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ADENOSINE TRIPHOSPHATASE AND p-NITROPHENYL PHOSPHATASE ACTIVITY IN ION TRANSPORT

I. The $Na^+ + K^+$-Dependent Adenosine Triphosphatase in the Isolated Mucosal Cells of Turtle Bladder

II. p-Nitrophenyl-phosphatase Activity in the Microsomal Fraction of Turtle Bladder Mucosal Cells

by

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INTRODUCTION

Transport of various ions, particularly sodium, occurs across single cell membranes in some cases (e.g. erythrocytes, muscle, nerve and axone); and across an entire cell in other cases (e.g. in frog skin, toad bladder, renal tubule, salivary glands, sweat glands, gastro-intestinal epithelium, as well as in the turtle bladder). The transport of sodium across a single membrane, or "transmembrane" transport, is concerned with volume regulation, with transmission of excitation, but not with net movement of the salts or water. On the other hand, transport of sodium across an entire cell including both membranes, or "transcellular" transport, is concerned with absorption or secretion accompanied by the net movement of large amounts of salts, water and other materials.

The present research began with a series of studies on the active transport of Na^+, Cl^-, and HCO_3^- from the mucosal (urine-containing side) to the serosal fluid (interstitial fluid and blood side) of the in-vitro turtle bladder. The turtle bladder was chosen as the working model because it was cheap, readily available, and had ion transport properties resembling those of the kidney tubule. The advantages of using such a living cell membrane system instead of a kidney in situ were: (a) the relative simplicity of the structure of the bladder compared to that of the renal tubules; (b) the ease of access to the in-vitro bladder (in contrast to an individual renal tubule) so that direct measurements of ion fluxes, transcellular electrical potential,

short-circuiting current density and resistance could be made by means of isotopes and precision macro-electrode equipment; and (c) the ability to separate the transporting cells (mucosal epithelial cells) from the underlying interstitium so that chemical analyses of composition and enzymatic activities could be made on various centrifugal fractions of the cell homogenates.

Other isolated cell membrane systems, such as amphibian skin or bladder, have been used as working models for certain of the renal functions. The turtle bladder was shown to transport salts and water as well as to acidify the mucosal fluid--a set of biological properties very similar to that of the distal renal tubule in mammals.

The term, active or "uphill" transport of an ion, means that there is a net transcellular movement of the ion against its transcellular electrochemical potential gradient. The process of active transport requires a source of metabolic energy whereas that of passive transport does not. In the case of the turtle bladder, the ions (Na^+, Cl^- and HCO_3^-) appear to be moved by an active transport mechanism, whereas water appears to move passively down its chemical potential gradient. The source of energy for active sodium transport in the nerve and erythrocytes is apparently the free energy of hydrolysis of an energy-rich phosphate ester, adenosine triphosphate (ATP). However, experimentally other energy-rich phosphate esters like inosine triphosphate (ITP), guanosinetriphosphate (GTP) or uridinetriphosphate (UTP) can replace ATP.

The source of energy for active sodium transport in the turtle bladder is apparently from anerobic as well as from aerobic metabolism. The maintenance of sodium transport under anerobic conditions with glucose as the main substrate, suggests that energy-rich phosphate esters such as ATP are involved in delivering metabolic energy for active transport.

Exogenously added ATP cannot replace glucose as the main metabolic substrate for the support of ion transport in the turtle bladder as it can in the case of nerve and erythrocyte when it is introduced inside but not outside the cell. The lack of effect of ATP was probably because of its inability to penetrate the serosal membrane and to reach the site of the transport-related enzyme or enzymes such as $Na^+ + K^+$ stimulated adenosine triphosphatase (ATPase).

The enzyme, $Na^+ + K^+$-ATPase has been correlated with sodium transport in crab nerve, erythrocytes, and in other tissues. The correlation between sodium transport and ATPase activity has been recently reviewed by Skou 1965, Heinz 1967, Albers 1967 and Whittam 1970. The ATPase activity, found in crude homogenates as well as in microsomal fractions of many tissues, is absolutely dependent upon Mg^{++}, is stimulated by addition of Na^+ and K^+ together, and the $Na^+ + K^+$ stimulated moiety is inhibited by ouabain.

Microsomal preparations from many tissues have other phosphatase activities, such as acetyl phosphatase and p-nitrophenyl phosphatase (p-NPPase). Both acetyl and p-nitrophenyl phosphatase activities

are inhibited by addition of ouabain, sodium and N-ethylmaleimide (NEM).

The microsomal fraction of the mucosal cells of the turtle bladder, a known sodium transporting system, possesses a Mg^{++}-dependent, $Na^+ + K^+$ stimulated, ouabain inhibited ATPase activity, satisfying five out of eight criteria of Skou for identification, isolation and correlation of enzymatic activity with sodium transport. The five criteria satisfied by the findings in the turtle bladder are: (a) the enzyme has been found in a system which transports sodium; (b) the enzyme activity detected in bladder cells does catalyze the hydrolysis of ATP, which is theoretically capable of transferring the free energy of hydrolysis into that needed for cationic transport; (c) there is a parallel inhibitory effect of cardiac glycosides on the activity of the isolated enzyme extract and on the sodium transport in the intact cell; (d) the pattern of increasing ATPase activity versus the concentration of $Na^+ + K^+$ in the microsomes resembles qualitatively the pattern of increasing sodium transport versus the concentration of mucosal sodium in the intact bladder; and (e) the $Na^+ + K^+$ ATPase is located in the microsomal fraction of mucosal cells. The $Na^+ + K^+$ stimulated ATPase catalyzes the hydrolysis of ATP as follows:

$$ATP + H_2O \longrightarrow ADP + P_i + H^+ \qquad 1)$$

The enzymatic activity has been shown to have an absolute requirement for Mg^{++} and is increased by the simultaneous addition of Na^+ and K^+. Mg^{++} is needed not only for activation of the enzyme, but as a part

of the substrate which has been shown to be a Mg^{++}-ATP complex. Evidence accumulated from many laboratories suggests that ATP hydrolysis occurs through formation of one or more intermediary complexes with the enzyme. The following reaction sequences have been postulated from studies (by Post et al) done on microsomes using γ^{32} phosphate labelled ATP.

$$ATP + E_1 \underset{}{\overset{Na^+ + Mg^{++}}{\rightleftharpoons}} E_1\text{-}P + ADP \quad , \qquad 2)$$

where E_1-P is a high energy phosphorylated intermediate; and

$$E_1\text{-}P \longrightarrow E_2\text{-}P, \qquad 3)$$

where E_2-P is a lower energy phosphorylated protein than is E_1-P.

$$E_2\text{-}P \xrightarrow[\text{ouabain}]{K^+} E_2 + P_i \qquad 4)$$

The phosphorylated enzyme (intermediary complex) can be estimated under different ionic conditions with and without various inhibitors such as ouabain.

The second phosphatase activity found in the microsomal preparations from many tissues including those from the turtle bladder is p-nitrophenyl-phosphatase which is activated by Mg^{++} and K^+, and inhibited by ouabain, NEM, Na^+ and ATP. It has been postulated that p-nitrophenylphosphate may phosphorylate the enzyme and that the phosphorylated enzyme may serve as a substrate for the reaction in equation No. 4 shown above for ATP hydrolysis. It has also been postulated that the two enzymatic activities, ATPase and p-NPPase,

are two conformational states of the same enzyme and that the inhibition of p-NPPase activity by addition of Na^+ or ATP is due to a change in the conformation of the enzyme from that characteristic of p-NPPase to that characteristic of ATPase. Attempts at further purification of these enzymatic activities have resulted in inactivation rather than purification of the enzymes. The estimated molecular weight of the enzyme protein from data on sedimentation after solubilization with different detergents, and from data on radiation inactivation varies from 250,000 to 670,000.

The purposes of the present studies were to investigate the existence of ATPase and p-NPPase in microsomal preparations from the turtle bladder, to evaluate their kinetic parameters such as K_m, K_i and V_m with respect to their substrates and ionic co-factors, to determine the effect of pH, and to determine the effect of various inhibitors such as ouabain, NEM, etc.

THE $Na^+ + K^+$-DEPENDENT ADENOSINE TRIPHOSPHATASE IN THE ISOLATED MUCOSAL CELLS OF TURTLE BLADDER

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SUMMARY

1. In the microsomal fraction of isolated mucosal cells of turtle bladders, addition of Na^+ and K^+ to Mg^{++}-containing mixtures increased ATPase activity by 100 to 150 percent; and the $Na^+ + K^+$-dependent activity was completely inhibited by ouabain.

2. Apparent K_m values, with respect to ATP were: 2.2×10^{-4}M for the $Na^+ + K^+$-dependent, and 1.8×10^{-4}M for the Mg^{++}-dependent activity. The apparent K_m for Na^+ was 4.5×10^{-3}M; and that for K^+, 0.36×10^{-3}M and 2.9×10^{-3}M.

3. No effects on activity were found after addition of amiloride, furosemide, and acetazolamide. In contrast, NEM, 10^{-4}M inhibited the $Na^+ + K^+$-dependent activity by 45% without affecting the Mg^{++}-dependent activity.

4. For Mg^{++}-dependent ATPase, the order of nucleotide preference was: ATP > ATP > ITP > CTP > UTP > ADP; and for $Na^+ + K^+$-dependent ATPase, the order was: ATP > ITP > CTP > GTP, while the activity with UTP or ADP was negligible.

5. The pH optimum of the activity with $Na^+ + K^+ + Mg^{++}$ was 7.3 - 7.5; while that with Mg^{++} alone was a weak function of the pH.

6. Activity of ATPase increased as a function of temperature

(15-45°C). The calculated activation energy was 8 KCal/mole for the Mg^{++}-dependent; and 13 KCal/mole for the Na^{+} + K^{+}-dependent activity.

INTRODUCTION

The isolated bladder of the fresh water turtle, Pseudemys scripts, possesses mechanisms for the active transport of sodium, chloride and bicarbonate under conditions of open-circuit (1) and under conditions of short-circuiting (2,3). Recently reported work has demonstrated that ouabain added to the serosal bathing fluid (final conc. 10^{-4}M) of the short-circuited bladder suppressed the net transport of sodium completely and irreversibly without affecting that chloride or bicarbonate (4). In crude homogenates and in microsomal fractions of the epithelial cells, there was demonstrated a Na^+ K^+ stimulatable, ouabain inhibitable ATPase activity (4).

The purposes of the present paper were to investigate further the distribution of the Na^+, K^+ ATPase activity in various fractions of the mucosal cells; to determine the effects of pH and temperature; to evaluate the kinetic parameters such as K_m and V_{max} of the ATPase activity with respect to ATP and with respect to Na^+ and/or K^+ as the subtrate; to determine the effect of various inhibitors of Na^+ transport; and to determine the substrate specificity for the nucleotides--ATP, ITP, CTP, GTP, UTP, and ADP.

METHODS

Isolation of mucosal cells. The necks of the ten bladders, in the form of closed sacs, were tied to the outlet of a Luer lock syringe, filled with and immersed in Ca-free Ringer solution (4) containing bicarbonate, 17 mM and EDTA, 2 mM, and incubated for 30-40 minutes at 25°C. After incubation, walls of the sacs were rubbed gently against one another for 2-3 minutes, as recommended by Lipman et al (5)--a maneuver which released the epithelial cells into the mucosal fluid. The cell-containing mucosal fluid was removed from the sacs and subjected to a series of homogenizations and centrifugations.

Isolation of microsomal fractions. During the procedure for obtaining the various centrifugal fractions from isolated mucosal cells, low temperatures (0-2°) were maintained throughout. Isolated cells, obtained by the EDTA treatment of 10 bladders and suspended in a total volume of about 250 ml of Ringer solution, were carried through the previously described (4) procedures of homogenization and differential ultracentrifugation in order to obtain the microsomal fractions.

A microsomal pellet obtained in the last centrifugation step (65,000 g for 60 minutes) was suspended in 15-20 ml of 1 mM EDTA, stored at -10°, and used as an enzyme source for all the experiments reported here. In some cases, the pellets obtained at 10,000 g and 20,000 g, as well as all of the supernatants, were examined for ATPase activity. Protein concentration (of the enzyme suspension used in the incubation procedure), determined by the method of Lowry et al (6)

was used as the normalizing parameter for enzyme activity.

Assay of ATPase Activity. In the assay for total ATPase activity (that in the presence of $Na^+ + K^+ + Mg^{++}$), the composition of reaction mixture, expressed in terms of final millimolar concentration in the incubation flask was: Tris or Na ATP, 2.0; $MgCl_2$, 3.0; EDTA, 0.2; imidazole, 40; histidine, 40; NaCl, 84; and KCl, 15. To this was added the enzyme-containing aliquot of 0.5 to 1.0 ml of the raw cell homogenate or of any one of the centrifugal pellets containing 50-100 µg protein. The final pH was 7.3; and the final volume in the flask was 5 ml.

In the assay for Mg^{++}-dependent ATPase activity, the composition of the incubation mixture was the same as that for Na^+, K^+, Mg^{++}-dependent activity (total ATPase) except that choline chloride, 100 mM was substituted for NaCl and KCl. In many cases, the Mg^{++}-dependent ATPase activity was estimated from that measured in the presence of Na^+, K^+, Mg^{++} and ouabain, 10^{-4}M.

After 5 minutes of preincubation, the reaction was started by addition of ATP, allowed to proceed for 10 minutes at 38°C., and stopped by addition of 5.0 ml of cold perchloric acid, 6%. Control tubes, carried through all incubations, were of two types--those without ATP and those without the enzyme. Aliquots of the final mixture were analyzed for inorganic phosphate by the method of Berenblum and Chain (7).

According to the current convention (8,9), the ATPase activities were defined operationally as: a) that which catalyzes the hydrolysis

of ATP in the presence of magnesium (Mg^{++}-dependent ATPase) and, b) that which catalyzes the hydrolysis of ATP in the presence of Na^{+}, K^{+}, and Mg^{++} (total ATPase). Total ATPase minus Mg^{++}-dependent ATPase was called "Na^{+}, K^{+} ATPase."

The initial reaction velocity was determined, as previously described (4), from the P_i released during the first 10 minutes of incubation. The initial concentrations of ATP varied from 0 to 2.0 mM; and no more than 20% of the ATP was degraded during the course of the incubation.

Sources of Material. Ouabain (G-strophanthidin), Tris ATP, disodium ATP, disodium ITP, disodium GTP, disodium CTP, disodium UDP, disodium UTP, N-ethyl-maleimide, L-Histidine, imidazole grade I, Tris (hydroxymethyl)aminomethane, and EDTA (ethylene-diamine tetra acetic acid) were obtained from Sigma Chemical Co., St. Louis, Mo. Furosemide was obtained from Hoechst Pharmaceutical Company, Cincinnati, Ohio, and amiloride was obtained from Dr. J.E. Baer at Merck-Sharpe and Dohme, West Point, Pennsylvania.

