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ROLE OF GILL CALCIUM-ATPASES IN BLOOD CALCIUM REGULATION IN THE KILLIFISH, (FUNDULUS HETEROCLITUS)

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ROLE OF GILL CALCIUM-ATPases IN BLOOD CALCIUM REGULATION IN THE KILLIFISH, (*FUNDULUS heteroclitus*)

by

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Abstract

ROLE OF GILL CALCIUM-ATPases IN BLOOD CALCIUM REGULATION IN THE KILLIFISH, (FUNDULUS heteroclitus)

by

Mary Jane Frances Paul

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Killifish, a euryhaline fish has the ability to maintain a constant blood calcium level in both seawater and freshwater environments. These fish face a constant calcium challenge which is closely related to the external media. Seawater-adapted fish are hypo-osmotic to the environment and have the problem of Ca^{2+} overload. Freshwater-adapted fish are hyperosmotic to the environment and have the problem of Ca^{2+} loss

Preliminary studies showed the presence of two Ca-ATPases in the gill epithelium microsomal fraction from killifish; Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase.

Characterization of the two Ca-ATPases revealed: 1) A Ca^{2+}+Mg^{2+}-ATPase which has a high affinity for Ca^{2+}, requires Mg^{2+} for activity and may be controlled by calmodulin; and 2) A Ca^{2+}+Na^{+}-ATPase which has a low affinity for Ca^{2+}, requires Na^{+} for activity, does not require Mg^{2+} and is probably not controlled by calmodulin.

Environmental calcium specifically controls the activities of the killifish branchial epithelium Ca-ATPases. The experiments provided evidence that the activity of Ca^{2+}+Mg^{2+}-ATPase was significantly higher in killifish adapted to freshwater and calcium-deficient seawater - both low calcium environments. On the other hand, Ca^{2+}+Na^{+}-ATPase activity was significantly higher in killifish adapted to seawater and calcium-enriched freshwater - both high calcium environments. These finding suggest that one gill epithelium Ca-ATPase is pumping calcium from the environment into fish adapted to a low calcium environment while the other gill epithelium Ca-ATPase is pumping calcium out of fish adapted to a high calcium environment.

The interrelationship between changes in plasma Ca^{2+} and Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities was investigated. The results indicated two types of mechanisms are involved in killifish Ca^{2+} regulation when killifish are transferred to a new environment. One is a fast adaptation mechanism which appears to occur within a few days of transfer not involving changes in Ca-ATPase activity and the other is a slow adaptation mechanism which appears to occur within two weeks following transfer and does not involve changes in enzyme activity.

It appears that the branchial epithelium Ca-ATPases are under the influence of the Ca^{2+} regulating hormones (pituitary gland and corpuscles of Stannius). Killifish adapted to seawater and injected with cod pituitary

homogenate had significantly decreased branchial Ca^{2+}+Na^{+}-ATPase activity and significantly increased gill epithelium Ca^{2+}+Mg^{2+}-ATPase activity. Killifish adapted to seawater and injected with ovine prolactin also had significantly decreased gill epithelium Ca^{2+}+Na^{+}-ATPase activity but there was no effect on branchial Ca^{2+}+Mg^{2+}-ATPase activity. These results suggest that Ca^{2+}+Na^{+}-ATPase activity is decreased by prolactin while Ca^{2+}+Mg^{2+}-ATPase activity is not affected by prolactin but may be stimulated by another hypercalcemic factor(s) in the pituitary gland.

Killifish adapted to calcium-deficient seawater and injected with cod corpuscles of Stannius homogenate had a significantly decreased Ca^{2+}+Mg^{2+}-ATPase activity while Ca^{2+}+Na^{+}-ATPase activity was significantly increased.

The data in this thesis provides a structure to support continuation of investigation of the role that Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase from gill epithelium microsomal fraction from seawater and freshwater-adapted killifish play in regulating blood Ca^{2+} levels.

This thesis is dedicated in
loving memory of my mother
and father,
Gertrude Burg Colella and
Charles L. Burg

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The satisfaction of the completion of the research and writing of this thesis must be shared by many. If I leave someone out, I hope he (she) will understand that his (her) contribution too, has been greatly appreciated.

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INTRODUCTION

The ability of euryhaline fish to maintain a constant serum level of calcium in both seawater and freshwater environments has been a subject of investigation for many years.

These fish face a constant calcium challenge which is closely related to the external medium. In seawater the calcium level may be four or more times higher than the fish's blood while the calcium level in soft freshwater may be one tenth that of the fish's body fluid. Seawater-adapted fish, therefore, face the problem of getting rid of excess calcium entering from external sources and of limiting its rate of entrance. Freshwater-adapted fish on the other hand, are challenged with obtaining calcium from the low calcium environment and prevention of calcium loss. Euryhaline fish are, however, successful in maintaining a constant blood calcium level under different environmental conditions.

The problems of calcium regulation in euryhaline fish are different than the problems encountered by terrestrial vertebrates. The main features of terrestrial vertebrate calcium metabolism are the absorption of calcium from food, the prevention of calcium loss from the body and the ability to mobilize calcium from bone in times of need. Fish on the other hand, respire through gills that are in constant contact with surrounding water, a reservoir of calcium which is readily available and virtually inexhaustible. Therefore, the calcium regulatory mechanism in the aquatic vertebrates might be expected to be different from those of

the terrestrial vertebrates.

Terrestrial vertebrates control body calcium levels through regulatory mechanisms involving intestine, kidney and bone. In fish, regulation occurs at the gills, kidney, intestine and in some fish, bone.

There are specific endocrine calcium regulating systems found in the vertebrates. These systems include: vitamin D-metabolizing tissues, the parathyroid gland, calcitonin-producing cells, the pituitary gland, the ovaries and the Stannius corpuscles.

A vitamin D-metabolizing system has been reported in mammals, birds, reptiles, amphibians and teleosts, but has not been identified in cyclostomes and elasmobranchs. Parathyroid glands are found in all tetrapods except aquatic urodeles and are completely absent from fish. Calcitonin-producing cells are found in the ultimobranchial glands in all vertebrate groups with the exception of the mammals. In mammals the calcitonin-producing cells are the thyroid "C" cells. The pituitary gland appears to play a role in calcium regulation in teleosts, aquatic urodeles and birds. There is a relationship between calcium regulation and vertebrate reproduction which exists predominantly in females. Estrogen has been demonstrated to induce hypercalcemia in fish, amphibians, reptiles and birds. Stannius corpuscles are found only in some bony fish.

I. Endocrine control of calcium in terrestrial vertebrates

A. Hormones with hypercalcemic effects

The following hormones have a hypercalcemic effect in terrestrial vertebrates (Figure 1).

1. Parathyroid hormone

In 1925, Collip reported the function of the parathyroid gland.

Parathyroid hormone has a pronounced hypercalcemic effect in terrestrial vertebrates. Parathyroidectomy produces hypocalcemia and tetany in dogs, cats and rats. Administration of parathyroid hormone (PTH) corrects this condition (Turner and Bagnara, 1976).

Hypocalcemia stimulates the rate of synthesis of prepro-PTH (1-115). the earliest biosynthetic product in parathyroid cells (Kemper et al., 1976; Habener et al., 1978). Prepro-PTH appears to be converted to pro-PTH (1-90) after completion of its synthesis on the rough endoplasmic reticulum (Habener et al., 1977). Pro-PTH is converted to PTH (1-84) by proteolytic cleavage within about 20 minutes after its first appearance in the tissue (Chu et al., 1973); this conversion takes place at the Golgi region (Habener et al., 1979).

The main targets for PTH are kidney and bone. At the kidney. parathyroid hormone prevents urinary calcium loss by increasing reaborption of calcium in the distal convoluted tubule (Agus et al., 1973).

PTH regulates the function of all known bone cell types (Rasmussen and Bordier, 1974). Osteoclasts, the multi-

nucleated agents of bone resorption, arise by fusion of mononuclear precursors which are derivatives of a hemopoietic stem cell (Horton et al., 1985). PTH facilitates this fusion of the mononuclear cells into multinucleate cells (Feldman et al;, 1980). There are changes in the cell surfaces and the osteoclasts are then said to present "ruffled borders" to resorption sites (Miller, 1978).

The bone-forming cell, the osteoblast, arises from local mesenchyme (Vaughan, 1981). PTH limits the growth of the cells and the synthesis of bone matrix (Talmage et al., 1975; Wong et al., 1978).

PTH converts "osteogenic osteocytes" to "osteolytic osteocytes" and along with PTH-invoked biochemical cellular changes in the "osteolytic osteocytes" promotes transfer of calcium from bone to blood plasma (Luben and Cohn, 1976).

Parathyroid hormone has a hypercalcemic function in nonmammalian vertebrates as well. Parathyroidectomy produces hypocalcemia in chickens ans ducks (Riddle and McDonald, 1945), which can be reversed by injections of mammalian parathroid hormone (Candlish and Taylor, 1970; Gonnerman et al., 1975; Clark and Wideman, 1977). During eggshell calcification in the chicken, PTH levels are elevated and after completion of the shell PTH levels fall to a low level but rise again 2 hours after ovulation (Van DeVelde et al., 1984).

In reptiles, hypocalcemia results from parathyroidectomy in lizards (Clark, 1968a; Clark et al., 1969) and snales (Oguro, 1970, 1972; Clark, 1971) and is corrected by injetion of mammalian parathyroid hormone. In two species of turtles, Geoclemys reevesii and Testude graeca, parathyroidectomy resulted in a fall of plasma calcium levels to 60% of control animals (Oguro and Tomisawa, 1972). However, in two other species of parathyroidectomized turtles, Chrysemys picta and Pseudemys scripta hypocalcemia was not observed (Clark, 1965).

Parathyroidectomy reduces plasma calcium levels in anuran amphibians which seems to be the result of a decreased rate of calcium resorption from bone (Cortelyou, 1967). Injections of parathyroid hormone have a hypercalcemic effect (Bentley, 1982).

2. Vitamin D-metabolizing system

The vitamin D-metabolizing system has been most extensively studied in mammals. Hypocalcemia increases the production of vitamin D ($1,25(OH)_2D$) while hypercalcemia decreases its production (Bentley, 1982).

There are two main sources of vitamin D: 1) dietary vitamin D_2 is absorbed in the proximal small intestine via the intestinal lymphatics, a process requiring bile acids (Bell, 1985); 2) previtamin D_3 is synthesized photochemically in the skin from 7-dehydrocholesterol. It has been found in man that 85-90% of the vitamin D pool is probably derived from solar irradiation, indicating sun-

light is the key determinant of vitamin D status (Haussler and Brickman, 1982).

After synthesis of previtamin D_3 in the skin, a temperature-dependent isomerization of previtamin D_3 to vitamin D_3 occurs over several days. The vitamin is removed by way of the dermal capillaries by vitamin D-binding protein (Bell, 1985). Vitamin D is metabolized in the liver to 25-hydroxyvitamin D (25-(OH)D) and the final enzymatic conversion to $1,25(OH)_2D$ occurs in the kidney. This activation of vitamin D_3 is catalyzed by the renal enzyme 25-hydroxyvitamin D-1a-hydroxylase (Fraser and Kodicik, 1970). This metabolite satisfies the criteria for being the functional form of vitamin D_3. When administered to animals that have received a vitamin D-deficient diet, it prevents rickets and maintains normal mineral metabolism (McNutt and Haussler, 1973). $1,25-(OH)_2D$ is at least five times as biologically active as vitamin D_3 or 25(OH)D and it functions three times faster than either of its precursors in promoting calcium absorption (Haussler et al., 1971).

One effect of vitamin D_3 is to facilitate calcium absorption from the intestine (Wasserman and Taylor, 1973). Vitamin D-dependent calcium absorption takes place primarily in villus cells of rat duadenum (Van Corven et al., 1985). Calcium absorption across the intestinal villus cells is highest in the mid to upper villus as compared to villi tip cells and crypt base cells

(Van Corven et al., 1985). The net result of the action of vitamin D on the gut is the raising of serum calcium (Haussler and Brickman, 1982).

Bone is another target tissue for vitamin D. Vitamin D functions to mobilize bone calcium. Here too, $1,25(OH)_2D$ is the active metabolite (Bentley, 1982). There is some evidence that PTH is required for the action of $1,25(OH)_2D$ to mobilize bone calcium in vivo (DeLuca, 1978), but Reynolds et al., (1976) found that PTH is not necessary for this effect in mice. The metabolites of vitamin D do not require the presence of PTH to resorb bone calcium in mice organ culture (Reynolds et al., 1976). It is therefore not certain whether $1,25(OH)_2D$ and PTH function independently or synergistically to mobilize skeletal calcium.

Vitamin D may also have a direct action on renal handling of calcium. Decreases in the urinary excretion of calcium have been observed following the administration of pharmacologic doses of vitamin D to thyroid-parathyroidectomized dogs (Gran, 1960).

The plasma transport proteins for 25-hydroxyvitamin D have been identified in birds and amphibians (Hayes and Watson, 1976); and the renal enzyme that converts 25-hydroxyvitamin D to its dihydroxy hormonally active form has been detected in reptiles and amphibians. However, the presence of the renal enzyme does not necessarily mean the existence of a functional endocrine

system and until the hormone can be demonstrated in the circulation in response to physiological needs then the physiological significance of the active metabolite can not be evaluated (Pang et al., 1980). The only direct evidence for a physiological function of vitamin D in lower vertebrates is that vitamin D has been shown to increase intestinal absorption of calcium in bullfrogs (Baksi et al., 1978).

3. Gonadal hormones

Estrogen is not released when blood calcium islow in humans (Rude and Singer, 1982) and estrogen injection does not increase plasma calcium levels (Urist and Scheide, 1961).

However, observations in postmenopausal women suggest that estrogen may influence calcium metabolism by an interaction with skeleton, intestine and kidney.

Following natural or artificial menopause, loss of bone and/or a decrease in bone mass has been demonstrated (Rude and Singer, 1982). Estrogen therapy in these patients results in a slowing or cessation of bone loss (Lindsay et al., 1980). The mechanism by which estrogen slows bone loss is unknown although there is experimental evidence that the sex steroids may modulate the peripheral effects of parathyroid hormone. In vivo studies have shown an increased sensitivity of bone to parathyroid hormone in ovariectomized rats (Orimo et al., 1972). In an in vitro bone culture system, estrogen was

found to decrease bone resorbtion response to parathyroid hormone (Aitkins et al., 1972). Progesterone, testosterone and androstenedione have also been observed to inhibit the resorptive action of parathyroid hormone in bone culture (Atkins and Peacock, 1975).

Intestinal calcium absorption has usually been found to be low in patients with postmenopausal or senile osteoporosis but following administration of sex steroids to these patients, intestinal absorption of radioactive calcium was improved (Jaworski et al., 1963). Impaired metabolism of vitamin D may contribute to the decrease in intestinal calcium absorption in osteoporotic patients and elderly subjects (Rude and Singer, 1982). A low serum concentration of $1,25(OH)_2D$ has been reported in these patients (Gallagher et al., 1979), along with a decreased conversion of 25(OH)D to $1,25(OH)_2D$ on aging rats (Armbrecht et al., 1980). Patients with osteoporosis associated with maladsorption of calcium were treated with small doses of vitamin D and calcium which resulted in suppression of bone resorption (Need et al., 1985).

Young and Nordin (1967) observed an increase in urinary calcium following natural or artificial menopause. They showed that estrogen administration caused a lowering of urinary calcium excretion to levels similar to those of the premenopausal state. However, the principal mechanism by which the sex steroids decreases ur-

inary calcium excretion is thought to be secondary to decreased bone resorption (Rude and Singer, 1982).

