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# In vitro effects of prolactin, cortisol, aldosterone, and cyclic amp on branchial Na+, K+ - activated ATPase of the killifish, Fundulus heteroclitus

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# IN VITRO EFFECTS OF PROLACTIN, CORTISOL, ALDOSTERONE, AND CYCLIC AMP ON BRANCHIAL $Na^+$, $K^+$ - ACTIVATED ATPASE OF THE KILLIFISH, FUNDULUS HETEROCLITUS

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ABSTRACT

*In vitro* effects of prolactin, cortisol, aldosterone, and cyclic adenosine monophosphate were studied on branchial $Na^+$, $K^+$ - activated ATPase of freshwater adapted killifish, *Fundulus heteroclitus*. Decreases in ATPase activity were found after treatment with prolactin and aldosterone. Cyclic AMP caused an increase in ATPase activity. The action of cortisol on this enzyme was not clear. A dose - response relationship was found only for cyclic AMP. It is proposed that cyclic AMP may be involved in the adaptation of *F. heteroclitus* to seawater.

# INTRODUCTION

The comparative physiology of salt and water homeostasis in fishes has long fascinated biologists interested in the mechanism of sodium transport, because the demands of the external environment are so stringent, and the evolutionary adjustments arrived at are so varied (Parry, 1966; Smith, 1930). The problem facing animal cells is that sodium enters the cell by diffusion (a passive process) with a concentration gradient and then must be extruded against that gradient (an active process). Potassium leaks from the cell with a concentration gradient and then must be actively reabsorbed (Fraser, 1970). This active process, known as the sodium pump, requires energy which is considered to be derived from a labile high energy phosphate group present in adenosine triphosphate (ATP), and which becomes available when ATP is broken down to adenosine diphosphate (Bentley, 1971). The release of energy from ATP and its use to drive the sodium pump is thought to be catalyzed by a specific adenosine triphosphatase (ATPase), activated by sodium and potassium (Skou, 1965). Sodium - potassium - activated adenosine triphosphatase ($Na^+$, $K^+$ - ATPase), first isolated from invertebrate nervous tissue by Skou in 1957, plays a key role in the active reciprocal transfer of sodium and potassium

across the plasma membrane of individual cells. There is increasing evidence that this enzyme is intimately involved in the secretion of sodium across teleost epithelial membranes in the kidney (Katz and Epstein, 1967; Pickford et al., 1970), the urinary bladder (Hirano et al., 1971; Lam, 1972), the intestine (Utida et al., 1972; Lam, 1972), and the gill (Epstein et al., 1967; Kamiya and Utida, 1969; Milne et al., 1971). The specific activity of $Na^+$, $K^+$ - ATPase in these tissues appears to be proportional to the level of sodium transport or pumping activity that the tissue would be expected to carry out in response to the osmotic stress placed on the animal by the environment (Jampol and Epstein, 1970).

Freshwater teleosts maintain a serum sodium level of about 140 - 160 mEq / liter by reducing the efflux of sodium to a minumum through the gill and kidney, by active inward transport of a small amount of sodium by the gills, and by excreting large quantities of water through the kidneys (Maetz, 1964; Parry, 1966; Potts, 1968; Jampol and Epstein, 1970). Consistent with these ion transport requirements, $Na^+$, $K^+$ - ATPase activity is low in the gills and high in the kidneys and renal tubules of freshwater teleosts such as the smallmouthed bass (Micropterus dolomieu) and the lake minnow (Notropis sp.) (Jampol and Epstein, 1970).

Saltwater teleosts maintain a serum sodium level of about 180 - 200 mEq / liter (Jampol and Epstein, 1970). Water lost by osmosis is replaced by drinking quantities of seawater varying from 7 to 40% of the body weight per day (Maetz and Skadhauge, 1968; Parry, 1966; Smith, 1930). The sodium contained in the ingested water is excreted by the gills against a concentration gradient (Parry, 1966; Maetz and Garcia - Romeu, 1964). In addition, the gills must excrete sodium that enters the body by diffusion across the gill surface (Maetz, 1964). Consistent with these ion transport requirements, $Na^+$, $K^+$ - ATPase activity is high in the gills and low in the kidneys and renal tubules of seawater teleosts such as the sea raven (Hemitripterus americanus), the long - horned sculpin (Myoxociphalus octodecimspinosus), and the goosefish (Lophius americanus) (Jampol and Epstein, 1970).