RESULTS

Fractional Distribution and Stability

Distribution: Table 1 presents data on the distribution and partial purification of Na^+, K^+, Mg^{++}-dependent ATPase activity in a representative experiment on a pool of epithelial cells from 10 turtle bladders. In this instance, the enzyme activities and protein contents were determined for each and every pellet and supernatant. No enzymatic activity was detectable in any of the supernatant fractions.

The three columns on the right side of table 1 present values for specific activity. In the crude homogenates of mucosal cells, the Na^+, K^+, Mg^{++}-dependent or total ATPase activity (A) and the ouabain-sensitive moiety of this activity (A-B) were 8.7 and 2.5 micromoles/mgm protein/hr, respectively. The activity increased progressively during the centrifugal separation of the different cell fractions. For example, the $Na^+ + K^+$-dependent moiety of the activity (A-B) increased from 2.5 in the crude homogenate to 64.5 μm P_i/mg protein/hr in the microsomal fraction (65,000 g x 1 hr)--a 26-fold increase; while the Mg^{++}-dependent moiety of activity increased from 6.2 in the homogenate to 42.5 in the microsomal fraction--a 7-fold increase. Similar increases in specific activity were found in the centrifugal fractions derived from 10 other pools of epithelial cells.

The 4 columns on the left of table 1 present values for volume; for activity of Mg^{++}, Na^+, K^+-dependent ATPase; and for amount of

protein in each of the designated fractions.

Of the total protein in the cell homogenate, 85% was recovered from all of the supernatants and pellet fractions. The microsomal pellet (65,000 g) contained 6% of the original total protein.

Whereas the total protein in all of the pellets and supernatants amounted to 85% of that in the original homogenate, the total ($Na^+ + K^+ + Mg^{++}$-dependent) activity amounted to 128%, and the Mg^{++}-dependent activity (derivable from values under column B) to 93% of its activity in the original homogenate. From these data, it was estimated that the recovery of Na^+, K^+-dependent ATPase activity was 213% of what it was in the original homogenate.

The 213% yield of Na^+, K^+-dependent ATPase recovered in all centrifugal fractions was interpreted as the consequence of an inhibition of this activity in the original homogenate. Such an inhibition could have been due to the existence of naturally occurring inhibitors such as histone-like substances released from the nuclei during homogenization of the cells (10); or to certain unspecified effects of high concentration of other proteins in the cell homogenate.

Stability. Having achieved the partial isolation and partial purification of a comparatively high specific activity of ATPase in the microsomal fraction, the next step was to determine the stability of the activity during storage at -10°C, and during the course of incubation at 38°C.

Figure 1 is a semi-log plot of values of activity of the Mg^{++}-dependent and of the Na^+, K^+-dependent enzyme as a function of time

of incubation at 38°C. The linear pattern of the plot indicates that both decay rates were exponential functions, from which the half life of the enzyme activities could be calculated. The half-times of decay of both enzyme activities (Na^+, K^+-dependent and Mg^{++}-dependent) were 300 and 240 minutes respectively. This means that less than 3% of the enzyme activity would be lost during a 10-15 minute interval at 38°C--which was the maximal time of incubation in any or all of the present experiments.

Not shown in the figure are data obtained on the corresponding activities after storage at -10°C. Every 4-5 days, aliquots of the microsomal pellet, stored at -10°C, were brought to 38°C and tested (during a 10-15 minute period) for the Mg^{++}-dependent and for the Na^+, K^+-dependent ATPase activity. Both activities yielded logarithmic patterns which were qualitatively, but not quantitatively similar to those in figure 1. The estimated half-life of either activity at -10°C was 35-36 days.

Enzymatic Properties

In the microsomal pellets isolated from the mucosal epithelial cells of the turtle bladder, the ATPase activity has an absolute requirement for Mg^{++} as well as a cation-sensitivity with respect to sodium and potassium together (4).

The pattern of microsomal ATPase activity versus simultaneous concentrations of sodium and potassium in the incubation mixture was similar to that first reported by Skou in crab nerve microsomes (11).

The maximal activity in this microsomal preparation was attained in the presence of Na^+, 60-90 mM together with K^+, 10-20 mM.

Cationic concentrations for measuring Mg^{++}, Na^+, K^+-dependent activity in the present work was: Mg^{++}, 3; Na^+, 85; K^+, 15 mM; while the pH of the incubation mixture was fixed at 7.3.

Accordingly, a series of experiments were performed in microsomal pellets in the presence of : (Mg^{++}); (Mg^{++} + Na^+); (Mg^{++} + K^+); (Mg^{++} + Na^+ + K^+); and (Mg^{++} + Na^+ + K^+ + ouabain).

Table 2 presents mean values for ATPase activity under the aforementioned conditions. As expected, the mean ATPase activity in the presence of Mg^{++} + Na^+ + K^+, (73.2 μmoles/mgm/hr) was greater than that in the presence of Mg^{++}; Mg^{++} + Na^+; or Mg^{++} + K^+ ($P \ll 0.001$). Moreover the baseline (Mg^{++}-dependent) rate was not perceptibly altered after addition of either Na^+ or K^+ along ($P > 0.90$ in both cases).

The table also shows that the activity of a ouabain-treated microsomal preparation in the presence of Na^+ + K^+ + Mg^{++} was the same as that of the preparation in the presence of Mg^{++} alone ($P > 0.8$). This suggested that the ouabain-treated preparation in the presence of all 3 cations could be taken as equivalent to the preparation in the presence of Mg^{++} alone.

Sodium iodide treatment. Exposure of microsomal pellets to sodium iodide as recommended by Nakao (12) and others (13) presumably reduces the Mg^{++}-dependent, but not the Na^+ + K^+-dependent ATPase activity. In

two experiments on turtle bladder microsomes, NaI decreased the activity of both the Mg^{++}-dependent and $Na^+ + K^+$-dependent ATPase by 10 to 20%--an effect much less than that found in other tissues (12, 13).

Km Values. The kinetic parameters for $Na^+ + K^+$-dependent and for Mg^{++}-dependent ATPase activity were determined with respect to: ATP concentration as substrate; Na^+ concentration as substrate; and K^+ concentration as substrate. In all of the kinetic calculations, the Mg^{++}-dependent moiety of ATPase activity was measured as the residual ATPase activity remaining after addition of ouabain (10^{-4}M) to a microsomal pellet incubated in the presence of Na^+, K^+, and Mg^{++}.

Figure 2 is a plot of values of reciprocal ATPase activity versus reciprocal ATP concentration for $Na^+ + K^+$-dependent and for Mg^{++}-dependent activities in a representative experiment on the microsomal fraction isolated from a pool of 10 turtle bladders. Estimating graphically from the Lineweaver-Burke plot of Figure 2, the Km for $Na^+ + K^+$ ATPase was 2.3×10^{-4}M, while that for Mg^{++} ATPase was 2.4×10^{-4}M.

Figures 3 and 4 show Lineweaver-Burke plots derived from data on the ouabain-sensitive moiety of ATPase activity (i.e., Na^+, K^+ stimulated ATPase). The form of the plots is similar to that of Figure 2, except that the reciprocal of the ATPase activity was plotted as a function of the reciprocal of the sodium concentration in the presence of constant levels of ATP, K^+, and Mg^{++} (Fig. 3); and as a function of the reciprocal of the potassium concentration in the pres-

ence of constant levels of ATP, Na^+, and Mg^{++} (Fig. 4).

Whereas the apparent Km with respect to sodium was clearly 4.0×10^{-3}M (see Fig. 3), the Km with respect to potassium appeared to have two values, 0.20×10^{-3} and 2.0×10^{-3}M (see Fig. 4). This double valued Km was derived graphically by making two straight lines approximate the curvilinear pattern of reciprocal ATPase activity versus reciprocal potassium concentration in Figure 4. Interestingly, the single valued Km's (with respect to potassium) reported for other tissues (9, 11, 13) as well as for the turtle bladder (14) ranged from 1.0×10^{-3} to 3.0×10^{-3}M--a range which fell between the two limits of the double valued Km shown in Figure 4.

Over and above the single experiments shown in Figures 2, 3, and 4, the Km values were determined in three experiments performed under each one of the three conditions; i.e. with ATP as substrate, with Na^+ as substrate, and with K^+ as substrate. The mean values for Km were as follows: 2.2×10^{-4}M (ATP); 4.5×10^{-3}M (Na^+); and 0.36×10^{-3}M and 2.9×10^{-3}M (K^+).

Inhibitors. The ouabain-induced inhibition of sodium transport and of Na^+, K^+ ATPase raised the question of the effect of other inhibitors of sodium transport on the Na^+, K^+ ATPase--a question which provided the basis for the next set of experiments.

As indicated previously, ouabain, 10^{-4}M, inhibited all of the $Na^+ + K^+$-dependent moiety but none of the Mg^{++}-dependent moiety of the microsomal ATPase. As a matter of fact, ouabain at a final concentration of 3×10^{-7}M resulted in the inhibition of 50% of the Na^+, K^+

ATPase activity. This degree of sensitivity to ouabain is greater (by 3 orders of magnitude) than that found in microsomes from other tissues (18, 19, 20).

Amiloride, 10^{-4}M, furosemide, 10^{-4}M, and acetazolamide, 10^{-4}M, had no inhibitory effects on Na^{+} + K^{+}-dependent or on Mg^{++}-dependent ATPase.

The lack of inhibition by amiloride is in accord with results of Baer et al (16) on ATPase activity. However, amiloride has been shown to block sodium transport in the toad bladders (17) and turtle bladders (Gentile and Brodsky--unpublished data).

The lack of inhibition of microsomal ATPase activity by furosemide, 10^{-4}M, is consistent with parallel findings in our laboratory, on the lack of effect of furosemide on sodium transport and short-circuiting current across the intact turtle bladders.

The sulfhydryl blocker, N-ethyl maleimide, 10^{-4}M, reduced the short-circuiting current of two turtle bladders to zero 30 minutes after its addition to the bathing media. However, NEM, 10^{-4}M inhibited the Na^{+} - K^{+}-dependent moiety of microsomal ATPase by 45 percent (without affecting the Mg^{++}-dependent moiety) after it had been incubated with the microsomal pellet for 30 minutes.

Substrate specificity. In order to determine the specificity of the substrate required for the nucleotidase activity in the microsomal pellets, comparative experiments on the rate of hydrolysis were performed in the presence of equimolar concentrations of ITP, CTP, GTP, ADP or UTP, instead of ATP. Ionic conditions in all reaction vessels were the

same as those described above--i.e. $Na^+ + K^+ + Mg^{++}$ in one reaction vessel, and $Na^+ + K^+ + Mg^{++}$ + ouabain in the paired vessel containing another aliquot of the same microsomal pellet.

Table 3 presents values of specific enzymatic activity of a selected microsomal pellet with respect to its ability to catalyze the rate of P_i release from six different nucleotides.

Considering the Mg^{++}-dependent hydrolytic activity with respect to ATP as 100%, the order of nucleotide preference was as follows: GTP, 91%; ITP, 60%; CTP, 35%; UTP, 29%; and ADP, 16%.

Considering the $Na^+ + K^+$-dependent activity (see column designated B-A), with respect to ATP as 100%, the order of nucleotide preference was: ITP, 60%; CTP, 32%; GTP, 10%; ADP, 1.4%; UTP, 0%.

The degree of stimulation of hydrolysis by addition of $Na^+ + K^+$ (activity ratio) was about 0.80 regardless of which nucleotide was used.

These data on absolute levels of activity and on degree of stimulation by $Na^+ + K^+$ were similar to those of Skou on the comparative hydrolytic activities of ITP and ATP in crab nerve (21), but differed from those of others working with beef kidney microsomes (22), calf heart microsomes (13), and bovine brain microsomes (23).

pH dependency. Figure 5 is a plot of values of Na^+, K^+-dependent and of Mg^{++}-dependent ATPase activity versus those of pH of the incubation mixture in a series of experiments on a single pool of microsomal pellets. Each point on the graph was taken from the average of at least three separate experiments on enzymatic activity.

In a separate set of experiments, increases in osmolality or ionic

strength of 5 to 10% (such as occasioned by addition of HCl or of NaOH) were found to have no effect on ATPase activity.

The Na^+-K^+-dependent activity reached a maximal value at pH levels of 7.3 to 7.5; and the Mg^{++}-dependent activity increased monotonically as a function of pH. The figure also shows that the activity ratio, $(Na^+ + K^+)/(Mg^{++})$, varied as a function of pH; and was 1.1 at pH 7.3, the pH used routinely in the present studies.

Temperature effects. The Mg^{++}-dependent and $Na^+ + K^+$-dependent ATPase activities were measured as functions of temperature between 15°C and 45°C. Under the conditions of of the present experiments, the initial reaction velocity (rate of P_i release) was assumed to be directly proportional to the specific rate constant, K_r, and may be substituted for it, as is commonly done in order to estimate the activation energy from the Arrhenius equation,

$$\ln K_r = -E/RT + C ,$$

where E is the activation energy; R, the gas constant; T, the absolute temperature; and C, the constant of integration.

Figure 6, a plot of values of log (rate of P_i release) versus those of reciprocal absolute temperature, shows the two functions--$Na^+ + K^+$-dependent activity and Mg^{++}-dependent activity. From the slope of the Arrhenius plot, -E/2.3R, one can estimate the activation energy for each of the two reactions.

The activation energy for the $Na^+ + K^+$-dependent reaction was 13 K Cals/Mole over the entire temperature range studied; while that for the Mg^{++}-dependent reaction was 8 K Cals/mole over the range,

23-45°C, and 38 K Cals/mole over the range 15-23°C. The Q_{10} values estimated between 30° and 40° C were: 2.2 for the Na^{+} + K^{+}-dependent function; and 1.8 for the Mg^{++}-dependent function. These values were similar in magnitude to those reported by Bonting and Carravagio for ATPase from several different tissues (15).

DISCUSSION

Coupling between ATPase and transport. Ouabain abolishes the net transport of sodium (4) and reduces the rate of oxygen consumption in the intact turtle bladder; and inhibits the $Na^+ + K^+$-dependent ATPase activity in the isolated microsomal pellet of this tissue. In other tissues, the binding of ouabain to microsomal protein is increased by sodium and decreased by potassium (24, 25). This suggests that the free energy of the $Na^+ + K^+$-dependent ATP hydrolysis is coupled to the sodium transport mechanism. Thus, ouabain may inhibit the reaction providing free energy for transport; or the mechanism coupling ATP hydrolysis to transport; or the pump-carrier operation in the membrane.

Estimates of the stoichiometry between sodium transport and ATP hydrolysis (15, 26), or between sodium transport and oxygen consumption (26) have been made without exact knowledge of the mechanism which couples sodium transport to the ATPase activity. Nevertheless, the sodium dependency of transport in the intact system is similar to the sodium dependency of the microsomal ATPase in red cells (27).