Estrogen injection does have a hypercalcemic effect in birds, reptiles and amphibians (Clark, 1967; Woodhead, 1969; Simkiss, 1974). This effect of estrogen is observed on oviparous vertebrates that produce large megalecithal eggs that contain a lot of calcium (Bentley, 1982).

B. Hormones with hypocalcemic effects

Calcitonin is the only hormone which has a hypocalcemic effect in terrestrial vertebrates (Figure 1).

Hypercalcemia promotes calcitonin release from the thyroid "C" cells in mammals and from the ultimobranchial glands of birds, reptiles and amphibians. Calcitonin exerts its biological effects by acting on three primary target organs - bone, kidney and gastrointestinal tract. These actions of calcitonin result in a decrease in blood calcium levels.

Calcitonin deceases the overall rate of bone resorption (Fisher, 1982). The hormone delays proliferation of osteoprogenitor cells and modulation of osteoblasts to osteocytes. The osteolytic activity of osteocytes is inhibited. Calcitonin accelerates modulation of precursrer cells to osteoblasts and lengthens the life span of these cells (Talmage et al., 1975).

Calcitonin increases urinary excretion of calcium and phosphorus (Ardaillou et al., 1967). There is some

evidence that calcitonin may also influence calcium transport across intestine. Administration of the hormone to sheep and parathyroidectomized pigs decreases the absorption of calcium from the intestine (Swaminathan et al., 1974).

In birds calcitonin is produced by the ultimobranchial glands and the hormone has been observed in the blood of several species. Injections of calcitonin have been found to have no effect on plasma calcium levels in intact birds (Urist,1967; Candlish and Taylor, 1970). However, hypocalcemia was observed in parathyroidectomized birds after calcitonin administration (Urist, 1967). Ultimobranchialectomy does not influence calcium metabolism during the egglaying cycle of the domestic hen (Speers et al., 1970).

In reptiles ultimobranchialectomy has not been performed and injection of calcitonin has no effect on turtles (Clark, 1968b, 1971b), lizards (Dix et al., 1970) or snakes (Clark, 1971b). There is some evidence to suggest that calcitonin may protect the reptilian skeleton from excessive resorption (Taylor, 1985).

II. Endocrine control of calcium in aquatic vertebrates

A. Hormones causing hypercalcemia

The following hormones have a hypercalcemic effect in aquatic vertebrates (Figure 2).

1. Hypophyseal hormones

There is evidence fro a hypercalcemic action

of the pituitary gland in teleosts. Hypophysectomy in calcium-deficient seawater-adapted fish results in hypocalcemia but this condition is not observed in hypophysectomized fish kept in an environment rich in calcium (Pang et al., 1971a, 1973a). Injection of pituitary gland homogenate (Pang et al., 1973b) or of prolactin (Pang et al., 1978,; Pang, 1981) or ACTH (Pang et al., 1973b), corrects the hypocalcemia in hypophysectomized killifish adapted to calcium-deficient seawater.

Fish pituitary glands may contain at least two hypercalcemic factors: prolactin and other unknoen factor(s). When killifish and cod pituitary gland were divided into different parts and tested separately for hypercalcemic actions in hypophysectomized killifish, both the part containing prolactin cells and the part containing the pars intermedia were hypercalcemic (Pang et al., 1973b, 1978). Ball et al., (1982) reported that killifish adapted to calcium-deficient seawater exhibited increased cellular activity in the cells of the pars intermedia while the prolactin cells in the pars distalis were only mildly stimulated. Parsons et al., (1978) suggested that the cod pituitary gland may contain a hypercalcemic factor other than prolactin after reporting a cross reactivity of a cod pituitary gland factor with mammalian PTH antibody. The name "hypercalcin" was suggested for this hypercalcemic factor.

2. Vitamin D

Henry and Norman (1975) detected the renal enzyme, 25-hydroxyvitamin D-1a-hydroxylase, that converts 25-hydroxyvitamin D to its dihydroxy hormonally active form in eight species of teleosts. Vitamin D injection produced hypercalcemia in the freshwater catfish Clarias hatrachus (Swarup and Srivastav, 1982). Bone resorption is stimulated in eels by $1,25(OH)_2D$ (Lopez et al., 1977); and Wendelaar Bonga et al., (1983) reported that $1,25(OH)_2D_3$ inhibited bone formation in the teleost Sarotherodon mossambicus. Injection of vitamin D or $1,25(OH)_2D$ induced hypercalcemia and stimulated uptake of ^{45}Ca from intestinal sacs from American eels Anguilla rostrata in situ (Fenwick et al., 1984).

In many teleosts pharmacological doses of vitamin D_3 metabolites do not effect calcium homeostasis. The role of vitamin D in fish remains unresolved.

3. Gonadal hormones

Ovarian maturation in most teleosts is associated with an increase in plasma bound calcium levels. Injections of estrogen can also produce an increase in plasma bound calcium in male and female fish (Bentley, 1982). This effect of estrogen is either absent or minimal in cyclostomes and elasmobranchs (Urist and Schejeide, 1961; Woodhead, 1969; Urist et al., 1972). There is little evidence that androgens play an important role in calcium regulation in aquatic vertebrates.

B. Hormones causing hypocalcemia

The following hormones have a hypocalcemic effect in aquatic vertebrates (Figure 2).

1. Calcitonin

Calcitonin has a hypocalcemic effect in aquatic vertebrates. Calcitonin is abundant in teleostean ultimobranchial bodies. Many species of teleosts have been injected with mammalian and fish calcitonin but most failed to show hypocalcemia after treatment (Pang and Pickford, 1967; Pang, 1971; Yamauchi et al., 1978; Wendelaar Bonga, 1980; Wendelaar Bonga and Lammers, 1982). Hypocalcemia has been reported in calcitonin-treated freshwater-adapted European eels, Anguilla anguilla and North American eels, Anguilla rostrata but the same treatment was ineffective in seawater-adapted animals (Chan, 1968; Pang, 1971).

Ultimobranchialectomy produced hypercalcemia in Anguilla anguilla (Lopez et al., 1976). Hypercalcemia was also observed in partially ultimobranchialectomized goldfish, Carassius auratus (Fenwick, 1975). Calcitonin stimulated bone formation in Anguilla anguilla (Lopez et al., 1976).

Calcitonin has been detected in elasmobranchs (Pang et al., 1971b) but injection of mammalian and salmon calcitonin has not shown a hypocalcemic response (Urist, 1967; Copp et al., 1970).

The studies suggest calcitonin may play a role in calcium regulation in some teleosts but not others, and in view of the inconsistenct of the data, calcitonin's role

in Ca^{2+} regulation is not as well defined as other endocrine systems.

2. Corpuscles of Stannius factor

The Stannius corpuscles are found only in some fish and have not been identified in cyclostomes or elasmobranchs. In teleosts there are, in most cases, two or more of these whitish glandular bodies closely associated with the kidneys. There is evidence for a hypocalcemic role of the Stannius corpuscles in most teleostean species studied. Stanniectomy in seawater-adapted teleosts leads to hypercalcemia (Pang et al., 1973c; Fenwick, 1974; So and Fenwick, 1977). Injection of corpuscles of Stannius homogenate produces hypocalcemia in stanniectomized seawater-adapted Fundulus heteroclitus (Pang et al., 1974) and in stanniectomized freshwater-adapted eels Anguilla rostrata (So and Fenwick, 1979).

III. Calcium regulating mechanisms

A. Intracellular calcium regulation

Most animal cells keep their ionic calcium concentration at a level three or four times lower than that in the extracellular fluid (Schatzmann, 1982). There is a continuous influx of Ca^{2+} into the cell driven by the concentration gradient across the plasma membrane. Longterm regulation of intracellular Ca^{2+} is influenced by calcium storage organelles - mitochondria (Carafoli, 1982) and endoplasmic reticulum (Bayerdorffer et al., 1984) and plasma membrane mechanisms (Schatzmann, 1982). The mito-

chondria and endoplasmic reticulum can accumulate Ca^{2+} while the plasma membrane $Ca^{2+}+Mg^{2+}$-ATPase and Na/Ca exchange remove calcium from the cell.

1. Ca-storage organelles

a. Mitochondria

Calcium can enter the mitochondria by two mechanisms: respiration-dependent and ATP-dependent. In the respiration-dependent mechanism, calcium enters mitochondria electrphoretically in response to the negative-inside membrane potential developed across the inner membrane by electron transport. Each Ca^{2+} ion carries two net positive charges that are electrically compensated for by extrusion of two H^+ ions from the matrix. A rise in internal pH is prevented by simultaneous influx of phosphate.

Vasington and Murphy (1962) reported the process of energy-linked uptake of Ca^{2+} by mitochondria. The mitochondira ATP synthase (also called ATPase complex) caralyzes both ATP synthesis (oxidative phosphorylation) and hydrolysis of ATP. Both of these processes are inhibited by the antibiotic oligomycin. The ATPase complex consists of 2 distinct components, an inner membrane-bound sector F_o, which functions as a proton-translocating channel and an extra membrane sector F_1, which has catalytic activity. An oligomycin sensitivity conferring protein (OSCP) is required for the interaction of the F_1 and F_o moieties of the mitochondrial ATPase to yeild a membrane-bound oligomycin-sensitive ATP synthase.

ATP hydrolysis by the mitochondrial ATPase generates a transmembrane potential that supports uptake of calciu. The negative-inside membrane potential developed across the inner membrane by extrusion of 3 H^+ ions formed during ATP hydrolysis, supports uptake of 1.5 Ca^{2+} ions (Fiskum and Lehninger, 1982).

b. Other Ca-storage organelles

(1) Endoplasmic reticulum

Fragments of endoplasmic reticulum (ER) from liver, kidney, brain, salivary glands and platelets have been shown to actively accumulate Ca^{2+} by a Ca^{2+}+Mg^{2+}-ATPase (Carafoli, 1982). In liver and kidney the ER seems to be a system with low capacity but high affinity fro Ca^{2+} (Carafoli, 1982). The Ca^{2+}+Mg^{2+}-ATPase in the ER fraction from smooth muscle differs in protein composition and in the characteristics of the Ca^{2+}-uptake compared to plasma membrane Ca^{2+}+Mg^{2+}-ATPase discussed below (Raeymaekers et al., 1985). Betlvmo et al., (1984) suggested that in hepatocytes the mitochondria play a more important role than the ER in the regulation of the cytosol free calcium levels when the plasma membrane Ca^{2+} pump is inhibited.

(2) Sarcoplasmic reticulum

The major protein component of sarc0-plasmic reticulum is a Ca^{2+}-transporting ATPase. The hydrolytic process is located on the outer membrane of the organelle. Two calcium ions are transported at the ex-

pense of one mole of ATP (Hasselbach, 1964). The enzyme is responsible for the cessation of muscle contraction cycles by removing calcium from the sarcoplasm into the sarcoplasmic reticulum cavities where the ion is stored until the arrival of the next excitatory stimulus.

2. Plasma membrane mechanisms

There is evidence in a number of systems that intracellular calcium regulation involves two energy-dependent systems in the plasma membrane. One is a Ca^{2+}+ Mg^{2+}-ATPase which is a system directly dependent on ATP hydrolysis as the energy supply. The other is a system that transports calcium in exchange fro sodium. This exploits the energy invested in the sodium gradient by outward calcium pumping (Bridge and Bassingthwaighte, 1983).

In some cells (the squid axon, cardiac muscle, renal tubular cells) both processes may be operating in parallel and in others the Ca^{2+}+Mg^{2+}-ATPase may be present alone (red blood cells, L cells) (Schatzmann, 1982).

a. Ca^{2+}+Mg^{2+}-ATPase

Ca^{2+}+Mg^{2+}-ATPase has been reported to be present in the sarcolemma of the muscle cell, in blood platelets, brain microsomes, rainbow trout gills, nerve cell, renal tubular cells, salivary glands, red blood membrane (Carafoli, 1982) and squid axon (Matsumura and Clark, 1980).

Matsumura and Clark (1980) reported Ca^{2+}+ Mg^{2+}-ATPase in the axon membrane from the retinal nerve of

the squid, Loligo pealei. The Ca^{2+}+Mg^{2+}-ATPase displayed a high temperature sensitivity (Q_{10}) of 3.78 between 20°C and 37°C) and a low optimal Ca^{2+} concentration (on the order of 10^{-5}M).

The best studied Ca^{2+}+Mg^{2+}-ATPase enzyme is in the red blood cell membrane. Ca^{2+}-stimulated ATPase (Ca-pump) activity was first observed in the erythrocyte membrane by Dunham and Glynn in 1961. Schatzmann (1966) showed an ATP-dependent Ca^{2+} efflux from resealed red cell ghosts. There was no evidence that the exiting calcium was exchanged for other ions and magnesium was essential for enzymatic activity. In 1969, Ca^{2+}+Mg^{2+}-ATPase was described and characterized by Schatzmann and Vincenzi for the erythrocyte. All of the energy for calcium extrusion from the red blood cell is apparently derived from ATP hydrolysis. One mole of ATP is hydrolyzed by the Ca^{2+}+Mg^{2+}-dependent ATPase for every mole of Ca^{2+} transported (Schatzmann, 1973). The hydrolytic process is located on the inner membrane side in the erythrocyte (Schatzmann, 1975).

The cytosol of red blood cells contains 3-5um calmodulin (CaM) (Foder and Scharff, 1981). Calmodulin is a Ca-binding protein that activates the plasma membrane Ca^{2+}-pumping ATPase (Carafoli, 1982). It is an acidic, fairly heat-stable, water-soluble protein of 16,700 molecular weight, ubiquitous in all eukaryotic cells and tissues (Schatzmann, 1983). Saturation of the four calcium

binding domains on calmodulin induces a number of structural changes (Niggli et al., 1979) including an increase in hydrophobicity (Crouch and Klee, 1980; Laporte et al., 1980; Tanaka and Hidaka, 1981). The calcium pump or other cytosolic calmodulin acceptor proteins (CAP) which mediate calcium-dependent cellular processes (Moore et al., 1984) have acomplimentary hydrophobic site which has a high affinity for the calcium-calmodulin site. The interaction results in alteration in CAP structure and activity. The activity is reversible by removing calcium causing a reduction in hydrophobicity and a separation of the calcium-calmodulin CAP complex. Trifluoperazine (TFP) and related phenothiazines bind to the hydrophobic regions and act by blocking calcium-CaM-CAP interaction (Dedman, 1984).

b. Ca^{2+}+Na^{+}-ATPase

Matsumura and Clark (1980) reported a Ca^{2+}+Na^{+}-ATPase (called ecto-Ca^{2+}-ATPase) in the axon membrane from the retinal nerve in the squid, Loligo pealei. The stimulation of this enzyme occurred only at high Ca^{2+} concentration (2mM) in the absence of Mg^{2+}. The Ca^{2+}+Na^{+}-ATPase had a low temperature sensitivity (Q_{10} of 2.23 between 16°C and 26°C) and the activity was affected by changes in Na^{+} and K^{+} concentrations.

c. Na/Ca exchange

A plasma membrane calcium transport system which appears to depend upon the external sodium concentra-

tion has been reported in nerve, muscle, epithelium and secretory cells (Blaustein, 1974; Bers et al., 1985; Matil et al., 1985). The evidence indicates that the exit of calcium is tightly coupled to the entry of sodium (Blaustein and Russel, 1975). It has been suggested that this process is driven by a Na^+ concentration gradient (Reuter and Seits, 1968). Indirect evidence indicates that there is an exchange of three Na^+ for one Ca^{2+} (Gmaj et al., 1979). ATP increases the affinity of the carrier transport system for external Na^+ and a phosphorylation step at high calcium concentration inside the cell could be involved in the ATP activation of the Na/Ca exchange (DiPolo and Beauge, 1983).