Ion influx and efflux are altered when these stenohaline teleosts are transferred to a different environmental salinity, disrupting their homeostasis and resulting in death. Some teleosts, referred to as euryhaline, can survive and adapt to abrupt changes in salinity (Zaugg and McLain, 1971). Such euryhaline teleosts are of special interest because of their physiological responses to varying salinities through changes in enzymatic activity. Jampol and Epstein (1970) reported that the activity of $Na^+$, $K^+$ - ATPase in gill filaments and intestinal mucosa doubles when the American eel (Anguilla rostrata) is adapted to seawater.

Sodium - potassium - ATPase activity increases fourfold in the gills of the Japanese eel (Anguilla japonica), the rainbow trout (Salmo gairdnerii irideus), and the goby (Acanthogobius flavimanus) when these fishes are transferred from freshwater to seawater (Jampol and Epstein, 1970). In the killifish, Fundulus heteroclitus, the ATPase activity in the gills increases during adaptation to seawater (Epstein et al., 1967), while it decreases in the kidneys (Epstein et al., 1969). Towle and Gilman (1973) also found that the $Na^+$, $K^+$ - ATPase activity in the gills of F. heteroclitus increases during adaptation to 30 ppt salt water and reaches a constant level within one - half hour after transfer from freshwater.

Osmoregulation in teleosts as well as other animals during adaptation to varying salinities has been shown to involve the endocrine system. The pituitary gland seems to play an important role in the osmoregulation of teleosts in freshwater. Burden (1956) showed that hypophysectomy destroyed the ability of F. heteroclitus to survive in freshwater, and speculated that prolactin or some unknown adeno - hypophysial factor is necessary for osmoregulation in freshwater. In later investigations, it was discovered that prolactin is the freshwater survival factor missing in the hypophysectomized killifish (Pickford and Phillips, 1959). Since these investigations, mammalian prolactin (usually ovine) has been used to restore freshwater tolerance

in the following hypophysectomized teleosts: the sailfin molly (Poecilia latipinna) (Ball and Olivereau, 1964; Pickford et al., 1966), the moonfish (Xiphophorus maculatus) (Schreibman and Kallman, 1966; Pickford et al., 1966), the mosquitofish (Gambusia sp.) (Chambolle, 1966), the cichlid (Tilapia mossambica) (Dharmamba et al., 1967; Pickford et al., 1966), and the medaka (Oryzias latipes) (Utida et al., 1971). Prolactin is essential for life in freshwater in the above species. However, after hypophysectomy, prolactin is not essential for life in the plains killifish (Fundulus kansae) (Stanley and Fleming, 1966), the European eel (Anguilla anguilla) (Maetz et al., 1967), the flounder (Platichthys flesis) (MacFarlane, 1971), and the goldfish (Carassius auratus) (Lahlou and Sawyer, 1969). Nevertheless, without prolactin, some osmoregulatory impairments occur, which may be corrected by prolactin treatment.

The pituitary - interrenal (adrenal) axis seems to play the important role in the osmoregulation of teleosts in seawater. Hypophysectomy or interrenalectomy reduces the sodium exchange rate as well as the net sodium efflux in the eel gill, and adrenocorticotropic hormone or cortisol restores them to the normal level (Mayer et al., 1967; Maetz, 1969). Cortisol has been shown to be involved in seawater adaptation in A. anguilla, and A. japonica (Maetz, 1969; Chester - Jones et al., 1969), A. rostrata (Epstein et al., 1971; Butler and Carmichael,

1972), C. auratus (Lahlou and Giordan, 1970), and F. heteroclitus (Epstein et al., 1967; Pickford et al., 1970).