Despite the limitations of such kinetic similarities, we estimated the ratio of sodium transported to A TP hydrolyzed, assuming that the estimated cell concentration of ATP, 0.25-0.30 mM (28) is near that corresponding to the Km of the $Na^+ + K^+$ ATPase reaction.

The mean value for net transport of sodium is 7.5 μ Eq/mgm of microsomal protein/hr (3); and present data show that the $Na^+ + K^+$ ATPase-catalyzed rate of release of P_i is 10-20 μ moles/mgm of microsomal

protein/hr at the assumed ATP concentration of 0.25 mM. Therefore, 3.5 to 7.0 equivalents of sodium ions are transported for each mole of P_i released--a range similar to that found (0.7-15) in a wide variety of Na^+ transporting tissues (23).

By an analogous procedure, it was estimated that 15 equivalents of sodium ion are transported for each mole of the "sodium-dependent" consumption of oxygen--a value slightly less than Na^+/O_2 ratio of 16-20 estimated for frog skin (29) and toad bladder (30).

"Sodium-dependent" oxygen consumption is defined as the measured decrement in oxygen consumption after replacing the ambient sodium with choline or after adding ouabain, 10^{-4}M to the incubation mixture of whole respiring bladder wall or mucosal layer (LeFevre, M.E. and W.A. Brodsky--unpublished data). The estimated ratio of the $Na^+ + K^+$-dependent P_i release to "sodium-dependent" oxygen consumed was 2.0 to 4.3.

Even if ATP were the sole source of transport energy, the stoichiometry cannot provide data showing the nature of coupling between a spatially oriented chemical reaction in the membrane phase and a directionally oriented transport process.

Properties of bladder ATPase. The presently reported values of the pH optimum, of the Q_{10}, and of the Km's for ATP and Na^+, but not those of Km for potassium were similar to corresponding values reported elsewhere (11, 13, 14).

The double valued Km for potassium (shown in Figure 4 of this report) is consistent with the existence of two potassium binding sites on the

microsomal protein. One site has a high affinity and low capacity for K^+, while the other has a low affinity and a high capacity for K^+ binding. Although not the unique explanation, this picture is similar to a previous hypothesis of Skou based on a large mass of kinetic data (31).

The broad spectrum of the Mg^{++}-dependent nucleotide hydrolyses is in accord with similar data obtained from several microsomal sources (11, 13, 22, 23)--although the degree of hydrolysis of each nucleotide was not exactly the same in comparing any one tissue with another.

The spectrum of the $Na^+ + K^+$-dependent nucleotide hydrolyses was narrower than that of the Mg^{++}-dependent hydrolyses. Whereas our data on ITP and ATP in the turtle bladder resembled those of Skou in the crab nerve (21), they differed from those of others in beef kidney (22), calf (13), and bovine brain (23).

The discontinuity in the Arrhenius plot of Mg^{++}-dependent activity vs. reciprocal absolute temperature in the neighborhood corresponding to 23°C is consistent with several possibilities suggested for such phenomena by Dixon and Webb (32)--e.g. with two parallel or two successive reactions between enzyme and the same substrate; or with two forms of the enzyme, or with a reversible inactivation of the enzyme at certain temperatures. Moreover, the discontinuity in the Arrhenius plot of the Mg^{++} ATPase of the turtle bladder is similar to that in the plot of myosin ATPase (also a Mg^{++}-dependent ATPase) at 16° with ATP or ITP as substrate (33).

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FRACTION	VOL (ml)	ACTIVITY $Mg^{++} + K^+ + Na^+$ (units/ml)	ACTIVITY $Mg^{++} + K^+ + Na^+$ (total units)	PROTEIN CONC. (mg/ml)	SPECIFIC ACTIVITY A $Mg^{++} + Na^+ + K^+$ no ouabain (units/mg)	SPECIFIC ACTIVITY B $Mg^{++} + Na^+ + K^+$ ouabain (units/mg)	SPECIFIC ACTIVITY A - B $Na^+ + K^+$ (units/mg)
Mucosal Cells Homogenate	90	16.2	1457	1.87	8.7	6.2	2.5
10,000g Pellet	20	19.2	389	0.98	19.6	16.2	3.4
20,000g Pellet	21	17.0	358	0.50	34.1	19.8	14.3
65,000g Pellet	21	53.7	1128	0.50	107	42.5	64.5

Table 1. Distribution and recovery of ATPase activity and of total protein in the designated centrifugal fractions originated from a pool of isolated mucosal cells from 10 turtle bladders. Fractions were obtained by technique illustrated in Fig. 1. Exact composition of each incubation mixture and definition of Mg^{++} and $Na^+ + K^+$-dependent ATPase can be found under "Assay of ATPase Activity" of METHODS section. The "unit" of activity is defined as the amount of inorganic phosphate (P_i) in micromoles released into the incubation fluid per hour.

Statistical Parameters	Mg	Mg + Na	Mg + K	Mg + K + Na	Mg + K + Na + ouabain
	(micromoles Pi/mgm protein/hr.)				
Mean	32.0	31.9	31.7	73.2	32.2
± S. E.	2.16	1.20	1.87	6.49	1.96
P (Mg)	—	P > 0.8	P > 0.8	P < 0.001	P > 0.8
n	9	5	5	9	9

Table 2. Mean values for the ATPase activity of microsomal pellets incubated under the five cationic conditions designated. In all cases, the composition of the reaction mixture, expressed in terms of final millimolar concentration in the 5 ml of incubation fluid was: Tris-ATP, 2.0; $MgCl_2$, 3.0; EDTA, 0.2; imidazole, 40; histidine, 40; and final pH, 7.3. The cations were added to each reaction mixture as designated at the top of each column, and the final millimolar concentrations of the salts were: NaCl, 85; and KCl, 15; while that of ouabain was 0.1 mM. The conditions and concentrations specified for the last two columns on the right were employed throughout the remainder of the present work, except where otherwise specified.

Substrate 2.0 mM	SPECIFIC ACTIVITY (A) $Mg^{++} + Na^{+} + K^{+}$ + ouabain	(B) $Mg^{++} + Na^{+} + K^{+}$	(B) - (A)	Activity Ratio $\frac{(B) - (A)}{(A)}$
	(u moles Pi/mg. Protein/hr)			
ATP	27.5	49.1	21.6	0.79
ITP	16.3	29.3	13.0	0.80
CTP	9.6	16.5	6.9	0.72
GTP	25.0	27.5	2.5	0.10
ADP	4.5	4.8	0.3	0.07
UTP	8.0	8.1	0.1	—

Table 3. Nucleotide preference with respect to hydrolytic activity of the microsomal pellet. All hydrolytic activities were compared to that obtained in the presence of Tris-ATP, by substitution of equimolar amountf os the Na^{+} salts of the five other nucleotides. Conditions were otherwise identical to those described under METHODS and for previous tables.

Fig. 1. Semi-log plot of Mg^{++}-dependent ATPase activity, —○—○—; and Na^+, K^+-dependent ATPase activity, —●—●—, versus time. Na^+, K^+-dependent activity is defined as that measured in the presence of Mg^{++}, Na^+, and K^+ less that in the presence of Mg^{++}, Na^+, K^+ and ouabain.

Fig. 2. Lineweaver-Burk plot of Na^+, K^+-dependent ATPase activity, —●—●—, and Mg^{++}-dependent ATPase activity, —○—○—, versus molar concentration of ATP. Concentration of non-ATP constituents were the same as those described for Figure 1 in the Methods section.

Fig. 3. Lineweaver-Burk plot of Na^+, K^+-dependent ATPase activity versus molar concentration of sodium. Concentration of all non-sodium constituents as listed in Methods section.

Fig. 4. Lineweaver-Burk plot of Na^+, K^+-dependent ATPase activity versus molar concentration potassium. Concentration of all non-potassium constituents as listed in Methods section.

Fig. 5. ATPase activity versus pH. Na^+, K^+-dependent activity calculated as described for Fig. 2. The desired pH was obtained by addition of either HCl or NaOH to the reaction mixture, the composition of which has been described under Fig. 1 and in the Methods section.

Fig. 6. Semi-logarithmic plot of Na^+, K^+-dependent, —●—●—, and of Mg^{++}-dependent, —○—○— ATPase activity versus reciprocal absolute temperature.

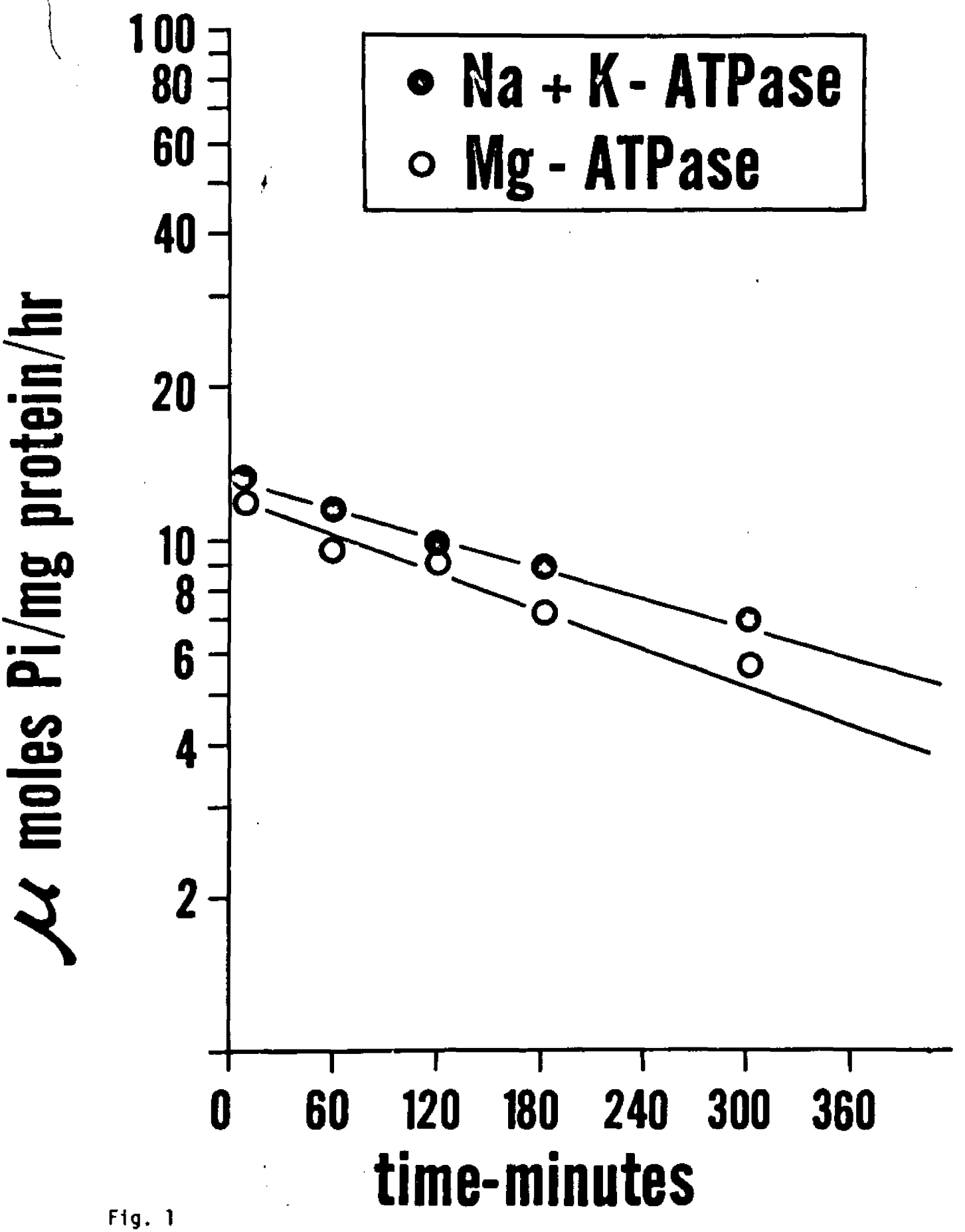
Na + K - ATPase
Mg - ATPase
μ moles Pi/mg protein/hr
100
80
60
40
20
10
8
6
4
2
0
60
120
180
240
300
360
time-minutes

Fig. 1

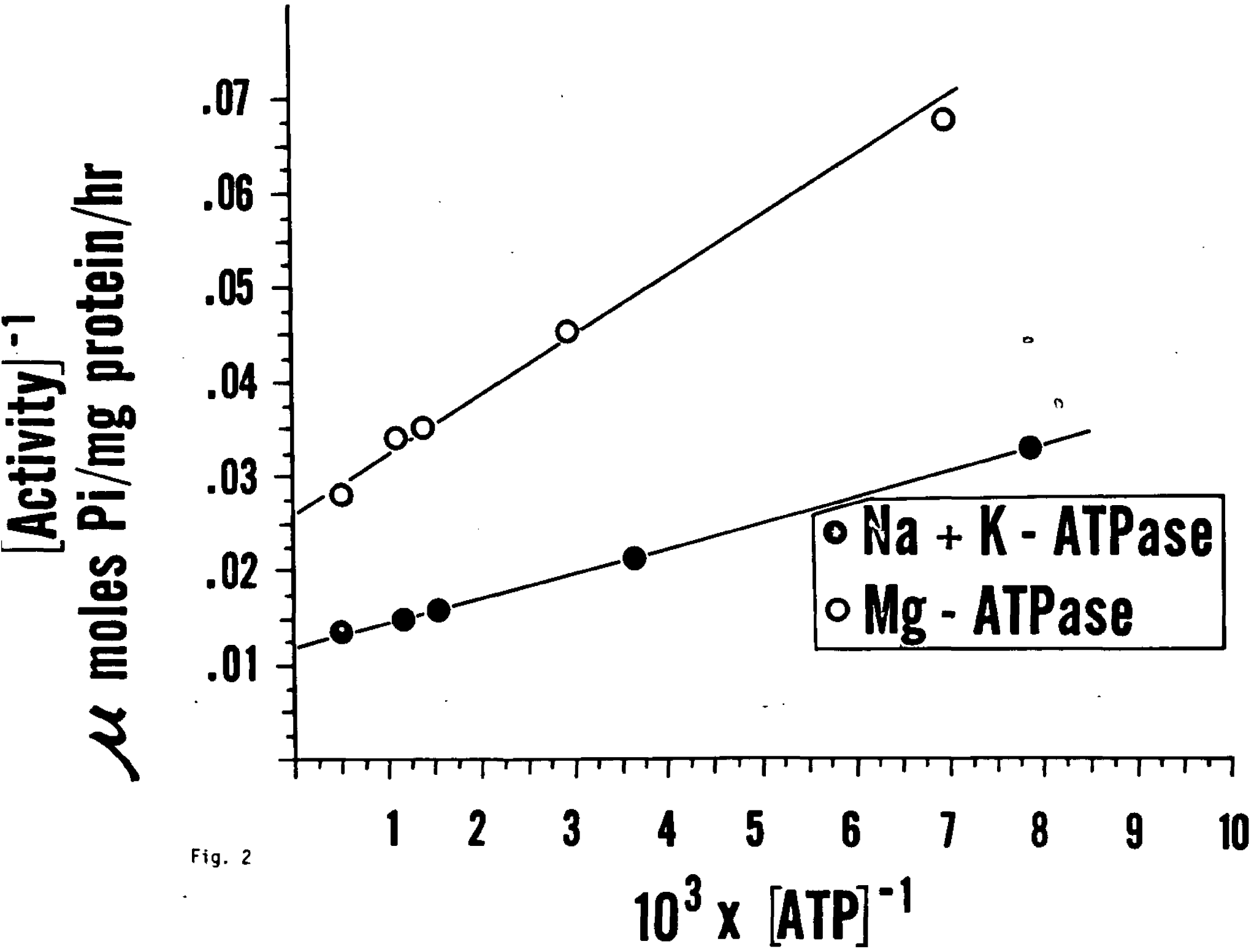
[Activity]$^{-1}$
μ moles Pi/mg protein/hr
.07
.06
.05
.04
.03
.02
.01
1
2
3
4
5
6
7
8
9
10
10^3 x [ATP]$^{-1}$
Na + K - ATPase
Mg - ATPase

