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Changes in the (Na+ K+) - dependent ATPase activity in gills of the euryhaline teleost, Fundulus heteroclitus, upon adaptation to freshwater and salt water as determined by specific activities and 3H - ouabain binding sites

Mary Elizabeth Gilman

CHANGES IN THE (Na^++K^+) - DEPENDENT ATPASE ACTIVITY IN GILLS OF THE EURYHALINE TELEOST, FUNDULUS HETEROCLITUS, UPON ADAPTATION TO FRESHWATER AND SALT WATER AS DETERMINED BY SPECIFIC ACTIVITIES AND ^3H - OUABAIN BINDING SITES

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ABSTRACT

Previous attempts to measure the $(Na^+ + K^+)$ - dependent ATPase in gills of euryhaline teleosts have yielded controversial results. In this study both the $(Na^+ + K^+)$ - dependent ATPase activity and the number of ^3H - ouabain binding sites, representing the number of ATPase molecules, were measured in gill tissue preparations of the killifish, Fundulus heteroclitus. The $(Na^+ + K^+)$ - dependent ATPase activity and ouabain binding sites were greater in gills of fish fully adapted to freshwater than in those adapted to salinities of 17 ppt or 24 ppt. On the other hand, in 30 ppt salinity the $(Na^+ + K^+)$ - dependent ATPase activity and ouabain binding sites were greater than in freshwater samples. Upon transfer of fish from freshwater to salt water (17 ppt) the $(Na^+ + K^+)$ - dependent ATPase activity declined significantly within one - half hour to a value which remained constant in salt water (17 ppt). Observed changes in $(Na^+ + K^+)$ - dependent ATPase activity were not correlated with any significant changes in the Mg^{++} - dependent ATPase activity.

INTRODUCTION

Evidence suggests that a (Na^++K^+) - dependent adenosine triphosphatase (ATPase) is responsible for Na^+ excretion in the gills of salt water teleosts (Motais and Garcia - Romeu, 1972; Maetz, 1971). The (Na^++K^+) - dependent ATPase has been reported to be higher in stenohaline teleosts in marine environments than in stenohaline teleosts in freshwater environments (Kamiya and Utida, 1969).

Euryhaline fish are of special interest, and much controversy has arisen from the results published concerning the changes in (Na^++K^+) - dependent ATPase activity upon adaptation to either salt water or freshwater. Jampol and Epstein (1970) reported a doubling of the specific activity of the (Na^++K^+) - dependent ATPase in the gills and intestinal mucosa of salt water adapted Anguilla rostrata (eel) coupled with no difference in kidney enzymatic activity in the two environments. In Fundulus heteroclitus (killifish) the specific activity of the (Na^++K^+) - dependent ATPase increased in the gills during adaptation to salt water (Epstein,

et al., 1967) while decreasing in the kidneys (Epstein, et al., 1969). In Anguilla japonica (eel), Salmo gairdnerii irideus (rainbow trout), and Acanthogobius flavimanus (goby) gill (Na^++K^+) - dependent ATPase activity increased in salt water to four times the activity in freshwater. These three teleosts are normally freshwater dwellers (Kamiya and Utida, 1969). No changes in the (Na^++K^+) - dependent ATPase activity was reported in salt water and freshwater specimens of Anguilla anguilla (eel) and Platychthys flesus (flounder) (Kirschner, 1969). Lasserre (1971) reported that in the gills of Crenimugil labrosus (thick lipped mullet) and Morone (Dicentrarchus) labrax (sea bass) the specific activity of the (Na^++K^+) - dependent ATPase of freshwater fish was twice that of salt water adapted fish. All the mentioned investigators reported no change in the Mg^{++} - dependent ATPase activity.

The purpose of this study was to attempt to clarify the confusion concerning reported changes in (Na^++K^+) - dependent ATPase activity in euryhaline teleosts adapting from one salinity to another. The time intervals required for adaptation from freshwater to salt water and vice versa were investigated in F. heteroclitus. To substantiate the enzymatic data

an independent technique utilizing ^{3}H - ouabain was employed.

LITERATURE REVIEW

Biologists have long been interested in the physiology of teleostean osmoregulation as these fish occupy varied environments and have made various evolutionary adaptations (Jampol and Epstein, 1970). Both water and ionic regulatory mechanisms are needed by teleosts for survival as freshwater and salt water habitats are hypotonic and hypertonic respectively to their blood (Motais and Garcia - Romeu, 1972).

In freshwater, water enters through the gills and is excreted in large quantities by the kidneys (Potts, 1968; Jampol and Epstein, 1970; Motais and Garcia - Romeu, 1972). Na^{+} and Cl^{-} are lost to some extent in the urine, but these losses are replenished by active salt absorption in the gills (Potts, 1968; Maetz, 1971; Motais and Garcia - Romeu, 1972). Active reabsorption of Na^{+} by the renal tubules is also needed for survival in freshwater

teleosts (Jampol and Epstein, 1971).

In salt water, teleosts lose water osmotically across the body surface. To compensate for this water loss, they ingest water and the water ingested is absorbed in the intestine along with the ions present (Maetz and Garcia - Romeu, 1964; Maetz, 1969). Monovalent ions also enter by diffusion through the gills (Potts, 1968; Motais and Garcia - Romeu, 1972). As the kidneys are unable to excrete a hypertonic urine, monovalent ions are excreted by the gills against a chemical gradient (Jampol and Epstein, 1970; Motais and Garcia - Romeu, 1972).