The relationship between $Na^+$, $K^+$ - ATPase activity and hormonal control has not been well defined and recent investigations have attempted to study this correlation. An increased concentration of plasma cortisol during seawater adaptation was found in A. rostrata (Forrest et al., 1973), A. japonica (Hirano, 1969), and A. anguilla (Ball et al., 1971). This burst of cortisol secretion occurs shortly after the eels make their first contact with seawater and plays an important role in conditioning the animal to withstand the osmotic stress (Forrest et al., 1973). In A. rostrata, hypophysectomy prevents the increase in ATPase activity observed upon transfer of control fish to seawater; the enzyme activity level can be restored by cortisol (Forrest et al., 1973; Epstein et al., 1971; Butler and Carmichael, 1972). Cortisol injections into intact freshwater A. japonica were found to induce a rise in the branchial $Na^+$, $K^+$ - ATPase activity (Kamiya, 1972). This increase in enzyme activity produced by cortisol is of special interest because it is most unlikely that transport of sodium across the gills was increased while the fish were in freshwater. Thus increased ATPase activity is not due to a secondary adaption of the enzyme to a primary increase in $Na^+$ transport as was previously speculated (Maetz, 1969). Epstein et al. (1971) observed striking

and unexpected pigmentation changes in specimens of A. rostrata that were injected with cortisol for more than 7 to 10 days. Such fish lost their yellow color and became silver, resembling the silver hue of A. anguilla migrating to seawater.

If cortisol exerts a direct effect on the gills of the eel, then one might expect that this hormone would be bound to the gills, as found in other cases of hormones binding to target tissues. Goodman and Butler (1972) found that gills of freshwater A. rostrata do bind large amounts of injected radioactive cortisol. Towle and Gilman (1973) showed control of the ATPase activity during adaptation of F. hetero - clitus to 30 ppt salt water does not depend on synthesis of new enzyme molecules and that control must occur by activation of preexisting enzyme. On the basis of studies cited above, they speculated that cortisol may be involved in the direct regulation of ATPase activity.

Aldosterone, like cortisol, is another hormone produced in the adrenal cortex and functions as a mineralocorticoid by affecting electrolyte metabolism. Most work with aldosterone was done on in vitro toad bladder in which aldosterone was shown to increase sodium transport (Crabbe, 1961), and on in vivo rat kidney where results were controversial. It is well established that the activity of the ATPase in membrane fragments of kidney tissue is reduced after adrenalectomy (Chignell and Titus, 1966; Jorgensen, 1968; Katz and Epstein, 1967;

Landon et al., 1966). However, mineralocorticoids were found to have no effect on ATPase activity in some cases (Chignell and Titus, 1966; Landon et al., 1966), while increasing activity in other cases (Jorgensen, 1968). There is little information on aldosterone as it effects sodium transport in teleosts and the results of the investigations are of conflicting nature (Mayer and Maetz, 1967; Favre, 1960; Motais, 1967; Henderson and Chester - Jones, 1967; Holmes et al., 1963).

Increasing evidence is now available to support the hypothesis that many hormones bring about intracellular effects through the mediation of a second messenger that has been shown to be 3', 5' - cyclic adenosine monophosphate (cyclic AMP) (Sutherland et al., 1968; Turner and Bagnara, 1971; Jost and Rickenberg, 1971). Through the action of adenyl cyclase, ATP is converted to cyclic AMP, which then acts within the effector cell to produce the appropriate hormonal response. Thus, specific hormonal events can possibly be mimicked in the effector system by the application of cyclic AMP. It has been established that cyclic AMP mimics the actions of antidiuretic hormone on the permeability of the toad urinary bladder to water and sodium (Orloff and Handler, 1965). Bastide and Jard (1968) also showed that cyclic AMP mimics the action of adrenalin by increasing sodium transport across the amphibian skin.

This investigation was undertaken to determine if the *in vitro* effects of prolactin and cortisol on the branchial $Na^+$, $K^+$ - activated ATPase activity in freshwater adapted *F. heteroclitus* concur with the *in vivo* responses found in the articles discussed above. The effects of aldosterone on ATPase activity were of interest because little work has been done using aldosterone on teleosts. Experiments with cyclic AMP were included to determine if it might be involved in the regulation of branchial ATPase activity.

## METHODS AND MATERIALS

### General

The Fundulus heteroclitus used in these experiments were adult males, collected from Mobjack Bay at Severn, Virginia. The fish were kept in an 80 - gallon Living Stream aquarium filled with tap water, which was constantly filtered and maintained at a temperature of 18 C. Overhead fluorescent laboratory lights served as the light source and were set for a 12 hour daylight period. Fish were fed Tetramin Fish Food daily. The fish were adapted to freshwater for at least two weeks before experimentation.