Fig. 2

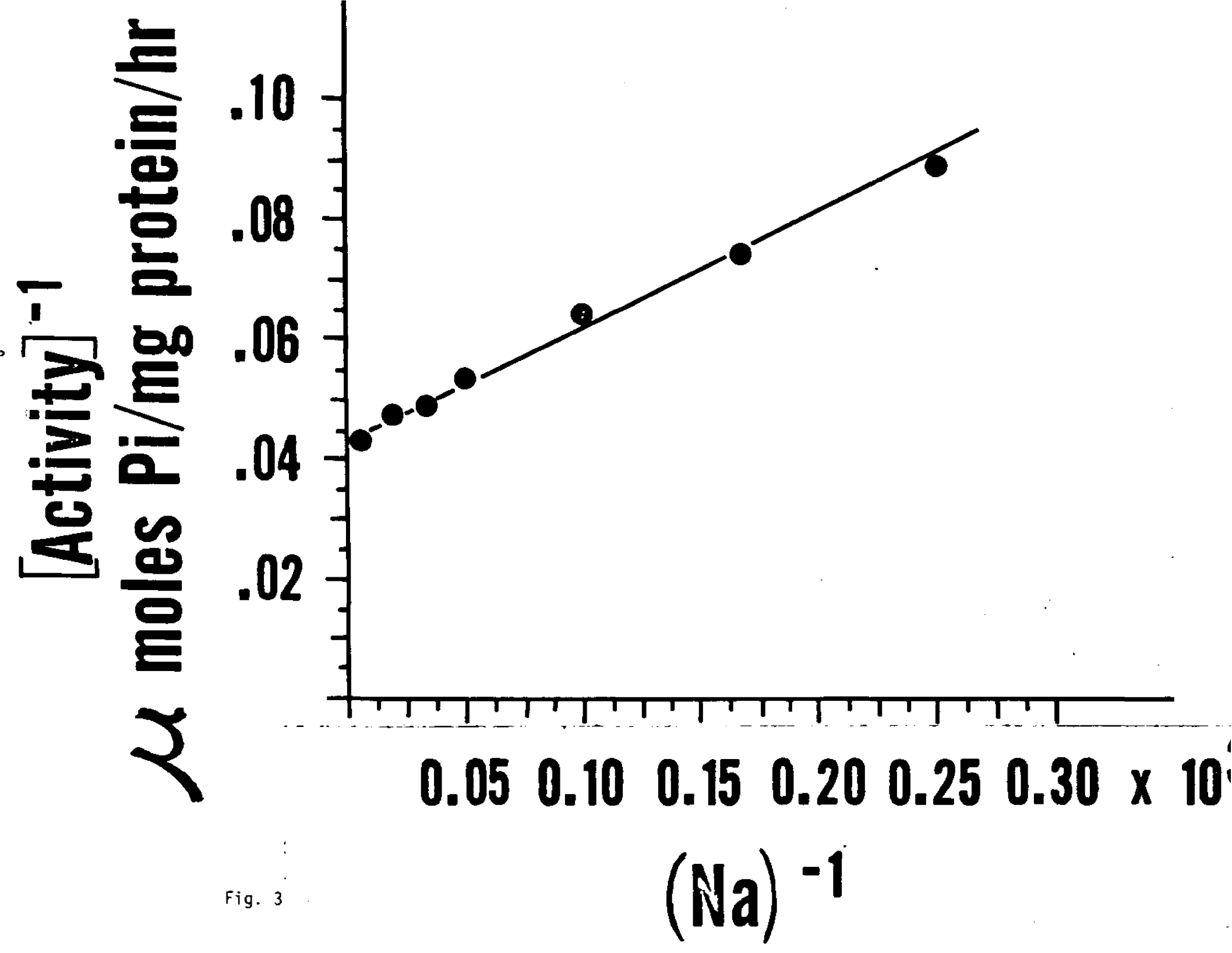
[Activity]-1
μ moles Pi/mg protein/hr
.10
.08
.06
.04
.02
0.05 0.10 0.15 0.20 0.25 0.30 x 10
(Na) -1

Fig. 3

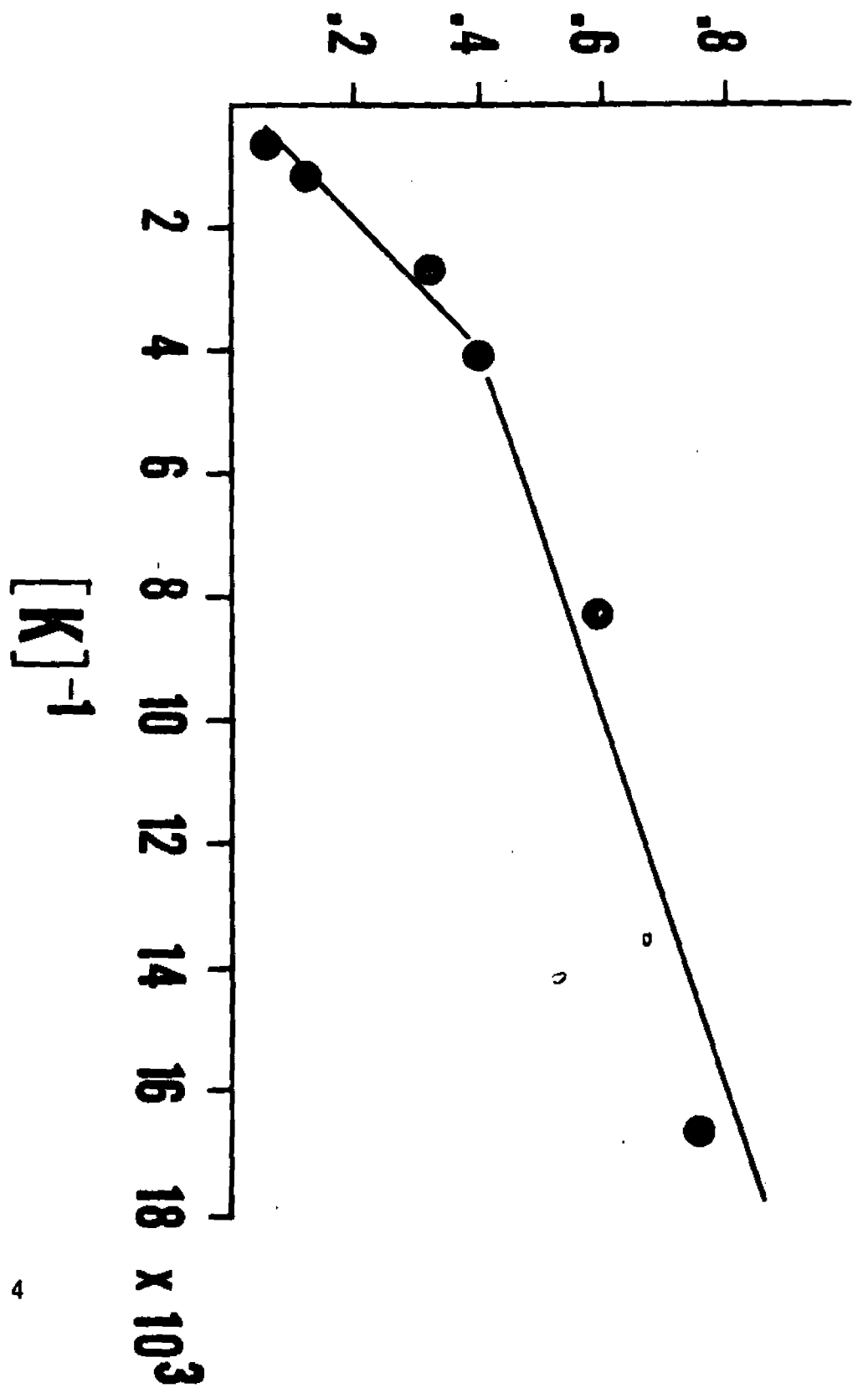
(Activity) -1
(μ moles Pi/mg protein/hr)-1
2
4
6
8
2
4
6
8
10
12
14
16
18
X 10³
[K]-1

Fig. 4

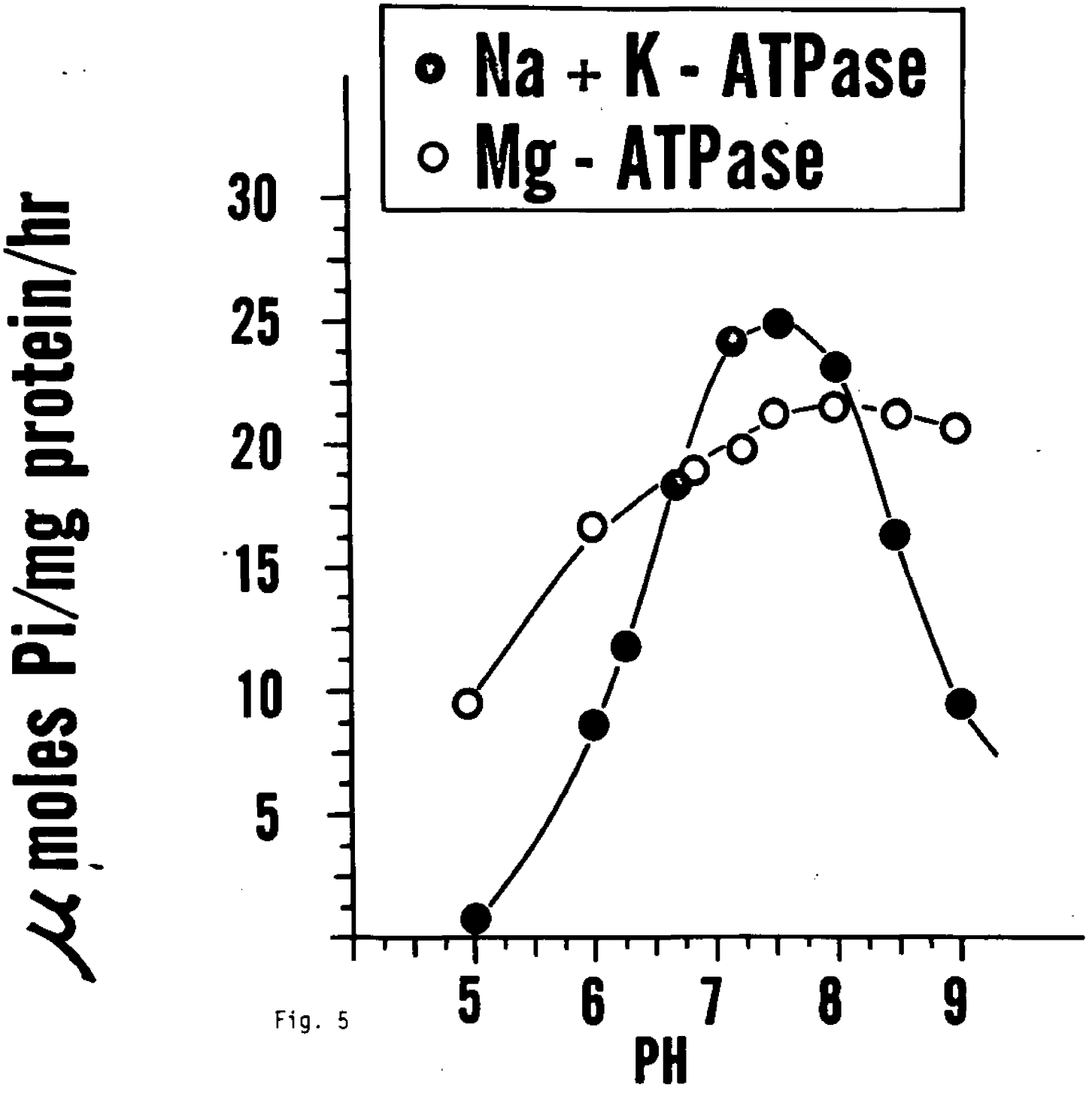
Na + K - ATPase
Mg - ATPase
μ moles Pi/mg protein/hr
30
25
20
15
10
5
5
6
7
8
9
PH