In salt water the gills of stenohaline teleosts are permeable to electrolytes. Upon transfer to freshwater the ion influx ceases and the ion outflux continues, resulting in a loss of internal electrolyte concentration and death. Some fish, referred to as euryhaline, can survive abrupt changes in salinity and adapt to such changes (Zaugg and McLain, 1971). The euryhaline gill has a high ionic permeability in salt water and a low ionic permeability in freshwater. Thus an important change during freshwater adaptation is the reduction of the high salt water gill permeability. To be able to fully adapt to freshwater from salt water, fish must already have or acquire a gill mechanism for active

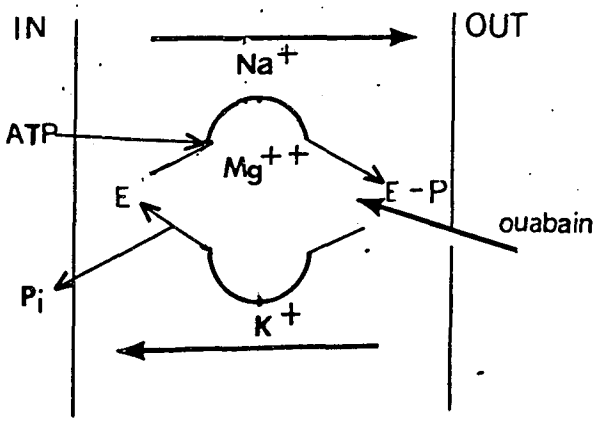
ion absorption (Motais and Garcia - Romeu, 1972). The $(Na^+ + K^+)$ - dependent ATPase appears to be responsible for this ion transport (Maetz, 1971).

The $(Na^+ + K^+)$ - dependent ATPase was first discovered by Skou (1957) in crab nerve homogenates. He found that crab nerve preparations hydrolyzed ATP, and that the rate of hydrolysis was increased by Na^+ ions plus K^+ ions. From these observations he suggested that ion transport was related to adenosine triphosphatase activity (Glynn and Chir, 1968).

It has been shown that a $(Na^+ + K^+)$ - dependent ATPase is found in almost all cell membranes, and its function is to transport Na^+ out of the cell and K^+ into the cell (Kinsolving, et al., 1963; Skou, 1965; Lehninger, 1970). Post (1968) defined this "salt pump" as a "membrane transport system in which transport is stoichiometrically coupled to breakdown (or synthesis) of a metabolic intermediate." The $(Na^+ + K^+)$ - dependent ATPase in human erythrocytes transports 3 Na^+ ions outward and 2 K^+ ions inward for each molecule of ATP split (Glynn and Chir, 1968; Post, 1968; Sen, et al., 1969).

The mechanism of the ATPase reaction has been shown to consist of at least two steps (Whittam and Wheeler, 1970). In the first step phosphate is

transferred from ATP in the presence of Mg^{++} and Na^{+} to some membrane protein (E in the diagram below) (Skou, 1965; Glynn and Chir, 1968; Kyte, 1971). The exact nature of this phosphorylated intermediate (E - P) is unknown although it appears in the lipoprotein fraction (Glynn and Chir, 1968). The phosphate of the intermediate is probably attached to the carboxyl group of a glutamyl residue in the protein (Hokin, et al., 1965). The second step involves the release of the phosphate as orthophosphate (Pi) into the cell in the presence of K^{+} ions (Glynn and Chir, 1968; Post, 1968). Thus, the formation of the phosphorylated intermediate is accelerated by Na^{+} ions, and dephosphorylation of the intermediate is accelerated by K^{+} ions (Sen, et al., 1969). NH_4^{+}, Rb^{+}, Cs^{+}, and Li^{+} can be substituted for K^{+} in order of decreasing efficiency, but Na^{+} cannot be replaced by any other ion (Katz and Epstein, 1968).

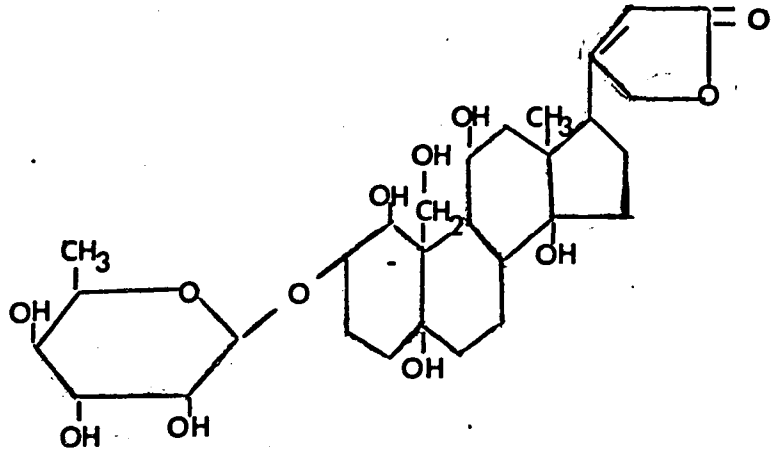


Kinetic studies indicate that there are two cation - binding sites on the ATPase molecule, one at the inner surface of the plasma membrane with a high affinity for Na^+ and one at the outer surface with a high affinity for K^+ ions (Skou, 1965; Katz and Epstein, 1968). There is also an ATP site on the inside membrane surface and a ouabain site on the outside surface (Albers, 1967). High concentrations of K^+ ions can competitively inhibit the Na^+ site. The assumption is that enzymatic activation needs Na^+ on the inside site and K^+ on the outside site of the cell membrane, yet the possibility still remains that there is only one site which has different cation affinities in different positions as it moves from inside to outside (Skou, 1965). It has been proposed recently that ATP plus Na^+ and K^+ ions bring about a conformational change in the enzyme thus causing a rotation of the active center (Somogyi, 1968; Koyal et al., 1971). The rotation of the active center could bring K^+ into the cell and take Na^+ out.

The (Na^++K^+) - dependent ATPase is thought to be a lipoprotein (Ahmed and Judah, 1964). It is known that it requires an organized phospholipid structure for activity (Skou, 1965). Phosphatidyl-serine has been shown to activate the (Na^++K^+) -

dependent ATPase molecule (Wheeler and Whittam, 1969; Harris, et al., 1971). Whether phosphatidylserine is a part of the ATPase structure is not known at this time.

Cardiac glycosides such as ouabain (structural formula below) inhibit (Na^++K^+) - dependent ATPase activity possibly by binding to and stabilizing the phosphorylated intermediate (Landon and Norris, 1963; Tobin and Sen, 1970; Towle, 1970), thus yielding it insensitive to the action of K^+ ions (Schwartz, et al., 1968). The"specificity of inhibition of the (Na^++K^+) - dependent ATPase by active cardiac glycosides is not mimicked by any other known inhibitor (Matsui and Schwartz, 1968)."



