# CELL SURFACE ATPase OF THE MAMMARY ADENOCARCINOMA

By

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1968

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1976

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MAMMARY ADENOCARCINOMA

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ACKNOWLEDGEMENTS

The author extends special appreciation to his major professor, Dr. K. L. Carraway, for his guidance and advice. Appreciation is also expressed to Dr. Richard C. Essenberg and Dr. Ulrich K. Melcher for their help as advisory committee members.

Special thanks are given to Mr. Glendon Jett for his assistance and to Dr. John Huggins for assistance and guidance during these studies.

Special gratitude is expressed to my wife, Linda, for her understanding, encouragement and many sacrifices.

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ADP | - adenosine 5'-diphosphate |
| ATP | - adenosine 5'-triphosphate |
| EDTA | - ethylenediaminetetra-acetate |
| Tris | - tris(hydroxymethyl)aminomethane |
| Con A | - concanavalin A |
| SBA | - soybean agglutinin |
| WGA | - wheat germ agglutinin |
| HEPES | - hydroxyethylpiperazineethanesulfonate |
| EGTA | - ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetate |

# CHAPTER I

## INTRODUCTION

Concanavalin A, a lectin from Canavalia ensiformis (jack bean), known more simply as Con A, is the most extensively studied of the plant lectins. The primary, secondary and tertiary structure of Con A have been determined, showing that it is composed of identical 25,500 molecular weight asymetric subunits that may exist as dimers, tetramers and polymers of high molecular weight. Con A contains one atom each of $Ca^{2+}$ and $Mg^{2+}$ per protomer. EDTA diminishes Con A's ability to bind oligosaccharide residues but it may be restored by the addition of $MgCl_2$ and $CaCl_2$. Below pH 5.6 Con A exists primarily as a dimer with a molecular weight of 55,000 containing one saccharide binding site on each subunit. Above pH 7.6 Con A forms a tetramer with a molecular weight of 112,000. Saccharide binding to Con A does elicit a conformational change in the Con A tetramer. Con A specifically binds to sugars with the D-arabinose configuration at carbons 3, 4 and 5 (1). Thus Con A binds specifically to a wide variety of oligosaccharides containing α-D-mannose or α-D-glucose residues. Since glycoconjugates are integral components of all mammalian cell surfaces (2-4), this ability of Con A to bind to glycoproteins and glycolipids has made it an interesting tool in the study of plasma membranes.

In studies of the cell surface of Dicotyostelium discoideum it has been estimated that the young growing cell possessed $4 \times 10^7$ binding

sites per cell, all of equal affinity (5). Con A has been shown to bind to 25% of the total detergent soluble liver plasma membrane proteins (6). This was demonstrated by using antibodies formed by injecting membranes into rabbits to form antibodies. Reactivity was then demonstrated to the proteins bound to the Con A-Sepharose column. Some of the antigens possessed ATPase activity.

The binding of Con A to the surface of the membrane causes a number of diverse effects. The binding of Con A has been shown to cause lymphoid cell transformations (7, 8). Con A has also been shown to mimic the effect of insulin on the adipocyte plasma membrane $Mg^{2+}$-ATPase (9), glucose oxidation (10) and inhibition of epinephrine stimulated lipolysis (11). Also of importance is the ability of Con A to kill selectively transformed cells (12, 13). Malignant cells are more prone to agglutination with Con A than are non-malignant cells in most of the systems studied (14). Con A has been reported to cause release of $K^{+}$ and to cause cellular agglutination of Ehrlich ascites tumor cells (15). These effects were only noticeable with tetrameric Con A binding to the surface glycoprotein receptors. The binding of Con A to T spleen lymphocytes increases $Ca^{2+}$ uptake in these cells which is measureable by 45 seconds and complete by 1 minute (16).

$Mg^{2+}$-ATPase is considered to be an ecto-enzyme, that is, one localized on the plasma membrane with the active site oriented toward the medium. The presence of an ATPase as an ecto-enzyme has been demonstrated in the guinea pig polymorphonuclear leukocyte (17, 18). The ecto-ATPase was found to be unrelated to the sodium and potassium ion stimulated ATPase present on the plasma membrane of many mammalian cells. It was insensitive to ouabain and stimulated by neither sodium nor

potassium ions. To establish this ATPase as an ecto-enzyme the following criteria were used. Firstly, detergent disruption doubled the activity of ATPase. Thus the membrane is acting as a barrier to ATP. Secondly, the cells were loaded with inorganic $^{33}P$. The cells were then allowed to hydrolyze $\gamma$ labeled $^{32}P$-ATP. After incubation $^{32}P_i$ was found outside the cell while $^{33}P_i$ was found only inside the cell. This is evidence against the argument that the ATP may be cleaved inside the cell and then transported outside the cell. Thirdly, the diazonium salt of sulfanilic acid (a reagent known not to penetrate the membrane) inhibited the enzyme.

The presence of three ATPases has been demonstrated in the red blood cell membrane based upon phosphorylated intermediates isolated from the membrane (19). Phosphorylation was detected in the presence of $Mg^{2+}$ alone ($Mg^{2+}$-ATPase), $Mg^{2+}$ and $Na^{+}$ (($Na^{+}$, $K^{+}$)-ATPase) and $Mg^{2+}$ and $Ca^{2+}$ ($Ca^{2+}$-ATPase). The $Mg^{2+}$-ATPase of the human erythrocyte has been shown to be stimulated by the very low density lipoproteins of plasma (20). The binding of the very low density lipoproteins alters the membrane and promote agglutination of the red blood cell by Con A. It is interesting to note that this phenomenon is not dependent upon the neutral lipids but rather upon the content of specific apolipoproteins. The presence of a $Mg^{2+}$-ATPase has also been demonstrated in adipocyte plasma membranes (9), muscle sarcolemma (21) and liver plasma membranes (22).

The function of the $Mg^{2+}$-ATPase remains unclear although there is some speculation that it is involved with the contractile proteins inside the cell. Since the cell surface has been identified as an important site in the growth, control and differentiation of mammalian cells,

information about the membrane bound enzymes should prove useful. Perturbations of the membrane that affect other cellular activities may be monitored by the alteration of activity of the membrane bound enzymes. In this system using Con A as the perturbing agent, one is able to observe the interaction of Con A with both intact cells and isolated membranes.

# CHAPTER II

# MATERIALS & METHODS

## Methods

### Maintenance of the 13762 MAT Cell Line

The strain of 13762 MAT cells were maintained by weekly transplantation by intraperitoneal injection of 0.3 ml of the ascites fluid into Fischer 344 strain female rats. The fluid for injection is obtained from an animal injected a week prior. The cells are then harvested weekly by aspirating the cells from the peritoneal cavity.

### Preparation of the 13762 MAT Plasma Membrane

A procedure (23) which yields whole envelopes and large sheets of plasma membrane was employed. The 13762 MAT cells were aspirated from the peritoneal cavity 6-8 days post-injection. The cells were then washed three times with ice cold HEPES to remove the peritoneal fluid and pelleted at 2000 r.p.m. for 3 min. in a Sorvall SS-34 rotor (1446 g•min). The washed cells were resuspended in 10 volumes of ice cold 40 mM Tris (pH 7.4) and allowed to swell for 4 minutes at 4°C. This step was necessary to lyse any red blood cells that may be present. The cells were centrifuged at 2000 r.p.m. for 4 min. in a Sorvall SS-34 rotor (1928 g•min). This step allowed pelleting of the partially swollen 13762 MAT cells while leaving the red cell ghost in the

supernatant. This cycle was repeated a second time and the cells were checked visually to determine the degree of swelling. For easy disruption the cells should swell to approximately twice their normal diameter. The cells may be washed additional times if necessary to achieve this state of swelling. The swollen cells were resuspended in 11 volumes of ice cold 1 mM $ZnCl_2$ for 2 minutes and homogenized in a Dounce homogenizer. The homogenization was continued until greater than 75% of the cells are disrupted. Again, this was checked visually by phase contrast microscopy.

The homogenate was diluted with an equal volume of 40 mM Tris (pH 7.4) and centrifuged at 1500 r.p.m. for 10 min. (3670 g•min) in a Sorvall HB-4 rotor. Exact conditions are critical here and the step was checked to insure that the plasma membranes remain in the supernatant. The supernatant was decanted and centrifuged at 4500 r.p.m. for 10 min. ($2.44 \times 10^5$ g•min) in a Sorvall HB-4 rotor. The pelleted membranes were washed three times with 40 mM Tris (pH 7.4).

