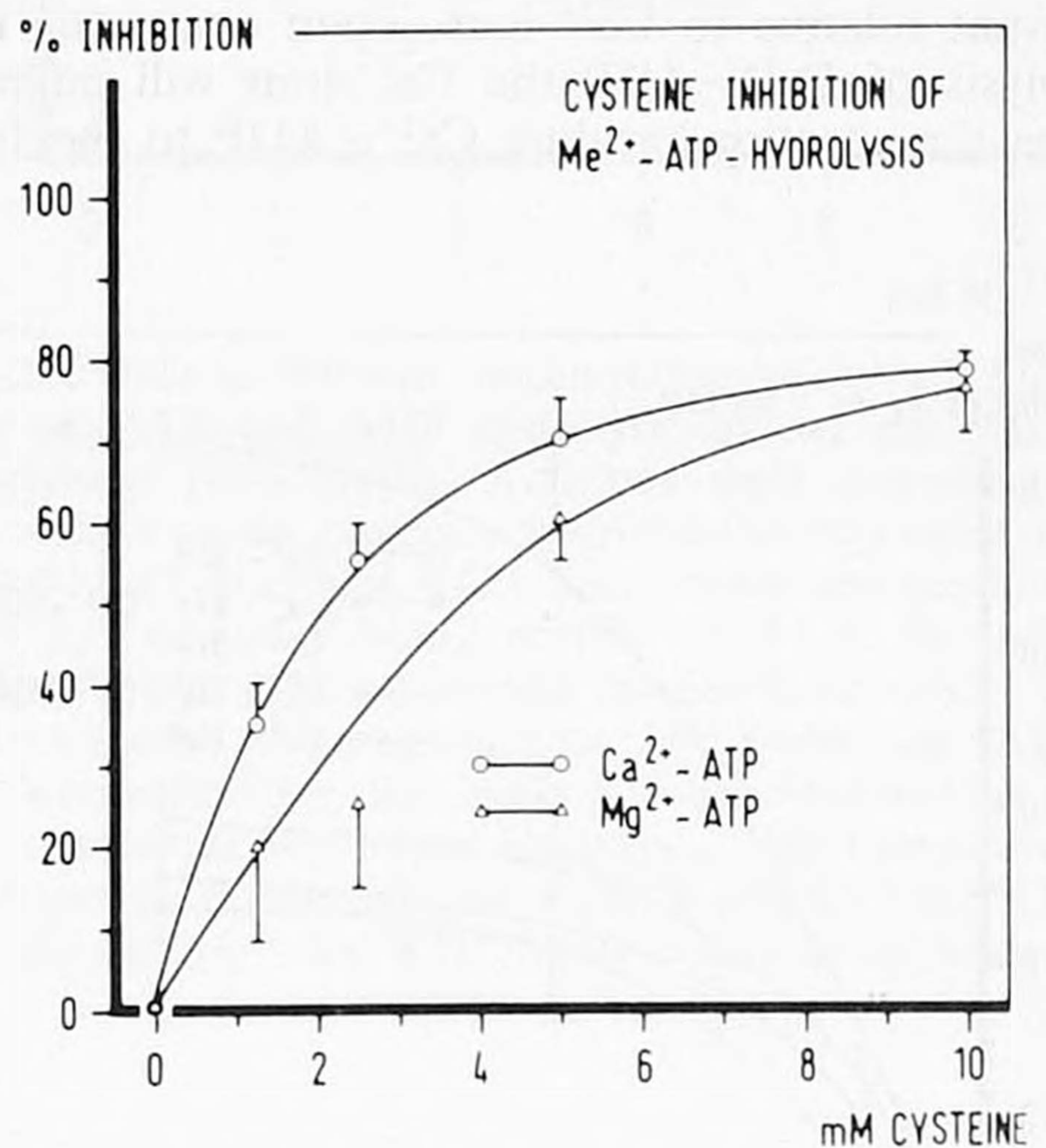


Fig. 4. L-Cysteine inhibition of Mg~ATP and Ca~ATP hydrolysis by eel gill plasma membranes ( $P_3$ -fraction). Half-maximal inhibition was calculated to occur at 2.2 mM L-cysteine for Ca~ATP and at 5 mM L-cysteine for Mg~ATP. Specific activities in the absence of L-cysteine amounted to  $38.2 \pm 4.7 \mu\text{mol P}_i/\text{hr}/\text{mg}$  protein for Ca~ATP (3 mM) and  $23.3 \pm 3.0 \mu\text{mol P}_i/\text{hr}/\text{mg}$  protein for Mg~ATP (3 mM). Mean values and standard errors of the mean are given;  $n = 6$ .