In the presence of ATP and Mg^{++}, ouabain appears to bind to the (Na^++K^+) - dependent ATPase at a site on the outside of the membrane (Matsui and Schwartz, 1966; Akera, 1971). Na^+ enhances

binding; K^+ depresses it (Matsui and Schwartz, 1968). The amount of ouabain bound per cell is proportional to the number of transport sites (Dunham and Hoffman,1970), and thus proportional to the number of (Na^++K^+) - dependent ATPase molecules (Dunham and Hoffman, 1971; Hendler, et al., 1972).

There is also a (Na^++K^+) - independent ATPase found in the cell membrane. This Mg^{++} - dependent ATPase hydrolyzes ATP and is insensitive to ouabain inhibition. Evidence suggests that the (Na^++K^+) - dependent and the Mg^{++} - dependent ATPases are located on different membrane fragments (Boegman, et al., 1970).

MATERIALS AND METHODS

Adult male killifish, F. heteroclitus, were collected on August 11, and December 14, 1972 from Mobjack Bay at Severn, Virginia. The fish were maintained at 20 C in filtered freshwater or salt water. The various salinities used were 17 ppt and 24 ppt as obtained from the collecting site in August and December respectively, and 30 ppt prepared synthetically from Instant Ocean

(Aquarium Systems, Inc., Eastlake, Ohio). All fish were fed Tetramin (fish food) daily.

Preparation of gill homogenates and microsomes

After decapitation, the gills were excised and rinsed in homogenizing solution. The gill tissue was then surgically removed from the cartilaginous arches. Gill tissue from ten to twelve fish was used in each experiment.

Samples to be used for $(Na^+ + K^+)$ - dependent ATPase assays only were homogenized in a solution that contained 0.25 M sucrose, 5 mM disodium ethylenediamine tetraacetic acid (EDTA), 30 mM histidine (pH 6.8), and 0.1% sodium deoxycholate (Epstein, et al., 1967) yielding a 2% (w/v) homogenate. Homogenization was carried out at 0 - 2 C in a Potter - Elvehjem homogenizer with a smooth Teflon pestle at 1,725 rpm using 10 strokes. Homogenate samples were prepared in this manner from fully adapted freshwater and salt water samples, from partially adapted salt water samples ($\frac{1}{2}$ hour to 36 hours), and from partially adapted freshwater samples ($\frac{1}{2}$ hour to 24 hours).

Samples to be used for $(Na^+ + K^+)$ - dependent ATPase assays and microsomal preparations were homogenized in a solution of 0.25 M sucrose,

6 mM EDTA, 20 mM imidazole (pH 6.8), and 0.1% sodium deoxycholate (added immediately before use) making a 5% (w/v) homogenate. Homogenization was carried out as previously described except that 18 strokes were used (Hendler, et al., 1972).

The homogenate was filtered through a double layer of cheesecloth to remove unhomogenized material. Part of the resulting homogenate was frozen at -20 C and assayed for enzymatic activity within 36 hours. The remainder was used to prepare the microsomal fraction according to Hendler, et al., (1972).

The whole homogenate was centrifuged at 10,800 x g at 0 C for 30 minutes in a preparative ultracentrifuge to remove unbroken cells, nuclei, and mitochondria. The supernatant was carefully removed, leaving a sufficient amount of fluid to avoid contamination, and was centrifuged at 105,000 x g at 0 C for 60 minutes. The supernatant was discarded, and the microsomal pellet was resuspended in 0.8 ml of the original homogenizing solution without sodium deoxycholate using a vortex mixer. A portion of this suspension was frozen at -20 C and assayed for enzymatic activity within 36 hours.

^{3}H - Ouabain binding assay

The amount of ^{3}H - ouabain bound to microsomal

membranes was determined by the method of Matsui and Schwartz (1968). Microsomes were incubated in polycarbonate centrifuge tubes in a reaction mixture containing 2 mM disodium adenosine triphosphate (ATP), 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 6 x 10^{-4}uM ^3H - ouabain, and 50 mM trishydroxymethylamino methane - HCl (Tris-HCl) (pH, 7.4). The reaction was initiated by the addition of 0.2 ml of the microsomal fraction yielding a final volume of 2 ml. Each reaction tube was incubated for 3 minutes at 37 C with shaking. Each tube was then quickly transferred to a pre-warmed rotor (Spinco 50Ti) and centrifuged for 3 minutes at 35 C at 105,000 x g. The supernatant was discarded, and the droplets were carefully wiped from the centrifuge tube wall. The pellet was resuspended in 0.5 ml of H_2O using a vortex mixer and the resulting suspension transferred to a scintillation vial. Another 0.5 ml of H_2O was added to the centrifuge tube, vortexed, and transferred to the scintillation vial. To the scintillation vial was added 10 ml of Aquasol scintillation medium. The vial was capped tightly, shaken vigorously, and stored overnight in the dark prior to counting. Counts were made on a Nuclear Chicago Liquid Scintillation System (Mark IV).

The results were expressed as micromicromoles of ^{3}H - ouabain bound per milligram of protein, and as micromicromoles of ^{3}H - ouabain bound per micromole of Pi (orthophosphate) liberated by the same preparation when its (Na^{+}+K^{+}) - dependent ATPase activity was measured. All experiments were run in duplicate; duplicate controls were used (reaction mixture without ATP); and freshwater and salt water samples were used in each experiment.

(Na^{+}+K^{+}) - Dependent ATPase assay

A modified procedure of Epstein, et al., (1967) and Janicki and Kinter (1971) was employed to measure the (Na^{+}+K^{+}) - dependent ATPase and Mg^{++} - dependent ATPase activities in the F. heteroclitus gill preparations. The homogenate and microsomal fraction prepared according to Hendler, et al., (1972) were diluted 2.5 X and 10 X respectively using the homogenizing solution without sodium deoxycholate. The reaction mixture contained 20 mM imidazole - HCl (pH 7.8); either 130 mM NaCl and 1.0 mM ouabain, or 30 mM KCl and 100 mM NaCl; and 0.1 ml of enzyme. A 30 mM K^{+} concentration was used. This was found by Slate (1972) to be the optimum concentration in F. heteroclitus gill

preparations. The reaction was initiated by the addition of 0.2 ml of 50 mM ATP and 50 mM $MgCl_2$ neutralized with Tris (pH 7.0) making a final reaction volume of 2.0 ml. Incubation blanks contained 0.1 ml of homogenizing solution instead of 0.1 ml of enzyme. All tests were in duplicate.