The crude membrane preparation was further purified by discontinuous sucrose density gradient centrifugation. The membrane pellet was resuspended in 40 mM Tris (pH 7.4) and layered over a sucrose gradient composed of 8 ml each of 40%, 45%, 50% and 55% (w/w) sucrose in 20 mM Tris (pH 7.4). The gradients were centrifuged at 15,000 r.p.m. for 60 min. ($1.38 \times 10^6$ g•min) at 4°C using a Beckman SW-27 rotor. The band at the 40-45% interface contains the plasma membranes as shown by a 13.8 fold increase in the 5'-nucleotidase activity and low glucose-6-phosphatase and succinate dehydrogenase activity. The band was removed using a bent needle and was washed 3 times with 40 mM Tris, pelleting each time by centrifugation at 12,000 r.p.m. for 10 min. ($1.74 \times 10^5$

g•min) in a Sorvall SS-34 rotor. The membranes were stored frozen at an approximate concentration of 5 mg/ml in 40 mM Tris until needed.

Preparation of the Intact Cells

Whole cells were removed from the peritoneal cavity and washed three times with HEPES buffer, centrifuging each time at 2000 r.p.m. for 4 min. (1928 g•min) in a Sorvall SS-34 head. The cells were then stored for up to 1 to 2 hours in the HEPES buffer until immediately before the assay. The cells were washed twice in the assay buffer (25 mM histidine-imidazole, 120 mM KCl, 5 mM $MgCl_2$ and 0.5 mM EGTA), pelleted and resuspended at a concentration of $5 \times 10^7$ cells/ml and the assay begun immediately. The washing of the cells with the histidine-imidazole buffer permits the removal of inorganic phosphate which would interfere with the phosphate assay and also "loads" the whole cell with $Mg^{++}$ so that the whole cell will not reduce the $Mg^{2+}$ level of the assay medium.

Assay of the $Mg^{2+}$ Activated ATPase

The membrane fragments, approximately 50 µg protein, were incubated at 37°C for 30 min in the presence of 100 µl histidine-imidazole buffer (containing 100 mM histidine, 100 mM imidazole, 480 mM KCl, 20 mM $MgCl_2$ and 2.0 mM EGTA), 20 µl of a saturated solution of ouabain and the lectin (when used) were also included in the preincubation medium. The final volume before the addition of 50 µl of substrate is adjusted to 350 µl with deionized water. Thus the final concentrations in the assay mixture after ATP addition are: 25 mM histidine, 25 mM imidazole, 120 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA and approximately 1 mM ouabain.

The reaction is initiated by the addition of substrate (50 µl of ATP solution) and the incubation continued at 37°C for a time interval of 8 to 20 minutes. The reaction is terminated by the addition of 200 µl of 0.32 M perchloric acid. One milliliter of distilled water is added and the inorganic phosphate released is measured by a modified method of Fiske and Subbarow (24). The modification made was the addition of 10 µl of 10% SDS to the assay tube prior to the addition of the 1-amino-2 naphthol-4-sulfonic acid (26). When using whole cells the amount of SDS added was 20 µl of 10% solution. This gives SDS concentrations of 0.05% and 0.1%, respectively. This minor modification was made in order to lower the turbidity (light scattering) of the sample.

Protein Determination of the Membranes and Whole Cells

Protein concentrations were estimated by the method of Lowry (25).

Activation by Divalent Cations Other Than $Mg^{2+}$

Assays were performed similarly to the $Mg^{2+}$-ATPase assay except for the absence of both the $Mg^{2+}$ and EGTA in the assay buffer.

## Materials

Tetrasodium ATP, imidazole, barium sulfate and ouabain were obtained from Sigma Chemical Company. Con A, WGA and SBA were from Miles Laboratories Inc. Histidine was purchased from National Biochemicals Corporation. Other chemicals were reagent grade or highest purity available.

# CHAPTER III

## RESULTS

Preliminary experiments have shown that the $Mg^{2+}$-ATPase assay, with and without the addition of Con A, is linear over the time ranges used (Fig. 1). Also the results of the assay under our incubation conditions compared favorably with the continuous spectrophotometric method used by Cunningham (27). The assay conditions give similar values to those reported for red blood cell ghost.

A Lineweaver-Burke plot of substrate versus velocity displays apparent substrate inhibition (Fig. 2). Substrate concentration was varied from 0.1 to 2.5 mM ATP. Apparent substrate inhibition first becomes noticeable at substrate concentrations above 0.3 mM ATP. In a similar experiment Con A was allowed to incubate with the membranes for 30 minutes prior to the addition of substrate. In Fig. 2 is shown what resembles an apparent release of the enzyme from substrate inhibition. The velocity values remain the same as without Con A at the lower substrate concentrations (less than 0.3 mM) but continue in a hyperbolic fashion at higher substrate concentrations (> 0.3 mM ATP).

In an effort to determine if the apparent substrate inhibition was due to the substrate binding of the $Mg^{2+}$, the $Mg^{2+}$ concentration was varied over a range of 0.0 to 5.0 mM in the presence and absence of Con A. The results are shown in Fig. 3. Since the enzyme incubation medium normally contains $Mg^{2+}$ concentration of 5.0 mM, lack of available

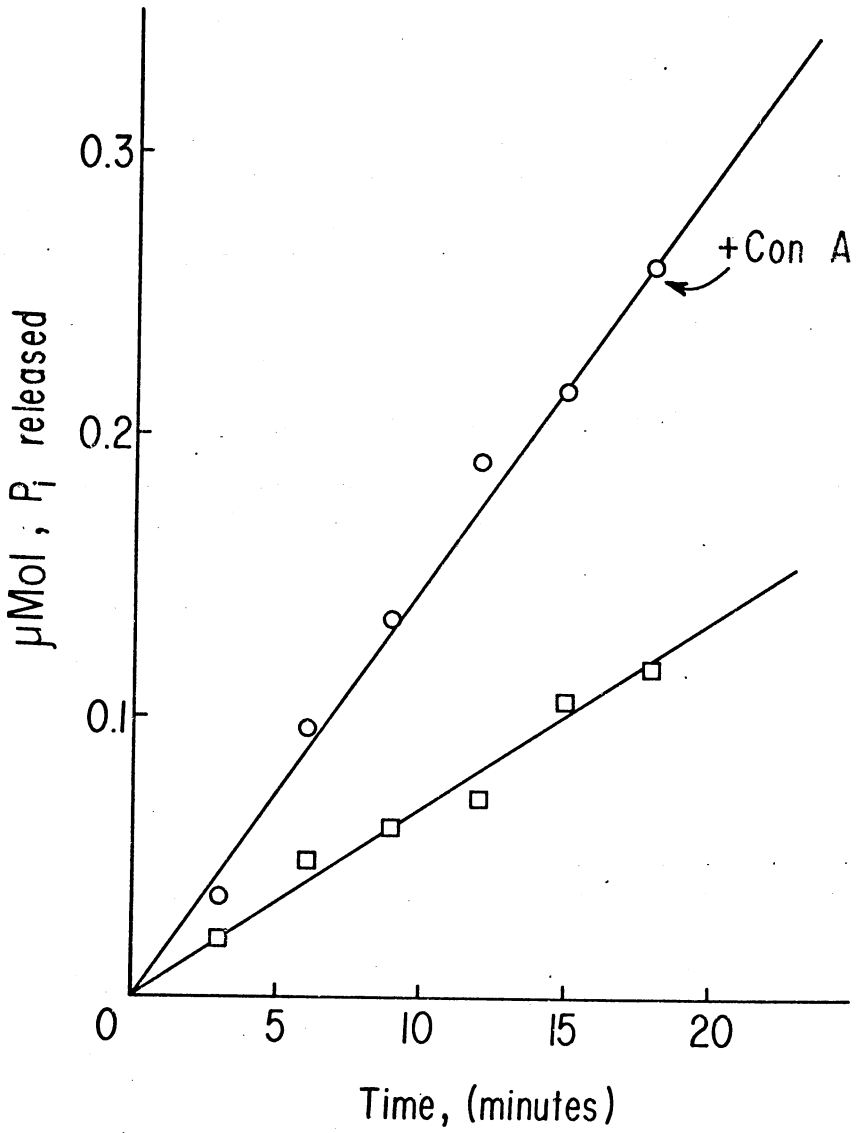


Figure 1. Linearity of $Mg^{2+}$-ATPase activity with time. (□) without Con A, (○) with Con A at a concentration of 660 µg Con A per milligram membrane protein. Samples were preincubated at 37°C for 15 minutes before addition of ATP.