B. Extracellular calcium regulation

There is some evidence that the Ca-regulatory systems discussed above may play a role in regulating extracellular (blood) calcium levels as well as intracellular Ca^{2+} levels.

1. Intestinal cells

The transcellular calcium movement in intestinal cells is thought to involve Ca^{2+} moving down its electrochemical gradient from the lumen into the cell. Calcium entry may be facilitated by a vitamin D-stimulated calcium binding protein (CaBP) in the intestinal brush border membrane (Miller et al., 1979), first isolated by Drescher and DeLuca (1971). Extrusion across the basolateral membrane into blood occurs by two routes:

a Ca-dependent ATPase and a Na/Ca exchange (Bronner, 1982).

2. Kidney tubule cells

All sections of the kidney tubule (except the medullary part of the thick ascending limb of Henle's Loop) seem to be able to transport Ca^{2+} actively (Suki and Rouse, 1980), but 60% of the filtered Ca^{2+} is removed from the tubular fluid in the proximal convoluted tubule (Suki, 1979). Gmaj et al., (1979) reported an ATP-driven Ca^{2+}-pump and a Na/Ca exchange system is responsible for the bulk flow of calcium across the epithelium into the blood, whereas the ATP-driven system may be involved in the regulation of intracellular calcium.

3. Gill epithelial cells

A calcium-activated ATPase has been demonstrated in the gills of rainbow trout (Ma et al., 1974; Parker et al., 1985) and freshwater and seawater-adapted eels (Fenwick, 1976, 1979; Shepard et al., 1978; Ho and Chan, 1980; Flick et al., 1984). Fenwick (1979) reported the gill epithelium Ca-ATPase had a lower activity in seawater-adapted than in freshwater-adapted eels. With the exception of the specific activity, the kinetic properties of the enzyme extracted from freshwater eels were identical to those of the enzyme extracted from the seawater eels. It was suggested that the increase in branchial Ca^{2+}-ATPase activity in the freshwater eel play a role in the direct acquisition of Ca^{2+} from the environment (Fenwick, 1979).

The activity of the branchial Ca-pump appears to be under hormonal control. Prolactin treatment of American eels, Anguilla rostrata resulted in a stimulation of gill epithelium Ca^{2+}-ATPase (Flick and Wendelaar Bonga, 1984). Branchial Ca^{2+}-ATPase activity was higher in stanniectomized eels than in sham-operated controls (Fenwick, 1976).

There also appears to be a Na/Ca exchange mechanism in teleostean branchial epithelium. Sodium flux across gill epithelium is dependent upon the external calcium concentration. This has been demonstrated in Fundulus kansae (Fleming et al., 1974) and in seawater-adapted eels (Bornancin et al., 1972).

IV. Purpose of this study

Matsumura and Clark (1980) reported two Ca^{2+}-dependent ATPases in the axon membrane from the retinal nerve of the squid, Loligo pealei; one was a Ca^{2+}+Mg^{2+}-ATPase which requires a low optimal Ca^{2+} concentration for activity; the other was a Ca^{2+}+Na^{+}-ATPase (called ecto-Ca^{2+}-ATPase) which requires a high optimal Ca^{2+} concentration for activity in the absence of Mg^{2+}. Preliminary studies showed the presence of two Ca-ATPases in the gill epithelium microsomal fraction from killifish similar to those reported in squid. These enzymes have neither been identified nor characterized in killifish branchial epithelium, although one Ca-ATPase has been described in the gill epithelium from other

teleosts as discussed above. However, all of the above investigators measured Ca-ATPase activity in an experimental medium that contained Ca^{2+}, Mg^{2+} and Na^{+}. At the concentrations of these ions used, both Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase would be active. It is therefore possible that they were measuring both Ca-ATPases rather than just one. Based on these experimental findings efforts to characterize killifish branchial epithelium Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase in seawater and freshwater-adapted killifish were warranted.

The presence of two Ca-ATPases in killifish gill epithelium suggest a role in Ca^{2+} regulation. A correlation between gill Ca-ATPase activity and environmental Ca^{2+} in other studies however, is controversial. Fenwick, (1979) reported gill epithelium Ca-ATPase activity to be higher in freshwater-adapted as compared to seawater-adapted North American eels. On the other hand, Ho and Chan (1980) showed that when freshwater-adapted Japanese eels were transferred to seawater, gill Ca-ATPase activity started to increase on day 4 after transfer and maintained a higher level of activity throughout the seawater adaptation. In addition, they reported serum Ca^{2+} levels rose for the first 4 days and then returned to control values. To obtain a clearer picture of the interrelationship between blood Ca-regulation and gill Ca-ATPase activities, the present study explores the effect of changes in environmental Ca^{2+} on Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase in killifish gill

epithelium. Whether changes in enzyme activity after transfer to seawater or freshwater correlated with changes in blood Ca^{2+} was also investigated as a further indication of a specific role of the gill Ca-ATPases in blood Ca^{2+} regulation. Of special interest was whether one enzyme activity is higher in seawater and the other enzyme in freshwater, suggesting that one enzyme may pump Ca^{2+} into the fish through the gills in a low calcium environment while the other enzyme pumps calcium out of the fish through the gills in a high calcium environment.

The pituitary gland has been implicated as playing a role in Ca^{2+} regulation in a low Ca^{2+} environment while the corpuscles of Stannius were shown to play a role in Ca^{2+} regulation in a high Ca^{2+} environment as discussed earlier in the Introduction. In eels there is endocrine regulation of gill Ca-ATPase. Prolactin treatment of American eels, Anguilla rostrata resulted in stimulation of gill epithelium Ca^{2+}-ATPase (Flick and Wendelaar Bonga, 1984). Fenwick (1976) reported that stanniectomy of American eels increased gill Ca-ATPase activity and Copp and Ma (1981) reported the glycopeptide isolated from salmon corpuscles of Stannius inhibited the enzyme. It was suggested that corpuscles of Stannius-induced hypocalcemia could be the result of an inhibition of active calcium uptake via a gill membrane-bound Ca-ATPase (Copp and Ma, 1981).

The effect of the pituitary gland and the corpuscles of Stannius on $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activity was investigated in this study to gain insight into the regulatory mechanisms by which the Ca-regulating hormones in killifish maintain a constant blood Ca^{2+} level in seawater and freshwater.

Killifish (Fundulus heteroclitus) were the animals of choice in this study for many reasons: 1) They are euryhaline fish that can adapte to low and high salinity environments so that effects of changing environmental Ca^{2+} concentrations can be studied; 2) They are hardy fish that can be maintained easily under laboratory conditions; 3) They can be easily sexed to enable investigation of only one sex; and 4) Killifish are large enough to obtain sufficient amounts of blood and gill tissue to study, yet small enough to be easily kept in the laboratory.

MATERIALS AND METHODS

I. Source and maintenance of experimental animals

Killifish, Fundulus heteroclitus, were puschased from a commercial dealer with stock from Jamaica Bay, New York. Males were used in all experiments due to the fact that during female ovarian maturation there is a significant elevation in blood calcium levels. The length of the fish utilized ranged from approximately 5cm to 8cm.

Stocks were maintained in the laboratory at Brooklyn College at 15°C in a 150 gallon Instant Ocean Culture System aquarium containing seawater from Long Island Sound, (obtained courtesy of the New York Aquarium). The fish were kept under ambient light-dark conditions. They were fed five times a week with Tetramin. For adaptation to freshwater, fish were transferred to small, clear plastic tanks containing 8 liters of aged tap water. The tanks were well aerated at room temperature. The fish were maintained under ambient light-dark conditions. The fish were fed with Tetramin and the water changed three times a week. The standard period for full adaptation to freshwater was two weeks.

II. Preparation of gill microsomes

The fish were sacrificed at approximately the same time each day to minimize diurnal effects. Fish were anaesthetized with MS-222 (Finquel, Ayerst Laboratories) at a concentration of 0.4g per liter distilled water.

The gill arches were dissected out and the gill filaments removed, blotted and weighed.

The microsomal fraction of the gill filaments was isolated by a modification of the technique described by Towle et al., (1976). The gill filaments were homogenized in a glass Potter-Elvehjem apparatus using 18 strokes with a teflon pestle at 3600 rev/min in a solution of 0.3M sucrose. The volume (ml) used was equal to 10 times the initial weight (g) of the tissue

The initial homogenate (H_I) was centrifuged for 10 minutes at 940 x g (Sorvall SMA 24 rotor). The pellet P_1, referred to as the nuclear fraction, was discarded. The supernatant after careful aspiration was centrifuged for 35 minutes at 11,116 x g (Sorvall SMA 24 rotor). The pellet P_2, referred to as the mitochondrial fraction, was discarded. The supernatant after careful aspiration was centrifuged for 60 minutes at 105,000 x g (Beckman SW 50.1 rotor). The final supernatant (FS) after careful aspiration was discarded. The final pellet P_3, referred to as the microsomal fraction, was resuspended in a solution of 0.3M sucrose and 0.1mM dithiothreitol (DTT) in a volume (ml) equal to 2.5 times the initial weight (g) of the tissue used. The pellet P_3 suspension was homogenized in a glass Potter-Elvehjem apparatus using 10 strokes with a teflon pestle at 3600 rev/min. This microsomal fraction , if not immediately assayed, was stored at -20^0C until use.

Storage at -20°C had no effect on enzyme activity (Table 1).

The H_I, P_1, P_2 and FS were not discarded when assaying marker enzymes (Section III).

III. Determination of purity of microsomal fraction

A. Succinic dehydrogenase assay

Succinic dehydrogenase was assayed as a marker enzyme for mitochondria by a modification of the technique described by Fried et al., (1961). Pellets P_1, P_2 and P_3 were resuspended in a solution of 0.3M sucrose and 0.1mM DTT in a volume (ml) equal to 2.5 times the initial weight (g) of the tissue used. The pellet suspensions were homogenized in a glass Potter-Elvehjem apparatus using 10 strokes with a teflon pestle at 3600 rev/min. The H_I, P_1, P_2, P_3, and the final supernatant were assayed fro succinic dehydrogenase (SDH) activity by measuring formation of INTH (formazan) from INT. The activity was assayed in a medium containing 0.3M phosphate buffer (ph 7.4), 30% di-iodo-di-nitrotetrazolum chloride (INT), 0.5M Na-succinate and 10^{-3}M NaCN. The reaction was started by addition of 0.1ml of the fraction being assayed to 1.9ml assay medium. A tube containing no succinate was also prepared to measure the amount of formazan produced by dehydrogenases other than SDH in gill tissue. Incubation was carried out at 37°C for 15 minutes. The reaction was stopped by adding 0.4ml of ice-cold 30% Trichloroacetic acid (TCA). Five ml of ethyl acetate was added to

each tube. The tubes were stoppered, shaken 50 times and centrifuged at 1500rpm for 10 minutes. The colored ethyl acetate layer was removed with a dropper and the absorbance read at 490nm using pure ethyl acetate as a blank. Final activity was expressed as µgm formazan produced/15'/gram tissue after subtracting the activity due to other dehydrogenases.

B. Na^++K^+-ATPase assay

Na^++K^+-ATPase was assayed as a marker enzyme for plasma membrane. Pellets P_1, P_2 and P_3 were resuspended in a solution of 0.3M sucrose and 0.1mM DTT in a volume (ml) equal to 5.0 times the initial weight (g) of the tissue used. The pellet suspensions were homogenized in a glass Potter-Elvehjem apparatus using 10 strokes with a teflon pestle at 3600 rev/min. The initial homogenate, P_1, P_2, P_3 and final supernatant were assayed for Na^++K^+-ATPase activity according to Towle et al. (1977). The activity was assayed in a complete assay medium containing 20mM imidazole, 100mM NaCL, 30 mM KCL, 5mM $MgCL_2$ and 5mM Na_2-ATP (Vanadium-free), and in a potassium-deficient assay medium containing 20mM imidazole, 130mM NaCL, 5mM $MgCl_2$, 5mM Na_2-ATP (Vanadium-free) and 1mM ouabain. Both media were adjusted to pH 7.8. The reaction was started by adding 0.04ml of the fraction being assayed to 2ml of either complete assay or deficient assay medium. Following incubation at 25^{0}C for 30 minutes, the reaction was stopped by adding 0.4ml of ice-cold 30% TCA, then centrifuged at 5,000rpm for 10 minutes. Inorganic phosphate in

the supernatant was determined according to Fiske and Subbarow (1925). Final activity was expressed as μmole Pi.$hr.^{-1}$. mg^{-1}protein, after subtracting deficient from complete activities. Protein concentration was determined by the procedure of Lowry et al. (1951).

IV. Characterization of gill Ca-ATPases

A. General assay procedures

Aliquots of the microsomal fraction were incubated in appropriate assay buffers for 10 minutes. The reaction was stopped by addition of 0.4ml of ice-cold 30% Trichloroacetic acid (TCA). The solutions were centrifuged for 10 minutes in a clinical centrifuge at 5,000rpm. The supernatant was poured into cold test tubes kept on ice. The amount of inorganic phosphate (Pi) released from ATP was determined by the method of Fiske and Subbarow (1925). A control containing assay medium and TCA to which no enzyme had been added under the same conditions showed no detectable release of inorganic phosphate from ATP. Protein was determined by the method of Lowry et al., (1951). The ATPase activity was expressed as micromoles of Pi released from ATP per hour per milligram of protein.

B. Characterization of Ca^{2+}+Mg^{2+}-ATPase

For characterizing Ca^{2+}+Mg^{2+}-ATPase activity a standard assay buffer was used containing: Tris-base (30mM), ouabain (10^{-4}M), KCL (160mM) and EGTA (0.5mM). The concentrations of $MgCl_2$, $CaCl_2$, Na_2-ATP (Vanadium-free) and the pH

of the solutions were varied as described below. Except in the section where optimal temperature was determined, assays were carried out at 37°C. For each sample, three assay buffers were used: 1) Complete (containing all components indicated above); 2) Basal Ca (containing all components except $MgCl_2$); 3) Basal Mg (containing all components except $CaCl_2$). Activity was calculated by subtracting the basal Ca and basal Mg assay buffer activities from the total activity in the complete Ca^{2+}+Mg^{2+}-ATPase assay buffer.

1. Effect of oligomycin B

To determine whether contamination by mitochondrial Ca^{2+}-ATPase was contributing significantly to plasma membrane Ca^{2+}+Mg^{2+}-ATPase activity being measured, oligomycin B (2µgm/ml) was added to the assay buffers of Ca^{2+}+ Mg^{2+}-ATPase. The standard assay medium described above contained in addition, 10mM Mg^{2+}, 0.1mM Ca^{2+}, 5mM ATP adjusted to pH 7.1. For Ca^{2+}+Mg^{2+}-ATPase activity assayed with and without oligomycin B, 3 to 5 separate determinations were done.

2. Magnesium

The optimal Mg^{2+} concentration was determined using the standard assay medium described above containing in addition, 0.1mM $CaCl_2$, 5mM ATP and a range of Mg^{2+} concentrations from 2mM to 12mM. The pH of the media was 7.1. For each Mg^{2+} concentration assayed, 2 to 5 separate determinations were done.

3. **Calcium**

The optimal Ca^{2+} concentration was determined using the standard assay medium described above containing in addition, 10mM Mg^{2+}, 5mM ATP and a range of Ca^{2+} concentrations from 0.0025mM to 20mM. The pH of the media was 7.1. For each Ca^{2+} concentration assayed, 2 to 11 separate determinations were done.