After 30 minutes at 25 C, the reaction was terminated by the addition of 2.0 ml of ice - cold 10% (w/v) trichloroacetic acid (TCA) to precipitate the protein. The tubes were allowed to cool in an ice bath for 10 minutes and then centrifuged at 10,000 x g for 10 minutes to precipitate the protein. Inorganic phosphate (Pi) in the supernatant was determined as described by Fiske and SubbaRow (1925).

As preliminary studies indicated that sodium deoxycholate interfered with the protein determinations, homogenate samples containing sodium deoxycholate were dialyzed against 0.9% NaCl for 24 hours. Protein determinations were performed by the method of Lowry, et al., (1951).

The specific activity of the enzyme was expressed as umoles of Pi released from ATP per mg of protein per minute. The $(Na^+ + K^+)$ - dependent ATPase specific activity was expressed as the difference between the total enzymatic activity

(without ouabain) and the Mg^{++} - dependent, ouabain - insensitive ATPase (with ouabain) specific activity.

Statistical analysis

A student's T - test of significance between means was employed (Ferguson, 1971).

RESULTS AND DISCUSSION

The initial experiment dealt with the changes in the $(Na^{+}+K^{+})$ - dependent ATPase in the gills of F. heteroclitus upon transfer from freshwater to salt water (17 ppt) and upon transfer from salt water (17 ppt) to freshwater. The $(Na^{+}+K^{+})$ - dependent ATPase activity in homogenized gill preparations from freshwater adapted F. heteroclitus is approximately twice ($p < .001$) the activity of 17 ppt salt water adapted fish (Table 1,2,3, Fig. 1). Statistical analysis indicates that the $(Na^{+}+K^{+})$ - dependent ATPase activity from gills of fish fully adapted to freshwater is significantly higher than activity in gills of fish both fully adapted (18 days or more) and partially adapted ($\frac{1}{2}$ hour to 36 hours) to salt water (Table 2,3, Fig. 1). Upon

transfer of F. heteroclitus from freshwater to salt water (17 ppt), the fish appear to be acclimated to salt water after ½ hour.

Lasserre (1971) found that in the gills of the euryhaline teleosts, C. labrosus (thick lipped mullet) and M. labrax (sea bass), the specific activity of the (Na^++K^+) - dependent ATPase declined significantly upon adaptation to salt water (33 ppt) and increased significantly upon freshwater adaptation. The specific activity of the (Na^++K^+) - dependent ATPase of freshwater fish was approximately twice as great as that of salt water fish, nearly the same relationship observed in the present comparison of enzyme activities from fish adapted to freshwater and salt water (17 ppt). Lasserre (1971) stated that when the "(Na^++K^+) - activated ATPase increases on transfer from salt water to freshwater this suggests an increase in the active absorption of Na^+ ions and K^+ ions at the gill level, and an active reabsorption of Na^+ in the kidney, during freshwater adaptation." (Na^++K^+) - dependent ATPase activity in gills of larval Rana catesbeiana (bullfrog) maintained in distilled water was twice as high as the activity of those maintained in a 25 mM NaCl solution. In larval bullfrogs salt depletion stimulates an active

absorption of Na^+ from a dilute medium (Boonkoom and Alvarado, 1971).

The fact that the (Na^++K^+) - dependent ATPase activity in fish transferred to freshwater (½ hour to 24 hours) was not significantly higher than either the fully adapted salt water fish (17 ppt) or the partially adapted salt water fish (½ hour to 36 hours) is unexplained (Table 3). It may be due to error introduced by the variance in the limited number of samples. The transfer from freshwater to salt water may involve a degradation of (Na^++K^+) - dependent ATPase protein; whereas the transfer from salt water to freshwater may involve a more complicated process of protein synthesis. At this time the freshwater data (½ hour to 24 hours) is not sufficient to evaluate.

No statistically significant changes were observed in the Mg^{++} - dependent ATPase activity in gill preparations of fish subjected to freshwater and salt water (17ppt) except in 1 comparison (Table 5, Fig. 1). A significant difference was seen when the fully adapted salt water samples were compared against the partially adapted salt water (½ hour to 36 hours) samples. Only four values for the Mg^{++} - dependent ATPase activities were obtained for the fully adapted salt water (17 ppt) fish. Thus,

the observed difference may be due to error introduced by the scatter in the limited number of samples.

The fact that no overall change in Mg^{++} - dependent ATPase activity was observed is in agreement with other researchers. Boonkoom and Alvarado (1971) and Lasserre (1972) found no significant differences in the Mg^{++} - dependent ATPase activity. Other workers have also observed no significant changes in the Mg^{++} - dependent ATPase activities of euryhaline teleosts (Epstein, et al., 1967; Kamiya and Utida, 1969; Jampol and Epstein, 1970). The absence of significant changes in the Mg^{++} - dependent ATPase activity indicates that upon adaptation to freshwater and salt water it is the $(Na^{+}+K^{+})$ - dependent ATPase that is changing in response to physiological needs.

Previous work by Epstein, et al., (1967) showed that the $(Na^{+}+K^{+})$ - dependent ATPase activity was approximately six times greater in gill homogenates of F. heteroclitus adapted to salt water (26 ppt) than in fresh water preparations. The present study, however, indicated that in freshwater F. heteroclitus the gill preparations had a $(Na^{+}+K^{+})$ - dependent ATPase activity approximately twice that of salt water (17 ppt) samples. Thus, an independent procedure

using ^{3}H - ouabain to label the $(Na^{+}+K^{+})$ - dependent ATPase molecules was utilized in order to evaluate the enzymatic data. These experiments were designed to see if a decrease in $(Na^{+}+K^{+})$ - dependent ATPase molecules as measured by ^{3}H - ouabain would correlate quantitatively to the observed decrease in enzymatic activity.

As the $(Na^{+}+K^{+})$ - dependent ATPase of certain tissues is not inhibited by ouabain (for example rat brain) (Akera, et al., 1969), a preliminary experiment was designed to determine if the enzyme from F. heteroclitus gills was sensitive to ouabain inhibition. The results shown in Table 6 indicate that even in the presence of both Na^{+} and K^{+} the $(Na^{+}+K^{+})$ - dependent portion of the ATPase activity is totally inhibited by 1 mM ouabain.