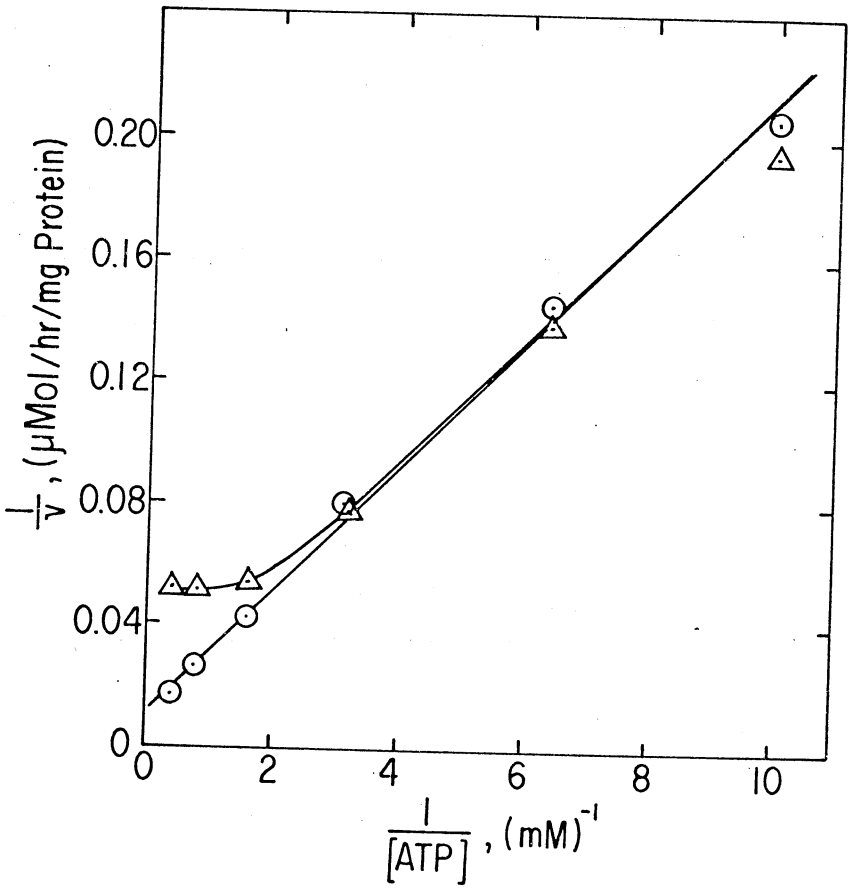


Figure 2. Lineweaver-Burke plot of 13762 membrane $Mg^{2+}$-ATPase in the presence (○) and absence (Δ) of Con A. Membranes were incubated with 660 μg Con A per mg membrane protein for 30 minutes before addition of substrate. Protein concentration was 43 μg/assay tube. Incubation time was 20 minutes.

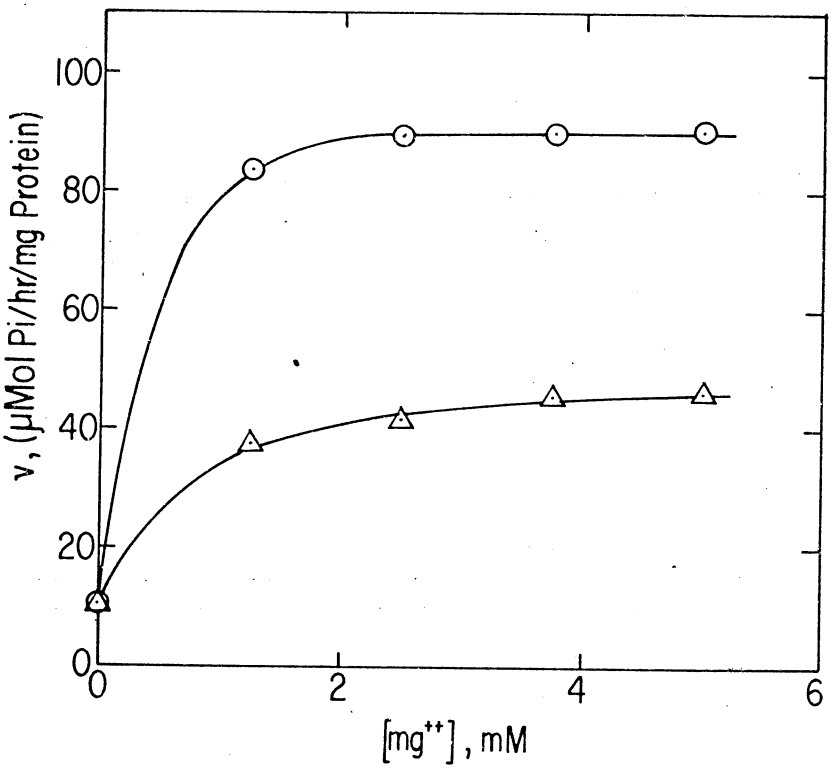


Figure 3. Plot of specific activity versus concentration of $Mg^{2+}$ in the incubation medium. $Mg^{2+}$ concentration was varied from 0.0 to 5.0 mM, in the absence of Con A ( Δ ), and in the presence of Con A ( o ). The Con A concentration was 660 μg Con A per milligram membrane protein. ATP concentration was 2.5 mMole. Samples were given a 20 minute preincubation at 37°C and continued for 10 minutes after addition of ATP.

$Mg^{2+}$ would not appear to be the limiting factor.

Intact 13762 MAT cells were incubated in the assay medium for 30 minutes prior to the addition of substrate. The substrate concentration was varied from 0.078 to 5.0 mM ATP (Fig. 4). Additional $Mg^{2+}$ is increased to 10 mM and added at the highest substrate concentration. As may be observed there is no appearance of substrate inhibition at even twice the highest substrate concentration used with the membranes alone. Whole cells were then incubated with and without Con A to observe any change in $V_{max}$. Results are shown in Table I. Thus incubation of whole cells with Con A appears to produce no significant difference in velocity.

In an attempt to determine the point at which the membranes develop their "sensitivity" to Con A activation the following experiment shown in Table II was performed. Cells from various stages of membrane preparation were incubated with and without Con A. As may be observed there appears to be no significant activation of the $Mg^{2+}$-ATPase at any step before the disruption of the plasma membrane.

The cation specificity of the $Mg^{2+}$-ATPase was investigated next. Calcium ions were substituted for the magnesium ions in the assay medium. As may be seen in Figs. 5 and 6 the enzyme is responsive to stimulation by $Ca^{2+}$. In Fig. 6 the enzyme demonstrates saturation kinetics with respect to both calcium and magnesium ions. The $V_{max}$ obtained is consistently greater for the calcium ion. The Lineweaver-Burke plot (Fig. 6) gives a $K_m$ value near 0.08 mM for both calcium and magnesium ions.

In an attempt to determine if the same enzyme was activated by both $Ca^{2+}$ or $Mg^{2+}$ ions the following experiment was performed. Mem-