4. ATP

The optimal ATP concentration was determined using the standard assay medium described above containing in addition, 10mM Mg^{2+}, 1mM Ca^{2+} and a range of ATP concentrations from 1mM to 10mM. The pH of the media was 7.1. For each ATP concentration assayed, 4 to 15 separate determinations were done.

5. pH

The optimal pH was determined using the standard assay medium described above containing in addition, 10mM Mg^{2+}, 1mM Ca^{2+}, 5mM ATP and a range of pHs from pH 6.0 to pH 8.0. For each pH determination assayed, 4 to 8 separate determinations were done.

6. **Temperature**

The optimal temperature was determined using the standard assay medium described above containing in addition, 10mM Mg^{2+}, 1mM Ca^{2+}, 5mM ATP, adjusted to a pH of 7.5 at a range of temperatures from 10°C to 65°C. For each temperature determination assayed, 4 to 8 separate

determinations were done.

7. Trifluoperazine

To determine whether Ca^{2+}+Mg^{2+}-ATPase is regulated by calmodulin, 50 μM trifluoperazine, an inhibitor of calmodulin was added to the complete, basal Ca and basal Mg assay media. The standard assay medium was utilized as described above containing in addition, 10mM Mg^{2+}, 1mM Ca^{2+}, 5mM ATP, adjusted to pH 7.5. For Ca^{2+}+Mg^{2+}-ATPase activity assayed with and without trifluoperazine, 8 to 12 separate determinations were done.

C. Characterization of Ca^{2+}+Na^{+}-ATPase

For characterization of Ca^{2+}+Na^{+}-ATPase activity a standard assay buffer was used containing: Tris-base (30 mM), ouabain (10^{-4}M), KCl (160mM) and EDTA (1mM). The concentrations of NaCl, $CaCl_2$, Na-ATP (Vanadium-free), the pH and temperatures of the solutions were varied as described below. For each assay, three assay buffers were used: 1) Complete (containing all components indicated above); 2) Basal Ca (containing all components except NaCl); 3) Basal Na (containing all components except $CaCl_2$). Activity was calculated by subtracting the basal Ca and basal Na assay buffer activities from the total activity in the complete Ca^{2+}+Na^{+}-ATPase assay buffer.

1. Effect of oligomycin B

To determine whether contamination by mitochon-

drial Ca^{2+}-ATPase was contributing significantly to plasma membrane $Ca^{2+}+Na^{+}$-ATPase activity being measured, oligomycin B (2µgm/ml) was added to the assay buffers of $Ca^{2+}+Na^{+}$-ATPase. The standard assay medium described above contained in addition, 160mM Na^{+}, 20mM Ca^{2+}, 5mM ATP adjusted to pH 7.1. For $Ca^{2+}+Na^{+}$-ATPase activity assayed 4 to 8 separate determinations were done.

2. Effect of sucrose

To determine whether marked differences in osmolarity could affect $Ca^{2+}+Na^{+}$-ATPase activity, the osmolarity of the basal assay medium was adjusted with sucrose to equalize the osmolarity of the complete assay medium (420 milliosmolar). The standard assay medium was utilized as described above containing in addition, 160mM Na^{+}, 20mM Ca^{2+} and 5mM ATP. The pH of the medium was 7.1. For $Ca^{2+}+Na^{+}$-ATPase activity assayed with and without sucrose, 10 to 12 separate determinations were done.

3. Sodium

The optimal Na^{+} concentration was determined using the standard assay medium described above containing in addition, 20mM $CaCl_2$, 5mM ATP and a range of Na^{+} concentrations from 10mM to 300mM. The pH of the medium was 7.1. For each Na^{+} concentration assayed, 4 to 12 separate determinations were done.

4. Calcium

The optimal Ca^{2+} concentration was determined using the standard assay medium described above containing in addition, 60mM Na^{+}, 5mM ATP and a range of Ca^{2+} concentrations from 0.1mM to 50mM. The pH of the medium was 7.1. For each Ca^{2+} concentration assayed, 4 separate determinations were done.

5. ATP

The optimal ATP concentration was determined using the standard assay medium described above containing in addition, 60mM Na^{+}, 20mM Ca^{2+} and a range of ATP concentrations from 0.5mM to 10mM. The pH of the medium was 7.1. For each ATP concentration assayed, 4 separate determinations were done.

6. pH

The optimal pH was determined using the standard assay medium described above containing in addition, 60mM Na^{+}, 20mM Ca^{2+}, 6mM ATP and a range of pHs from 6.0 to 8.0. For each pH determination assayed, 4 separate determinations were done.

7. Temperature

The optimal temperature was determined using the standard assay medium described above containing in addition, 60mM Na^{+}, 20mM Ca^{2+}, 6mM ATP adjusted to a pH of 7.2 at a range of temperatures from 10°C to 65°C. For each temperature determination assayed, 4 separate

determinations were done.

8. Trifluoperazine

To determine whether Ca^{2+}+Na^{+}-ATPase activity is inhibited by trifluoperazine (TFP), 50μM TFP was added to the complete, basal Ca and basal Na assay media. The standard assay medium was utilized as described above containing in addition, 60mM Na^{+}, 20mM Ca^{2+}, 6mM ATP adjusted to pH 7.2. For Ca^{2+}+Na^{+}-ATPase activity assayed with and without trifluoperazine, 4 to 12 separate determinations were done.

V. Effect of environmental calcium on Ca^{2+}+Mg^{2+}-ATPase And Ca^{2+}+Na^{+}-ATPase activities

Fish were adapted to seawater and calcium-enriched freshwater, both of which contained 10mM calcium. Fish were also adapted to freshwater and calcium-deficient seawater, both of which contained 0.2mM calcium. The number of fish in each group were as follows: seawater - 4; freshwater - 14; calcium-deficient seawater - 16 to 20; and calcium-enriched freshwater - 9 to 27. The adaptation period was two weeks. The branchial epithelium Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities were assayed using optimal conditions determined above. The optimal conditions for assaying Ca^{2+}+Mg^{2+}-ATPase were: 1) Complete assay medium; Components of the standard assay buffer (Section IV,B) plus 10mM

Mg^{2+}, 1mM Ca^{2+}, 5mM ATP adjusted to pH 7.5 and assayed at 37°; 2) Basal Ca: All components except 10mM mg^{2+}; 3) Basal Mg: All components except 1mM Ca^{2+}. The optimal conditions for assaying Ca^{2+}+Na^{+}-ATPase were: 1) Complete assay medium: Components of the standard assay buffer (Section IV, C) plus 60mM Na^{+}, 20mM Ca^{2+}, 6mM ATP adjusted to pH 7.2 and assayed at 37°; 2) Basal Ca: All components except 60mM Na^{+}; 3) Basal Na: All components except 20mM Ca^{2+}.

VI. Relationship between changes in plasma Ca^{2+} and Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities

Killifish fully adapted to seawater were transferred to freshwater and fully adapted freshwater fish were transferred to seawater. At 0, 3, 7, 10, 13, 15, 17 and 21 days following transfer, blood Ca^{2+} levels and gill epithelium Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities were determined from 2 to 7 fish. The enzyme assays utilized the optimal conditions described above (Section V.). To measure blood calcium levels, the tail of the killifish was severed and blood collected from the caudal artery in heparinized capillary tubes. The tubes were centrifuged in a microhematocrit centrifuge for 10 minutes. Total plasma calcium was determined with a commercial Ca kit (Sigma 586-A).

VII. Endocrine studies

A. Pituitary homogenate

1. Preparation of pituitary homogenate

Cod (Gadus morhus) were obtained as splits from

Reliable Filet Company, Fulton Fish Market, NY and kept frozen until dissected. The pituitary glands were removed, transferred to a weighed vial on ice and wet weight recorded. The pituitary glands were kept frozen until use. The glands were thawed on ice and homogenized in 0.6% saline solution in a glass Potter-Elvehjem apparatus using a teflon pestle. The volume of the homogenate was adjusted so that 0.01ml of the solution contained 4% of a cod gland portion. The homogenates were divided into aliquots sufficient for each day's injection and were frozen immediately. Prior to each day's injection, one aliquot was thawed on ice.

2. Injection procedure

Fully adapted seawater fish were divided into two groups: 1) Control, consisting of 31 fish - injected intraperitoneally with 0.01 ml 0.6% saline/g body weight; 2) Experimental, consisting of 48 fish - injected intraperitoneally with 0.01 ml pituitary gland homogenate/g body weight. A total of five daily injections were given. Two hours after the last injection, blood was collected from the caudal artery for plasma Ca^{2+} determination as described above (Section VI.). Gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities were determined using optimal conditions as described above (Section V.).

B. Prolactin

Ovine prolactin was purchased from Signma (St. Louis, MO) and dissolved in 0.6% saline solution at a concentration of 5ug/0.01 ml. Fully adapted seawater fish were

divided into two groups: 1) Control, consisting of 64 fish - injected intraperitoneally with 0.01 ml 0.6% saline/g body weight; 2) Experimental, consisting of 67 fish - injected intraperitoneally with ovine prolactin at a dose of 5ug/g body weight in a volume of 0.01ml/g body weight. A total of five daily injections were given. Two hours after the last injection, blood was collected from the caudal artery. Plasma Ca^{2+} determination and gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities were determined as described above (Section V. and VI.).

C. Corpuscles of Stannius

1. Preparation of corpuscles of Stannius homogenate

Cod (Gadus morhus) were obtained as splits from Reliable Filet Company, Fulton Fish Market, NY and kept frozen until dissected. The corpuscles of Stannius were removed, transferred to a weighed vial on ice and the wet weight recorded. The corpuscles of Stannius were kept frozen until use. The corpuscles were thawed on ice and homogenized in 0.6% saline solution in a glass Potter-Elvehjem apparatus using a teflon pestle. The volume of the homogenate was adjusted so that 0.01 ml of the solution contained 0.17mg of cod corpuscles of Stannius. The homogenates were divided into aliquots sufficient for each day's injection and were frozen immediately. Prior to the injection each day, one aliquot was thawed.

2. Injection procedure

Killifish were adapted to Ca-deficient seawater for two weeks. The fish were then divided into two groups: 1) Control, consisting of 43 fish - injected intraperitoneally with 0.01 ml 0.6% saline/g body weight; 2) Experimental, consisting of 48 fish - injected intraperitoneally with 0.01 ml corpuscles of Stannius homogenate/g body weight. A total of four daily injections were given. Two hours after the last injection, blood was collected from the caudal artery for Ca^{2+} determination and gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities were assayed as described above (Section V. and VI.).

VIII Statistics and calculations

Statistical analysis of the data was carried out applying Student's "t" test. Significant difference was accepted at p 0.05. Apparent K_m-values were calculated by means of the Lineweaver-Burke transformation of the Michaelis-Menten equation. Apparent energy of activation (E_a) was calculated by means of the Arrhenius Equation. Apparent Q_{10} was determined as the ratio of the reaction rate at $(T + 10)^{0}K$ compared with that at $(T)^{o}K$, between $20^{o}C$ and $30^{o}C$ for $Ca^{2+}+Mg^{2+}$-ATPase and between $15^{o}C$ and $25^{o}C$ for $Ca^{2+}+Na^{+}$-ATPase.

RESULTS

I. Preparation and determination of purity of the microsomal fraction

The objective of the studies in this thesis was to measure the activity of plasma membrane-bound CaATPase in the gill epithelium. An ATP-dependent Ca^{2+} transport mechanism in mitochondria is well known (Carafoli, 1982). Therefore it was necessary to assay gill Ca-ATPase activity in a plasma membrane-encriched fraction that was virtually free of mitochondria. The gill epithelium was fractionated and the initial homogenate (H_I), nuclear fraction (P_1), mitochondrial fraction (P_2), microsomal fraction (P_3) and the final supernatant (FS) were assayed for succinic dehydrogenase, a mitochondrial marker for Na^++K^+-ATPase, a plasma membrane marker. The P_3 fraction contained the highest concentration of membranes with little mitochondrial contamination. The P_2 fraction also contained a high concentration of membranes but had more mitochondrial contamination (Table 2). The P_3 fraction was therefore utilized in all subsequent studies even though it meant losing some plasma membrane.

Since there there was some mitochondrial contamination in the P_3 fraction, oligomycin B, an inhibitor of mitochondrial Ca-ATPase activity, was added to the assay medium to determine if the mitochondrial ATPase activity was contributing to the Ca-ATPase activity values being obtained.

Table 3 shows the activity of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in the gill epithelium microsomal fraction (GEMF) from seawater(sw) and freshwater (fw)-adapted killifish with and without oligomycin B. No effect of oligomycin B was observed on the Ca-ATPase activities. Therefore, oligomycin B was not utilized in subsequent studies.

II. Characterization of gill Ca-ATPases

A. $Ca^{2+}+Mg^{2+}$-ATPase

1. Magnesium

The optimal Mg^{2+} concentration was determined for $Ca^{2+}+Mg^{2+}$-ATPase in the GEMF from sw and fw-adapted killifish (Figure 3). The enzyme from sw-adapted fish has one Mg^{2+} peak at 10mM. The enzyme from fw-adapted fish has two Mg^{2+} peaks at 3mM and 10mM. The activity at 3mM for fw fish was significantly different from 2mM, 4mM and 10mM. The highest $Ca^{2+}+Mg^{2+}$-ATPase activity however, was at 10mM Mg^{2+} concentration for both sw and fw-adapted fish and therefore this Mg^{2+} concentration was utilized in all subsequent $Ca^{2+}+Mg^{2+}$-ATPase assays.

The K_m values for Mg^{2+} were determined from the Lineweaver-Burke graphs shown in Figure 4. The values obtained were 1.31mM for sw fish and 2.54mM for fw fish. There was no significant difference between the K_ms ($0.3>p>0.2$).

2. Calcium

The optimal Ca^{2+} concentration was determined for $Ca^{2+}+Mg^{2+}$-ATPase in the GEMF from sw and fw-adapted killi-

fish (Figure 5).

There was a broad peak of Ca^{2+}+Mg^{2+}-ATPase activity from 0.0025mM to 5.0mM Ca^{2+} for sw-adapted fish. For fw-adapted fish the peak of Ca^{2+}+Mg^{2+}-ATPase activity was between 0.1mM to 1.0mM Ca^{2+}. Since 1.0mM Ca^{2+} was in the optimal range for both sw and fw-adapted fish, this Ca^{2+} concentration was arbitrarily chosen as the Ca^{2+} concentration to be utilized in all subsequent Ca^{2+}+Mg^{2+}-ATPase assays. EGTA is a specific Ca^{2+} chelator. Having EGTA (0.5mM) and $CaCl_2$ (1.0mM) in the assay medium, the concentration of free ionized Ca^{2+} was stabilized at $10^{-7.7}$M (Portzehl et al., 1964).

The K_m values for Ca^{2+} were determined from the Lineweaver-Burke graphs shown in Figure 6. The values obtained for the K_ms were 0.6uM for sw fish and 0.8uM for fw fish. There was no significant difference between the K_ms ($0.7>p>0.5$).

3. ATP

The optimal ATP concentration was determined for Ca^{2+}+Mg^{2+}-ATPase activity in the GEMF from sw and fw-adapted killifish (Figure 7).

There was no peak of activity for Ca^{2+}+Mg^{2+}-ATPase at the ATP concentrations tested for sw fish. For fw fish there was no significant difference in activities from 4mM to 7mM concentrations indicating an optimal range of activity. Since the greatest significant difference between sw and fw fish Ca^{2+}+Mg^{2+}-ATPase activities occurred

at 5mM ATP, this concentration was utilized in subsequent $Ca^{2+}+Mg^{2+}$-ATPase assays.