Gill microsomes of fish fully adapted to fresh-water bound 103 uumoles of ^{3}H - ouabain per mg protein; whereas gill microsomes of fish adapted to salt water (17 ppt) bound 45 uumoles of ^{3}H - ouabain per mg of protein (Table 10, Fig. 2). As the amount of ouabain bound indicates the number of $(Na^{+}+K^{+})$ - dependent ATPase molecules, this data validates my earlier results which indicated that the $(Na^{+}+K^{+})$ - dependent ATPase activity was twice as high in

freshwater gill homogenates as it was in the 17 ppt salt water gill homogenates. The (Na^++K^+) - dependent ATPase activity of the freshwater gill microsomes was also approximately twice as great as the activity of the salt water (17 ppt) gill microsomes (Table 7, Fig. 2).

Two other experiments were performed to determine if salinities higher than 17 ppt caused increased (Na^++K^+) - dependent ATPase activities in the gills of F. heteroclitus. It was found that the freshwater gill microsomes were approximately twice as high as the 24 ppt salt water gill microsomes with respect to the uumoles of ^3H - ouabain bound per mg protein and the specific activity of the (Na^++K^+) - dependent ATPase activity (Table 7, 10; Fig. 2). However, the freshwater F. heteroclitus gill microsomes showed a lower amount of uumoles of ^3H - ouabain bound and a lower (Na^++K^+) - dependent ATPase activity than did salt water gill microsomes of 30 ppt (Table 7, 10; Fig. 2).

These results indicate that at a high salinity (30 ppt) gill (Na^++K^+) - ATPase activity increases, reflecting the greater requirement of the "pump" to eliminate excess internal Na^+. The intermediate salinities (17 and 24 ppt) may not cause as much stress on the organism and therefore may permit a

lower pump activity. In freshwater, the need to bring Na^+ into the organism demands greater pump activity.

The last test that was performed was ^3H - ouabain binding of fish fully adapted to freshwater compared with fish adapted to salt water (17 ppt) for ½ hour. This was an attempt to validate the enzymatic data which indicated that such fish were fully adapted to salt water within ½ hour (Table 1). The fish gill preparations from the salt water (17 ppt, ½ hour) showed both a higher (Na^++K^+) - dependent ATPase activity and a greater amount of ^3H - ouabain bound than did the freshwater samples (Table 7, 10; Fig. 2). This does not corroborate the previous work. This could be due to seasonal variation (Zaugg and Mc Lain, 1971), as the initial enzymatic data was taken in September and the ^3H - ouabain binding experiment conducted in January. Another factor that must be considered is stress. The salt water (17 ppt) fish had not been handled for several months,and there were only twenty fish in the tank. On the other hand, the freshwater fish had been handled,and there were over one hundred in the tank. It was observed during the experiment that the salt water fish were excited. Such excitement or stress could cause a cortisol

release and thus elevate the (Na^++K^+) - dependent ATPase activity (Pang, Columbia University, pers. commun.; Pickford, Hiram College, pers. commun.; Umminger and Gist, 1973).

The (Na^++K^+) - dependent ATPase of teleosts' osmoregulatory organs appears to be at least partially under endocrine control. Maetz (1969) stated that " cortisol plays the role of a salt - excretingfactor in the fish living in hypertonic media, and as a salt absorbing factor in the fresh-water adapted teleosteans." Cortisol, a mineralo-corticoid, concerned with improving extrarenal Na^+ excretion favors salt water adaptation (Pickford, et al., 1970; Epstein, et al., 1971). As (Na^++K^+) - dependent ATPase is related to ion transport it is "likely that the influence of cortisol on Na^+ excretion might be associated with changes in the activity of this enzyme (Pickford, et al., 1970)." The (Na^++K^+) - dependent ATPase increases in cortisol treated F. heteroclitus in the gills, kidneys, and intestinal mucosa (Pickford, et al., 1970). The Na^+ outflux in the gills of A. rostrata is depressed by hypophysectomy or adrenalectomy yet returned to normal by cortisol (Epstein, et al., 1971). Thus the pituitary - adrenal axis is quite important in teleostean osmoregulation (Pickford, et al., 1970).

The mean specific activity for the freshwater $(Na^+ + K^+)$ - dependent ATPase ± one standard error and the mean uumoles of ^3H - ouabain per mg protein were calculated. It was observed (Table 8, 11; Fig.2) that the standard error was quite small for these data. The experiment was reproduced four times, and thus the salt water data must be valid. This totally contradicts the earlier work of Epstein, et al., (1967). Their data was highly variable and could not be repeated.

Ratios of the amount of bound ^3H - ouabain to the $(Na^+ + K^+)$ - dependent ATPase activity were calculated according to the formula (ratio = $\frac{\text{uumoles } ^3H \text{ - ouabain / mg protein}}{\text{Pi / mg / min}}$) . If these ratios are constant, then ^3H - ouabain binding is a reliable index of $(Na^+ + K^+)$ - dependent ATPase activity. The data in Table 11 show that the ratios are constant.

There are two theories that can explain the results of this study. In A. rostrata (eel) a positive linear correlation has been observed between gill $(Na^+ + K^+)$ - dependent ATPase activity and the difference in environmental and plasma Na^+ ion concentrations throughout a range of salinities (Butler and Carmichael, 1972). In the same fish,

when the plasma Na^+ is greater than the environmental Na^+ the active transport of Na^+ is reversed. That is, the transport is inward through the gills, as opposed to outward transport of Na^+ when the environmental Na^+ is higher than the plasma Na^+. The $(Na^+ + K^+)$ - dependent ATPase activity in the gills of this eel is greater in distilled water than in tap water, greater in tap water than in salt water (8.25 ppt) (Butler and Carmichael, 1972). Butler and Carmichael (1972) stated that the increase in enzyme activity in dilute media might by caused by "an intrinsic capacity of the Na^+ pump to increase where there are only trace amounts of Na^+ in the external medium." Thus, in a freshwater environment or in a 30 ppt salt water environment the gills of F. heteroclitus could be pumping Na^+ in and pumping Na^+ out, respectively; whereas in intermediate salinities (17 ppt and 24 ppt) the strain could be less, and the pump needed less.