(0.01–1.0  $\mu\text{M}$ ) or by affecting the calmodulin-independent basal activities at higher concentrations (1.0–10.0  $\mu\text{M}$ ). Maximum inhibition of Ca~ATP or Mg~ATP hydrolysis occurred at the higher inhibitor concentrations but no significant differences were observed between the effects of these inhibitors at low or high concentrations. Mg~ATP hydrolysis was, however, significantly more sensitive (14% inhibition) towards these inhibitors than Ca~ATP hydrolysis (6% inhibition).

These results confirm that the Ca~ATP or Mg~ATP hydrolytic activities in eel gill plasma membranes are not homogeneous.

#### DISCUSSION

Four major findings are presented in this paper. (1)  $\text{Ca}^{2+}$ -activated ATPase activities occurring in the



gills of American eels are located in the plasma membranes of this epithelium. (2) The assay procedures for fish gill  $\text{Ca}^{2+}$ -ATPase previously described in the literature, and retested in this study did not allow the determination of a single, specific  $\text{Ca}^{2+}$ -ATPase. (3) Eel gill plasma membranes contain more than one type of enzyme that hydrolyzes complexes of  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ions and adenosinephosphate-esters, as indicated by substrate specificity tests and effects of various inhibitors on these hydrolytic activities. (4) These  $\text{Ca}^{2+}$ -activated ATPase activities do not represent high affinity, transport  $\text{Ca}^{2+}$ -ATPase activity but rather non-specific "alkaline" phosphatase activities.

#### *Location of $\text{Ca}^{2+}$ - or $\text{Mg}^{2+}$ -activated ATPase activities*

The procedure used for isolating the plasma membranes of eel gills in this study yielded a highly enriched plasma membrane fraction, as indicated by the  $\text{Na}^+/\text{K}^+$ -ATPase specific activities, with only minor mitochondrial contamination. To exclude any interference of mitochondrial ATPase activities, oligomycin and sodium azide were routinely added to the assay media. We therefore conclude that the observed  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -activated ATPase activities reside in the plasma membranes of the gill epithelium. But we do acknowledge that a portion of these activities may have originated from enzyme activities of endoplasmic reticulum origin as we did not screen for marker enzyme activities for these membranes during the isolation procedure. The activation curves observed for ATP hydrolysis at increasing concentrations of  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ions with this preparation resembled closely those previously reported for gill membranes of rainbow trout (Ma *et al.*, 1974), American eels (Fenwick, 1976), roach (Shephard, 1981), and tilapia (our unpublished observations). The calculated  $K_m$ -values for  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -activation of ATP hydrolysis are similar to previously published values (Fenwick, 1979). The differences in  $V_{\text{max}}$ -values presented here and those in the earlier literature may be the result of the use of more highly purified membrane fractions in the present study and from differences in incubation temperatures.

#### *Characteristics of the $\text{Ca}^{2+}$ -activated ATPase activities*

One of the results of this study we wish to emphasize is that the assay procedures previously employed for  $\text{Ca}^{2+}$ -activated ATPase activities in fish gills (among others) did not fulfil the requirements for the determination of transport  $\text{Ca}^{2+}$ -ATPases. Rather they yielded non-specific activities of  $\text{Ca}\sim\text{ATP}$  or  $\text{Mg}\sim\text{ATP}$  hydrolyzing enzymes. To support this contention we propose four major criticisms. (1) The affinity of the reported ATPase for  $\text{Ca}^{2+}$  was too low for an enzyme that must be stimulated by  $\text{Ca}^{2+}$  at intracellular  $\text{Ca}^{2+}$ -concentrations. (2) This  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis, even measured at suboptimal pH (Fenwick, 1979), exceeds the  $\text{Na}^+/\text{K}^+$ -ATPase activity and, therefore, would indicate  $\text{Ca}^{2+}$ -transport rates that would surpass the  $\text{Na}^+$ -transport rates by several orders of

magnitude. (3) No evidence was provided that ATP was the preferential substrate. (4) The pH-optima for the activities were reportedly in the alkaline trajectory (Ma *et al.*, 1974; Fenwick, 1976) and were thus characteristic for alkaline phosphatase.