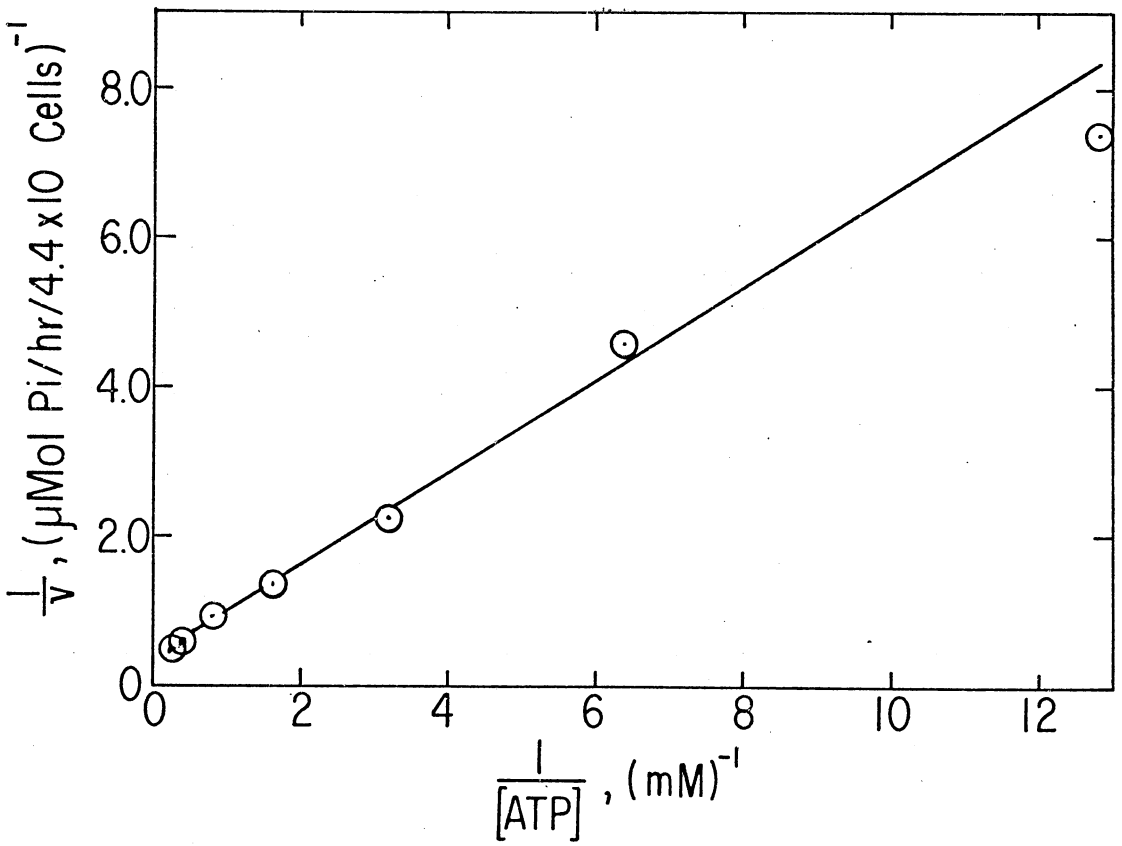


Figure 4. Lineweaver-Burke plot of intact 13762 MAT cells. ATP concentration varied from 0.078 to 5.0 mM. The cells were "pre-loaded" with $Mg^{2+}$. At the highest substrate level additional $Mg^{2+}$ was added to the incubation medium giving a final $Mg^{2+}$ concentration of 10 mM. Cells were incubated for 15 minutes at 37°C before and after addition of ATP. Whole cell concentration in the assay was $7.5 \times 10^5$ cell/ml.

TABLE I

EFFECTS OF CON A CONCENTRATION ON THE $Mg^{2+}$-ATPase OF INTACT 13762 MAT CELLS

| Concentration ATP | Concentration Con A | Velocity µmol Pi/hr |
|---|---|---|
| 0.0 mM | 75 µg/ml | 0.0 |
| 2.5 " | 0.0 " | 2.8 |
| " " | 37.5 " | 2.2 |
| " " | 75.0 " | 2.3 |
| " " | 112.5 " | 2.3 |
| " " | 150 " | 2.2 |
| 5.0 mM | 0.0 " | 3.0 |
| " " | 37.5 " | 2.7 |
| " " | 75.0 " | 2.8 |
| " " | 112.5 " | 2.9 |
| " " | 150 " | 2.7 |

The cell suspension ($1.5 \times 10^6$ cells/ml) was incubated with Con A for 30 minutes at 37°C prior to the addition of ATP and the activity assayed.

TABLE II

13762 MAT CELLS RESPONSE TO ADDED CON A AT VARIOUS STAGES OF MEMBRANE PREPARATION

| | Velocity |
|---|---|
| Whole Cells (HEPES washed) | 1.82 µmol/hr |
| Whole Cells (HEPES washed) + Con A | 1.70 " |
| Tris Swollen Cells | 1.71 " |
| Tris Swollen Cells + Con A | 1.66 " |
| Zn Treated Cells | 1.55 " |
| Zn Treated Cells + Con A | 1.71 " |
| Isolated Membrane | 31 µMol/hr/mg membrane protein |
| Isolated Membrane + Con A | 75.8 " |

The 13762 MAT cell suspension, $4.7 \times 10^6$ cells/ml, was subjected to the various stages of membrane preparation as outlined in Experimental section. Con A, when present, was at a final concentration of 75 µg/ml. ATP concentration was 2.5 mM.

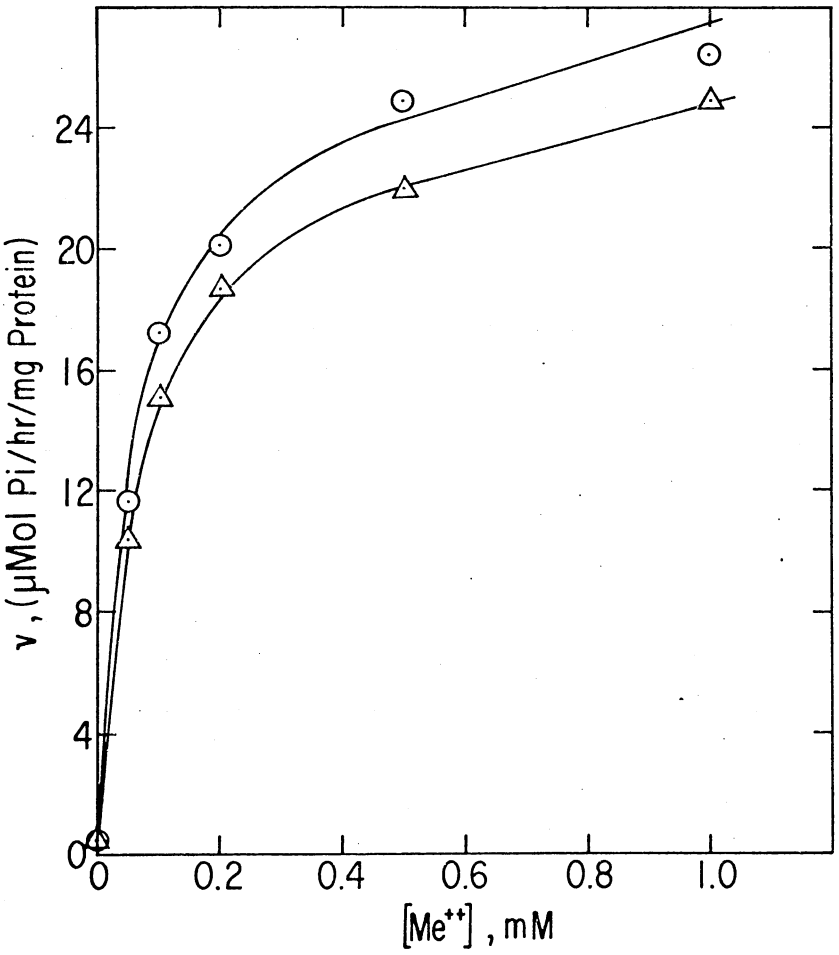


Figure 5. Plot of rate of ATP hydrolysis versus concentration of the divalent cation. Cation concentration varied from 0.05 to 1.0 mM. Calcium ions are represented by ( o ). Magnesium ions by ( Δ ). ATP concentration was 1.25 mM. Incubation time was 10 minutes at 37°C.

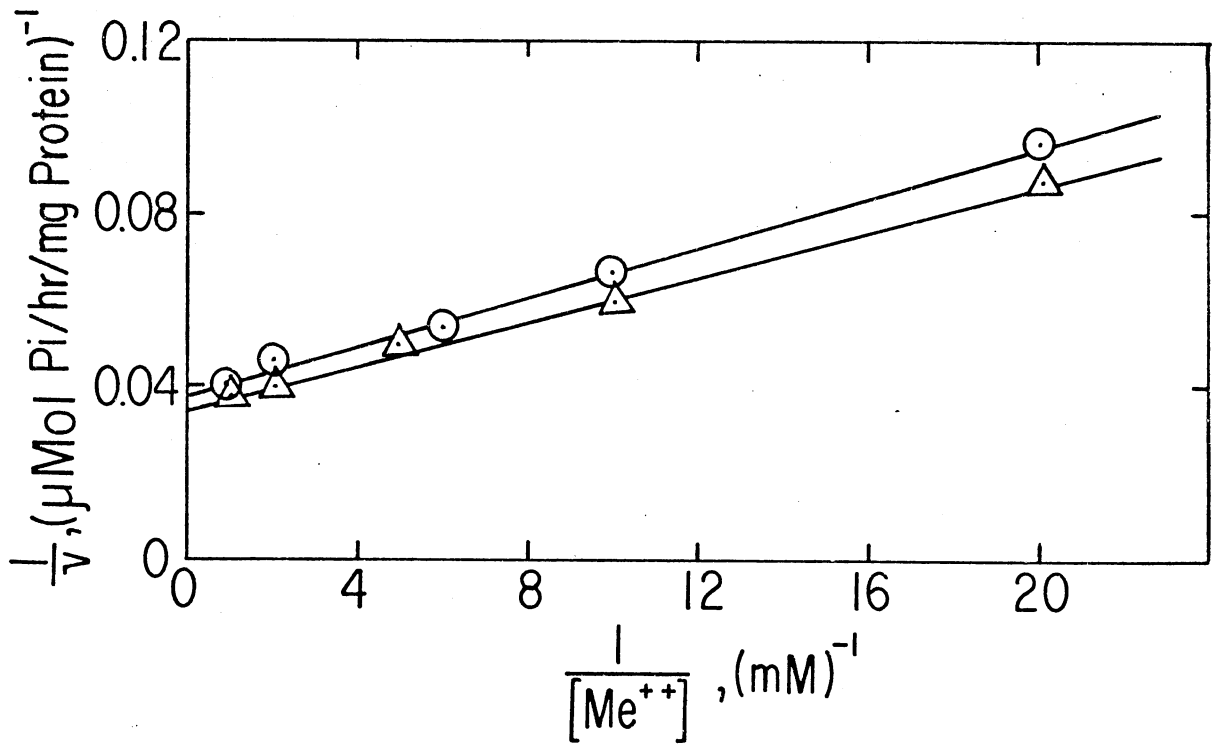


Figure 6. Lineweaver-Burke plot of velocity versus concentration of $Ca^{2+}$ ( Δ ) and $Mg^{2+}$ ( o ). Concentration ranges of cation are 0.05 to 1.0 mM. ATP concentration was 1.25 mM.