Lasserre (1971) proposed the theory that if an euryhaline teleost is of marine origin, a freshwater environment would be a stressful environment and thus the $(Na^+ + K^+)$ - dependent ATPase activity would increase. Similarly, a teleost originating in freshwater would exhibit a higher enzymatic activity in salt water. This type of adaptation is referred to as "ecobiochemical" (Lasserre, 1971).

In summary, in gills of F. heteroclitus the specific activity of (Na^++K^+) - dependent ATPase is significantly higher in freshwater adapted fish than in fish adapted to salinities of 17 ppt or 24 ppt, but as the salinity becomes higher (30 ppt) the (Na^++K^+) - dependent ATPase also becomes higher. Upon transfer from fresh water to 17 ppt salt water, killifish appear to be acclimated completely within ½ hour in samples measured in September. These studies ellucidate molecular mechanisms which may allow F. heteroclitus to survive in a dynamic estuarine environment.

Table 1. Specific activities of $(Na^+ + K^+)$ - dependent ATPase and Mg^{++} - dependent ATPase in gill homogenates (2%) of F. heteroclitus.

Salinity	$(Na^+ + K^+)$ - ATPase umoles Pi/mg/min	Mg^{++} - ATPase umoles Pi/mg/min
SW	.0598	.1350
SW	.0565	-----
SW	.0510	.0610
SW	.0360	.0765
SW	.0495	.0906
SW ½ hr.	.0610	.1280
SW ½ hr.	.0340	.0980
SW 1½ hr.	.0545	.1750
SW 1½ hr.	.0820	.1756
SW 12 hr.	.0562	.1580
SW 12 hr.	.0510	.1450
SW 16 hr.	.0261	.1190
SW 16 hr.	.0645	.1550
SW 36 hr.	.0517	.1330
FW	.0920	.1770
FW	.0870	.1310
FW	.0985	.1380
FW	.1260	.0580
FW	.0925	.0925
FW ½ hr.	.0635	.1070
FW 1½ hr.	.1380	.1570
FW 16 hr.	.0480	.0915
FW 24 hr.	.0768	.1460

SW = salt water (17 ppt)

FW = freshwater

Table 2. The mean specific activity ± S. E. of $(Na^+ + K^+)$ - dependent ATPase in gill homogenates (2%) of F. heteroclitus.

Salinity	Number of samples	umoles Pi/mg/min $\bar{X}$ ± S. E.
FW (fully adapted)	5	.0992 ± .0069
FW (½ hr. to 24 hr.)	4	.0815 ± .0197
FW (fully adapted + ½ hr. to 24 hr.)	9	.0914 ± .0093
SW (fully adapted)	5	.0505 ± .0041
SW (½ hr. to 36 hr.)	9	.0534 ± .0054
SW (fully adapted + ½ hr. to 36 hr.)	14	.0524 ± .0037

FW = freshwater
SW = salt water (17 ppt)

Table 3. Results of T - test for significance between means of (Na^++K^+) - dependent ATPase in gill homogenates (2%) of F. heteroclitus.

X	Y	df	T
SW - F	FW - F	8	*6.037
SW - T	FW - F	12	*5.102
SW - F + SW - T	FW - F	17	*6.292
SW - F	FW - F + FW - T	12	**3.108
SW - F	FW - T	7	0.002
SW - T	FW - T	11	0.244
SW - F + SW - T	FW - F + FW - T	21	*4.450
SW - F	SW - T	12	0.360
FW - F	FW - T	7	0.927

SW - F = fully adapted salt water (17 ppt) samples
SW - T = salt water (17 ppt) samples ½ hr. to 36 hr.
FW - F = fully adapted freshwater samples
FW - T = freshwater samples ½ hr. to 24 hr.

* Significant at the .001 level
** Significant at the .01 level

Table 4. The mean specific activity ± S. E. of Mg^{++} - dependent ATPase in gill homogenates (2%) of F. heteroclitus.

Salinity	Number of samples	umoles Pi/mg/min $\bar{X}$ ± S. E.
FW (fully adapted)	5	.1193 ± .0203
FW (½ hr. to 24 hr.)	4	.1254 ± .0155
FW (fully adapted + ½ hr. to 24 hr.)	9	.1220 ± .0125
SW (fully adapted)	4	.0908 ± .0159
SW (½ hr. to 36 hr.)	9	.1429 ± .0086
SW (fully adapted + ½ hr. to 36 hr.)	13	.1269 ± .0101

FW = freshwater
SW = salt water (17 ppt)

Table 5. Results of T - test for significance between means of Mg^{++} - dependent ATPase in gill homogenates (2%) of F. heteroclitus.

$\bar{X}$	$\bar{Y}$	df	T
SW - F	FW - F	8	0.056
SW - T	FW - F	12	1.256
SW - F + SW - T	FW - F	17	0.371
SW - F	FW - F + FW - T	11	1.439
SW - F	FW - T	6	1.553
SW - T	FW - T	11	1.065
SW - F + SW - T	FW - F + FW - T	21	0.363
SW - F	SW - T	11	*3.136
FW - F	FW - T	7	0.226

SW - F = fully adapted salt water (17 ppt) samples
SW - T = salt water (17 ppt) samples ½ hr. to 36 hr.
FW - F = fully adapted freshwater samples
FW - T = freshwater samples ½ hr. to 24 hr.

* Significant at the .01 level

Table 6. Ouabain inhibition of (Na^++K^+) - dependent ATPase in gill preparations of F. heteroclitus.

Source of microsomes	Na^+ (mM)	K^+ (mM)	Ouabain (mM)	ATPase activity umoles Pi/mg/min
FW	100	30	0	.788
	130	0	1	.178
	100	30	1	.179
SW	100	30	0	.472
	130	0	1	.176
	100	30	1	.176

FW = freshwater
SW = salt water (17 ppt)

Table 7. Specific activities of $(Na^{+}+K^{+})$ - dependent ATPase and Mg^{++} - dependent ATPase in microsomal gill fractions of F. heteroclitus.