### Gill Microsome Preparation

Microsomes were prepared according to a modified procedure of Hendler, et al. (1972). Groups of four to six specimens were sacrificed on various days and at approximately the same time of day. After decapitation, gills were excised and rinsed in cold homogenizing solution (0.25 M sucrose, 6 mM ethylenediamine tetraacetic acid, and 20 mM imidazole pH 6.8) and blotted. The gill tissue was then removed surgically from the cartilaginous arches and pooled to give the 500 - 800 mg gill tissue weight required for preparation of the microsomal fraction. The gill preparation was maintained at 0 - 4 C throughout

the rest of the procedure. To each 100 mg of tissue, 2 ml of homogenizing solution containing 0.1% (w/v) sodium deoxycholate was added. The tissue was homogenized in a Potter - Elvehjem apparatus with a smooth Teflon pestle for 18 strokes at 1,725 rpm. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 10,800 x g at 0 C for 35 minutes. The supernatant was carefully removed and centrifuged at 105,000 x g at 0 C for 60 minutes. The microsomal pellet was resuspended in 0.3 ml of homogenizing solution per 100 mg original gill weight and homogenized for three strokes at 1,725 rpm. This microsomal preparation was frozen and maintained at -20 C until used for the measurement of $Na^+$, $K^+$ - ATPase activity.

ATPase Assay

The assay procedure of Epstein et al. (1967) as modified by Gilman (1973) was used to measure the $Na^+$, $K^+$ - activated ATPase activities in the microsomal fractions of the gills of F. heteroclitus. Total ATPase activity was measured in a medium containing 20 mM imidazole - HCl (pH 7.8), 30 mM KCl, 100 mM NaCl, and 0.1 ml of the gill microsome preparation. To this mixture was added prolactin (7.5 IU) or varying concentrations ($10^{-7}$ - $10^{-3}$ M) of cortisol, aldosterone, or cyclic AMP. The ouabain - insensitive activity was measured in a mixture containing the same ingredients as the total ATPase activity mixture except that 130 mM NaCl and 1.0 mM ouabain were substituted for 30 mM KCl and

100 mM NaCl. All tests were run in duplicate. The tubes were incubated in a water bath at 25 C for five minutes. The reaction was then initiated by the addition of 0.2 ml of 50 mM ATP and 50 mM $MgCl_2$ (adjusted with Tris to pH 7.0), making a final reaction volume of 2.0 ml. All tubes were vortexed. The mixture was then incubated at 25 C for exactly 30 minutes after which the reaction was stopped by the addition of 2.0 ml of 10% trichloroacetic acid. The tubes were placed in an ice bath for 10 minutes and then centrifuged at 10,000 x g at 0 C for 10 minutes. Inorganic phosphate (Pi) in the supernatant was then measured by the Fiske - SubbaRow (1925) method. Protein concentrations of the microsome preparations were measured by the Lowry et al. (1951) method. The ouabain - sensitive $Na^+$, $K^+$ - activated ATPase specific activity was defined as the difference between the total enzymatic activity (without ouabain) and the $Mg^{++}$ - dependent, ouabain - insensitive ATPase activity (with ouabain). The specific activity of the enzyme was expressed as u moles of Pi released from ATP per mg of protein per minute.

## RESULTS AND DISCUSSION

Prolactin injections have been shown to reduce sodium efflux by decreasing the activity of $Na^+$, $K^+$ - activated ATPase in hypophysectomized F. heteroclitus (Pickford et al., 1970; Utida et al., 1971), A. japonica (Kamiya, 1972), and F. kansae (Fleming and Ball, 1972) which had just been transferred from seawater to freshwater. This suggests that prolactin is involved in freshwater adaptation, an idea that is widely accepted. However, the effects of prolactin on fish already adapted to freshwater are not clear. Although Stanley and Fleming (1967) found that prolactin injections had no effect on ATPase activity in intact freshwater adapted F. kansae, Fleming and Ball (1972) found that a higher dose of prolactin reduced enzyme activity in such fish which had been hypophysectomized.