branes were incubated with $Mg^{2+}$ alone, $Ca^{2+}$ only and in combination with both $Ca^{2+}$ and $Mg^{2+}$ in the presence and absence of Con A. The results may be seen in Table III. While the enzyme is activated by both $Mg^{2+}$ and $Ca^{2+}$, the combination of the two show no further increase in the rate of hydrolysis and behave similarly in the presence of Con A.

When comparing the velocity of the membrane $Mg^{2+}$-ATPase with the concentration of ATP, in the presence of $Ca^{2+}$ when substituted for $Mg^{2+}$ a similar kinetic plot was found (Fig. 7). Once again substrate inhibition is apparent at ATP concentrations above 0.3 mM. When using whole cells and $Ca^{2+}$ as the divalent cation, varying the concentration of ATP again gives similar kinetic results to those obtained using $Mg^{2+}$ (Fig. 8). The ATPase of the whole cell using $Ca^{2+}$ was again different from that in membranes in that it did not display apparent substrate inhibition as previously found for the membrane.

Barium was also used to check for the specificity of the ATPase. The results are shown in Table IV. Barium is much less effective than either Ca or Mg ions in the stimulation of the ATPase. Also, when barium is used to the divalent cation very slight or no increase in velocity is noticed in the presence of Con A.

## Hill Plot of Activation of the $Mg^{2+}$-ATPase by Con A

In another experiment using the membrane preparation possible cooperative effects of Con A on the $Mg^{2+}$-ATPase were considered. Fig. 9 shows a plot of specific activity versus concentration of Con A as µg Con A per milligram membrane protein. The substrate concentration was 0.4 mM ATP. This concentration is barely within the region of

TABLE III

COMPARISON OF ACTIVATION OF THE ATPase BY $Ca^{2+}$ AND $Mg^{2+}$ BOTH IN PRESENCE AND ABSENCE OF CON A

| Divalent Cations | Con A | Velocity (μmol/mg protein) |
|---|---|---|
| None | + | 8.3 |
| $Mg^{2+}$ | - | 36 |
| $Mg^{2+}$ | + | 86 |
| $Ca^{2+}$ | - | 45 |
| $Ca^{2+}$ | + | 72 |
| $Mg^{2+}$ + $Ca^{2+}$ | - | 44 |
| $Mg^{2+}$ + $Ca^{2+}$ | + | 79 |

The membrane suspension (approx. 50 μg protein/assay) was incubated in the presence of 5 mM $Ca^{2+}$. 5 mM $Mg^{2+}$ or a combination of 2.5 mM $Ca^{2+}$ + 2.5 mM $Mg^{2+}$. The ATP concentration was 2.5 mM. Con A when present was at a concentration of 75 μg/ml (600 μg Con A/mg membrane protein).

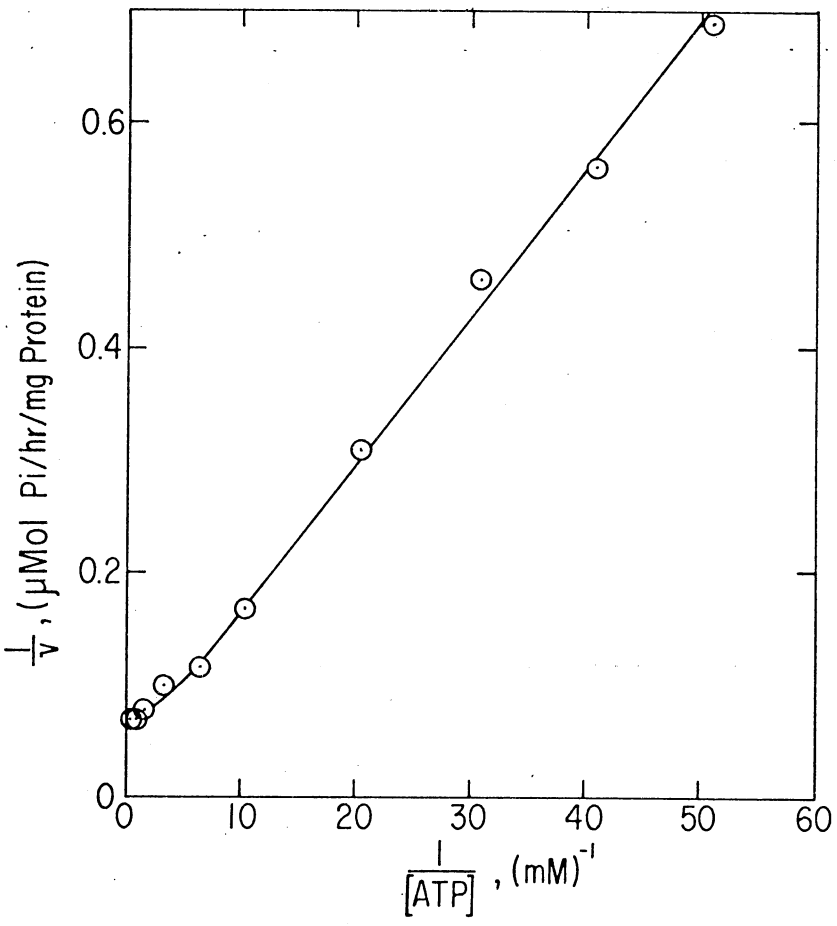


Figure 7. Lineweaver-Burke plot of velocity versus substrate concentration using $Ca^{2+}$ as the divalent cation. Membrane concentration was 125 μg protein/ml. ATP concentration was varied from 0.02-2.5 mM. Incubation time of 10 minutes at 37°C.

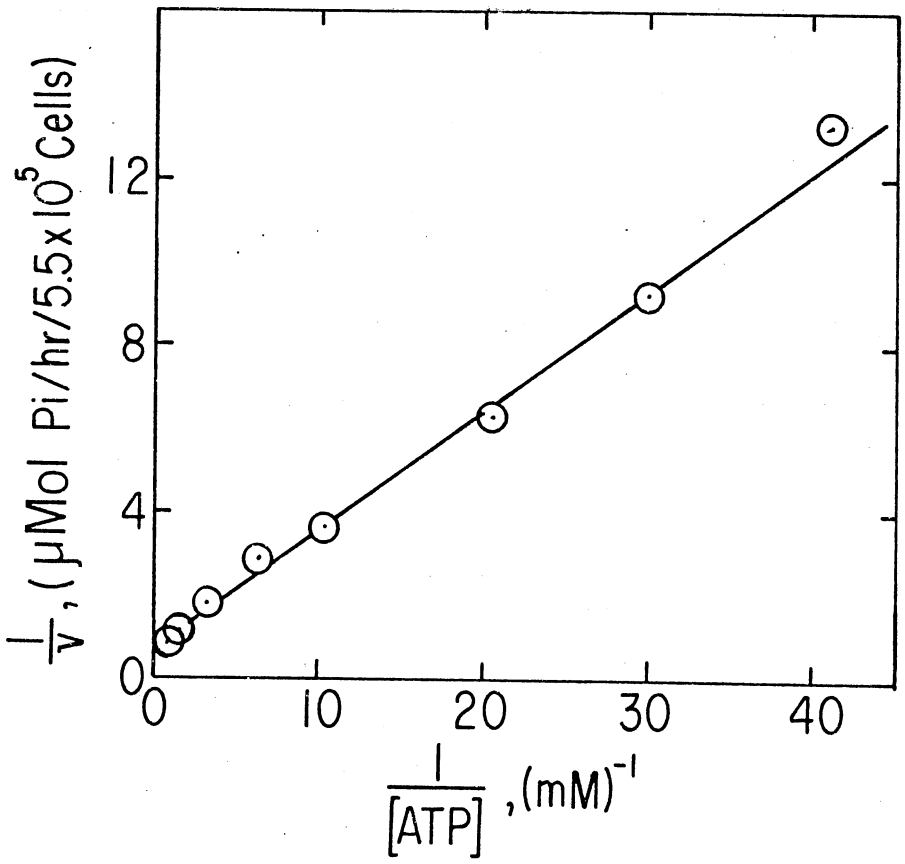


Figure 8. Lineweaver-Burke plot of velocity versus substrate concentration using $Ca^{2+}$ as the divalent cation with whole cells. Whole cell concentration was 1.4 x $10^6$ cells per milliliter. Cells were preincubated at 37°C for 20 minutes before and for 12 minutes after addition of ATP. ATP concentration was varied from 0.024-5 mM. $Ca^{2+}$ concentration was 5 mM.