Salinity	$(Na^{+}+K^{+})$ - ATPase umoles Pi/mg/min	Mg^{++} - ATPase umoles Pi/mg/min
FW	.610	.178
SW - 17 ppt	.296	.176
FW	.550	.177
SW - 24 ppt	.200	.191
FW	.540	.144
SW - 30 ppt	.985	.193
FW	.565	.233
SW - 17 ppt, ½ hr.	.770	.194

FW = freshwater
SW = salt water

Table 8. The mean specific activity ± S. E. of $(Na^{+}+K^{+})$ - dependent ATPase and Mg^{++} - dependent ATPase in microsomal gill preparations of F. heteroclitus.

Sample	Number of samples	ATPase activity umoles Pi/mg/min	$\bar{X}$ ± S. E.
FW	4	$(Na^{+}+K^{+})$-dependent	.5662 ± .0154
FW	4	Mg^{++}-dependent	.1830 ± .0184

FW = freshwater

Table 9. Results of T - test for significance between means of Mg^{++} - dependent ATPase and $(Na^{+}+K^{+})$ - dependent ATPase in microsomal gill preparations of F. heteroclitus.

ATPase	$\bar{X}$	$\bar{Y}$	df	T
Mg^{++}-dependent	FW	SW(17 ppt, 17 ppt ½ hr., 24 ppt, and 30 ppt)	6	.2907
$(Na^{+}+K^{+})$-dependent	FW	SW(17 ppt, 17 ppt ½ hr., 24 ppt, and 30 ppt)	6	*15.9242

* Significant at the .001 level

Table 10. uumoles ^{3}H - ouabain bound per mg protein in microsomal gill preparations of *F. heteroclitus*.

Salinity	uumoles ^{3}H -ouabain / mg protein
FW	103.0
SW - 17 ppt	45.0
FW	96.3
SW - 24 ppt	41.5
FW	90.0
SW - 30 ppt	116.0
FW	96.0
SW - 17 ppt, ½ hr.	157.0

FW = freshwater
SW = salt water

Table 11. The mean uumoles ^{3}H - ouabain bound / mg protein ± S. E. in microsomal gill preparations of F. heteroclitus.

Sample	Number of samples	uumoles ^{3}H-ouabain/mg protein $\bar{X}$ ± S. E.
FW	4	96.32 ± 2.65

FW = freshwater

Table 12. Ratios as determined by the formula:

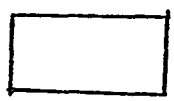
$$\text{Ratio} = \frac{\text{uumoles } {}^{3}H \text{ - ouabain/ mg protein}}{\text{Pi/ mg/ min}}$$

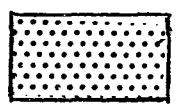
Sample	Ratio	$\bar{X} \pm$ S. E.
FW	167	
SW - 17 ppt	154	
FW	173	
SW - 24 ppt	203	
FW	167	
SW - 30 ppt	116	
FW	169	
SW - 17 ppt ½ hr.	157	
		163.25 ± 8.55

FW = freshwater
SW = salt water

Figure 1. Mean specific activity of (Na^++K^+) - dependent ATPase and Mg^{++} - dependent ATPase in gill homogenates (2%) of F. heteroclitus.

SW = fully adapted salt water (17 ppt) fish
SWT = salt water (17 ppt) ½ hr. to 36 hr.
FW = fully adapted freshwater fish
FWT = freshwater ½ hr. to 24 hr.
( ) = number of different samples