Because of these criticisms we conclude that the observed activation curves for  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -induced ATP hydrolysis represent saturation curves for complexes of ATP with either metal ion rather than  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -activation of ATP-hydrolyzing enzymes. Hydrolysis of ATP is strictly dependent on the presence of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , suggesting that ATP alone is not (detectably) used as a substrate. Upon addition of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  to ATP-containing media, these metal ions will be chelated by ATP (Walaas, 1958; Sapper *et al.*, 1980). Although  $\text{Mg}^{2+}$  is chelated more strongly than  $\text{Ca}^{2+}$  by ATP, both interactions have the same modifying effect on the conformation of the adenosine-phosphate-ester and thus allow effective binding of the phosphate group to the active site of enzymes. In this context it should be mentioned that  $\text{Na}^+/\text{K}^+$ -ATPase (Bonting and Caravaggio, 1963) as well as the typical transport  $\text{Ca}^{2+}$ -ATPases of rat intestine (Ghijsen *et al.*, 1980) are dependent on  $\text{Mg}\sim\text{ATP}$  although they may be inhibited by  $\text{Ca}\sim\text{ATP}$  (Epstein and Whittam, 1966).

Maximum velocities are found at equimolar concentrations of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  and ATP or ADP. However, when  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ion concentrations exceeded the ATP-concentration by 2.5 or 5.0 mM, ATP-hydrolysis was inhibited. This indicates that  $\text{Ca}\sim\text{ATP}$  and  $\text{Mg}\sim\text{ATP}$  hydrolysis is inhibited by high levels of free  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  respectively.

The possibility cannot be excluded that in our assay procedure, stimulation of free or complexed ATP hydrolysis occurs by micromolar concentrations of free  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . But if we assume that the presence of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the assay medium is a prerequisite for the determination of a transport  $\text{Ca}^{2+}$ -ATPase, it is clearly not possible to show such an enzyme with the assay procedures applied in this and many other studies. Additionally, we want to stress that free  $\text{Ca}^{2+}$ -ion concentrations of the assay media in the micromolar range cannot be obtained reliably unless appropriate buffering with EGTA or NTA is employed (Sharff, 1979, 1981; Ghijsen *et al.*, 1980).

Moreover, when a surplus of ATP relative to  $\text{Ca}^{2+}$  is used, new substrate will constantly be produced in the assay medium as the free ATP will react with the reaction product  $\text{Ca}\sim\text{ADP}$  to produce  $\text{Ca}\sim\text{ATP}$  and ADP. When this  $\text{Ca}^{2+}$ -dependent substrate production occurs, the activation curve represents the effects of  $\text{Ca}^{2+}$ -defined substrate concentrations rather than the effects of  $\text{Ca}^{2+}$ -concentrations on ATP-hydrolytic activities. When ADP was substituted for ATP in this type of experiment, comparable results were obtained. No phosphate release occurred in the absence of  $\text{Ca}^{2+}$ . The shape of the activation curve observed resembled the one for ATP and suggested that the hydrolysis of ADP is also dependent on the chelation of  $\text{Ca}^{2+}$ .

As  $\text{P}_i$ -release was measured as an indication of substrate hydrolysis in all cases, no straightforward answer can be given as to whether these activities were the result of the hydrolysis of a single sub-



strate-complex or of a combination of the original substrate and the reaction products.  $K_m$ -values calculated for  $\text{Ca}^{2+}$ -activation of ATP or ADP hydrolysis by the enzyme preparations used, may therefore be incorrect due to the continuous production of new substrate. If such was the case the affinities for complexes of ATP and ADP with  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  were probably overestimated.