TABLE IV

EFFECTIVENESS OF $Ba^{2+}$ AS AN ACTIVATOR OF THE $Mg^{2+}$-ATPase

| Divalent Cation | Con A | Velocity (μmol Pi/hr/mg protein) |
|---|---|---|
| None | - | 1.2 |
| None | + | 2.8 |
| $Mg^{2+}$ | - | 14.0 |
| $Mg^{2+}$ | + | 35.8 |
| $Ba^{2+}$ | - | 5.38 |
| $Ba^{2+}$ | + | 5.84 |

The membrane suspension was incubated in the presence of the divalent cation and with or without Con A for 30 minutes @ 37°C prior to the addition of ATP. In the assay the protein concentration was 75 μg/ml and the Con A when present at a concentration of 75 μg per milliliter. Concentration of $Mg^{2+}$ and $Ba^{2+}$ was 5 mM. ATP concentration was 2.5 mM.

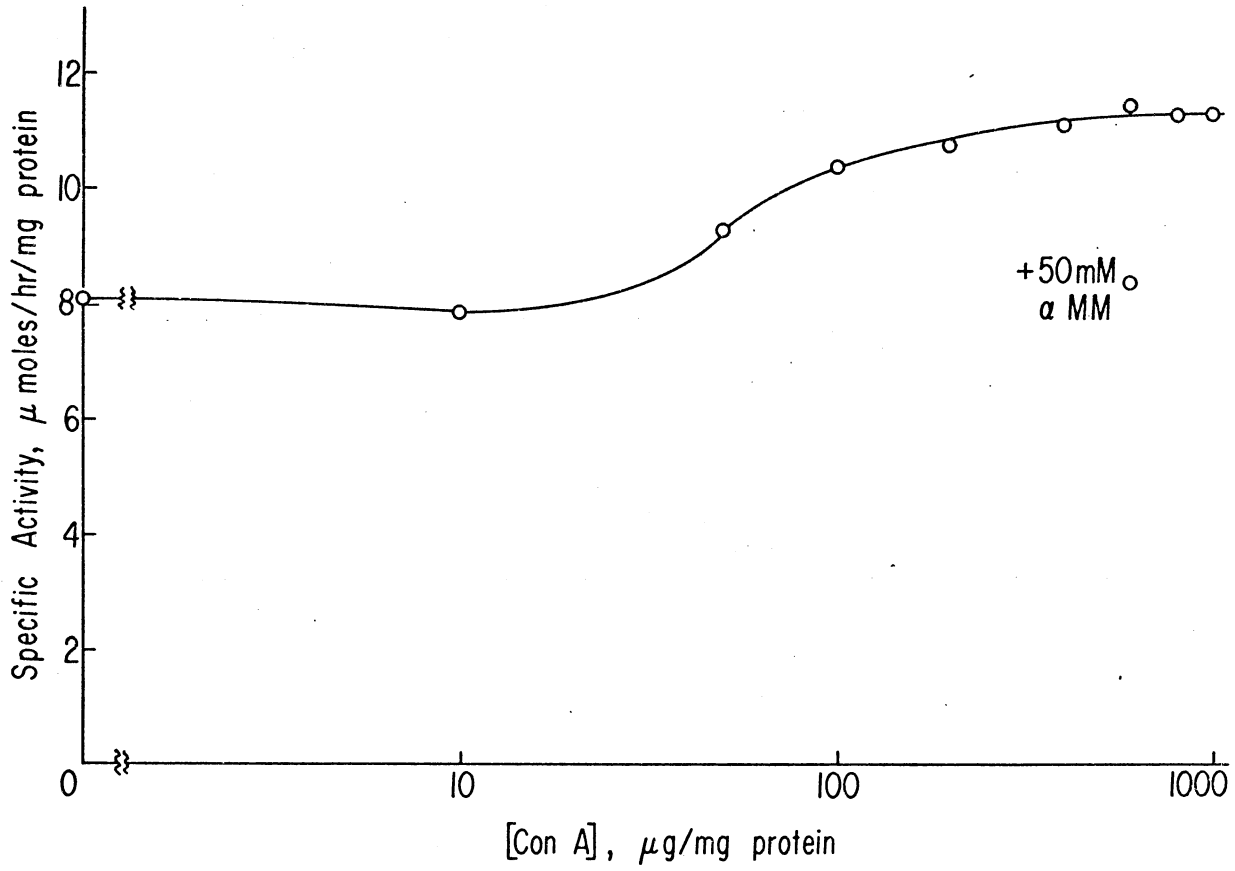


Figure 9. Concentration dependence of Con A activation of the $Mg^{2+}$-ATPase. Membranes incubated with Con A for 30 minutes at 37°C before addition of ATP. ATP concentration was 0.4 mM. Reaction terminated after 15 minutes.

substrate inhibition. In other determinations where the ATP concentration was 2.5 mM a much more pronounced activation was observed. That this effect (activation) was due specifically to the binding of the lectin was demonstrated by the versibility with α-methylmannoside. A Hill plot of the data is presented in Fig. 10. A Hill coefficient of 1.6 is obtained. Experiments were also performed using wheat germ agglutinin (WGA) and soybean agglutinin (SBA). WGA showed some ability to activate while SBA demonstrated very limited if any capacity to stimulate the $Mg^{2+}$-ATPase.

## Inhibition of the $Mg^{2+}$-ATPase by $NaN_3$

The membrane was incubated in the presence of $NaN_3$ with and without Con A for 30 minutes at 37°C prior to the addition of substrate. The results are shown in Table V. $NaN_3$ appears to cause no detectable decrease in the activity of the enzyme. In the presence of Con A there is a slight decrease in the activity as the concentration of azide is increased.

## Time Course of Activation of $Mg^{2+}$-ATPase by Con A

To determine the length of time necessary for Con A to activate the membrane bound $Mg^{2+}$-ATPase fully the following experiment was performed. At timed intervals Con A was added to the pre-incubation assay medium containing the 13762 MAT membranes. ATP was then added, and the reaction was terminated after an appropriate incubation. While the velocities at the shorter time intervals reflect a contribution from both the Con A stimulated and the non-Con A stimulated $Mg^{2+}$-ATPase,