Fig. 5

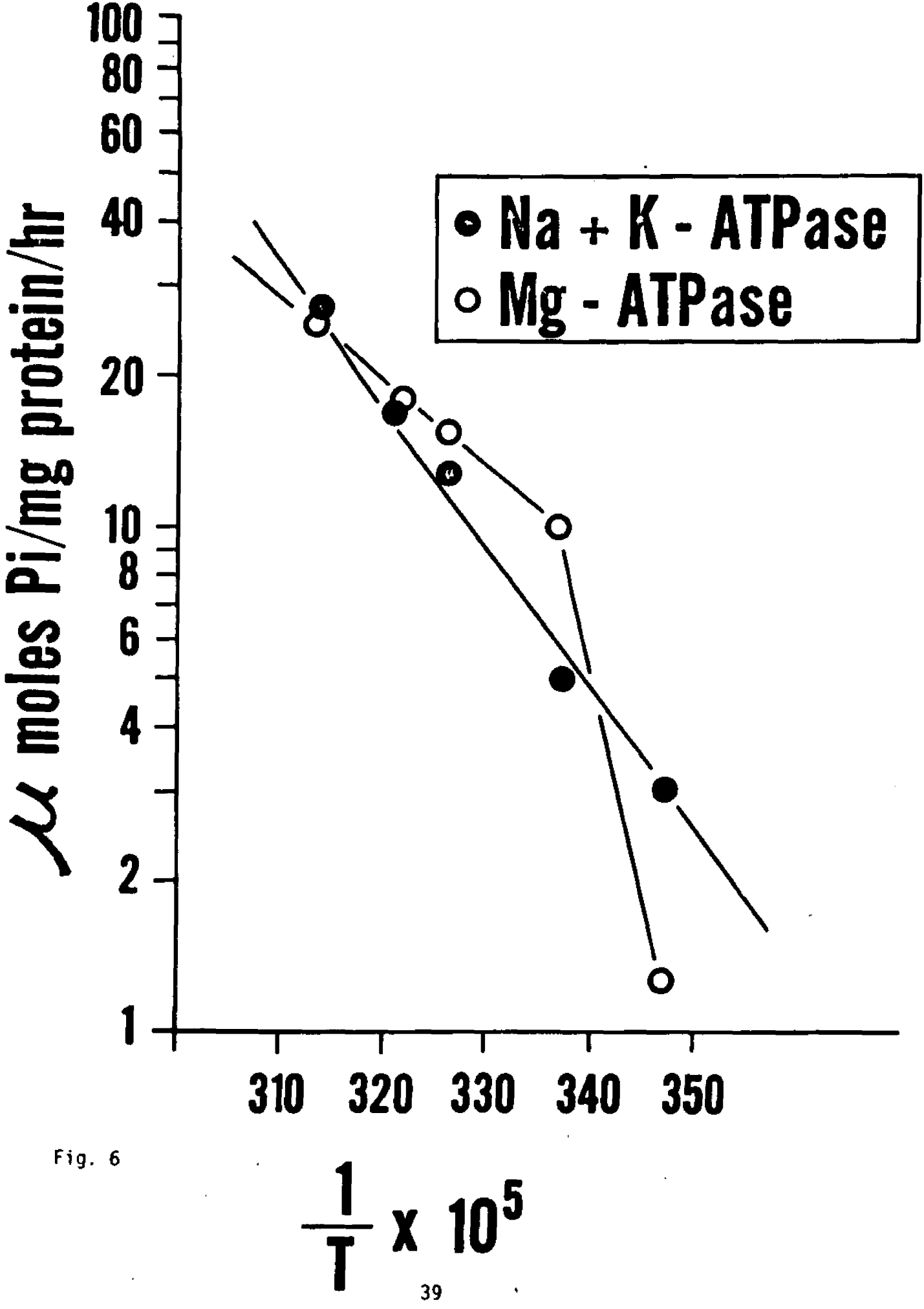
100
80
60
40
20
10
8
6
4
2
1
μ moles Pi/mg protein/hr
Na + K - ATPase
Mg - ATPase
310
320
330
340
350
1/T x 10^5

Fig. 6

P-NITROPHENYL-PHOSPHATASE ACTIVITY IN THE MICROSOMAL FRACTION OF TURTLE BLADDER MUCOSAL CELLS

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SUMMARY

The microsomes of turtle bladder epithelium contain a potassium-stimulatable, ouabain-and/or sodium-inhibitable p-nitrophenylphosphatase (p-NPPase) with an absolute requirement for Mg^{++}. The optimal pH range was 7.2 - 7.3 in the imidazole-histidine buffer system used. Treating the data in terms of Michaelis-Menten kinetics, the apparent Km's were as follows: for p-NPP, 0.6 mM; for Mg^{++}, 0.6 mM; and for K^+, 1.0. Substrate inhibition was observed for p-NPP, Mg^{++}, and K^+ at concentrations exceeding 4.0, 4.0, and 10 mM respectively. Ouabain, 10^{-4}M or Na^+, 100 mM completely inhibited the p-NPPase activity, and each inhibitor reacted competitively with potassium for enzyme sites (K_I for ouabain = 2.5 x 10^{-8}M and K_I for sodium = 3.0 mM). The inhibitory action of NEM was dependent upon the time of pre-incubation of the microsomes with NEM. Related to the question of whether p-NPPase is the same as or distinct from $Na^+ + K^+$-ATPase, is the fact that p-NPPase activity is inhibited competitively in the presence of ATP.

INTRODUCTION

Microsomal preparations from many tissues have a $Na^+ + K^+$-dependent ATPase (1-4), which has been related to the transport of Na^+ and K^+ across the cell membrane on the basis of evidence recently reviewed by Skou 1965 (5), Heinz 1967 (6) and Albers 1967 (7). Microsomes also contain other enzymatic activities including a K^+-stimulated acetyl phosphatase and a K^+-stimulated p-nitrophenyl phosphatase (p-NPPase). Acetyl phosphatase has been found in kidney and brain microsomes (8-9), and p-NPPase has been found in the electric organ of eels (10), red blood cells (11) and white blood cells (12).

In all cases reported (8-12), both acetyl-phosphatase and p-nitrophenylphosphatase were inhibited by ouabain and sodium. Whereas the relationship between either of the two phosphatases and transport is not yet clarified, Woodin has claimed that the K^+-dependent p-NPPase may be related to K^+ transport in the white blood cells (12).

The purposes of the present paper were: to investigate the K^+-dependent p-NPPase in the microsomal fraction isolated from mucosal cells of turtle bladder; to evaluate the kinetic parameters such as K_m, K_i and V_m of p-NPPase activity with respect to the concentration of p-NPP and with respect to the concentrations of Mg^{++} and K^+; to determine the effect of pH; and to determine the effect of various inhibitors such as sodium, n-ethyl-maleimide (NEM), ATP, and sodium azide.

METHODS

The procedures for isolating mucosal cells from freshly excised turtle bladder together with those for the ultracentrifugation, fractionation, and isolation of the microsomal pellet have been described in detail previously (1). Protein concentration of the microsomes, determined by the method of Lowry et al (13), was used as the normalizing parameter for enzyme activity.

The composition of reaction mixture, expressed in terms of final millimolar concentration was: the di-sodium salt of p-NPP, 4.0 (only in case of Figure 1); or usually the imidazole-histidine salt of p-NPP, 4.0; $MgCl_2$, 4.0; imidazole, 20; histidine, 20; KCl, 10 mM. The final pH was 7.3, and an enzyme aliquot of 0.5 ml containing 150-300 µg of protein was added to the incubation mixture, the total volume of which was 5 ml. The final concentrations of imidazole and histidine were increased from 20 to 30 mM by the addition of substrate, the imidazole-histidine salt of p-NPP. The final concentrations of imidazole and histidine were also 30 mM when the disodium salt of p-NPP was used as substrate.

After 5 minutes of pre-incubation, the reaction was started by addition of p-NPP, and incubated at 38°C for 20-30 minutes. The reaction was stopped by addition of 5 ml of cold perchloric acid 6%. Control tubes, carried through all incubations, were of two types--those without p-NPP and those without the enzyme. The non-catalytic rate of hydrolysis of p-NPP was zero, but the lot of p-NPP used

contained inorganic phosphate amounting 2-3% of the concentration of p-NPP. Aliquots of the final mixture were analyzed routinely for inorganic phosphate by the method of Berenblum and Chain (14).

The initial reaction velocity was determined by measuring the increment of inorganic phosphorous (P_i) released into the incubation flask after 30 minutes of incubation of the appropriate amount of microsomes with p-NPP. For an initial concentration of p-NPP of 4 mM, no more than 15% of total p-NPP was hydrolyzed during the first 30 minutes of incubation. The amount of P_i released was proportional to the time of incubation for as long as 40 minutes of incubation at 38°C. For a given batch of microsomes at a given time, the rate of P_i release was directly proportional to the amount of protein (150 to 300 μgm) in each incubation flask. After storage of the microsomes at -20°C, the p-NPPase activity decreased with time. The half-life of p-NPPase, estimated from the time-dependent logarithmic decrease of the activity, was 18-19 days.

During the experiments on the ATP-induced inhibition of p-NPPase, the p-nitrophenol released (instead of the P_i) was measured spectrophotometrically in order to insure that the hydrolytic product originated solely from p-NPP, and not from the added ATP. The spectrophotometric measurements were made at 25°C. in a Beckman DU monochromator fitted with a Gilford Model 220 Absorbance Indicator and a Varian Model G-14 strip chart recorder. The concentrations of the reactants were the same as in the phosphate experiments. The substrate was added, mixed, and the change in absorbance measured

at 348 mu (15), the isosbestic point for p-nitrophenol and p-nitrophenolate ion, where $\epsilon = 5.4 \times 10^3\ M^{-1}cm^{-1}$ for both species. The reaction was linear, and the initial slope was taken for the calculation of the enzymatic rate.

Preparation of the imidazole-histidine salt of p-nitrophenyl phosphate. One gm of the disodium salt of p-NPP (Sigma) was dissolved in 20-30 ml of water and added gradually to a 10 cm column of Dowex 50 x 8 (H^+ form). Then the column was washed three times with 10 ml aliquots of water. Flame photometric measurements indicated that passage through the column replaced more than 99% of the sodium with hydrogen ions. The entire effluent (pH, 2.0) was collected, and its pH adjusted to 7.3 by the addition of a mixture containing equimolar amounts of imidazole and histidine-OH.

RESULTS

Table 1 presents values on mean rates of hydrolysis of p-nitrophenyl phosphate (p-NPP) in four experiments on microsomes under the conditions denoted in the first column.

Under all conditions except condition d, small increments of P_i (over and above the non-enzymatic blank) were released into the incubation fluid. Apart from the significance of the small, but detectable releases of P_i, the largest of the small increments (condition e) was but 3% of the amount of P_i released, 12.2 μmoles/mg/hour, in the presence of Mg^{++} + K^+ (condition d).

The activity of the Mg^{++} + K^+-dependent, ouabain-sensitive p-nitrophenyl phosphatase (hereafter called either K^+-p-NPPase or p-NPPase) of the turtle bladder microsomes, was considerably greater than that found in the microsomes of other tissues (10-12). Not shown in Table 1 are similar results from 16 other experiments on freshly prepared bladder microsomes where the mean value ± se for K^+-dependent p-NPPase was 10.1 ± 0.7 μmoles/mgm prot./hr. The mean values for the Mg^{++} and Na^+ + K^+-dependent ATPase, concomitantly determined in two of the four experiments of Table 1, were 26.9, 40.8 μmoles/mg/hr. respectively. The next set of experiments was designed to determine the kinetic parameters of the K^+-dependent, ouabain-sensitive microsomal p-NPPase.

Kinetic parameters

Figure 1, a Lineweaver-Burk plot of values of reciprocal activity in arbitrary units versus reciprocal concentration of p-NPP, was

obtained from a set of incubation mixtures in which the concentrations of microsomes, Mg^{++} and K^+, were fixed while those of p-NPP varied from 0.4 to 15 mM. Treating the data as if they followed simple Michaelis-Menten kinetics, the apparent K_m (K_s) (between the limits of concentration of 0 and 4 mM p-NPP) was 0.6 mM; and the V_{max} between the same concentration limits was 8.3 μmoles/mgm/hr. The plot also indicates a pronounced substrate inhibition for concentrations of p-NPP in excess of 4 mM. Not shown is a plot of the same data in the form of 1/V versus substrate concentration from which the apparent K_I (or K_{SS}) for p-NPP was estimated to be 16 mM.

Figure 2, a plot of values of reciprocal activity in arbitrary units versus reciprocal Mg^{++} concentration, was obtained from a set of incubation mixtures in which the concentration of microsomes, potassium and p-NPP were fixed, while those of Mg^{++} varied from 0.4 to 25 mM. Between the limits of Mg^{++} concentration of 0 to 4 mM, the apparent K_m was 0.6 mM and the V_{max} was 7.2 μmoles/mg/hr. Pronounced substrate (Mg^{++}) inhibition was observed for Mg^{++} concentrations in excess of 4 mM and the apparent K_I for Mg^{++} was 16 mM.

On the basis of data in Figures 1 and 2, the optimal activity in 10 mM K^+ occurs when the concentration ratio $[Mg^{++}]/[p\text{-NPP}] = 1.0$. This is similar to the $[Mg^{++}]/[ATP]$ ratio required for optimal activity of the Mg^{++} and $Na^+ + K^+$-dependent ATPase activity in several tissues (4, 5, 7).

Figure 3, a plot of values of reciprocal activity in arbitrary

units versus reciprocal concentration of potassium, was obtained from a set of incubation mixtures in which the concentrations of microsomes, magnesium, and p-NPP were kept constant while those of potassium varied from 0.5 to 100 mM.

Between the limits of potassium concentration of 0.5 to 10 mM, the apparent K_m was 1 mM and the V_{max} was 14.1 μmoles/mg/hr. Substrate (potassium) inhibition was observed for potassium concentrations in excess of 10 mM, and the apparent K_I for potassium was 100 mM.

Since the addition of 100 mM Na^+ resulted in nearly complete inhibition of the K^+-p-NPPase (see Table 1), we decided to examine the effect of lower concentrations of sodium upon the kinetic pattern of the K^+-dependent p-NPPase illustrated in Figure 3. Therefore, the p-NPPase activity versus potassium concentration in the presence of a fixed concentration of sodium was determined for a family of such sodium concentrations--e.g. in the presence of 0, 5, 10, 20 and 50 mM. The potassium concentration was kept below 10 mM to avoid the inhibitory effect of potassium itself.

Figure 4 shows five Lineweaver Burk plots of values of reciprocal activity in arbitrary units versus reciprocal potassium concentration in the presence of no sodium (the lowermost line which is in the same activity range as that of Figure 3), and in the presence of 5, 10, 20, and 50 mM sodium. Data shown were taken from two consecutive experiments, one covering Na^+ concentrations of 0, 5 and 10 mM; and the other covering Na^+ concentrations of 0, 20, and

50 mM. The line for 100 mM Na^+, not shown, is co-linear with the ordinate.

The five plots shown in the figure indicate that the higher the Na^+ concentration, the lower the p-NPPase activity over the range of potassium concentrations used (1 to 10 mM); and that the V_{max} for all five concentrations of Na^+ was 10.4 μmoles/mg/hr., which suggests that sodium competes with potassium for the K^+-activation sites on the microsomal protein. The K_I for sodium, 3 mM, was estimated graphically from the extrapolation of the plot of apparent Km for potassium versus sodium concentration. This suggests that the affinity of the p-NPPase-related protein for sodium was ca. one-third of that for potassium (Km for potassium = 1.0 mM; see Figure 3).

Effect of pH

The effect of pH (over the range 5 to 8.5) on p-NPPase activity was determined in the presence of 8 mM Na^+ and 20 mM K^+. The concentration of K^+ used, 20 mM, was twice that of the other experiments on Mg^{++} + K^+-dependent p-NPPase. This K^+ concentration was found sufficient to eliminate the inhibitory effect of the 8 mM Na^+ contained in the disodium salt of p-NPP; and provided a convenient means of varying pH (by substitution of equimolar amounts of KOH for KCl) without changing the final concentrations of K^+, imidazole, histidine, Mg^{++}, Na^+ or p-NPP.

Figure 5 is a plot of values of p-NPPase activity versus final

pH of the incubation mixture in one of three parallel experiments on microsomes. It can be seen that a pH of 7.2-7.4 was required for optimal activity under the conditions used. At pH 5, the activity was 38% of that at 7.3; and at pH 8.5 the activity 52% of that at 7.3. Data from the two other experiments, not shown in figure, fell about a closely similar pattern.