To test the preferences for  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -substrate complexes we measured the activation curves for these complexes, assuming a 1:1 ratio for  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -chelation by the adenosinephosphate-esters.  $K_m$ -values were found to be much higher than the values found in the presence of unchelated ATP, probably because substrate concentrations become limiting at low concentrations. Surprisingly,  $K_m$ -values calculated from Lineweaver-Burk plots for the different complexes were highest for the ATP-complexes and lowest for the AMP-complexes. As the affinity for ADP and AMP, which appear as reaction products during the hydrolysis of ATP, were higher than for the original ATP it was to be expected that hydrolysis of the first-given substrate would continue until dephosphorylation was complete. Therefore the  $V_{\max}$ -values for ATP- and ADP-complexes calculated on the basis of  $\text{P}_i$ -release are overestimated. Thus when  $\text{P}_i$ -release is used as a measure for substrate hydrolysis, the rate of ATP hydrolysis is better estimated as the difference between ATP and ADP hydrolysis, and ADP hydrolysis as the difference between ADP and AMP hydrolysis. Corrected in this way, the  $V_{\max}$ -values for  $\text{Ca}\sim\text{ATP}$ ,  $\text{Ca}\sim\text{ADP}$  and  $\text{Mg}\sim\text{ATP}$  were not significantly different from each other. The values for  $\text{Mg}\sim\text{ADP}$  were significantly lower; those for  $\text{Mg}\sim\text{AMP}$  and  $\text{Ca}\sim\text{AMP}$  were lower still. This would clearly point again to the non-specific character of these phosphatase activities.

However, care should be taken with results obtained from Lineweaver-Burk plots when complicated samples such as plasma membrane-preparations are used. When the same results are plotted according to Eadie-Hofstee, no straight lines were observed for any substrate tested. This would mean then that the data do not fulfil the requirements for kinetic analysis by means of a Lineweaver-Burk plot (Borst Pauwels, 1973). This finding might also explain why the calculated  $V_{\max}$ -values were found to be lower than the observed values. Furthermore, the fact that no straight lines were observed in Eadie-Hofstee plots for substrate-complexes supports again the postulate that more than one phosphatase activity is present in eel gill plasma membranes. Another complicating factor is the observation that the high  $\text{P}_i$ -levels, which were yielded with high concentrations of substrate-complexes, may have inhibited phosphatase activities. This would result in an underestimation of  $V_{\max}$ -values.

We conclude that the non-specific  $\text{Ca}\sim\text{ATP}$ -phosphatase activity in the eel gill hydrolyzed  $\text{Ca}\sim\text{ADP}$  and  $\text{Mg}\sim\text{ATP}$  equally well (equal  $V_{\max}$ -values), when substrate hydrolysis is estimated properly. These data are also more consistent with the thesis that the activation of ATP or ADP hydrolysis by  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  is more likely a result of chelation of these metal ions by the substrates rather

than a direct stimulation of enzymes by  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . But it cannot be excluded that the  $\text{P}_i$  measured when  $\text{Ca}\sim\text{ATP}$  is the substrate, originates from a  $\text{Ca}\sim\text{ATP}$ , an ATP hydrolyzing activity (or both) after stimulation by micromolar concentrations of free  $\text{Ca}^{2+}$  present in the reaction media. For the determination of such "high-affinity"  $\text{Ca}^{2+}$ -ATPases the assay media must contain  $\text{Ca}^{2+}$ -buffers to establish reliably micromolar concentrations of  $\text{Ca}^{2+}$ .

#### *Effects of various inhibitors on $\text{Ca}\sim\text{ATP}$ and $\text{Mg}\sim\text{ATP}$ hydrolysis*

As the previously reported pH-optima for  $\text{Ca}^{2+}$ -activated ATPase activities approximated a value of 8.0 (Ma *et al.*, 1974; Fenwick, 1976) we deduced that most of the  $\text{P}_i$ -release was due to non-specific alkaline phosphatases and we therefore tested the effects of various inhibitors on  $\text{Ca}\sim\text{ATP}$  and  $\text{Mg}\sim\text{ATP}$  hydrolysis. L-Phenylalanine, theophylline and L-cysteine are reported to affect non-specific phosphatases with alkaline pH-optima. Chlorpromazine, phenothiazine and calmidazolium (R24571) are reported to act as calmodulin-antagonists in a concentration-dependent way and to inhibit calcium transport in rat enterocytes and human erythrocytes by inhibition of the high-affinity transport  $\text{Ca}^{2+}$ -ATPases (Ghijsen *et al.*, 1982; Gietzen *et al.*, 1981).