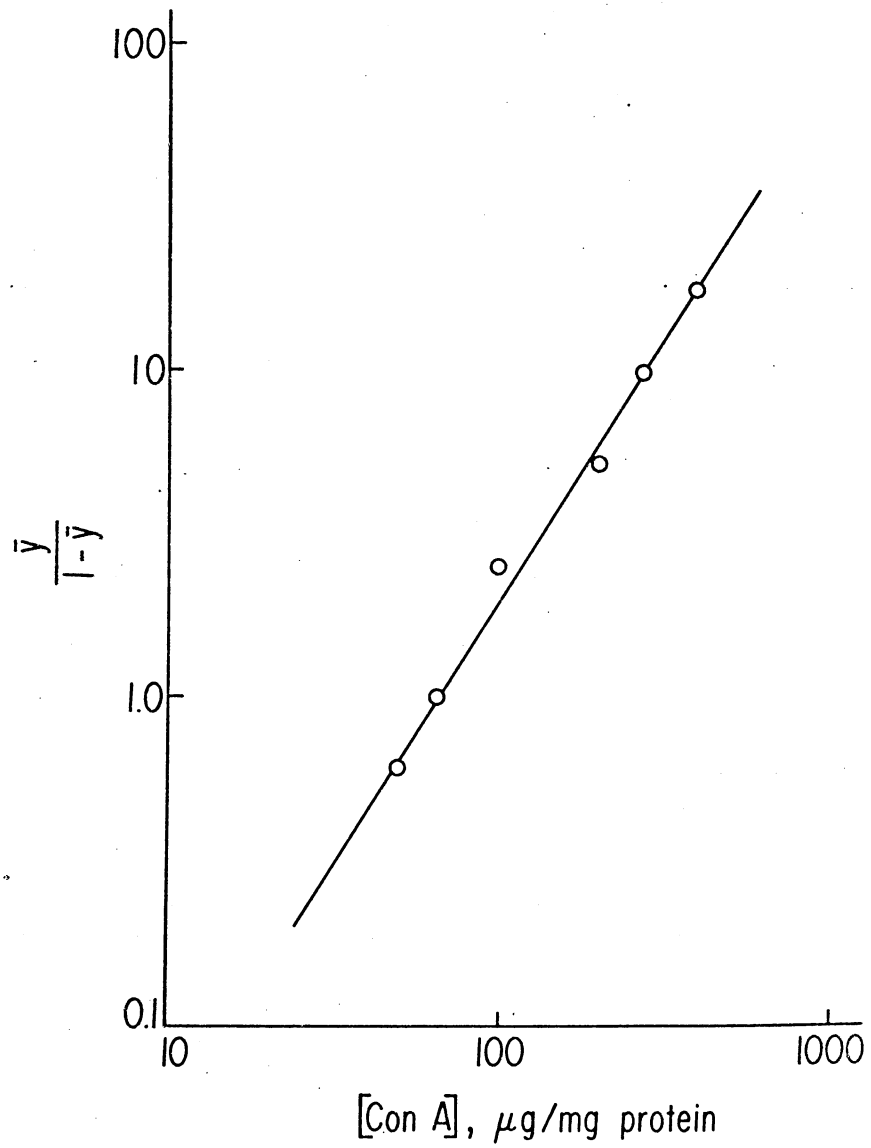


Figure 10. Hill plot for Con A activation of 13762 MAT membrane $Mg^{2+}$-ATPase. ATP concentration was 0.4 mM. Sample was pre-incubated for 30 minutes before addition of Con A. Assay was allowed to continue for 15 minutes before termination. $\bar{y}$ is defined as fractional activation.

TABLE V

THE EFFECT OF $NaN_3$ ON INHIBITION OF 13762 MAT MEMBRANE $Mg^{2+}$-ATPase

| Concentration of $NaN_3$ | Con A | Velocity (μmol Pi/hr/mg protein) |
|---|---|---|
| 0.0 | - | 15.5 |
| 2.5 | - | 16.0 |
| 5.0 | - | 15.5 |
| 10.0 | - | 15.5 |
| 0.0 | + | 42.1 |
| 2.5 | + | 41.2 |
| 5.0 | + | 40.3 |
| 10.0 | + | 37.1 |

ATP concentration was 2.5 mM. $Mg^{2+}$ was used as the only divalent cation. The azide was added prior to the addition of Con A. The samples were incubated at 37°C for 30 minutes prior to the addition of ATP.

there is a maximum velocity reached before 8 minutes (Fig. 11). Since there is no further increase in velocity after this time it is assumed that all the $Mg^{2+}$-ATPase is now fully stimulated by the Con A.

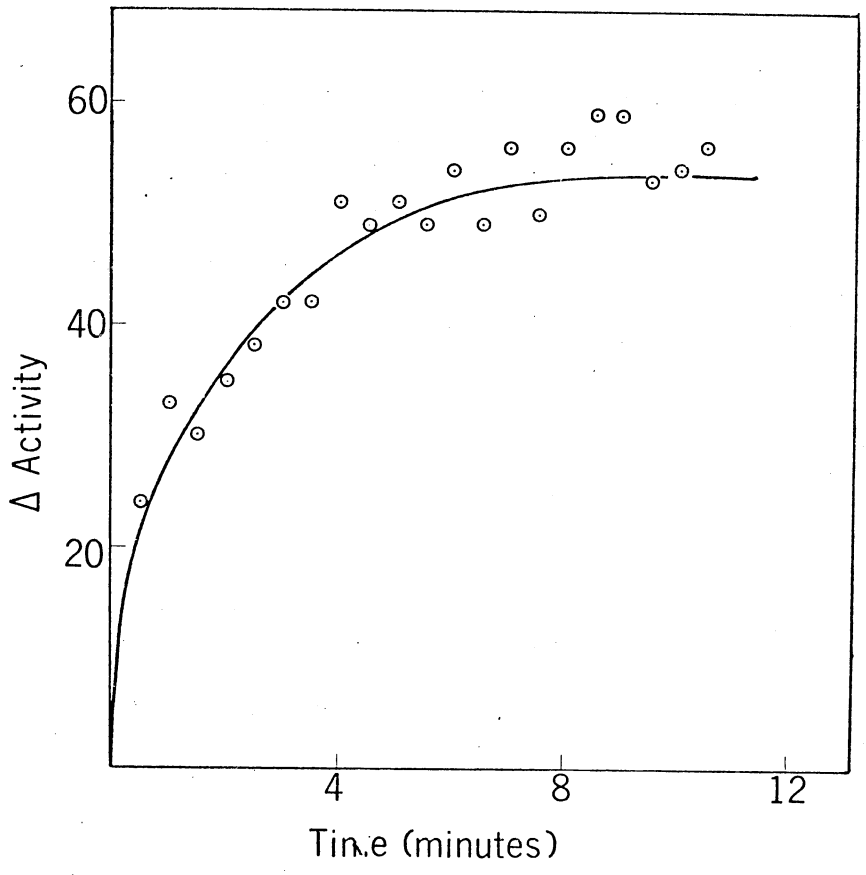


Figure 11. Time course of Con A activation of 13762 membrane $Mg^{2+}$-ATPase. Con A concentration of 75 µg/ml and ATP concentration of 2.5 mM. Membrane protein concentration of 95 µg per ml. Reaction time was 7.5 min. before termination with $HClO_4$.

# CHAPTER IV

## DISCUSSION

It has recently been reported that while ouabain strongly inhibits the ($Na^+$, $K^+$)-ATPase, only a slight but reproducible inhibition of the $Mg^{2+}$ and $Ca^{2+}$ stimulated ATPase in the Ehrlich ascites tumor cells is observed (28). In the plasma membrane of cells from the mouse liver, ouabain has been shown to inhibit the ($Na^+ + K^+$)-ATPase by 97% (29). The 13762 MAT cell line also has a low ($Na^+ + K^+$)-ATPase activity. This fact, combined with the low activity in the absence of divalent cation, assures that the activity present is not due to a ($Na^+$, $K^+$)-ATPase but due to the $Mg^{2+}$-ATPase. It has also been shown in the eel electric organ where Con A inhibits the ($Na^+ + K^+$)-ATPase that the binding of Con A interfered with neither the rate of nor final degree of inhibition by ouabain (30). This is in contrast to the 13762 MAT $Mg^{2+}$-ATPase which is stimulated by Con A. That the enzyme is activated by $Mg^{2+}$ and $Ca^{2+}$ in a saturable manner is shown in Fig. 5.

At high substrate concentration the $Mg^{2+}$-ATPase from the membrane preparations show apparent substrate inhibition. The apparent inhibition is not due to the binding of $Mg^{2+}$ by the increased ATP concentration as shown by Fig. 3. Since $Mg^{2+}$ concentration is 5.0 mM in all assays it is well above the saturation point. The effect (inhibition) is apparent only at substrate concentrations greater than 0.3 mM. This is similar to the effect of high substrate concentration on renal

cortical adenylate cyclase (31). These data seem to suggest a homotrophic allosteric binding site whose location may be: 1) on the enzyme itself; 2) on another protein (transducer system); 3) on either side of the membrane. In both heart and liver mitochondrial ATPase there are two types of binding sites distinguished by the inhibitor AMP-P(HN)P (32). It was further demonstrated that the binding of Mg-ATP appears to diminish the affinity for Mg-ATP at the catalytic site. It has been demonstrated that Con A and insulin have operationally different receptor sites in adipose tissue (10). Thus it was suggested a common coupler or transducer system connecting different receptors to a common pathway (enzyme) existed in adipose tissue (9). This is also a possibility in the 13762 MAT $Mg^{2+}$-ATPase.