 = (Na^++K^+) - dependent ATPase

 = Mg^{++} - dependent ATPase

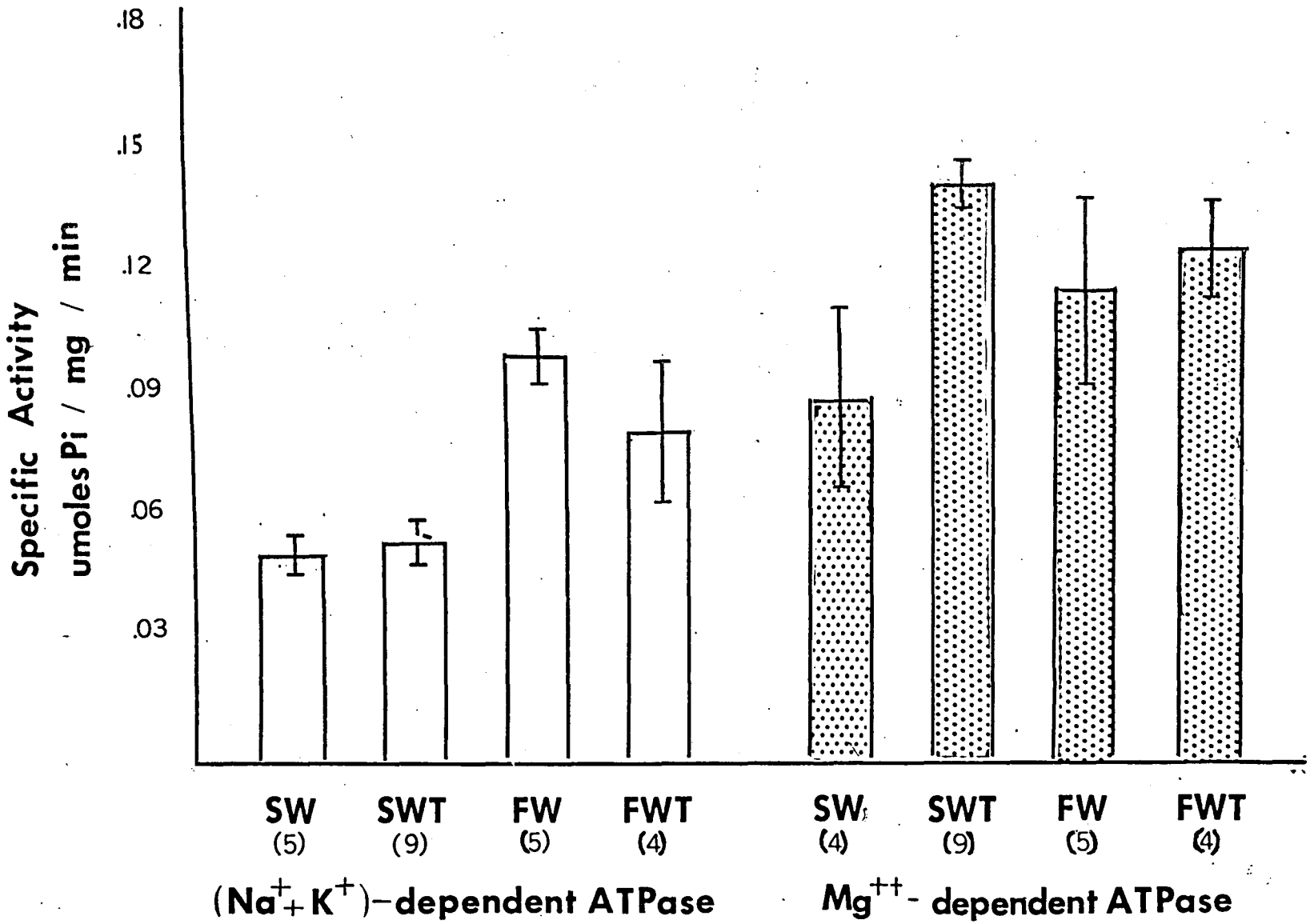
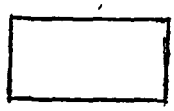
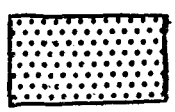
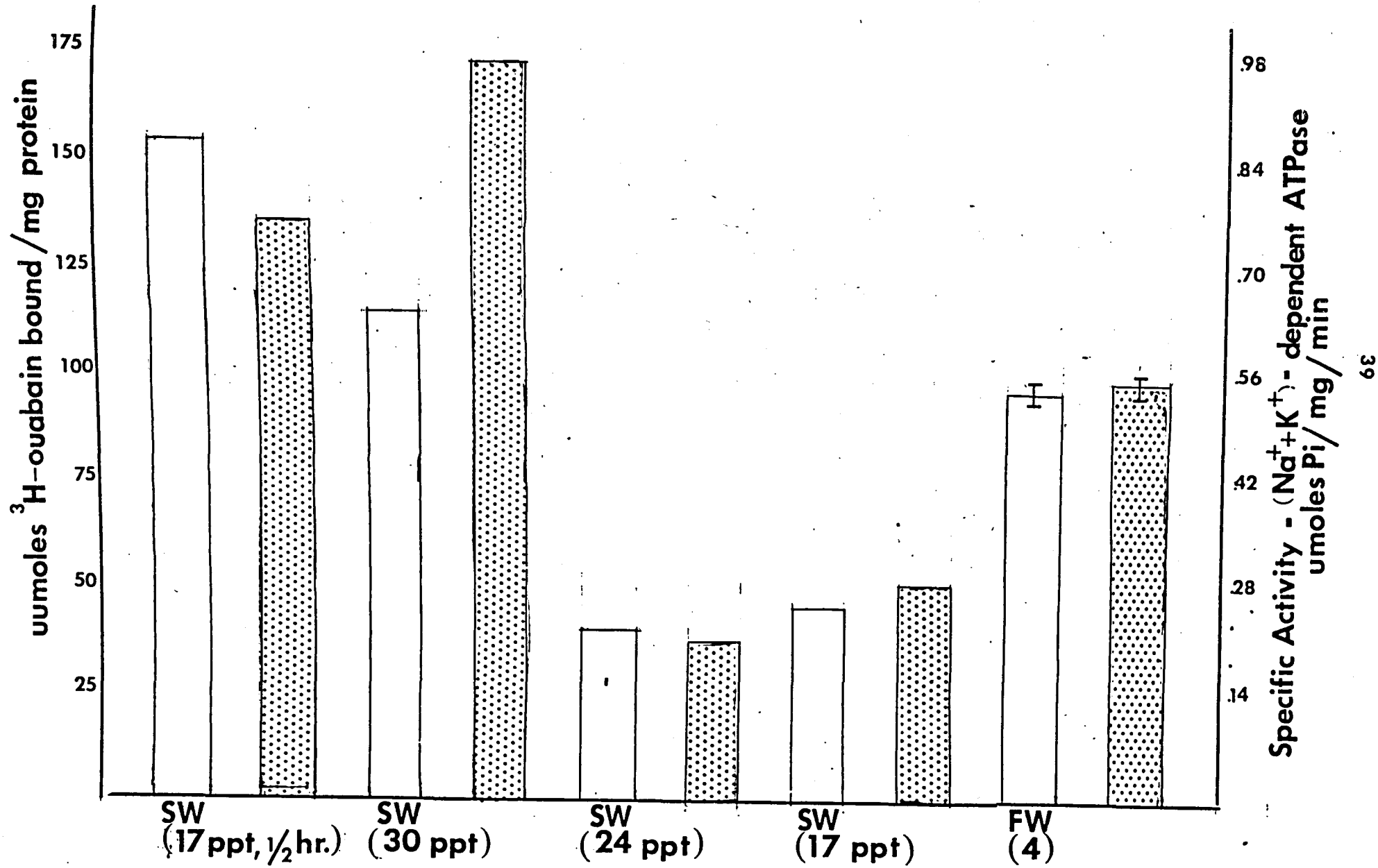

Specific Activity
umoles Pi / mg / min
.18
.15
.12
.09
.06
.03
SW
(5)
SWT
(9)
FW
(5)
FWT
(4)
SW
(4)
SWT
(9)
FW
(5)
FWT
(4)
(Na+ + K+)–dependent ATPase
Mg++ - dependent ATPase

Figure 2. uumoles of ^{3}H - ouabain bound per mg protein and specific activity of the $(Na^{+}+K^{+})$ - dependent ATPase in microsomal gill preparations of F. heteroclitus.

SW = salt water adapted fish
FW = freshwater adapted fish
( ) = salinity in ppt or number of different samples

 = uumoles ^{3}H - ouabain / mg protein

 = $(Na^{+}+K^{+})$ - dependent ATPase Specific Activity


umoles ^{3}H-ouabain bound/mg protein
175
150
125
100
75
50
25
Specific Activity - $(Na^{+}+K^{+})$- dependent ATPase
umoles Pi/mg/min
.98
.84
.70
.56
.42
.28
.14
SW (17 ppt, ½ hr.)
SW (30 ppt)
SW (24 ppt)
SW (17 ppt)
FW (4)

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VITA

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