Effect of ouabain

The next set of experiments was designed to examine the dependence of ouabain inhibition upon the concentration of potassium in the incubation mixture.

The microsomal pellet in the presence of Mg^{++}, K^{+}, appropriate buffer, and ouabain was pre-incubated at 38°C for five minutes before starting the reaction by addition of p-NPP. Increasing the time of the aforementioned pre-incubation from 5 to 30 minutes did not significantly change the degree of ouabain-induced inhibition.

Figure 6 presents a plot of normalized values of p-NPPase activity versus the logarithm of ouabain concentration for a family of three different potassium concentrations. Data presented were taken from one of two identically designed experimental sets. In each set, the potassium concentrations of the incubation mixtures were fixed at the three levels (1, 10 and 25 mM) denoted in the figure.

For each of the three concentrations of K^{+} used, the plots of percent activity vs. log ouabain concentration were linear over the

range of ouabain concentrations denoted along the abscissa. The linear nature of the three logarithmic functions indicates that the greater the potassium concentration (from 1 to 25 mM) the greater the concentration of ouabain required for any degree of inhibition between the limits of 10% to 90% of the control level. This pattern resembled that expected of a competitive inhibition between potassium and ouabain. As anticipated, the plot of 1/activity versus 1/potassium concentration (for each of the ouabain concentrations used) generated a set of straight lines intersecting at a common value on the 1/activity ordinate, where V_{max} for potassium was approximately the same as that in Figures 2 and 3; and where the graphic extrapolation of the plot of Km for potassium vs. ouabain concentration indicated that the K_I for ouabain was 2.5×10^{-8}M. Nearly complete inhibition of K^+-p-NPPase was achieved in the presence of 10^{-4}M ouabain, no matter what potassium concentration was used.

Effect of N-Ethylmaleimide (NEM)

The addition of NEM is known to inhibit $Na^+ + K^+$-ATPase, K^+-stimulated acetylphosphatase, and K^+-stimulated p-NPPase from various tissue sources (2, 7, 8). Moreover, the degree of inhibition was partly dependent upon the time of pre-incubation of microsomes with the inhibitor.

Current experiments on NEM in the turtle bladder microsomes were first focused on the relation between the time of pre-incubation of the microsomes with NEM and the subsequent degree of inhibition

of p-NPPase activity. Two sets of experiments, each involving a control and four concentrations of NEM, were performed. For each level of NEM, the time of exposure of enzyme to NEM, Mg^{++}, K^{+} and buffer, (before starting the reaction with addition of p-NPP) was 5, 20 and 30 minutes. In each case, the same times of pre-incubation and incubation were applied to the three control mixtures which contained eznyme and all of the aforementioned constituents except NEM.

Figure 7 presents a plot of values of percent activity versus concentration of NEM for a family of three pre-incubation periods (5, 20 and 30 minutes) in one of the two sets of aforementioned experiments.

It can be seen that the degree of inhibition of the K^{+}-dependent p-NPPase activity increased with increasing time of pre-incubation of the enzyme with the inhibitor over the entire range of concentrations ($1x10^{-5}$ to $5x10^{-3}M$) of NEM used. For a concentration of inhibitor of $10^{-3}M$, the inhibition was 25% after five minutes, 52% after 20 minutes, and 73% after 30 minutes of pre-incubation. The concentrations of NEM required to induce 50% inhibition of activity were: $>5x10^{-3}M$ for 5 minutes of pre-incubation; $1.25x10^{-4}M$ for 20 minutes; and $3.8x10^{-4}M$ for 30 minutes of pre-incubation.

Of interest are previously reported data (1) showing that 45% inhibition of the Na^{+} + K^{+}-ATPase was obtained with $10^{-4}M$ NEM after

30 minutes of pre-incubation of enzyme with inhibitor--a result quite similar to that shown (40%) for inhibition of the K^+-dependent p-NPPase with the same amount of inhibitor and the same time of pre-incubation (see the lower of figure 7).

Of further interest is the fact, reported previously from this laboratory (1), that NEM (final concentration, 10^{-4}M) reduced the short-circuiting current of the turtle bladder to zero 30 minutes after its addition to the bathing media.

Effect of ATP

Because of certain similarities between the reactions of the inhibitors, ouabain and NEM, on K^+-dependent p-NPPase and $Na^+ + K^+$-ATPase, it was decided to measure the activity of the former (p-NPPase) in the presence of ATP. In each of two experimental sets, p-NPPase activity was measured in the absence and in the presence of two concentrations of ATP. The p-NPPase activity in the presence of ATP was assayed by measuring the p-nitro phenol released as described in the Methods section.

Figure 8, a plot of values of reciprocal activity in arbitrary units versus reciprocal p-NPP concentration, was obtained from a set of incubation mixtures in a sodium-free medium in which the concentrations of microsomes, potassium, and magnesium were fixed for two levels of ATP (1.7×10^{-4}M and 1.2×10^{-3}M) while the concentration of p-NPP varied from 0.33 to 6 mM. The three lines generated

between these limits of substrate concentration intersected at the same point on the ordinate ($1/V_{max}$), suggesting that ATP competes with p-NPP for occupation sites on the enzyme. The value of V_{max} shown here was obtained at 25°C., and consequently was smaller than that estimated in other experiments at 38°C. (see figure 1).

Effect of sodium azide

Addition of sodium azide to a final concentration range of 10^{-5} to 10^{-3}M had no detectable effect on the K^+-dependent p-NPPase, even after 30 minutes of pre-incubation.

DISCUSSION

Some of the properties of K^+-dependent p-NPPase reported herein are analogous in certain respects to those described by us (1) and others (2, 5) on certain properties of $Na^+ + K^+$-ATPase.

In the case of the turtle bladder microsomes, the pH, the Mg^{++}/substrate ratio, the Mg^{++} and K^+ concentrations required for optimal activity of either p-NPPase or ATPase were about the same (1). At the optimal potassium concentration, the range of ouabain needed for 50% inhibition of either activity, K^+-p-NPPase or Na^++K^+-ATPase, was 3-5 x 10^{-7}M--a sensitivity to ouabain of 10 to 100-fold greater than that reported for 50% inhibition of either microsomal activity in other tissues (8) (19) (17). The ouabain sensitivity of acetyl phosphatase was only 20% less than that of Na^++K^+-ATPase in guinea pig kidney and brain (9), but was ca. 10-fold greater than that of Na^++K^+-ATPase in beef brain (8).

Other similarities between the two activities can be made with respect to the effects of NEM and the competition between ATP and p-NPP. Thus, pre-incubation of microsomes with 10^{-4}M NEM for 30 minutes resulted in a 40-45% inhibition of either Na^++K^+-ATPase or K^+-p-NPPase; and ATP appeared to compete with p-NPP for occupation sites on the protein (see Figure 8).

The main difference in the cationic effect on the two enzyme activities was that of sodium which stimulated the activity of the Na^++K^+-ATPase, but which inhibited the activity of K^+-p-NPPase.

This qualitative difference suggests that the hydrolysis of ATP and p-NPP is catalyzed via two mechanisms despite all of the aforementioned similarities. However, data reported here and elsewhere are not sufficient to determine whether each substrate, ATP or p-NPP, binds with a common site on the enzyme protein or even whether such binding entails two sites on the same protein, two sub-unit sites on a polymeric protein or two independent protein units. All possibilities can be made consistent with any of the available data. The solution of the problem will probably require the isolation, solubilization, and characterization of one or more pure enzymatically active proteins.

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Acknowledgment is gratefully accorded to Carol Faye Sorokin for her expert technical assistance.

Additions	p-NPPase Activity µmoles Pi/mg protein/hr
a - Buffer along	0.15 ± 0.08
b - Buffer + Mg	0.37 ± 0.20
c - Buffer + K	0.20 ± 0.10
d - Buffer + Mg + K	12.2 ± 0.84
e - Buffer + Mg + K + Na	0.40 ± 0.25
f - Buffer + Mg + K + ouabain	0.45 ± 0.15

Table 1. Effect of cations and ouabain on p-NPPase activity of microsomes. Each value represents the mean ± SE in four experiments. Final concentrations of additions in incubation mixtures were: imidazole, 20 mM; histidine, 20 mM; Mg, 4.0 mM; K, 10 mM; Na, 100 mM; and ouabain, 10^{-4}M. In all cases, final concentration of the imidazole-histidine salt of pNPP was 4.0 mM and the final pH was 7.3 (see methods section).

FIGURES

Fig. 1. Lineweaver-Burk plot of reciprocal p-NPPase activity in arbitrary units versus reciprocal millimolar concentration of p-NPP. The concentrations of buffer, Mg^{++}, K^{+} and the pH were the same as those described in Table 1 and in the Methods section.

(Activity)$^{-1}$ is in arbitrary units, taken from raw optical densities prior to correcting for dilution, aliquot, time of incubation and protein concentration, all of which were constant in any single set of experiments.

Fig. 2. Lineweaver-Burk plot of reciprocal p-NPPase activity in arbitrary units versus reciprocal millimolar concentration of Mg^{++}. Other than the Mg^{++}, the concentrations of the constituents were the same as those described in Table 1 and in the Methods section.

Fig. 3. Lineweaver-Burk plot of reciprocal p-NPPase activity in arbitrary units versus reciprocal millimolar concentration of potassium. Other than that of potassium, the concentrations of the constituents were the same as those described in Table 1 and in the Methods section.

Fig. 4. Lineweaver-Burk plot of reciprocal p-NPPase activity versus reciprocal millimolar concentration of potassium for a family of Na^{+} concentrations. *—* Na^{+} = 0; 0—0 Na^{+} = 5 mM; ■—■ Na^{+} = 10 mM; □—□ Na^{+} = 20 mM; 0—0 Na^{+} = 50 mM.

Fig. 5. p-NPPase activity versus pH of the incubation mixture. The concentrations of cations were: Mg^{++}, 4; K^{+}, 20; Na^{+}, 8; and p-NPP, 4 mM.

Fig. 6. Semi-log plot of percentage activity of p-NPPase versus concentration of ouabain for three levels of potassium concentration--1, 10, and 25 mM. Other than ouabain and potassium, the concentrations of the constituents in the incubation mixtures were the same as those described in Table 1 and in the Methods section.

Fig. 7. Percentage activity of p-NPPase versus concentration of NEM. Pre-incubation times are shown within the parentheses over each curve.

Fig. 8. Lineweaver-Burk plot of reciprocal p-NPPase activity in arbitrary units versus reciprocal millimolar concentration of p-NPP in the absence and in the presence of ATP, the concentration of which is indicated next to each line.

NOTE

Figures 1--8 are for the benefit of the reviewer and are not intended for publication. Similar figures compatible with the brochure of instructions for BBA were also submitted with this manuscript for publication.

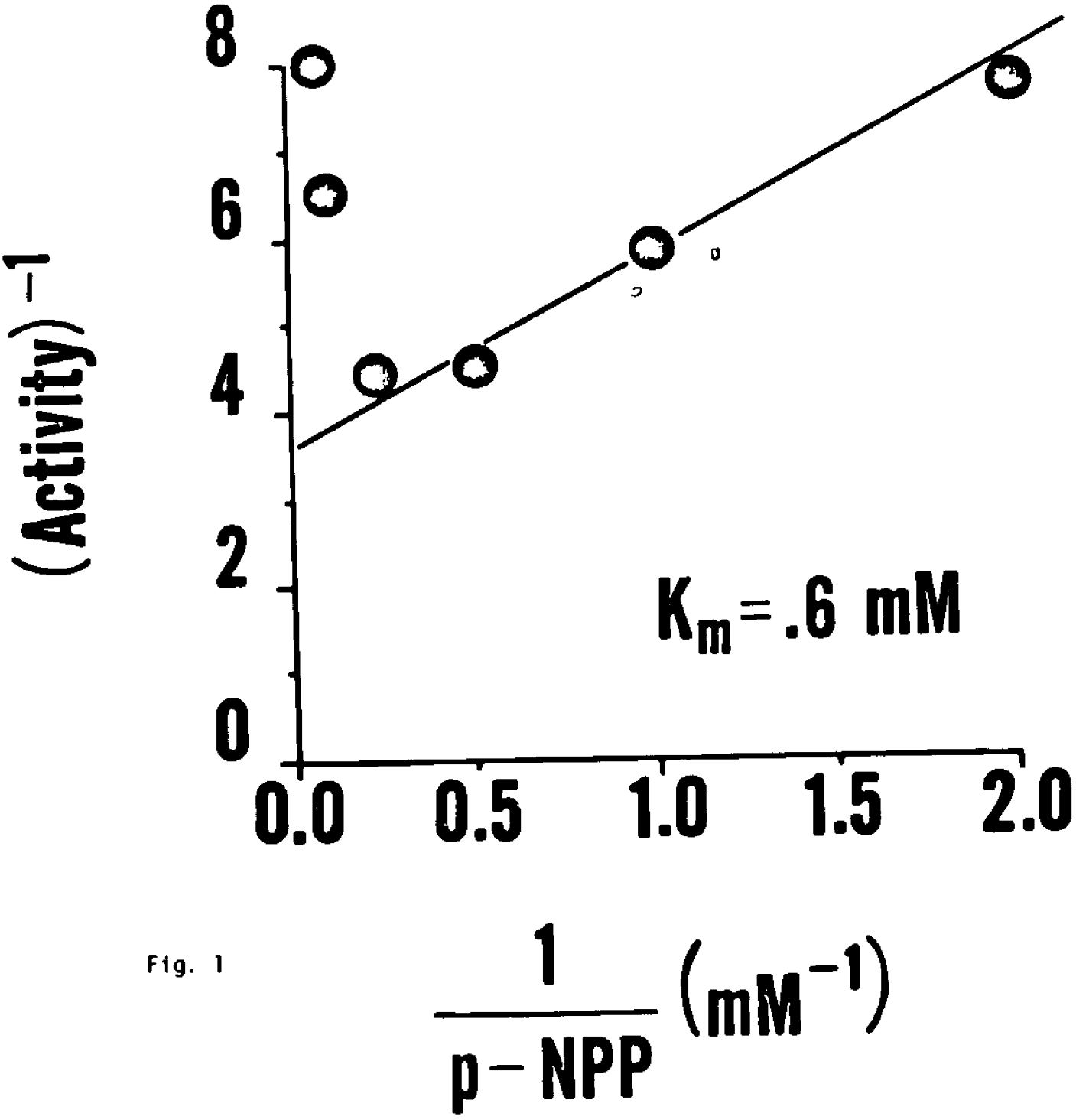
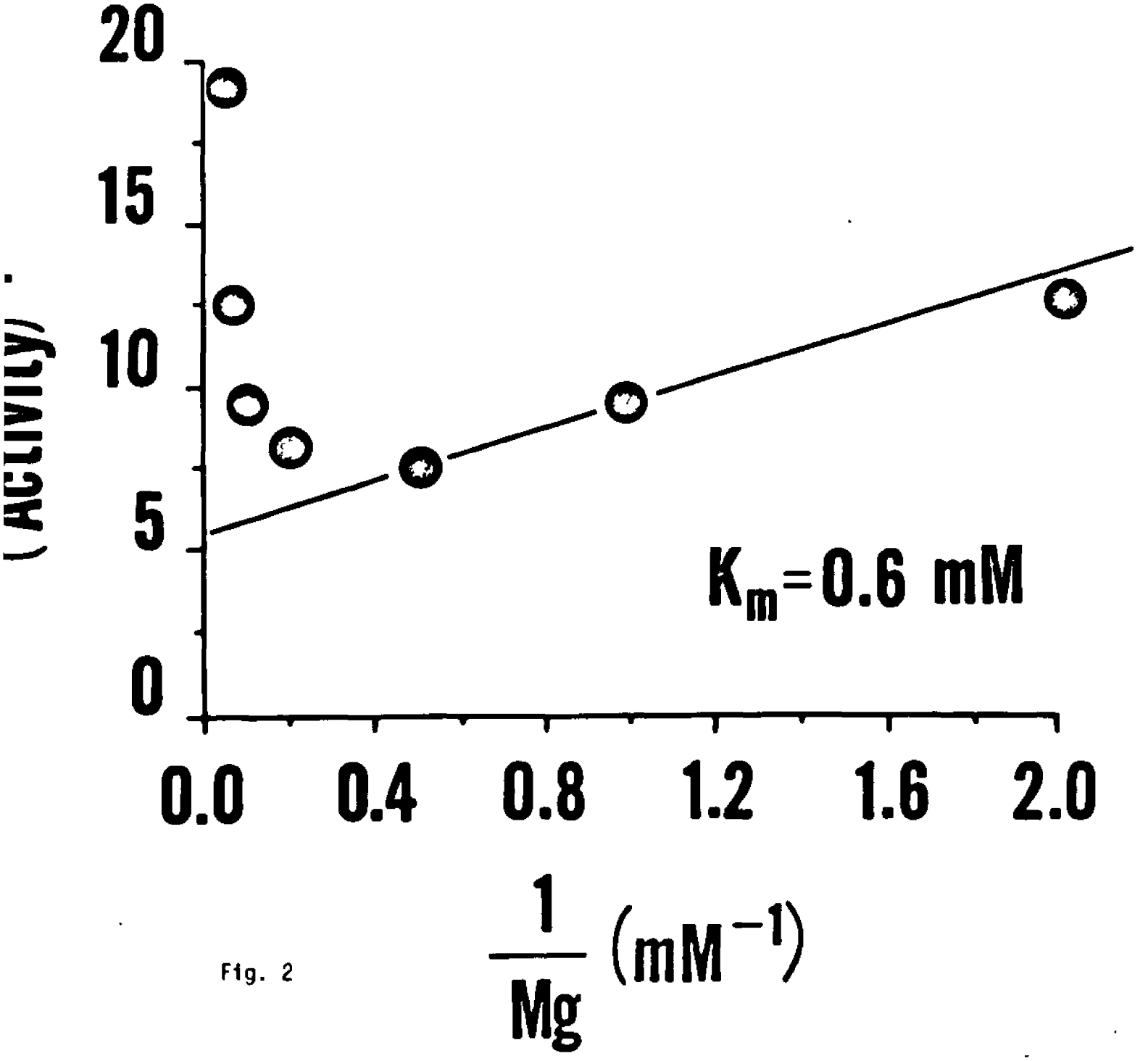