The K_m values for ATP were determined from the Linewaever-Burke graphs shown in Figure 8. The values obtained for the K_ms were 3.87mM for sw fish and 1.77mM for fw fish. There was no significant difference between the K_ms ($0.3>p>0.2$).

4. pH

The optimal pH was determined for $Ca^{2+}+Mg^{2+}$-ATPase in the GEMF from sw and fw-adapted killifish (Figure 9).

There was a peak of $Ca^{2+}+Mg^{2+}$-ATPase activity between pH 7.5 and pH 8.0 for sw-adapted fish and for fw fish the peak of $Ca^{2+}+Mg^{2+}$-ATPase activity was at pH 7.5. Since pH 7.5 was in the optimal range for sw fish, and the enzyme had the highest activity at pH 7.5 for fw fish, this pH was utilized in all subsequent $Ca^{2+}+Mg^{2+}$ -ATPase assays

5. Temperature

The optimal temperature was determined for $Ca^{2+}+Mg^{2+}$-ATPase in the GEMF from sw and fw-adapted killifish (Figure 10).

There was a broad peak of activity from 30°C to 50°C for sw fish and an optimal range of activity for fw fish from 30°C to 37°C. Since 37°C was in the optimal range for both sw and fw fish, this temperature was utilized in all subsequent $Ca^{2+}+Mg^{2+}$-ATPase assays.

The energy of activation (E_a) was calculated

from the Arrhenius equation. The E_a was 11.4Kcal/mol for both sw and fw-adapted fish. The Q_{10} for sw and fw fish was 2.19 between 20°C and 30°C.

6. Trifluoperazine

Trifluoperazine (TFP) has been reported to inhibit plasma membrane-bound Ca^{2+}+Mg^{2+}-ATPase by competition with calmodulin (Vincenzi, 1982) at a concentration of 50μM (Weiss, 1983). Table 4 shows that TFP significantly inhibited the gill Ca^{2+}+Mg^{2+}-ATPase from both sw and fw fish.

B. Ca^{2+}+Na^{+}-ATPase

1. Effect of osmolarity on enzyme activity

The osmolarity of the complete assay medium for Ca^{2+}+Na^{+}-ATPase was calculated to be 420 milliosmolar while the osmolarity of the basal medium from which Na^{+} was omitted was 60 milliosmolar and the basal medium from which Ca^{2+} was omitted was 360 milliosmolar. It was therefore possible that these marked differences in osmolarity could affect enzyme activity. To determine this, the osmolarity in the complete and basal medium was equalized by adding the appropriate amount of sucrose to the two basal media. Table 5 shows there was no significant difference in the Ca^{2+}+Na^{+}-ATPase activity with and without sucrose added to the basal medium, therefore, sucrose was not utilized in subsequent studies.

2. Sodium

The optimal Na^{+} concentration was determined for Ca^{2+}+Na^{+}-ATPase in the GEMF from sw and fw-adapted killifish (Figure 11).

There was a broad peak of Ca^{2+}+Na^{+}-ATPase activity from 10mM to 60mM Na^{+} for both sw and fw-adapted fish. Since 60mM Na^{+} was in the optimum range for both sw and fw-adapted fish, this Na^{+} concentration was arbitrarily chosen as the Na^{+} concentration to be utilized in all subsequent Ca^{2+}+Na^{+}-ATPase assays.

The K_m values for Na^{+} were determined from the Lineweaver-Burke graphs shown in Figure 12. The value for the K_ms were 1.05mM for sw fish and 1.85mM for fw-adapted fish. There was no significant difference between the K_ms ($p>0.7$).

3. Calcium

The optimal Ca^{2+} concentration was determined for Ca^{2+}+Na^{+}-ATPase in the GEMF from sw and fw-adapted killifish (Figure 13). The highest activity for Ca^{2+}+Na^{+}-ATPase was at 20mM Ca^{2+} concentration for sw fish. There was a broad peak of activity from 10mM to 20mM Ca^{2+} concentration in fw fish. Since 20mM Ca^{2+} was in the optimal range for fw fish and the enzyme had the highest activity at 20mM for sw fish, the Ca^{2+} concentration was utilized in all subsequent Ca^{2+}+Na^{+}-ATPase assays.

The K_m values for Ca^{2+} were determined from the Lineweaver-Burke graphs shown in Figure 14. The values obtained for the K_ms were 0.36mM for sw fish and 0.44mM for fw fish. There was no significant difference between the K_ms ($0.9>p>0.7$).

4. ATP

The optimal ATP concentration was determined for Ca^{2+}+Na^{+}-ATPase activity in the GEMF from sw and fw-adapted killifish (Figure 15). An optimal value of activity was obtained between 5mM and 6mM ATP for sw fish. A much broader range of maximal activity, between 4mM and 9mM ATP was observed for fw fish. Since 6mM ATP was in the optimal range for both sw and fw fish, 6mM ATP concentration was chosen arbitrarily as the ATP concentration to be utilized in all subsequent Ca^{2+}+Na^{+}-ATPase assays.

The K_m values for ATP were determined from the Lineweaver-Burke graphs shown in Figure 16. The values obtained for the K_ms were 0.29mM for sw fish and 0.54mM for fw fish. There was no significant difference between the K_ms ($0.3>p>0.2$).

5. pH

The optimal pH was determined for Ca^{2+}+Na^{+}-ATPase in the GEMF from sw and fw-adapted killifish (Figure 17).

There was a broad peak of enzyme activity from pH 7.0 to pH 7.5 for sw fish. There also was an optimal range of activity from pH 6.5 to pH 7.4 for fw fish. Since pH 7.2 was in the optimal range for both sw and fw fish, this pH was arbitrarily chosen as the pH to be utilized in all subsequent Ca^{2+}+Na^{+}-ATPase assays.

6. Temperature

The optimal temperature was determined for Ca^{2+}+Na^{+}-ATPase in the GEMF from sw and fw-adapted killigish (Fig-

ure 18).

There was a peak of activity from 30°C to 50°C for sw fish. There was a peak of activity for fw fish from 37°C to 50°C. Since 37°C was in the optimal range for both sw and fw fish, 37°C was arbitrarily chosen as the temperature to be utilized in all subsequent Ca^{2+}+Na^{+}-ATPase assays.

The energy of activation (E_a) was calculated from the Arrhenius equation. The E_a for Ca^{2+}+Na^{+}-ATPase was 12,59 Kcal/mol for sw fish and 11.77Kcal/mol for fw fish. The Q_{10} for the sw fish was 2.10 between 15°C and 25°C. The Q_{10} for fw fish was 2.00 between 15°C and 25°C.

7. Trifuoperazine

Trifluoperazine (TFP) is known to inhibit plasma membrane-bound Ca^{2+}+Mg^{2+}-ATPase by competition with calmodulin (Vincenzi, 1982). Ca^{2+}+Na^{+}-ATPase is also a plasma membrane-bound Ca-ATPase. To determine if Ca^{2+}+Na^{+}-ATPase might also be calmodulin-dependent, TFP (50μM) was added to the assay medium to note any change in enzyme activity. TFP significantly increased Ca^{2+}+Na^{+}-ATPase activity in the GEMF from sw and fw-adapted killifish (Table 4).

III. Effect of environmental calcium on Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities in gill epithelium

Table 6 shows Ca^{2+}+Mg^{2+}-ATPase activity to be higher in fw-adapted fish than in sw-adapted fish, while Ca^{2+}+Na^{+}-ATPase activity is higher in sw-adapted fish than in fw-adapted fish. Since Ca^{2+}+Mg^{2+}-ATPase activity is higher in a low Ca^{2+} environment and Ca^{2+}+Na^{+}-ATPase activity is high-

er in a high Ca^{2+} environment, these observations suggest that environmental Ca^{2+} may be regulating enzyme activity.

To gain further insight into the specific role of environmental Ca^{2+} in regulating the activity of these enzymes, one group of killifish was adapted to calcium-deficient seawater (Ca-D-sw), having a calcium concentration equal to that of freshwater (0.2mM). Another group of killifish was adapted to calcium-enriched freshwater (Ca-E-fw), having a calcium concentration equal to that of seawater (10mM).

In Ca-E-fw, as in sw, Ca^{2+}+Na^{+}-ATPase activity was significantly higher than Ca^{2+}+Mg^{2+}-ATPase actvity (p 0.001). There was no significant difference for either enzyme activity from that in sw-adapted fish ($0.9>p>0.7$). This clearly shows that Ca^{2+}+Na^{+}-ATPase acttivity is stimulated and Ca^{2+}+Mg^{2+}-ATPase is inhibited specifically by a high concentration of Ca^{2+} in the environment.

In Ca-D-sw, as in fw Ca^{2+}+Mg^{2+}-ATPase activity is significantly higher than Ca^{2+}+Na^{+}-ATPase activity ($p<0.001$). Even though Ca^{2+}+Mg^{2+}-ATPase activity was lower in Ca-D-sw than fw ($p<0.001$), the enzyme activity in Ca-D-sw was still significantly increased from sw and Ca-E-fw activities ($p<0.001$). This suggests that Ca^{2+}+Mg^{2+}-ATPase activity is stimulated and Ca^{2+}+Na^{+}-ATPase activity is inhibited specifically by low Ca^{2+} in the environment.

IV. <u>Interrelationship between changes in plasma Ca^{2+} and Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities in gill</u>

epithelium

To gain insight into the possible involvement gill epithelium Ca-ATPases may have in regulating plasma Ca^{2+} levels, blood Ca-levels and the gill epithelium Ca-ATPase activities were determined at various time intervals after transferring fw-adapted fish to sw and sw-adapted fish to fw.

A. Freshwater-adapted killifish transferred to seawater

Figure 19 shows the temporal changes in Ca^{2+}+ Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities and plasma calcium concentration following transfer of freshwater-adapted fish to seawater. The gill epithelium Ca^{2+}+Mg^{2+}-ATPase activity was significantly decreased by day 3 ($p<0.01$) following transfer and reached minimal activity level 13 days after transfer ($p<0.001$). There was no significant increase in Ca^{2+}+Na^{+}-ATPase activity until 15 days after transfer ($p<0.05$) when there was a sharp increase in activity to levels characteristic of fully adapted sw fish.

Plasma Ca^{2+} concentration increased significantly at day 2 ($p<0.05$), then returned to pretransfer level 4 days after transfer and remained constant for the remainder of the period studied ($p>0.9$).

B. Seawater-adapted killifish transferred to freshwater.

Figure 20 shows the temporal changes in Ca^{2+}+

Mg^{2+}-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities and plasma calcium concentration following transfer of sw-adapted fish to fw. Gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly increased 13 days after transfer ($p<0.001$) while $Ca^{2+}+Na^{+}$-ATPase activity was significantly decreased 13 days after transfer ($p<0.05$). There was no significant difference in both enzyme activity levels from day 13 to days 15,17 and 21 ($p>0.3$).

Plasma Ca^{2+} concentration decreased significantly at day 2 ($p<0.02$). Then returned to pretransfer level 5 days after transfer and remained constant for the remiander of the period studied ($p>0.9$).

V. Endocrine control of gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase

The pituitary gland has a hypercalcemic action in a low calcium environment (Pang et al., 1971a). On pituitary gland hormone that has been implicated in this action is prolactin (Pang et al., 1973b). A corpuscles of Stannius factor has been reported to have a hypocalcemic action in teleosts adapted to a high calcium environment (Pang et al., 1973c; Fenwick, 1974; So and Fenwick, 1977). To determine if these Ca-regulating hormones act by regulating gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase, one group of killifish was adpated to seawater and injected with cod pituiary homogenate or prolactin while another group of killifish was adpated to calcium-deficient seawater, fed low calcium

food and injected with cod corpuscles of Stannius homogenate. Branchial Ca-ATPases were assayed at the end of the experimental period.

A. Cod pituitary homogenate

Table 7 shows the activities of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in the GEMF and the plasma Ca^{2+} level in fish adapted to sw and injected with saline or cod pituitary gland homogenate.

The plasma Ca^{2+} level was significantly increased in the cod pituitaty gland homogenate-injected fish as compared to saline-injected controls ($p<0.001$), showing the pituitary gland homogenate had a hypercalcemic effect.

The $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly higher in the cod pituitary gland homogenate-injected fish than in the saline-injected fish ($p<0.001$). The $Ca^{2+}+Na^{+}$-ATPase activity was significantly decreased in the cod pituitary gland homogenate-injected fish ($0.02>p>0.01$) (Table 7).

B. Ovine prolactin

Table 7 shows the activities of the $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in the gill epithelium microsomal fraction and plasma Ca^{2+} levels in killifish adapted to seawater and injected with ovine prolactin or saline.

The plasma Ca^{2+} level was significantly increased in the ovine prolactin-injected fish as compared to saline-injected controls ($p<0.001$), showing that prolactin

had a hypercalcemic effect.

There was no significant difference in the activity of $Ca^{2+}+Mg^{2+}$-ATPase between prolactin-injected and saline-injected controls (0.5>p 0.3). The $Ca^{2+}+Na^{+}$-ATPase activity, on the other hand, was significantly decreased in the prolactin-injected fish compared to the controls (0.01>p>0.001) (Table 7).

C. Cod corpuscles of Stannius

Table 8 shows the activities of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in the GEMF and plasma Ca^{2+} levels in killifish adapted to calcium-deficient seawater and injected with saline or cod corpuscles of Stannius homogenate.

The plasma Ca^{2+} levels were significantly lower in the cod corpuscles of Stannius homogenate-injected fish as compared to saline-injected fish ($p<0.05$), showing the corpuscles of Stannius homogenate had a hypocalcemic effect.

The $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly decreased in the cod corpuscles of Stannius homogenate-injected fish as compared to the saline-injected controls ($p<0.001$). The $Ca^{2+}+Na^{+}$-ATPase activity was significantly increased in the cos corpuscles of Stannius homogenate-injected fish ($p<0.001$) (Table 8).

DISCUSSION

The mechanism by which constant blood Ca^{2+} levels in different environments are maintained ineuryhaline fish has been an enigma for many years. Seawater fish are hypocalcemic to the environment. Calcium enters the blood from external sources by diffusion and excess calcium has to be actively removed. Freshwater fish on the other hand are hyperosmotic to the environment. Freshwater fish face the problem of diffusional loss of calcium from the blood; calcium must be replaced by active intake. Since the gills are in constant contact with the surrounding water, it has been suggested that these organs may beinvolved in regulating blood Ca^{2+} levels. There have been reports of a calcium-activated ATPase in gill epithelium from rainbow trout (Ma et al., 1974; Parker et al., 1985) and seawater and freshwater-adapted eels (Fenwick, 1976; Shepard et al., 1978; Fenwick, 1979; Ho and Chan, 1980; Flick et al., 1983; Flick et al., 1984), that may have a role in Ca^{2+} regulation.

Matsumura and Clark (1980) reported two Ca^{2+}-dependent ATPases in the axon membrane from the retinal nerve of the squid, Loligo pealei, one was a Ca^{2+}+Mg^{2+}-ATPase requiring Mg^{2+} and a low Ca^{2+} concentration for activity and the other was a Ca^{2+}+Na^{+}-ATPase (called ecto-ATPase) requiring Na^{+} and a high Ca^{2+} concentration for activity.

These findings suggested that similar enzymes might

exist in killifish gill epithelium. It was speculated that in fw fish a high Ca^{2+} affinity enzyme such as $Ca^{2+}+Mg^{2+}$-ATPase might be responsible for active uptake of Ca^{2+} from a low Ca^{2+} environment. A low Ca^{2+} affinity enzyme like $Ca^{2+}+Na^{+}$-ATPase in sw-adapted killifish might be responsible for pumping Ca^{2+} out of the body to maintain Ca^{2+} homeostasis. In the present study a high Ca^{2+} affinity $Ca^{2+}+Mg^{2+}$-ATPase with significantly higher activity in fw fish than in sw fish, and a low Ca^{2+} affinity $Ca^{2+}+Na^{+}$-ATPase with significantly higher activity in sw fish than in fw fish have been identified in killifish gill epithelium. These two enzymes have been characterized in sw and fw-adapted fish and their role in Ca^{2+} regulation has been studied. Whether Ca^{2+} regulating hormones act by regulating these gill epithelium Ca-ATPases has also been investigated.