In the present study, prolactin reduced branchial $Na^+$, $K^+$ - activated ATPase of freshwater adapted killifish to 79% and 85% of the controls in two different experiments (Table 1). There was no change in activity in a third experiment, possibly due to the low activity already present. The results presented here compare favorably with the effects observed in vivo by Fleming and Ball (1972).

In seawater adapted F. heteroclitus, prolactin injections decrease

branchial ATPase activity (Mayer, 1970) but have no effect on enzyme activity in A. japonica (Kamiya, 1972) or A. anguilla (Mayer, 1970). The ATPase of F. heteroclitus thus appears to be more sensitive to prolactin control than the enzyme from other species.

Injections of cortisol into intact freshwater adapted A. japonica induce an increase in branchial $Na^+$, $K^+$ - ATPase activity (Kamiya, 1972). Several investigators have reported similar increases in ATPase activity in hypophysectomized seawater adapted teleosts injected with cortisol (Epstein et al., 1967; Epstein et al., 1971; Pickford et al., 1970). It was thus hypothesized that cortisol might increase enzyme activity in the present in vitro studies. As presented in Table 2, cortisol produced an increase and decrease in branchial ATPase activity of freshwater adapted killifish and on one occasion had no effect. The reason for these conflicting results are unknown. In some instances, it has been reported that such varying results may occur due to differences in animal husbandry, experimental techniques, stress in handling, seasonal variations, or differences in the natural adaptation of the fish (Milne et al., 1971; Lam, 1972). In any case, the present study offers no evidence to support a direct effect of cortisol on $Na^+$, $K^+$ - ATPase activity.

Administration of aldosterone has been shown to increase (Henderson and Chester - Jones, 1967) or decrease (Holmes et al, 1963)

active sodium absorption by gills of freshwater adapted eels. It was found in the present study that aldosterone decreases gill $Na^+$, $K^+$ - ATPase activity of freshwater adapted killifish (Table 3), suggesting that this hormone may reduce sodium influx *in vivo*. Although no dose - response relationship was found, aldosterone decreased enzyme activity in every case, ranging from 95% to 50% of the control.

The concentration of aldosterone is very low (about $10^{-8}$ M) in the circulation of some freshwater teleosts (Chavin and Singley, 1972) and has not been found in many other fishes (Bentley, 1971). In the experiments presented here, the aldosterone concentrations used ($10^{-7}$ to $10^{-3}$ M) may thus have been too high to give an accurate picture of the *in vivo* situation. Within this concentration range, however, aldosterone appears to have a direct effect on $Na^+$, $K^+$ - ATPase activity. The only previous indication of such a direct effect was obtained by Hendler *et al.* (1972) with preparations from mammalian kidney.

Cyclic AMP has been shown to increase sodium transport in toad bladder (Kirchberger *et al.*, 1971; Orloff and Handler, 1965; Sugita *et al.*, 1973) and amphibian skin (Bastide and Jard, 1968). In addition, Riddick *et al* (1971) found that dibutyryl cyclic AMP increases potassium transport in duck erythrocytes. No studies have been done,

however, on the direct effect of cyclic AMP on $Na^+$, $K^+$ - ATPase. The results reported here clearly show that cyclic AMP stimulates branchial $Na^+$, $K^+$ - ATPase from freshwater adapted F. heteroclitus (Table 4 and Figure 1). Maximal stimulation (158% of the control) was obtained with $10^{-3}$ M cyclic AMP, and a clear dose - response relationship was observed in two out of three experiments (Figure 1).

Cyclic AMP is known to activate several enzymatic processes involving protein phosphorylation (Jost and Rickenberg, 1971). One of the steps in the reaction mechanism of $Na^+$, $K^+$ - ATPase requires the phosphorylation of a membrane protein (Bader et al., 1968). The stimulation of ATPase activity by cyclic AMP, as reported here, is consistent with these observations.

The actions of many hormones appear to be mediated by cyclic AMP (Jost and Rickenberg, 1971). Whether cyclic AMP mediates the in vivo action of a hormone on sodium transport in the gills of F. heteroclitus requires further investigation.