Fig. 1



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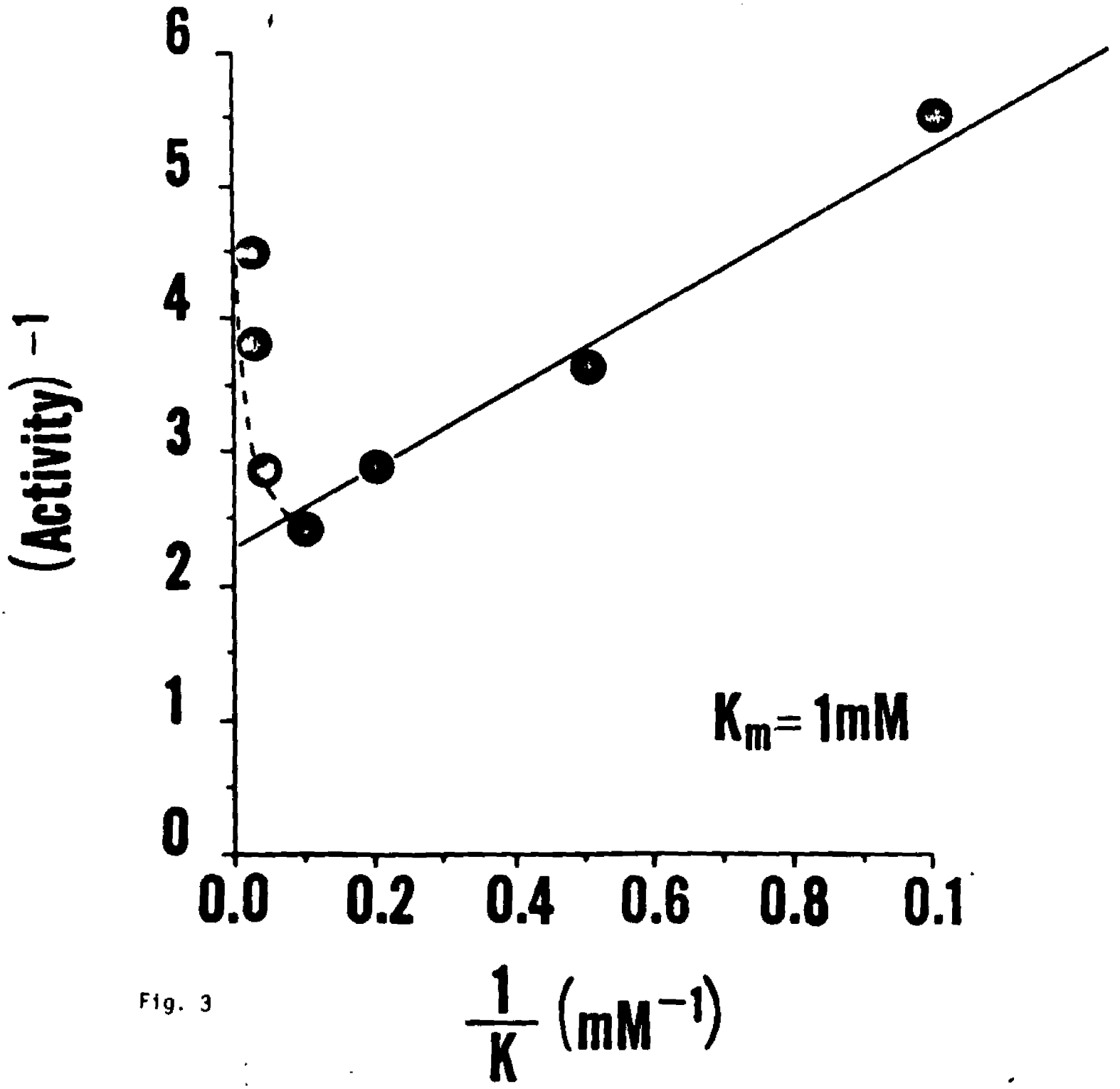

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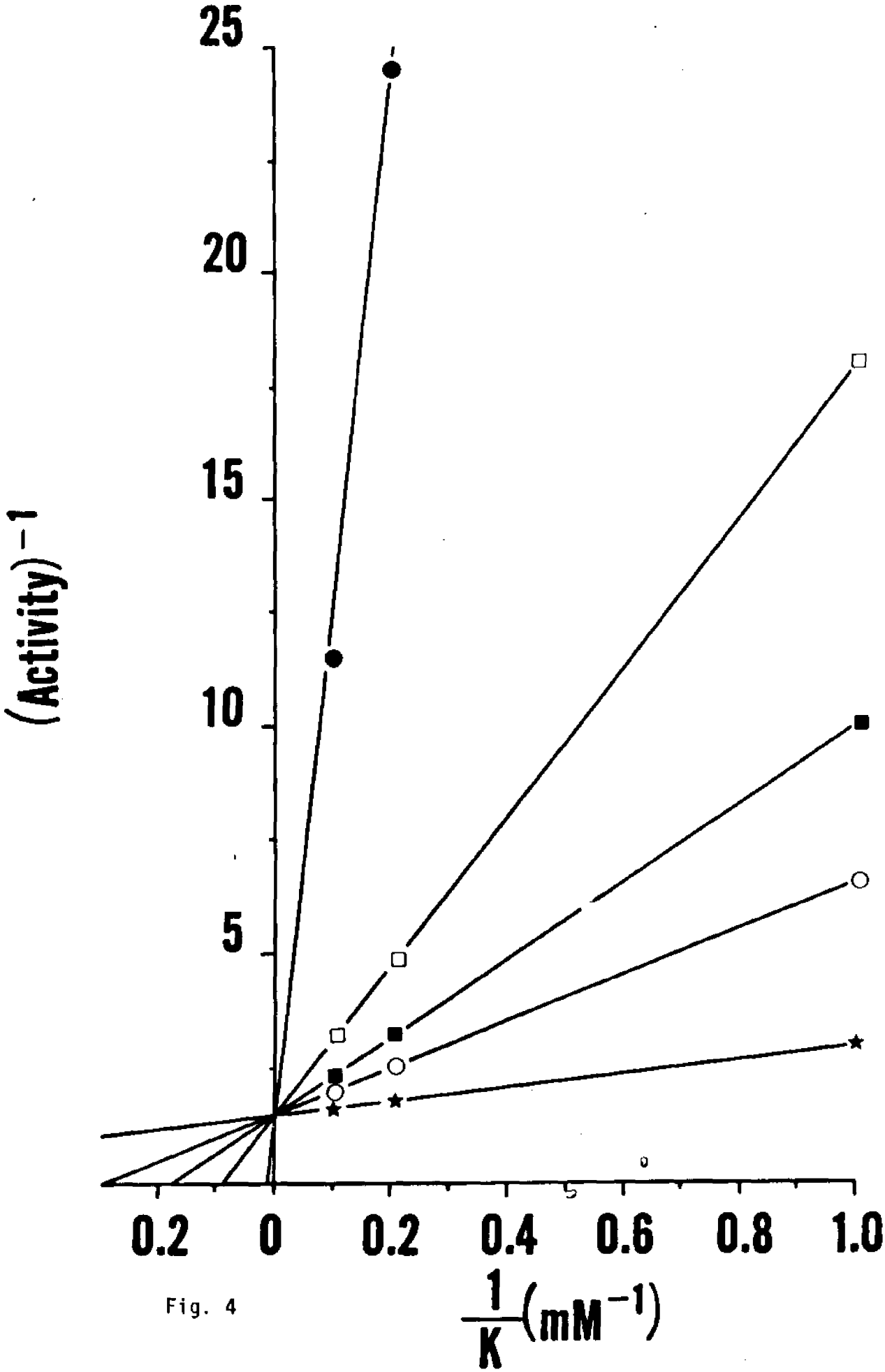

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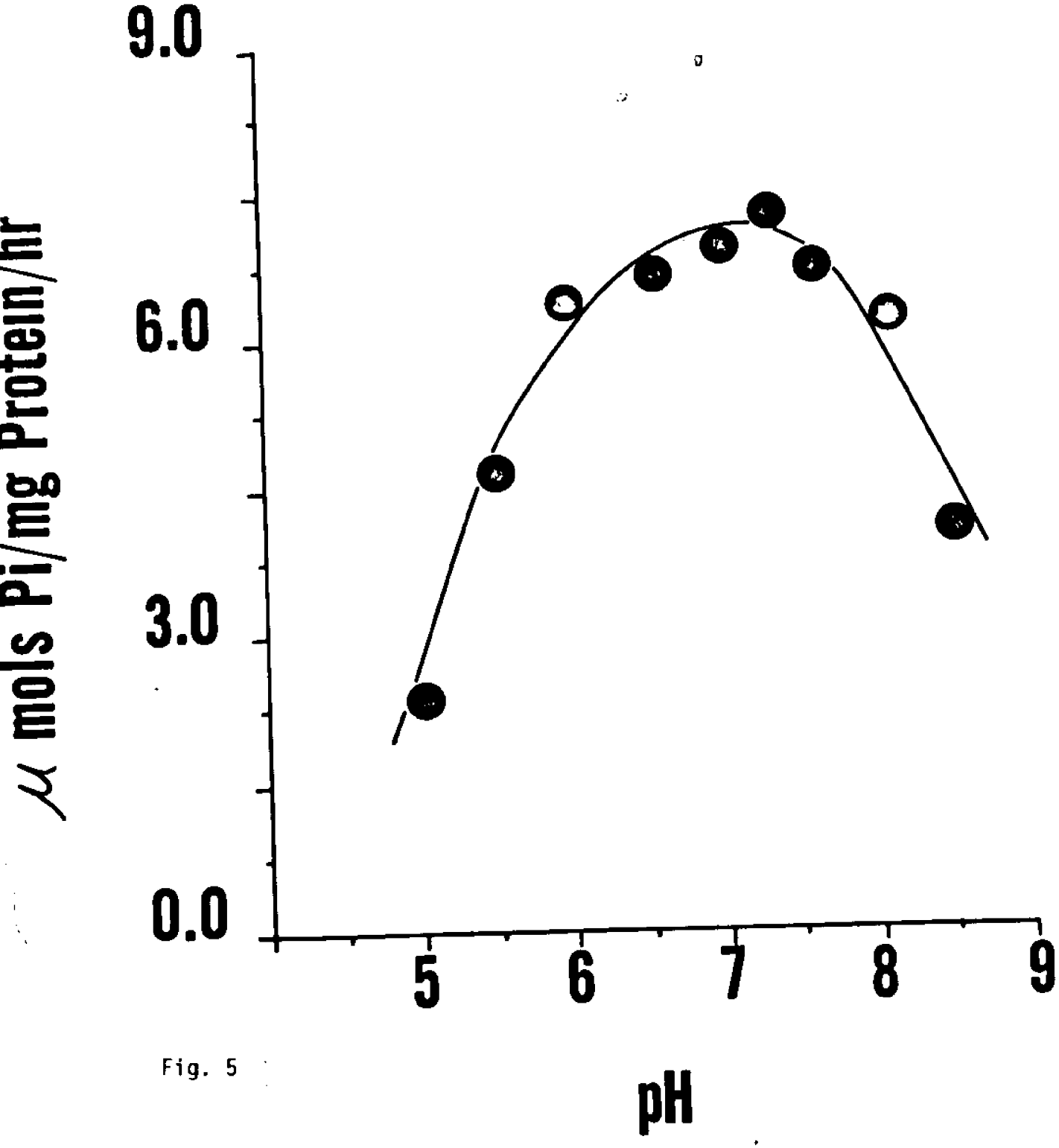