I. Characterization of the Ca-ATPases

A. $Ca^{2+}+Mg^{2+}$-ATPase

The criteria for distinguishing a plasma membrane $Ca^{2+}+Mg^{2+}$-ATPase are: 1) Location in the plasma membrane; 2) A requirement for a low concentration of free Ca^{2+}; 3) An apparent K_m for Ca^{2+} of less than 10uM in the presence of EGTA; 4) A requirement for Mg^{2+} in addition to Ca^{2+} for enzyme activity; and 5) Stimulation by calmodulin (Penniston, 1982).

This study demonstrated that the $Ca^{2+}+Mg^{2+}$-ATPase in killifish gill epithelium meets all these criteria.

The characteristics of this enzyme are summarized in Table 9. The criteria for this enzyme was: 1) The enzyme is found in a plasma membrane-rich fraction from gill epithelium. The Ca-ATPase from mitochondria contamination in this fraction did not contribute to the measured activity of the enzyme. Calmodulin does not stimulate the activity of the mitochondrial Ca-ATPase (Schwerzmann, 1985) as it does Ca^{2+}+Mg^{2+}-ATPase from plasma membrane. Oligomycin B an inhibitor of mitochondrial Ca-ATPase did not affect the Ca^{2+}+Mg^{2+}-ATPase in the gill microsomal fraction from sw and fw-adapted killifish in this study. This indicates mitochondrial Ca-ATPase was not being measured. A portion of the observed Ca^{2+}+Mg^{2+}-ATPase activity however may have originated from the endoplasmic reticulum (ER). It is known that the ER contains Ca^{2+}+Mg^{2+}-ATPase (Moore et al., 1974) and therefore contamination with this fragment could lead to overestimation of the Ca^{2+}+Mg^{2+}-ATPase activity of the plasma membrane. The optimal uptake of Ca^{2+} into the ER requires a Ca^{2+} concentration of 2μM (Bayerdorffer et al., 1984). During the isolation procedure the fractions were not screened for marker enzyme activities from the ER; 2) A low concentration of free Ca^{2+} ($10^{-7.7}$M) for activity was also observed; 3) The apparent K_m for Ca^{2+} in sw (0.6μM) and fw (0.8μM) fish was less than 10uM in the presence of 0.5mM EGTA; 4) The Ca^{2+}+Mg^{2+}-ATPase

characterized in this experiment required Mg^{2+} for activity. The optimal Mg^{2+} for sw and fw fish was 10mM; and 5) The gill epithelium Ca^{2+}+Mg^{2+}-ATPase may require calmodulin for stimulation of activity since trifluoperazine, an inhibitor of calmodulin, significantly decreased enzyme activity in sw and fw fish.

Other characteristics of Ca^{2+}+Mg^{2+}-ATPase arbitrarily chosen since there were broad peaks of activity 1) The enzyme had optimal activity in an ATP range from 4mM to 7mM; 2) The enzyme had an optimal range of activity from pH 7.5 to pH 8.0; and 3) The enzyme had an optimal range of activity from 30°C to 50°C. The energy of activation was 11.4Kcal/mol and the Q_{10} was 2.19 between 20°C and 30°C for both sw and fw-adapted fish. This Q_{10} value indicates a temperature dependent reaction.

In comparing the characteristics of the Ca^{2+}+ Mg^{2+}-ATPase from killifish gill epithelium with those described for Ca-ATPases in other teleosts it was found that the reported K_m values for Ca^{2+} (0.6μM for sw fish and 0.8μM for fw fish) are not in good agreement with Ca^{2+} K_m values reported for other teleost Ca-ATPases. In sw and fw-adapted gill epithelium from Anguilla japonica, the reported K_m value for Ca^{2+} was 0.45mM and 0.32mM respectively (Ho and Chan, 1980). Fenwick (1979) reported a K_m value fro Ca^{2+} of 0.4mM for both sw and fw-adapted Anguilla rostrata.

The difference in the K_m values for Ca^{2+} in Ca-ATPase from this study compared to that determined by the above investigators could be due to the fact that the assay conditions were different. The above investigators when assaying the Ca-ATPase from Anguilla gill epithelium utilized Ca^{2+},Mg^{2+} and Na^{+} in the assay medium at concentrations in which both Ca-ATPases characterized in killifish gill epithelium would be active.

In both Japanese and American eels 5mM ATP concentration was utilized for Ca-ATPase activity. In this experiment Ca^{2+}+Mg^{2+}-ATPase had a broad range of optimal activity from 4mM to 7mM ATP concentration and the apparent K_m value for ATP was 3.87mM for sw fish and 1.77mM for fw fish. This was not in good agreement with the reported ATP K_m values of 0.2mM from sw-adapted eels and 0.3mM from fw-adapted eels for Ca-ATPase in gill epithelium (Ho and Chan, 1980).

The calculated energy of activation (E_a) for branchial Ca^{2+}+Mg^{2+}-ATPase in sw and fw-adapted fish in this experiment was 11.4Kcal/mol. This value was in good agreement with the E_a of 13.84Kcal/mol reported from fw-adapted Anguilla rostrata gill epithelium (Flick et al., 1984). A Q_{10} of 2.2 was reported for fweel gill epithelium Ca-ATPase (Fenwick, 1979), which was in good agreement with the Q_{10} value observed in this experiment of 2.19 for both sw and fw-adapted killifish gill epithelium Ca^{2+}+Mg^{2+}-ATPase.

There were minor differences in the characteristics of the sw and fw-adapted gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase. However, there were no significant differences between the K_ms for Ca^{2+}, Mg^{2+} and ATP for the enzyme in both sw and fw-adapted fish. Both sw and fw-adapted fish had a Q_{10} and and E_a value of 11.4Kcal/mol. These observations strongly suggest the $Ca^{2+}+Mg^{2+}$-ATPase characterized in this experiment is the same enzyme in sw and fw-adapted killifish that has a significantly higher activity in fw fish compared to the activity in sw fish as discussed below.

B. $Ca^{2+}+Na^{+}$-ATPase

In all the literature, the only investigators that have studied a membrane-bound $Ca^{2+}+Na^{+}$-ATPase are Matsumura and Clark (1980). They reported a $Ca^{2+}+Na^{+}$-ATPase in plasma membrane from the retinal nerve of squid (Loligo pealei). For activation the enzyme had a requirement for a high Ca^{2+} concentration, Na^{+}, no Mg^{2+} and a Q_{10} value of 2.23. This experiment demonstrated a membrane-bound $Ca^{2+}+Na^{+}$-ATPase in gill epithelium from sw and fw-adapted killifish which fit the criteria established in squid. The characteristics are summarized in Table 10. The criteria for this enzyme was: 1) The optimal range of enzyme activity was between 10mM to 20mM Ca^{2+}. The K_m values for Ca^{2+} in sw fish was 0.36mM and in fw fish was 0.44mM; 2) The optimal range of enzyme activity was between 10mM to 60mM Na^{+}. The Na^{+} K_m values for sw fish was 1.05mM

and for fw fish was 1.85mM; 3) EDTA is a chelator more specific for Mg^{2+} than Ca^{2+} (Portzehl et al., 1964). Since 1.0mM EDTA was utilized in all homogenizing medium and all assay medium, this strongly suggests the enzyme does not require Mg^{2+} for activity; 4) The Q_{10} observed for sw fish was 2.1 and the Q_{10} observed for fw fish was 2.0. This Q_{10} value indicates a temperature-dependent reaction; and 5) The gill epithelium Ca^{2+}+Na^{+}-ATPase does not appear to be controlled by calmodulin since trifluoperazine, an inhibitor of calmodulin, singificantly increased enzyme activity in both sw and fw fish. A possible explanation for the increase is given in the following paragraph.

The phenothiazines such as trifluoperazine (TFP) and chloropromazine (CPZ) are antipsychotic drugs that are used a pharmacological tools to assess the physiological functions of calmodulin, an activator of the erythrocyte membrane-bound Ca^{2+}+Mg^{2+}-ATPase (Hinds and Vincenzi, 1983). As hydrophobic molecules, phenothiazines may interact non-specifically with Ca^{2+}-independent and Ca^{2+}-dependent membrane proteins thus perturbing membrane structure (Lohr et al., 1984). The phenothiazines have also been reported to interact with calmodulin-dependent enzymes (Wallace et al., 1983) and numerous other cellular constituents including several types of receptors for neurotransmitters, phospholipids, and other calcium-binding proteins, such as troponin C (Weiss, 1983). Martonsi et al., (1982) added TFP to muscle cells in which it activated the synthesis of two

proteins. Volpe et al., (1984) reported addition of TFP to skeletal muscle sarcoplasmic reticulum (SR) which inhibited Ca^{2+}-loading and $Ca^{2+}+Mg^{2+}$-ATPase activity but did not affect the Ca^{2+} efflux mechanism. Chanberlain et al., (1983) found TFP enhanced the Ca^{2+}-loading rate in cardiac SR vesicles and inhibited Ca^{2+} efflux from the vesicles. Ho et al., (1983) suggested that TFP acts by partitioning into the lipid phase of the SR membrane, allowing structural perturbations to occur. Adding chloropromazine to erythrocyte ghosts induced holes of constant size but variable number in the membrane (Lieber et al., 1984).

These observations show that the phenothiazines inhibit some proteins and enzymes while activating others. One way these drugs may work is by partitioning into membrane structure thereby causing alteration in membrane architecture. Since this investigation revealed a significant increase in $Ca^{2+}+Na^{+}$-ATPase activity when TFP was added it is suggested that the phenothiazines partitioned into the membrane allowing structural pertubations to occur which may have freed more of the enzyme from the membrane thus accounting for the higher activity.

Other characteristics of $Ca^{2+}+Na^{+}$-ATPase arbitrarily chosen since there were broad peaks of activity at different concentrations, pHs and temperatures were: 1) The enzyme had optimal activity in an ATP range from 4mM to 9mM; 2) The enzyme had an optimal rane of activity from pH 6.5 to pH 7.5; and 3) The enzyme had an optimal range of

activity from 30°C to 50°C. The energy of activation (E_a) was 12.59Kcal/mol for sw fish and 11.77Kcal/mol for fw fish.

There were minor differences in the characteristics of the sw and fw-adapted gill epithelium Ca^{2+}+Na^{+}-ATPase. However, there was no significant difference between the K_ms for Ca^{2+}, Na^{+} and ATP for the enzyme in both sw and fw-adapted fish. The Q_{10} for sw fish was 2.1 and for fw-adapted fish was 2.0. The E_a value for sw fish was 12.59Kcal/mol and for fw fish was 11.77Kcal/mol. These observations strongly suggest the Ca^{2+}+Na^{+}-ATPase characterized in this experiment is the same enzyme in sw and fw-adapted killifish that has a significantly higher activity in sw fish as compared to the activity in fw fish as discussed below.

II. Role of gill epithelium Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase in calcium regulation

The Ca^{2+}+Mg^{2+}-ATPase activity in killifish gill epithelium was found to be significantly higher in fish adapted to freshwater, a low Ca^{2+}-environment, compared to seawater fish, a high Ca^{2+}-environment. These findings suggest that gill epithelium Ca^{2+}+Mg^{2+}-ATPase may be involved in the branchial influx of Ca^{2+} in killifish adapted to a low Ca^{2+} environment. Calcium uptake rates in intact killifish have been determined in a medium containing various concentrations of calcium. The uptake rates were found to be higher in killifish adapted to low calcium environments (Pang and Pang, 1986). Ca^{2+}+Na^{+}-ATPase activity is significantly higher in sw fish than in fw fish, suggesting

this enzyme may be involved in the branchial outflux of Ca^{2+} in fish adapted to a high Ca^{2+} environment.

Both enzymes, Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase appear to be regulated specifically by environmental Ca^{2+}. Fish were adapted to calcium-enriched freshwater (Ca-E-fw) in which the ion concentrations are the same as in fw except for the Ca^{2+} concentration. In fw the Ca^{2+} concentration equals 0.2mM while Ca-E-fw contained a Ca^{2+} concentration comparable to sw (10mM). As in sw, the Ca^{2+}+Mg^{2+}-ATPase activity was significantly lower and the Ca^{2+}+Na^{+}-ATPase activity was significantly higher in Ca-E-fw as compared to fw activities. Fish were also adapted to calcium-deficient seawater (Ca-D-sw), in which all the ion concentrations are the same as sw except for the Ca^{2+} concentration. In sw, the Ca^{2+} concentration equals 10mM while the Ca-D-sw contained a Ca^{2+} concentration comparable to fw (0.2mM). As in fw, in Ca-D-sw the Ca^{2+}+Mg^{2+}-ATPase activity was significantly higher and the Ca^{2+}+Na^{+}-ATPase activity was significantly lower as compared to sw activities.

These results support the hypothesis that Ca^{2+}+Mg^{2+}-ATPase is pumping Ca^{2+} into the fish across branchial epithelium when fish are adapted to a low Ca^{2+} environment and that Ca^{2+}+Na^{+}-ATPase is pumping Ca^{2+} out of the fish across branchial epithelium when fish are adapted to a high Ca^{2+} environment. They do not,

however, give insight into the physiological importance of these enzymes in regulation of blood calcium.

To determine whether there is a correlation between plasma calcium levels and changed in gill epithelium Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities, killifish were transferred from fw to sw and from sw to fw. The blood Ca^{2+} levels were measured and the activities of the branchial Ca-ATPase were assayed on different days during a three week period.

When killifish were transferred from fw to sw, the plasma Ca^{2+} levels were significantly increased from pretransfer levels at day 2. The plasma Ca^{2+} levels returned to pretransfer level by day 4. Ca^{2+}+ Na^{+}-ATPase activity, on the other hand, did not begin to increase until day 15. The Ca^{2+}+Mg^{2+}-ATPase activity began to decrease significantly at day 3 and reached lowest activity level at day 13.

When killifish were transferred from sw to fw, the plasma Ca^{2+} levels were significantly decreased from pretransfer levels at day 2. The blood Ca^{2+} levels returned to pretransfer level by day 5. The Ca^{2+}+ Na^{+}-ATPase activity decreased significantly at day 7 and reached minimal activity level at day 13. However, Ca^{2+}+Mg^{2+}-ATPase activity did not significantly increase until day 10, reaching maximal level by day 13.

These results suggest the gill Ca^{2+}-ATPases are

involved in long term Ca^{2+} regulation and there may be other short term mechanisms not involving these enzymes for rapidly returning blood Ca^{2+} levels to normal values.

Short and long term control of ion regulation is not unknown in teleosts. Upon transferring tilapia (Sarotherodon mossambicus) from fw to sw, the short term mechanism of increased Cl^- conductance was activated within 24 hours in existing chloride cells, the cells responsible for salt extrusion by the gills. While the long term mechanism of branchial salt secretion involves differentiation of new chloride cells which occurs 1-2 weeks after transfer. Increased differentiation of chloride cells appears to be responsible for enhancement and maintenance of high chloride secretion rates (Foskett, 1981; Foskett et al., 1983).