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Table 1. Effect of prolactin (7.5 IU) on $Na^+$, $K^+$ - activated ATPase activity in gill microsomes of freshwater adapted Fundulus heteroclitus.

| Control Activity (umoles Pi/mg/min) | Activity with Hormone (umoles Pi/mg/min) | % of Control |
|---|---|---|
| .525 | .415 | 79 |
| .274 | .274 | 100 |
| .418 | .355 | 85 |

Table 2. Effect of cortisol on $Na^+$, $K^+$ - activated ATPase activity in gill microsomes of freshwater adapted Fundulus heteroclitus.

| Experiment 1 | Cortisol Conc (M) | $Na^+$, $K^+$ - ATPase Activity (umoles Pi/mg/min) | % of Control |
|---|---|---|---|
| | 0 | .301 | 100 |
| | $10^{-7}$ | .167 | 55 |
| | $10^{-6}$ | .301 | 100 |
| | $10^{-5}$ | .267 | 89 |
| | $10^{-4}$ | .267 | 89 |
| | $10^{-3}$ | .267 | 89 |
| Experiment 2 | | | |
| | 0 | .753 | 100 |
| | $10^{-7}$ | 1.050 | 139 |
| | $10^{-6}$ | .904 | 120 |
| | $10^{-5}$ | .602 | 80 |
| | $10^{-4}$ | .979 | 130 |
| | $10^{-3}$ | 1.050 | 139 |

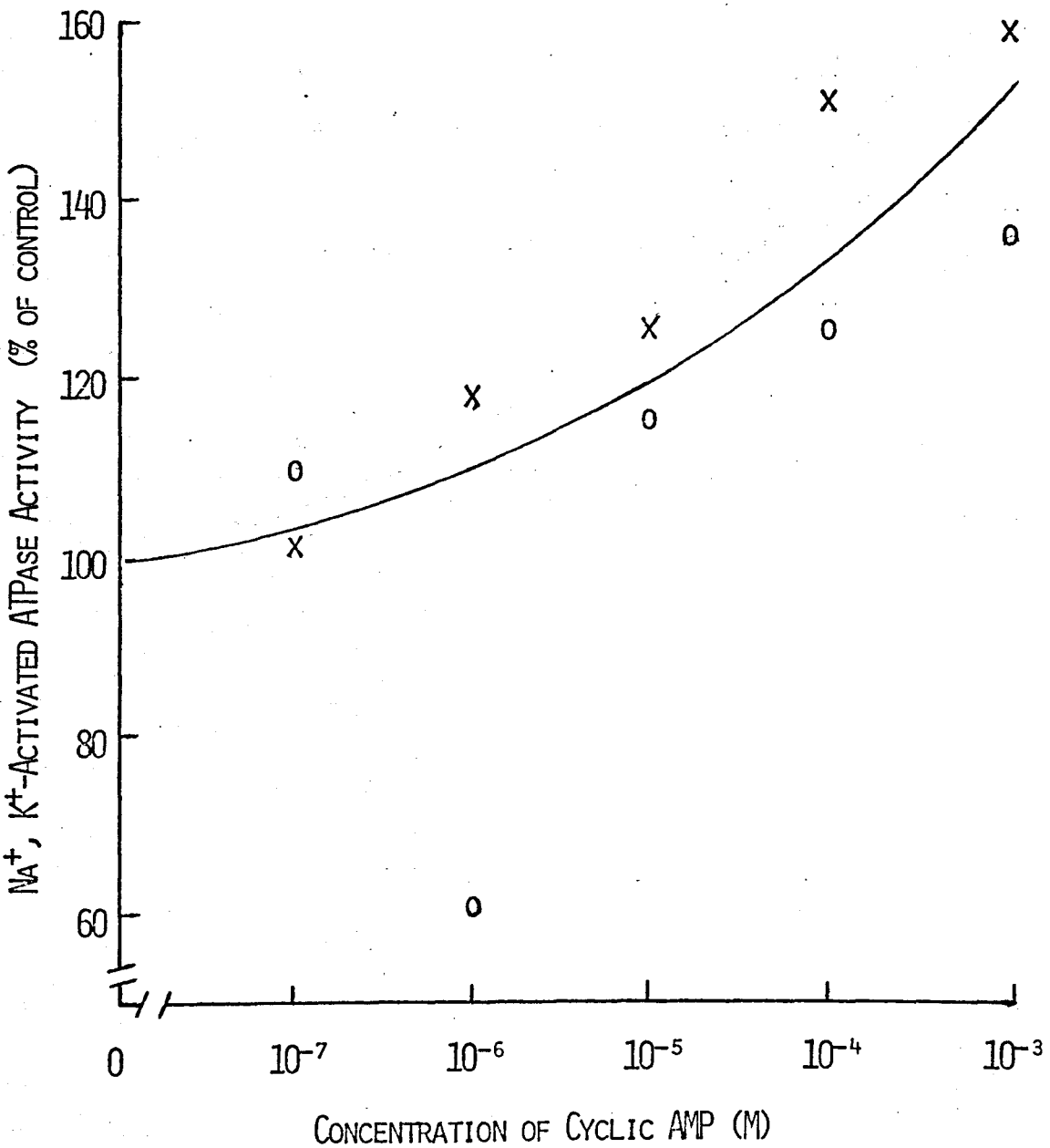
Table 3. Effect of aldosterone on $Na^+$, $K^+$ - activated ATPase activity in gill microsomes of freshwater adapted Fundulus heteroclitus.