Fig. 5

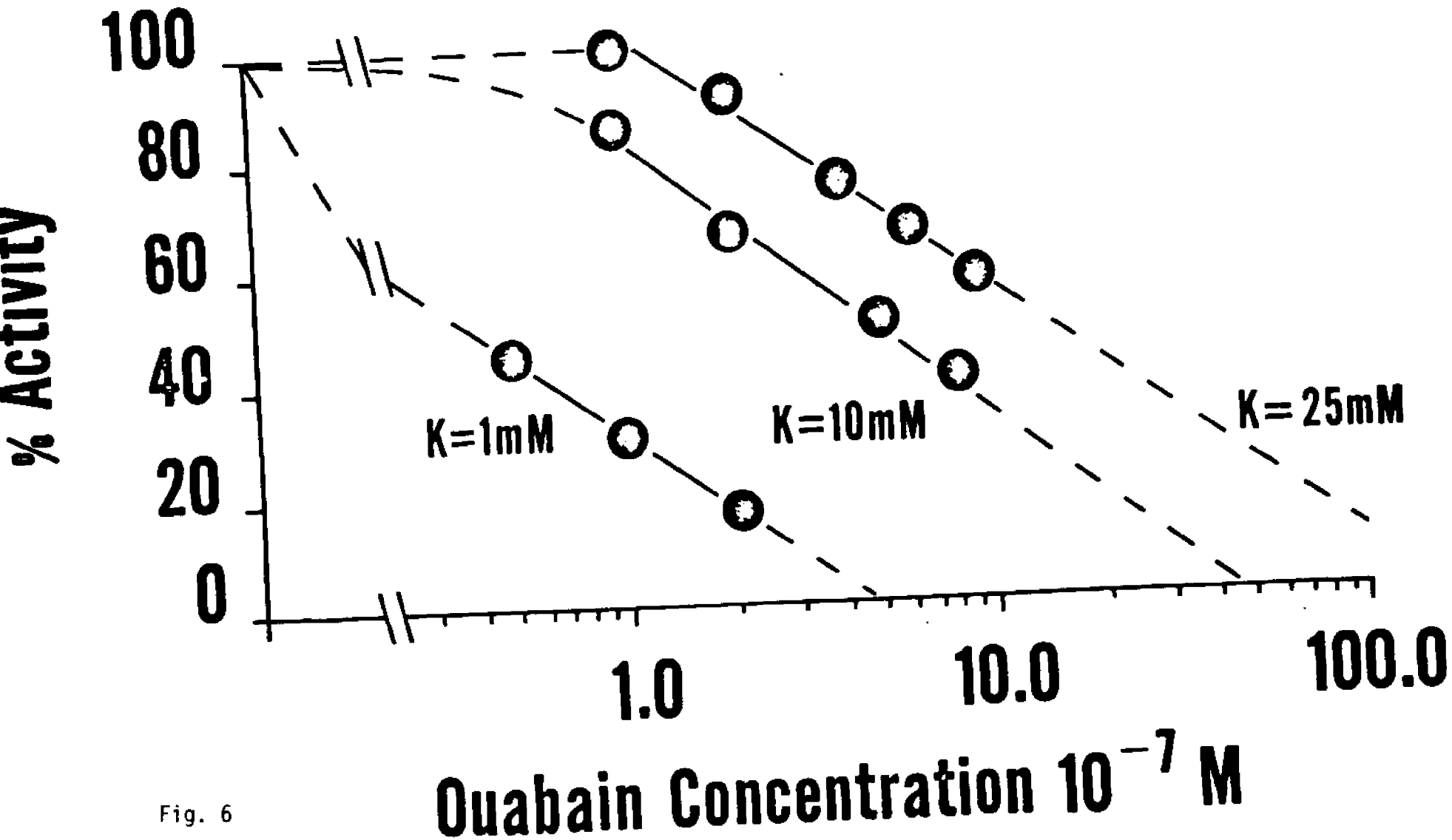
100
80
60
40
20
0
% Activity
K=1mM
K=10mM
K=25mM
1.0
10.0
100.0
Ouabain Concentration 10^{-7} M

Fig. 6

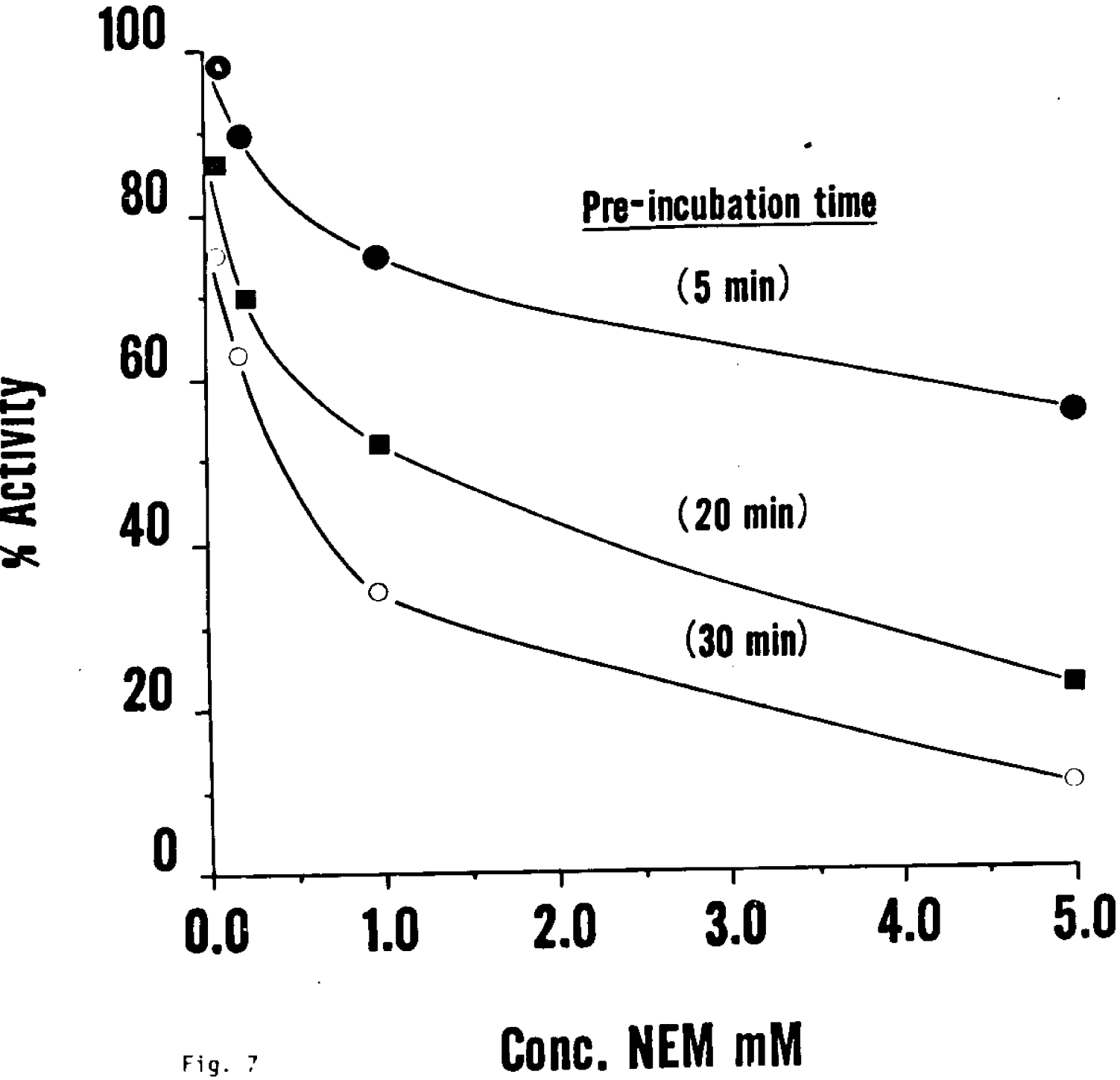
100
80
60
40
20
0
% Activity
Pre-incubation time
(5 min)
(20 min)
(30 min)
0.0
1.0
2.0
3.0
4.0
5.0
Conc. NEM mM

Fig. 7

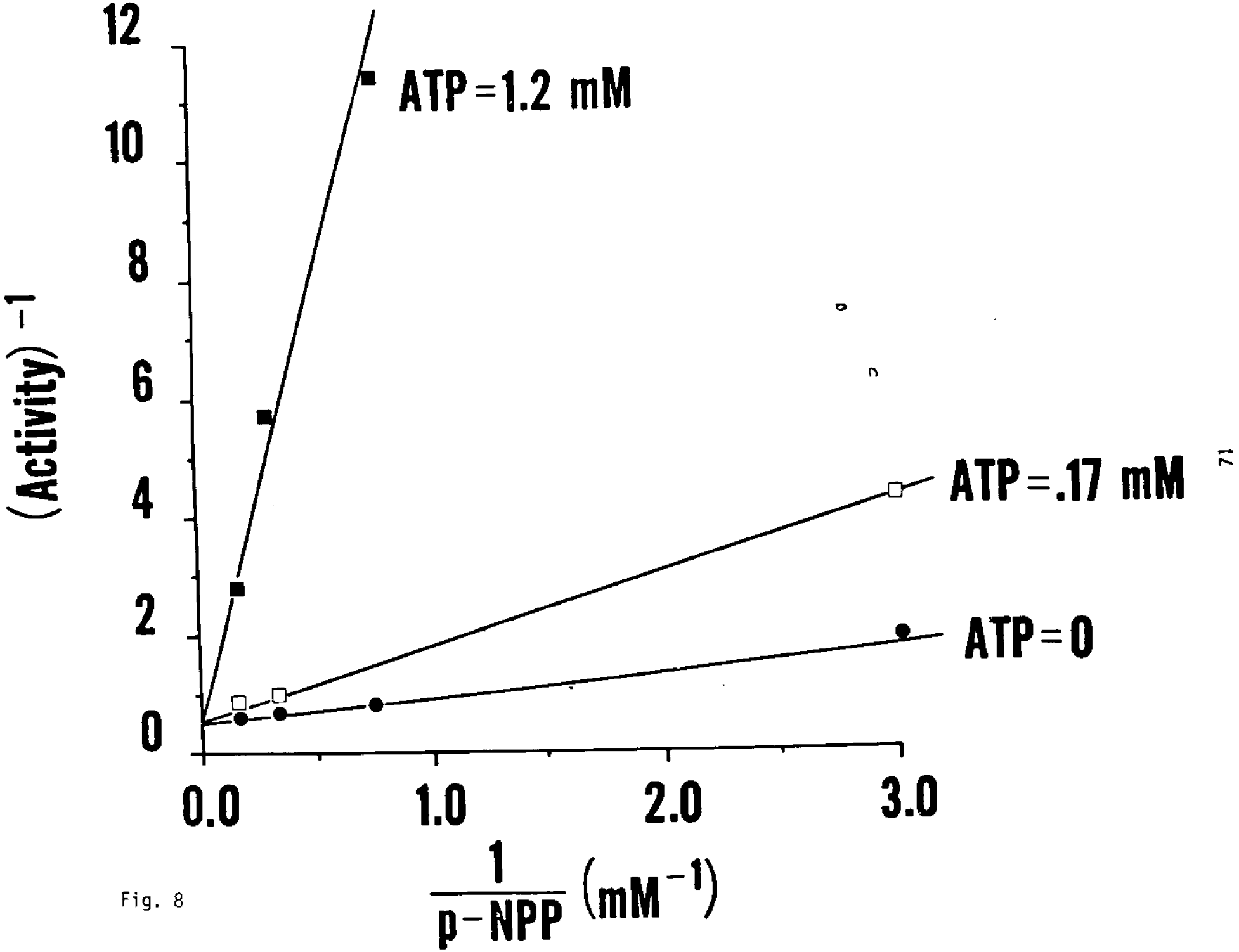
12
10
8
6
4
2
0
(Activity) $^{-1}$
ATP = 1.2 mM
ATP = .17 mM
ATP = 0
0.0
1.0
2.0
3.0
$\frac{1}{p-NPP}$ (mM^{-1})

Fig. 8

CONCLUSION

Turtle bladders possess an active mechanism for the transport of sodium from the mucosal to the serosal bathing fluid. Ouabain, 10^{-4}M in the serosal fluid, completely inhibits sodium transport, and concomitantly reduces oxygen consumption by 30-50%. Oxygen consumption is also reduced to the same extent (30-50%) by removal of ambient sodium. Sodium transport is dependent on glucose and inhibited by ouabain under anerobic as well as under aerobic conditions. Moreover, sodium transport is approximately doubled on going from an anerobic to an aerobic environment. The source of energy during anerobiasis is from glycolytic breakdown of glucose to lactic acid with the concomitant production of ATP; and it is reasonable to suppose that the ATP formed may be used to energize the anerobic transport of sodium. The effect of ouabain on transport and on oxygen consumption is paralleled by its effect on the $Na^+ + K^+$ stimulated ATPase and on the $Mg^{++} + K^+$ dependent p-nitrophenylphosphatase in microsomes from mucosal cells of the turtle bladder. Such parallelism suggests that the free energy of phosphate ester hydrolysis is coupled, in some manner, to the active transport mechanism for sodium. However, this does not show the exact nature and the degree of coupling between a spatially oriented chemical reaction in the membrane phase and a directionally oriented transport process.

The similarities between $Na^+ + K^+$ dependent ATPase and p-NPPase with respect to the pH optimum, the Mg^{++}/substrate ratio, the potassium con-

centration, the effect of ouabain and NEM, and the effect of ATP on p-NPPase suggest the possibility that we are dealing with one enzyme or a multienzyme complex which have more than one conformation, manifested either as ATPase or p-NPPase. However, Na^{+} inhibits p-NPase and stimulates ATPase in the presence of Mg^{++} and K^{+}. Thus, data reported here and elsewhere are not sufficient to determine whether we are dealing with one or more enzyme or an enzyme with more than one active site. The problems remaining to be solved include:

(a) further separation and purification of the enzyme or of the enzyme-proteins complexes;

(b) further studies on the nature of the intermediary complexes between ATP and the protein;

(c) reconstitution of the membrane from its protein, lipid, and phospholipid components and the subsequent incorporation of the enzyme (without loss of activity) into the re-constituted membrane; and finally,

(d) the identification of the exact role (e.g. carrier, energy transducer, etc.) of membrane ATPase and p-NPPase in the active process of sodium transport across either one or both cell membranes.

AUTOBIOGRAPHY

The author Yousif E. Shamoo, was born May 25, 1931 in Baghdad Iraq. On September 10, 1960 he was married to the former Tonia H. Kaminski in Louisville, Kentucky. He attended Health Official School beginning in 1949 and graduated in 1951, and worked as Health Official in the Ministry of Health in Baghdad-Iraq from 1951 to 1957.

In January 1958 he entered the United States and enrolled at the University of Louisville, graduating in 1965 with the degree of Bachelor of Science in Medical Technology.

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