III. Endocrine control of gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase

There is evidence in the literature that Ca^{2+} regulation in teleosts is under endocrine control. In low Ca^{2+} environments the pituitary gland appears to play a role in Ca^{2+} regulation (Pang et al., 1971a; Pang et al.,1973a,b; Ball et al., 1982). In high Ca^{2+} environments the corpuscles of Stannius appears to play a role in Ca^{2+} regulation (Pang, 1971b; Pang, 1981; Pang et al., 1974; Pang et al., 1975; Fenwick, 1976, 1979). The findings suggest that the killifish gill

epithelium Ca-ATPases may be under control of the hormones from these glands.

A. Pituitary gland

The pituitary gland appears to have an important hypercalcemic function in low Ca^{2+} environments. Hypophysectomy in fw fish results in hypocalcemia accompanied by tetanic seizures and invariably leads to freshwater failure (Pang et al., 1973,b). If sw-adapted fish are hypophysectomized no changes are seen in serum calcium levels (Pang, 1973), while hypophysectomy produces hypocalcemia in fish adapted to calcium-deficient sw (Pang et al., 1971a, 1973a,b, 1978; Chan and Chester Jones, 1968; Bjornssen and Hansson, 1983). Replacement therapy with pituitary gland homogenate corrects the hypocalcemia in hypophysectomized fish (Pang et al., 1973c, 1978).

The results of this study confirm the hypercalcemic function of the pituitary gland in teleosts. Injection of cod pituitary gland homogenate into intact killifish adapted to artifical seawater resulted in significantly increased blood Ca^{2+} levels. The branchial Ca-ATPases were shown to be regulated by the pituitary gland. $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly higher than saline injected control values and the $Ca^{2+}+Na^{+}$-ATPase activity was significantly lower than saline injected control activity when killifish were given 5 daily injections with cod pituitary gland

homogenate or saline solution. These findings are similar to those observed when killifish are adapted to a low Ca^{2+} environment like freshwater in which pituitary gland is active in calcium regulation.

Prolactin, a pituitary gland hormone, is known to be important in osmoregulation in freshwater fish. In the long term adaptation process, prolactin causes de-differentiation of chloride cells (Potts, 1984) and in the short term it reduces the active transport of chloride via $Na^{+}+K^{+}$-ATPase (Foskett, 1981). It also acts to stimulate sodium uptake and limit sodium loss, thus maintaining plasma osmolarity (Loretz and Bern, 1982). Prolactin's role in low salinity adaptation by fish has been substantiated by studies demonstrating that prolactin secretory cells are activated by low salinities (Dharmamba and Maetz, 1972; Wendelaar Bonga and Van der Meij, 1981) and that the blood levels of the hormone increase when fish are acclimated to a reduced saline environment (Nicol et al., 1981).

Prolactin injection is known to induce hypercalcemia in intact teleosts (Pang et al., 1978; Wendelaar Bonga and Greven, 1978) and in hypophysectomized Fundulus heteroclitus adapted to calcium-deficient seawater (Pang et al., 1973b). There was no effect of prolactin injection in intact killifish adapted to freshwater or calcium-enriched freshwater while fish adapted to seawater or calcium-deficient seawater exhibited hypercalcemia (Pang, 1981). The data suggest that environmental calcium does not affect prolactin release and that prolactin may not

be a specific Ca^{2+} regulating hormone. The effect of prolactin on Ca^{2+} regulation may be secondary to the effects on Na^{+} and Cl^{-} regulation.

In the present study prolactin injection into intact killifish adapted to seawater resulted in hypercalcemia. There was no significant increase in $Ca^{2+}+Mg^{2+}$-ATPase activity from saline injected control activities. On the other hand, $Ca^{2+}+Na^{+}$-ATPase activity was significantly decreased and $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly increased by pituitary gland homogenate injection, while prolactin injection significantly decreased $Ca^{2+}+Na^{+}$-ATPase activity but had no effect on $Ca^{2+}+Mg^{2+}$-ATPase activity. These results suggest that there may be two or more hypercalcemic factors secreted by the teleost pituitary gland. It has been reported that when male killifish were adapted to calcium-free seawater for nearly 5 months they developed hypocalcemia. The cells of the pars intermedia were found to be increased in number, became enlarged and more active while only a minor response was observed of the prolactin cells in the anterior pituitary gland (Ball et al., 1982). The authors thus suggest that there are two hypercalcemic factors secreted by the teleost pituitary gland, one being prolactin and the other originating from the pars intermedia. Parsons et al., (1978) reported that fish pituitary glands contain a hypercalcemic factor which cross-reacts immunologically with mammalian parathyroid hormone antibodies.

They named the factor hypercalcin. However, hypercalcin is reported to be a fast acting hormone which produces hypercalcemia within hours (Pang and Pang, 1986), but the results of this study do not support such a suggestion but does suggest the gill Ca-ATPases are regulated by a long term nechanism and therefore are not regulated by hypercalcin.

B Corpuscles of Stannius

Removing the corpuscles of Stannius from teleosts adapted to high calcium environments produces hypercalcemia whereas this effect is reduced or absent in Stanniectomized fish adapted to a low calcium medium (Fenwick and Forster, 1972; Pang et al., 1973c). Replacement therapy with corpuscles of Stannius transplant corrected the hypercalcemia in stanniectomized fish (Pang et al., 1973c). Injection of corpuscles of Stannius extract into intact fish induced hypocalcemia in fw-adapted eels (Bailey and Fenwick, 1974) and in sw-adapted and Ca-deficient sw-adapted killifish (Pang, 1971; Pang et al., 1974).

An electron microscopy study of Fundulus heteroclitus corpuscles of Stannius revealed that when the fish were adapted to calcium-deficient seawater the corpuscles of Stannius appeared inactive with an accumulation of secretory granules while the corpuscles of Stannius from killifish adapted to seawater showed a depletion of secretory granules, hyperactivity of the Golgi complex and dilation of the endoplasmic reticulum. These observations

indicated that the corpuscles of Stannius are actively secreting a substance in sw-adapted killifish. The name "hypercalcin" was proposed for the hypocalcemic factor(s) in the corpuscles of Stannius by Pang et al., 1974). Copp and Ma (1981) proposed the name teleocalcin for this same factor.

The hypocalcemic function of the corpuscles of Stannius was confirmed in the present study by the significant decrease in plasma Ca-levels in killifish adapted to calcium-deficient seawater inject with cod corpuscles of Stannius homogenate. The activity of Ca^{2+}+Mg^{2+}-ATPase in the branchial epithelium from killifish injected with cod corpuscles of Stannius was significantly decreased compared to saline control values while Ca^{2+}+Na^{+}-ATPase activity was significantly increased.

The results from this study sugges that a hormone from the corpuscles of Stannius is inhibiting the activity of the branchial epithelium Ca^{2+}+Mg^{2+}-ATPase and stimulating the activity of Ca^{2+}+Na^{+}-ATPase in a high Ca^{2+} environment. These findings support the hypothesis put forth in these studies that a Ca^{2+}+Na^{+}-ATPase is responsible for Ca^{2+} extrusion in killifish epithelium.

The findings in these studies correlate with the findings reported with isolated perfused eel gills. Stanniectomy increased the active uptake of calcium from the environmental media (Fenwick and So, 1974; So and Fenwick, 1977) and decreased calcium efflux (Milet et al.,

1979). These effects were reversed by the addition of corpuscles of Stannius extract to the perfusion fluid (So and Fenwick, 1979). This finding suggests that the corpuscles of Stannius factor may be stimulating efflux and inhibiting influx of Ca^{2+}. Fenwick (1976) reported stimulation in activity of a branchial epithelium Ca-ATPase in Stanniectomized Anguilla rostrata. When a preparation of eel gill membrane Ca-ATPase was added to a corpuscles of Stannius extract, Copp and Ma (1981) reported a significant decrease in the Ca-ATPase activity.

In conclusion, the results of this study sugges that the branchial epithelium Ca-ATPases are under hormonal control. The hypercalcemic factor(s) in the pituitary gland stimulate Ca^{2+}+Mg^{2+}-ATPase activity and inhibit Ca^{2+}+Na^{+}-ATPase activity while prolactin inhibits Ca^{2+}+Na^{+}-ATPase activity but did not affect Ca^{2+}+Mg^{2+}-ATPase activity. The corpuscles of Stannius factor(s) inhibited Ca^{2+}+Mg^{2+}-ATPase activity and stimulated Ca^{2+}+Na^{+}-ATPase activity.

Conclusions

The results of experiments presented in this thesis provide evidence for the first time that at least two different ATP-driven transport systems for calcium - Ca^{2+}+Mg^{2+}-ATPase and a Ca^{2+}+Na^{+}-ATPase - are present in killifish gill epithelium. Characterization of the two Ca-ATPases revealed: 1) A Ca^{2+}+Mg^{2+}-ATPase which has a high affinity for Ca^{2+}, requires Mg^{2+} for activity and may be controlled by calmodulin; and 2) A Ca^{2+}+Na^{+}-ATPase which

has a low affinity for Ca^{2+}, requires Na^{+} fpr activity, does not require Mg^{2+} and is probably not controlled by calmodulin.

The Ca^{2+}+Mg^{2+}-ATPase has a significantly higher activity in freshwater-adapted fish. The Ca^{2+}+Na^{+}-ATPase has a significantly higher activity in seawater-adapted fish.

Environmental calcium specifically controls the activities of the killifish branchial epithelium Ca-ATPases. The experiments provided evidence that the activity of Ca^{2+}+Mg^{2+}-ATPase was significantly higher in killifish adapted to freshwater and calcium-deficient seawater - both low calcium environments. On the other hand, Ca^{2+}+Na^{+}-ATPase activity was significantly higher in killifish adapted to seawater and calcium-enriched freshwater - both high calcium environments. These findings suggest that one gill epithelium Ca-ATPase is pumping calcium from the environment into fish adapted to a low calcium environment while the other gill epithelium Ca-ATPase is pumping calcium out of fish adapted to a high calcium environment.

The interrelationship between changes in plasma Ca^{2+} and Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase activities was investigated. When killifish were transferred from freshwater to seawater, blood Ca^{2+} levels significantly increased at day 2 and by day 4 returned to pretransfer level. Ca^{2+}+Na^{+}-ATPase activity did

not reach maximal activity until day 13 and $Ca^{2+}+Mg^{2+}$-ATPase reached minimal activity at day 13. When killifish were transferred from seawater to freshwater, blood Ca^{2+} levels significantly decreased at day 2 and by day5 returned to pretransfer levels. The activity of $Ca^{2+}+Mg^{2+}$-ATPase did not reach maximal activity until 15 days after transfer while $Ca^{2+}+Na^{+}$-ATPase activity reached minimal level 13 days after transfer. The results indicate two types of mechanisms are involved in killifish Ca^{2+} regulation when killifish are transferred to a new environment. One is a fast adaptation mechanism which appears to occur within a few days of transfer, not involving enzymes and the other is a slow adaptation mechanism which seems to occur within 2 weeks following transfer and involves changes in enzymes.

The pituitary gland and the corpuscles of Stannius have been implicated in Ca^{2+} regulation in killifish. The possible role of the Ca^{2+} regulating hormones on the branchial Ca-ATPase activities was investigated.

Killifish adapted to seawater and injected with cod pituitary homogenate had significantly decreased branchial $Ca^{2+}+Na^{+}$-ATPase activity and significantly increased gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase activity. Killifish adapted to seawater and injected with ovine prolactin also had significantly decreased gill epithelium $Ca^{2+}+Na^{+}$-ATPase activity but there was no effect

on branchial $Ca^{2+}+Mg^{2+}$-ATPase activity. These results suggest that $Ca^{2+}+Na^{+}$-ATPase activity is decreased by prolactin while $Ca^{2+}+Mg^{2+}$-ATPase activity is not affected by prolactin but may be stimulated by another hypercalcemic factor(s) in the pituitary gland.

Killifish adapted to calcium-deficient seawater and injected with cod corpuscles of Stannius homogenate had a significantly decresed $Ca^{2+}+Mg^{2+}$-ATPase activity while $Ca^{2+}+Na^{+}$-ATPase activity was significantly increased. These observations suggest that the branchial epithelium Ca-ATPases from killifish are under the influence of the Ca-regulating hormones.

The results of this experiment combined with results from other studies of vertebrate calcium transporting epithelium have been used to devise a hypothetical model for a slow adaptation mechanism for Ca^{2+}-regulation in a gill epithelium cell from killifish adapted to seawater and freshwater (Figure 21).

Based on the findings in this experiment several assumptions will be made: 1) $Ca^{2+}+Mg^{2+}$-ATPase activity is higher in fw fish than in sw-adapted fish. Therefore this enzyme may be responsible for transporting Ca^{2+} from the environment into the blood; and 2) $Ca^{2+}+Na^{+}$-ATPase activity is higher in sw fish than in fw-adapted fish. Therefore this enzyme may be responsible for transporting Ca^{2+} out of the gill epithelium cell into the environment.

It is known that an ATP-driven calcium transport mechanism is found in the basolateral membrane of the kidney and duodenal epithelial cells (Gmaj et al., 1979; Ghysen and Van Os, 1979). In addition, Na^++K^+-ATPase is located in the basolateral membrane of the chloride cell of teleost gill epithelium (Karnaky, 1986). These findings indicate that a transporting enzyme can be located on a specific side of an epithelial cell. This model of Ca-transport in killifish gill epithelium suggests that Ca^{2+}+Mg^{2+}-ATPase is located on the basolateral membrane while Ca^{2+}+Na^+-ATPase is located on the apical membrane of gill epithelial cells (Figure 21).

Seawater fish:

Seawater-adapted fish are hypo-osmotic to the environment and have the problem of Ca^{2+} overload. The Ca^{2+} concentration in seawater equals 10mM while the Ca^{2+} concentration inside the gill epithelial cell equals approximately 10^{-7}M and the Ca^{2+} concentration in the blood is equal to approximately 2mM. Ca^{2+} enters the cell by diffusion at the apical and basolateral membrane (1). The diffusion rate across the apical membrane however would be greater than across the basolateral membrane because of the high environmental Ca^{2+} concentration. One way to maintain a low cytosolic Ca^{2+} level in the epithelial cell would be by actively

pumping Ca^{2+} out of the cell to the environment by $Ca^{2+}+Na^{+}$-ATPase (2). The corpuscles of Stannius (CS) factor may be responsible for increasing the activity of the $Ca^{2+}+Na^{+}$-ATPase in sw-adapted fish since it was shown in this experiment that injection of fish adapted to Ca-D-sw with CS homogenate significantly increased $Ca^{2+}+Na^{+}$-ATPase activity.

Another way to maintain a low level of cytosolic Ca^{2+} could involve temporary storage of Ca^{2+} in the mitochondria and endoplasmic reticulum (3) (Carafoli, 1982); or Ca^{2+} might bind to a cytosolic Ca-binding protein (4). In intestinal cells a calcium-binding protein that is vitamin D-dependent is thought to carry out transepithelial Ca^{2+} transport from the brush border to the basolateral membrane (Bronner, 1982).

At the basolateral membrane $Ca^{2+}+Mg^{2+}$-ATPase would pump the Ca^{2+} into the blood but at a low rate (5). This enzyme activity also appears to be under the control of the corpuscles of Stannius factor. The results of this experiment show that $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly decreased when fish adapted to calcium-deficient seawater were injected with cod CS homogenate.

Thus in sw-adapted fish the combination of increased activity of $Ca^{2+}+Na^{+}$-ATPase pumping Ca^{2+} out of the cell into the environment and decreased activity of $Ca^{2+}+Mg^{2+}$-ATPase limiting Ca^{2+} entry into the blood would help solve the problem of Ca^{2+} overload in sw-

adapted fish.