| Experiment 1 | Aldosterone Conc (M) | $Na^+$, $K^+$ - ATPase Activity (umoles Pi/mg/min) | % of Control |
|---|---|---|---|
| | 0 | .335 | 100 |
| | $10^{-7}$ | .169 | 50 |
| | $10^{-6}$ | .246 | 73 |
| | $10^{-5}$ | .231 | 69 |
| | $10^{-4}$ | .277 | 83 |
| | $10^{-3}$ | .262 | 78 |
| Experiment 2 | | | |
| | 0 | .726 | 100 |
| | $10^{-7}$ | .630 | 87 |
| | $10^{-6}$ | .458 | 63 |
| | $10^{-5}$ | .687 | 95 |
| | $10^{-4}$ | .687 | 95 |
| | $10^{-3}$ | .687 | 95 |

Table 4. Effect of cyclic AMP on $Na^+$, $K^+$ - activated ATPase activity in gill microsomes of freshwater adapted Fundulus heteroclitus.

| Experiment 1 | Cyclic AMP Conc (M) | $Na^+$, $K^+$ - ATPase Activity (umoles Pi/mg/min) | % of Control |
|---|---|---|---|
| | 0 | .320 | 100 |
| | $10^{-7}$ | .352 | 110 |
| | $10^{-6}$ | .192 | 60 |
| | $10^{-5}$ | .368 | 115 |
| | $10^{-4}$ | .400 | 125 |
| | $10^{-3}$ | .432 | 135 |
| Experiment 2 | | | |
| | 0 | .795 | 100 |
| | $10^{-7}$ | .795 | 100 |
| | $10^{-6}$ | .663 | 83 |
| | $10^{-5}$ | .596 | 72 |
| | $10^{-4}$ | .861 | 108 |
| | $10^{-3}$ | .954 | 120 |
| Experiment 3 | | | |
| | 0 | .428 | 100 |
| | $10^{-7}$ | .428 | 100 |
| | $10^{-6}$ | .500 | 117 |
| | $10^{-5}$ | .535 | 125 |
| | $10^{-4}$ | .642 | 150 |
| | $10^{-3}$ | .678 | 158 |

Figure 1. The dose - response curve for Experiments 1 (O) and 3 (X) on the effects of cyclic AMP on *in vitro* $Na^+$, $K^+$ - activated ATPase activity in gill microsomes of freshwater adapted *Fundulus heteroclitus*.


160
140
120
100
80
60
0
$10^{-7}$
$10^{-6}$
$10^{-5}$
$10^{-4}$
$10^{-3}$
$Na^+$, $K^+$-Activated ATPase Activity (% of control)
Concentration of Cyclic AMP (M)

## VITA

Stephen Burke Kerby was born on November 11, 1949 in Washington, D. C. He attended parochial school in Arlington County, Virginia, and public schools in Loudoun County, Virginia, and was graduated from Loudoun County High School in June 1967. He received an Associate of Science degree from Northern Virginia Community College in June 1969, and his Bachelor of Science degree from George Mason University in December, 1971, with a major in biology. He entered the University of Richmond Graduate School in February, 1972. While at Richmond, he became a member of Beta Beta Beta Honorary Biological Society. He completed the requirements for the Master of Science degree from the University of Richmond in May 1974.