The enzyme shows release from the apparent substrate inhibition with addition of Con A. This release is shown to be virtually complete in 7 to 8 minutes. The kinetics are identical up through 0.3 mM ATP but at concentrations greater than 0.3 mM the kinetics show no substrate inhibition (Fig. 2) in contrast to the membranes alone. This extropolates to give approximately the same $V_{max}$ and $K_m$, which would indicate one of two things. 1) Con A binds at or near enough to the secondary binding site of ATP to block the binding of ATP physically. 2) Con A causes a conformational change such that substrate inhibition is no longer observed. The latter effect could arise through two possibilities: a conformational change in the coupler protein if effect of secondary site is through a coupler; conformational change through subunit submit interaction of the enzyme. For example, if a high concentration of ATP causes a conformational change in the enzyme but Con A is bound prior to the addition of ATP, it causes a conformational

change such that ATP is unable to bind at the secondary site or negates the effect of ATP if bound prior to the addition of Con A. In support of the former it has been suggested in the case of the eel electric organ ($Na^+$-$K^+$)-ATPase that the Con A binding site is at either the $Na^+$ or ATP binding site or that the Con A effect is through a regulation of conformational change (30).

Whole cells display linear Lineweaver-Burke plots at substrate ranges of 0.04 to 5.0 mM ATP, the latter value being twice the greatest value used with isolated membranes. The number of cells used in the assay is approximately $5 \times 10^5$ cells of approximately 150 µg total protein per determination. Addition of Con A to the $Mg^{2+}$-ATPase assay (2.5-5.0 mM ATP) does not alter its activity in the whole cell. There are at least two explanations for this observation. One, the secondary binding site of ATP is on the inside of the membrane inaccessible from the ATP in the incubation medium in the whole cell as compared with its accessability in the membrane preparation. Two, the secondary binding site is in a cryptic form and is exposed during preparation of the membrane. If the first possibility were true, this would tend to contradict a former postulate that Con A might block the secondary binding site for ATP physically since Con A is not known to bind to the inner cell surface of membranes. The second possibility is still quite plausible. A site which is cryptic in the cell under some conditions may not be cryptic at all stages of the cell cycle. It has been demonstrated that during differentiation there are different sites exposed (5).

When the intact cells are washed with HEPES and later swollen in 40 mM Tris, there is no indication of an increase in ATPase activity at

either step upon the addition of Con A. When the Tris swollen cells are exposed to the 1 mM $ZnCl_2$ and then incubated with Con A, there is consistantly observed an approximate 10% increase in activity as compared with the non-Con A stimulated cells. Whether this slight enhancement in activity may be due to loss of integrity of the cell membrane has not been fully investigated.

The enzyme appears to lack an absolute specificity for the divalent cation. Calcium consistantly proved to give a 15-20% higher $V_{max}$ when used as the divalent cation in place of magnesium. This compares well with previously reported data on the Ehrlich ascites carcinoma cells in which $Ca^{2+}$ proved to be 12% more effective in the hydrolysis of ATP (33). The reported order of effectiveness was $Ca^{2+} > Mg^{2+} > Mn^{2+} > Co^{2+}$. Using guinea pig polymorphonuclear leukocytes, the ATPase has been shown to have the following preference for the divalent cation, $Mg^{2+} > Ca^{2+} > Mn^{2+} = Co^{2+}$ (18). Using the present system $Ba^{2+}$ was shown to be only 38% as effective as $Mg^{2+}$ in activation of the ATPase. It is interesting however than when Con A is included in the preincubation medium that the degree of activation as well as the final velocity reached is consistantly 15-20% greater when $Mg^{2+}$ is used as when $Ca^{2+}$ is used as the divalent cation. $Ba^{2+}$ shows only slight activation upon addition of Con A.

Both $Ca^{2+}$ and $Mg^{2+}$ appear to follow hyperbolic first order kinetics. $Ca^{2+}$ and $Mg^{2+}$ appear to give nearly equal values for Km of 0.07 to 0.08 mM. This compares with the system of Ehrlich ascites carcinoma cells in which a divalent cation concentration of $5 \times 10^{-4}$ M gave maximal activation (33). In the $(Ca^{2+} + Mg^{2+})$-ATPase of the plasma membrane of mouse liver cells, maximal activation by $Ca^{2+}$ occurs at 0.3 mM while

maximal stimulation by $Mg^{2+}$ occurs at 3 mM (34). The ATPase activity of bovine milk fat globule membranes gave maximal activation with $Mg^{2+}$ at concentrations of 5.0 mM (35).

$Ca^{2+}$ also gives similar results to $Mg^{2+}$ with respect to substrate inhibition at high substrate levels when using membrane preparations. As is the case when using $Mg^{2+}$, the enzyme does not show apparent substrate inhibition when the intact cell is assayed.

The Con A stimulation when plotted by a Hill plot does show positive cooperativity giving a Hill coefficient of 1.6 suggesting site-site interaction. That this was reversible with α-methylmannoside at a concentration of 7.5 mM demonstrates that the effect is specifically due to the binding of Con A to a membrane oligosaccharide. The decreased effectiveness of WGA and SBA are similar to the effect of the lectins on 5'-nucleotidase of 13762 MAT membranes (23) and milk fat globule membranes (36). Con A in these systems causes an inhibition of the 5'-nucleotidase in a cooperative fashion.

The enzyme appeared to be insensitive to inhibition by $NaN_3$ at concentrations less than 10 mM. This is similar to the binding of Con A to T spleen lymphocytes in which there is an increased $Ca^{2+}$ uptake which is not inhibitable by $NaN_3$ (37). In contrast in the rat adipocyte plasma membrane there has been demonstrated a $Mg^{2+}$-ATPase which is strongly inhibited by $NaN_3$ (38). The inhibition by $NaN_3$ of the Con A activated membrane bound enzyme is most interesting. Both $NaN_3$ and oligomycin were found to inhibit the ability of low concentration of Con A to stimulate the plasma membrane $Mg^{2+}$-ATPase of the adipocyte but not at the higher levels (9). The $Mg^{2+}$-ATPase of the adipocyte membrane was shown to have a biphasic response to Con A stimulation.

The point at which inhibition of the Con A stimulation by $NaN_3$ occured was at a much lower Con A level than used in the present assay. Also, the $NaN_3$ concentration was 20 mM in contrast to 10 mM maximum in the present experiment. This could explain why they observed a much more pronounced inhibition of the Con A stimulation by $NaN_3$. These results may again add credence to the postulate of the existence of a transducer or coupler protein.

It is also interesting to consider how a system similar to that suggested by Moyle and Mitchell could be operative in the present system (39). They suggest that the microsomal ATPase could exist as a mixture of molecules in two states, one being active and the other inactive. Thus defining $\alpha = \frac{\text{active}}{\text{inactive}}$. They suggest that activators (e.g. aurovertin) affect the value of $\alpha$ rather than the velocity of the enzyme. It is stated that it seems probable that the active/inactive state transitions involves some change of conformation of the individual subunits of $F_1$ or some change of packing of the subunits or both. None of the present data appears to rule out such a possibility for the $Mg^{2+}$-ATPase in the present system. This would simply mean that ATP would decrease the value of $\alpha$ while Con A would suppress its action or increase $\alpha$ through another means in the isolated membrane system.

# CHAPTER V

## SUMMARY

The membrane bound ATPase of the 13762 MAT cell line has been shown to demonstrate apparent substrate inhibition at substrate concentrations above 0.3 mM. This effect has been shown not to be due to substrate binding of the divalent cation. Preincubation of the plasma membrane with Con A results in apparent release of the enzyme from substrate inhibition. Intact 13762 MAT cells do not show substrate inhibition even at concentrations as high as 5.0 mM ATP. Incubation of whole cells with Con A appears to make no significant difference in the $V_{max}$.

In an attempt to determine the point at which the ATPase develops its "sensitivity" to Con A activation, cells from various stages of membrane preparation were incubated with and without Con A. There appears to be no significant activation of the ATPase at any step before the disruption of the plasma membrane.

Cation specificity for the enzyme appears to be $Ca^{2+} > Mg^{2+} > Ba^{2+}$ without Con A and $Mg^{2+} > Ca^{2+} > Ba^{2+}$ with Con A, the difference between $Ca^{2+}$ and $Mg^{2+}$ in both cases being approximately 15%. $Ba^{2+}$, however, was only 38% as effective as an activator.

With a substrate concentration of 2.5 mM, Con A consistently gave an activation of 2.6 fold. A Hill plot yields a Hill coefficient of 1.6. The activation of the ATPase by Con A is completed within 10

minutes.

SBA and WGA showed limited ability to release the enzyme from substrate inhibition. $NaN_3$ appears to cause no detectable decrease in the activity of the enzyme in the absence of Con A. In the presence of Con A there is a slight decrease in the activity as the concentration of azide is increased.

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