L-Phenylalanine (up to 10 mM) did not affect  $\text{Ca}\sim\text{ATP}$  or  $\text{Mg}\sim\text{ATP}$  hydrolysis under our standard conditions (pH 7.4). These results concur with the results of Ghosh and Fishman (1966), who similarly reported that phosphatase activity was not inhibited by L-phenylalanine at pH-values below 7.8. On the other hand, Ghijsen *et al.* (1980) did report significant L-phenylalanine-induced inhibition of alkaline phosphatase activity in rat enterocyte plasma membranes, at a pH of 7.4. This discrepancy may reflect the organ-specificity of L-phenylalanine suggested by Ghosh and Fishman (1966). Other explanations for this discrepancy are that the kind of substrate used was not appropriate to show the effects of the inhibitor, or the fact that membrane preparations were used instead of pure enzymes. Theophylline, a more potent inhibitor of alkaline phosphatase activities than L-phenylalanine, had maximal effects at a concentration of 1.25 mM on both  $\text{Ca}\sim\text{ATP}$  and  $\text{Mg}\sim\text{ATP}$  hydrolysis, but the  $\text{Ca}\sim\text{ATP}$  hydrolysis was inhibited to a greater extent than the  $\text{Mg}\sim\text{ATP}$  hydrolysis. This suggests that  $\text{Ca}\sim\text{ATP}$  was the preferred substrate for a gill phosphatase activity. However, both  $\text{Ca}\sim\text{ATP}$  and  $\text{Mg}\sim\text{ATP}$  can be hydrolyzed by theophylline-sensitive enzyme activities. Maximum inhibition produced by theophylline was 20% of the total hydrolytic activity indicating again heterogeneity of enzyme activity. Cysteine, an alkaline phosphatase inhibitor that acts by binding the intrinsic  $\text{Zn}^{2+}$ -ion gave up to 80% inhibition of both  $\text{Ca}\sim\text{ATP}$  and  $\text{Mg}\sim\text{ATP}$  hydrolysis. Calculated  $I_{50}$ -values, however, differed significantly for these substrates:  $I_{50}\text{-Ca}\sim\text{ATP}$ : 5.0 mM,  $I_{50}\text{-Mg}\sim\text{ATP}$ : 2.2 mM. Thus, the cysteine would seem to act by dissociation of the substrate-complexes rather than



directly via the enzymes. Therefore, these results do not allow the conclusion that the phosphate release is due to the presence of Zn<sup>2+</sup>-containing enzymes.

The phenothiazines inhibited Mg~ATP and Ca~ATP hydrolysis to a different extent: maxima were only 6% for Ca~ATP and 15% for Mg~ATP. This finding suggests that a large quantity of non-specific phenothiazine insensitive phosphatase activity was present. But whether the effects of these inhibitors at high concentrations resulted from competition with calmodulins, which are abundantly present in these membrane preparations (unpublished observation) or resulted from an effect on membrane integrity, causing reduced hydrolytic activity (Van Belle, 1981), remains to be elucidated.

Tentatively, we conclude that at least a portion of the Ca~ATP and Mg~ATP hydrolytic activity is calmodulin dependent. This calmodulin-sensitive component preferentially hydrolyzes Mg~ATP. However, the calmodulin-antagonists attenuate Ca<sup>2+</sup>-activated ATPase activity only to a small extent. The latter conclusion indicates that a major part of the presumptive Ca<sup>2+</sup>-ATPase activity cannot be involved in active Ca<sup>2+</sup>-transport.

In further studies on Ca<sup>2+</sup>-stimulated phosphatases in eel gill plasma membranes we were able to discriminate between non-specific, Ca<sup>2+</sup>-stimulated phosphatases and high-affinity Ca<sup>2+</sup>-ATPase activity that may represent the calcium pump (data to be published separately). In fresh water adapted eels, both activities are correlated negatively with the environmental Ca<sup>2+</sup>-concentration. Although the non-specific Ca<sup>2+</sup>-stimulated phosphatase activity does not fulfil the requirements of a Ca<sup>2+</sup>-transport ATPase, some relation of this activity with Ca<sup>2+</sup>-transport phenomena seems to exist. Thus, more than one enzyme activity located in the plasma membrane may be involved in gill transepithelial Ca<sup>2+</sup>-transport.

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