Freshwater fish:

Freshwater-adapted fish are hyperosmotic to the environment. They also have the problem of Ca^{2+} loss. In these fish the rate of diffusion from the blood into the cell would be greater than the diffusion rate into the cell from the environment at the apical membrane because of the greater concentration difference of Ca^{2+} across the basolateral membrane. To maintain a constant blood calcium level the $Ca^{2+}+Mg^{2+}$-ATPase at the basolateral membrane would pump the Ca^{2+} out of the cell back into the blood (5). This enzyme activity appears to be under the control of a hypercalcemic factor of the pituitary gland. The results of this experiment show that $Ca^{2+}+Mg^{2+}$-ATPase activity was significantly increased when fish adapted to sw were injected with cod pituitary homogenate. At the apical membrane $Ca^{2+}+Na^{+}$-ATPase would pump Ca^{2+} into the environment but at a low rate (2). The activity of this enzyme appears to be under the control of prolactin. $Ca^{2+}+Na^{+}$-ATPase activity was significantly decreased when fish adapted to sw were injected with ovine prolactin or cod pituitary homogenate.

Thus in fw-adapted fish the combination of increased activity of $Ca^{2+}+Mg^{2+}$-ATPase pumping Ca^{2+} out of the cell into the blood and decreased activity of $Ca^{2+}+Na^{+}$-ATPase limiting Ca^{2+} pumping into the environment would

help solve the problem of Ca^{2+} loss in these fish.

Further studies to gain insight into the postulated mechanism for maintaining a constant blood calcium level in killifish adapted to either seawater or freshwater should involve attempts to localize the Ca-ATPases on specific sides of the gill epithelial cell membrane as hypothesized in the model. $Na^{+}+K^{+}$-ATPase has been localized on the basolateral membrane of the chloride cell using tritiated oubain, a specific inhibitor of $Na^{+}+K^{+}$-ATPase (Karnaky et al., 1976). At this time there are no known specific inhibitors for either $Ca^{2+}+Mg^{2+}$-ATPase or $Ca^{2+}+Na^{+}$-ATPase. However, since calmodulin stimulates $Ca^{2+}+Mg^{2+}$-ATPase activity an autoradiographic study utilizing tritiated calmodulin might localize $Ca^{2+}+Mg^{2+}$-ATPase in the gill epithelium cell membrane.

Perfusion studies with whole body preparations would also help elucidate the specific role of these Ca-ATPases in branchial Ca^{2+} exchange. The influx and efflux of Ca^{2+} can be measured by varying perfusate composition. The effects of hormones on Ca^{2+} fluxes can also be determined by this method. Once the Ca^{2+} optimum for Ca^{2+} efflux or influx is determined the results can be correlated with the Ca^{2+} optimum for the respective Ca-ATPase in gill epithelium.

The results of this experiment also indicate a fast adaptation mechanism for Ca^{2+} regulation in killifish. This mechanism appears to occur within a few days of transfer to a new environment (sw to fw or fw to sw) because blood calcium levels returned to normal before a change in enzyme activity ocurred.

One postulated mechanism involced in this fast adaptation mechanism of blood calcium regulation following transfer from sw to fw could involve phosphoinositides.

The breakdown of phosphoinositides in the plasma membrane has been hypothesized to be stimulated by Ca-mediated agonists, e.g., epinephrine, acetylcholine, histamine, vasopressin and some of the hypothalamic releasing hormones. These agonists exert some of their effects by raising the Ca^{2+} concentration in the cytosol of their target cells. The hypothesis postulates that interaction of a Ca^{2+}-mediated agonist with its receptor stimulate the breakdown of phosphatidylinositol 4,5 - bisphosphate (PIP_2) by a phosphodiesterase (phospholipase C) to yeild two intracellular "messengers" myoinositol 1,4,5 - triphosphate (IP_3) and 1,2 - diacylglycerol (DAG). IP_3 enters the cytosol and releases Ca^{2+} from endoplasmic reticulum. Then Ca^{2+} binds to calmodulin and there is a subsequent interaction of the Ca^{2+}-calmodulin complex with specific or multifunctional calmodulin-dependent protein kinases or other enzymes

and proteins. This results in specific cellular responses such as contraction of smooth, cardiac and skeletal muscle, cell shape changes, exocytosis, breakdown of glycogen and neurotransmitter release. When DAG increases in the plasma membrane it activates protein kinase C, a Ca^{2+}-phospholipid-dependent protein kinase (Exton, 1985; Ochs et al., 1985).

One pituitary gland hypercalcemic factor "hypercalcin", is reported to be a fast acting hormone (Pang and Pang, 1986). The hormone may be involved in the fast adaptation mechanism of killifish Ca^{2+} regulation. Hypercalcin could account for blood Ca^{2+} levels returning to pretransfer level by day 5 when killifish are transferred from sw to fw. The hormone may bind to a receptor on the killifish gill epithelium cell which could result in Ca^{2+} being released from the endoplasmic reticulum causing an increase in cytosolic Ca^{2+}. The calcium could then diffuse into the blood returning blood Ca^{2+} levels to pretransfer level. The result of these events could be a possible explanation to the findings reported in this experiment regarding the fast adaptation mechanism when killifish were transferred from sw to fw.

The kidney and intestine may also play a role in short term and/or long term Ca-regulation in killifish.

Stanniectomy in teleosts results in hypercalcemia and

also in a reduced Ca^{2+} excretion in the urine (Chan, 1972). This suggests that the kidney is involved in Ca^{2+} regulation in killifish. The Ca-ATPase activities in these organs should be investigated. In fw-adapted fish one of the ATPases may be involved in the active reabsorption of calcium from the glomerular filtrate into the blood to prevent Ca^{2+} loss while in sw-adapted fish the other Ca-ATPase may be involved in the active secretion of calcium from the blood into the filtrate to limit the rate of calcium entry into the body fluids. In fw fish intestine a Ca-ATPase may be actively pumping calcium across the intestinal epithelium and into the blood to obtain calcium from food. In the sw-adapted fish the activity of the Ca-ATPases in the intestinal cells may be decreased to limit Ca^{2+} entry into the blood from food and water. These Ca^{2+} regulating mechanisms in killifish kidney and intestine may be activated within days upon transfer to a different environment to maintain blood Ca^{2+} levels and be responsible for the short term mechanisms as discussed above.

The role that Ca^{2+}+Mg^{2+}-ATPase and Ca^{2+}+Na^{+}-ATPase from the gill epithelium microsomal fraction from sw and fw-adapted killifish play in regulating blood Ca^{2+} levels is far from being understood. The data in this thesis provides a structure to support continuation of this work.

Table 1. Effect of storage at -20°C on the activities of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase from seawater and freshwater-adapted Fundulus heteroclitus

	$Ca^{2+}+Mg^{2+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) M±SEM(n)		$Ca^{2+}+Na^{+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) M±SEM(n)	
	Stored (-20°C)	Not Stored	Stored (-20°C)	Not Stored
Seawater Fish	3.96±0.74(15)	6.66±1.16(4)*	8.92±0.13(4)	9.38±0.16(4)*
Freshwater Fish	16.15±0.35(8)	17.08±0.93(8)*	6.16±0.82(4)	5.03±0.56(4)*

* No significant difference in activity from stored preparations ($p>0.3$)

Table 2. Fractionation and recovery of marker enzymes in gill epithelium from *Fundulus heteroclitus*

Fraction	Succinic dehydrogenase ((μgm formazan.15 $min^{-1}.mg^{-1}$protein).10^3) M±SEM(n)	Na^++K^+-ATPase (μmoles Pi.$hr^{-1}.mg^{-1}$protein) M±SEM(n)
H_I	0.55±0.03(4)	2.21±0.09(4)
P_1	0.56±0.03(4)	1.59±0.07(4)
P_2	1.09±0.04(4)	6.02±0.22(4)
P_3	0.18±0.02(4)	8.23±0.12(4)
FS	0.02±0.00(4)	0.70±0.06(4)

H_I = initial homogenate
P_1, referred to as the nuclear fraction
P_2, referred to as the mitochondrial fraction
P_3, referred to as the microsomal fraction
FS = final supernatant

Table 3. Effect of oligomycin B on the activities of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in the gill epithelium microsomal fraction from seawater and freshwater-adapted Fundulus heteroclitus

	$Ca^{2+}+Mg^{2+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) $M\pm SEM(n)$		$Ca^{2+}+Na^{+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) $M\pm SEM(n)$	
	With Oligomycin B (2μg/ml)	Without Oligomycin B	With Oligomycin B (2μg/ml)	Without Oligomycin B
Seawater Fish	$8.89\pm0.16(4)$	$8.03\pm0.38(5)$*	$13.39\pm0.39(8)$	$12.66\pm0.99(4)$*
Freshwater Fish	$11.85\pm0.91(3)$	$11.20\pm0.71(4)$*	$12.17\pm0.97(8)$	$10.91\pm1.06(4)$*

* No significant difference from activity with oligomycin B ($p>0.5$)

Table 4. Effect of trifluoperazine (TFP) on $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities in the gill epithelium microsomal fraction from seawater and freshwater-adapted Fundulus heteroclitus

	$Ca^{2+}+Mg^{2+}$-ATPase (μmoles Pi.hr^{-1}.mg^{-1}protein) M$\pm$SEM(n)		$Ca^{2+}+Na^{+}$-ATPase (μmoles Pi.hr^{-1}.mg^{-1}protein) M$\pm$SEM(n)	
	With TFP (50μM)	Without TFP	With TFP (50μM)	Without TFP
Seawater Fish	$3.62\pm0.38(11)$	$10.17\pm0.56(8)$*	$11.50\pm0.20(9)$	$8.42\pm0.13(4)$*
Freshwater Fish	$3.35\pm0.29(12)$	$16.15\pm0.35(8)$*	$18.06\pm0.50(12)$	$6.16\pm0.82(4)$*

* Significant difference from activity with TFP ($p<0.001$)

Table 5. Effect of osmolarity on $Ca^{2+}+Na^{+}$-ATPase activity in gill epithelium microsomal fraction from seawater and freshwater-adapted Fundulus heteroclitus

	$Ca^{2+}+Na^{+}$-ATPase (μmoles Pi.hr^{-1}.mg^{-1}protein) M$\pm$SEM(n)	
	With Sucrose	Without Sucrose
Seawater Fish	8.19 ± 0.31(12)	8.42 ± 0.76(10)*
Freshwater Fish	6.83 ± 0.48(12)	5.58 ± 0.47(10)*

* No significant difference from activity with sucrose ($p>0.7$)

Table 6. Effect of environmental Ca^{2+} on the activity of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in the gill epithelium microsomal fraction from Fundulus heteroclitus

Fish adapted to:	$Ca^{2+}+Mg^{2+}$-ATPase (μmoles Pi.hr^{-1}.mg^{-1}protein) M$\pm$SEM(n)	$Ca^{2+}+Na^{+}$-ATPase (μmoles Pi.hr^{-1}.mg^{-1}protein) M$\pm$SEM(n)
Seawater	$5.66\pm0.35(4)^{*}$	$8.92\pm0.13(4)^{*}$
Freshwater	$10.98\pm0.94(14)$	$6.16\pm0.82(4)$
Calcium-deficient seawater	$7.15\pm0.30(20)^{**}$	$5.50\pm0.29(16)^{++}$
Calcium-enriched freshwater	$5.37\pm0.16(9)^{+}$	$8.35\pm0.21(27)^{+}$

* Significant difference from activity in freshwater fish ($p<0.02$)

** Significant difference from activity in freshwater and seawater fish ($p<0.001$)

$^{+}$ Significant difference from activity in freshwater fish ($p<0.001$)
No significant difference from activity in seawater fish ($0.9>p>0.7$)

$^{++}$ Significant difference from activity in seawater fish ($p<0.02$)
No significant difference from activity in freshwater fish ($p>0.3$)

Table 7. Effect of cod pituitary gland homogenate and ovine prolactin on plasma Ca^{2+} levels and gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities

	$Ca^{2+}+Mg^{2+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) M±SEM(n)	$Ca^{2+}+Na^{+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) M±SEM(n)	Plasma Ca^{2+} (mM) M±SEM(n)
Control*	3.41±0.24(19)	4.38±0.19(12)	1.50±0.02(13)
Pituitary gland homogenate injected**	5.25±0.23(25)+	3.73±0.17(23)+	1.68±0.03(13)+
Control*	3.89±0.42(28)	10.04±0.40(36)	1.50±0.02(13)
Prolactin injected***	4.40±0.28(33)	8.64±0.23(34)+	2.50±0.08(14)+

* Fish injected with o.01ml 0.6% saline solution/g body weight

** 0.01ml of a 0.6% saline solution contained 4% cod pituitary gland, fish injected with 0.01ml of 4% gland homogenate/g body weight

*** 0.01ml of a 0.6% saline solution contained 5ug ovine prolactin, fish injected with 5µg ovine prolactin/g body weight

\+ Significant difference from control activity ($p<0.05$)

Table 8. Effect of cod corpuscles of Stannius homogenate on plasma Ca^{2+} levels and gill epithelium $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase activities

	$Ca^{2+}+Mg^{2+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) M±SEM(n)	$Ca^{2+}+Na^{+}$-ATPase (μmoles $Pi.hr^{-1}.mg^{-1}$protein) M±SEM(n)	Plasma Ca^{2+} (mM) M±SEM(n)
Control*	7.15±0.30(20)	5.42±0.20(23)	1.60±0.08(7)
Corpuscles of Stannius homogenate injected**	3.61±0.10(28)+	7.20±0.37(20)+	1.33±0.08(9)+

* Fish injected with 0.01ml 0.6% saline solution/g body weight

** 0.01ml of a 0.6% saline solution contained 0.17mg corpuscles of Stannius, fish injected with 0.17mg corpuscles of Stannius/g body weight

\+ Significant difference from saline control activity ($p<0.05$)

Table 9. Summary of characterization of Ca^{2+}+Mg^{2+}-ATPase

	Seawater fish	Freshwater fish
Mg^{2+}		
Optimal concentration	10mM	10mM
K_m	1.31mM	2.54mM
Ca^{2+}		
Optimal concentration	0.0025mM-5mM	0.1mM-1mM
K_m	0.6μM	0.8μM
ATP		
Optimal concentration	no peak of activity	4mM-7mM
K_m	3.87mM	1.77mM
pH		
Optimum pH	7.5-8.0	7.5
Temperature		
Optimum temperature	30°C-50°C	30°C-37°C
Q_{10}(between 20°C-30°C)	2.19	2.19
E_a	11.5Kcal/mol	11.5Kcal/mol
Effect of trifluoperazine	Significant decrease in enzyme activity	Significant decrease in enzyme activity

Table 10. Summary of characterization of Ca^{2+}+Na^{+}-ATPase

	Seawater fish	Freshwater fish
Na^{+}		
Optimal concentration	10mM-60mM	10mM-60mM
K_m	1.05mM	1.85mM
Ca^{2+}		
Optimal concentration	20mM	10mM-20mM
K_m	0.36mM	0.44mM
ATP		
Optimal concentration	5mM-6mM	4mM-9mM
K_m	0.29mM	0.54mM
pH		
Optimal pH	7.0-7.5	6.5-7.4
Temperature		
Optimum temperature	$30^{o}C$-$50^{o}C$	$37^{o}C$-$50^{o}C$
Q_{10} (between $15^{o}C$-$25^{o}C$)	2.10	2.00
E_a	12.59Kcal/mol	11.77Kcal/mol
Effect of trifluoperazine	Significant increase in enzyme activity	Significant increase in enzyme activity

Figure 1. Effect of Ca^{2+} regulating hormones on target organs in terrestrial vertebrates.

+ = stimulates
- = inhibits
? = uncertain

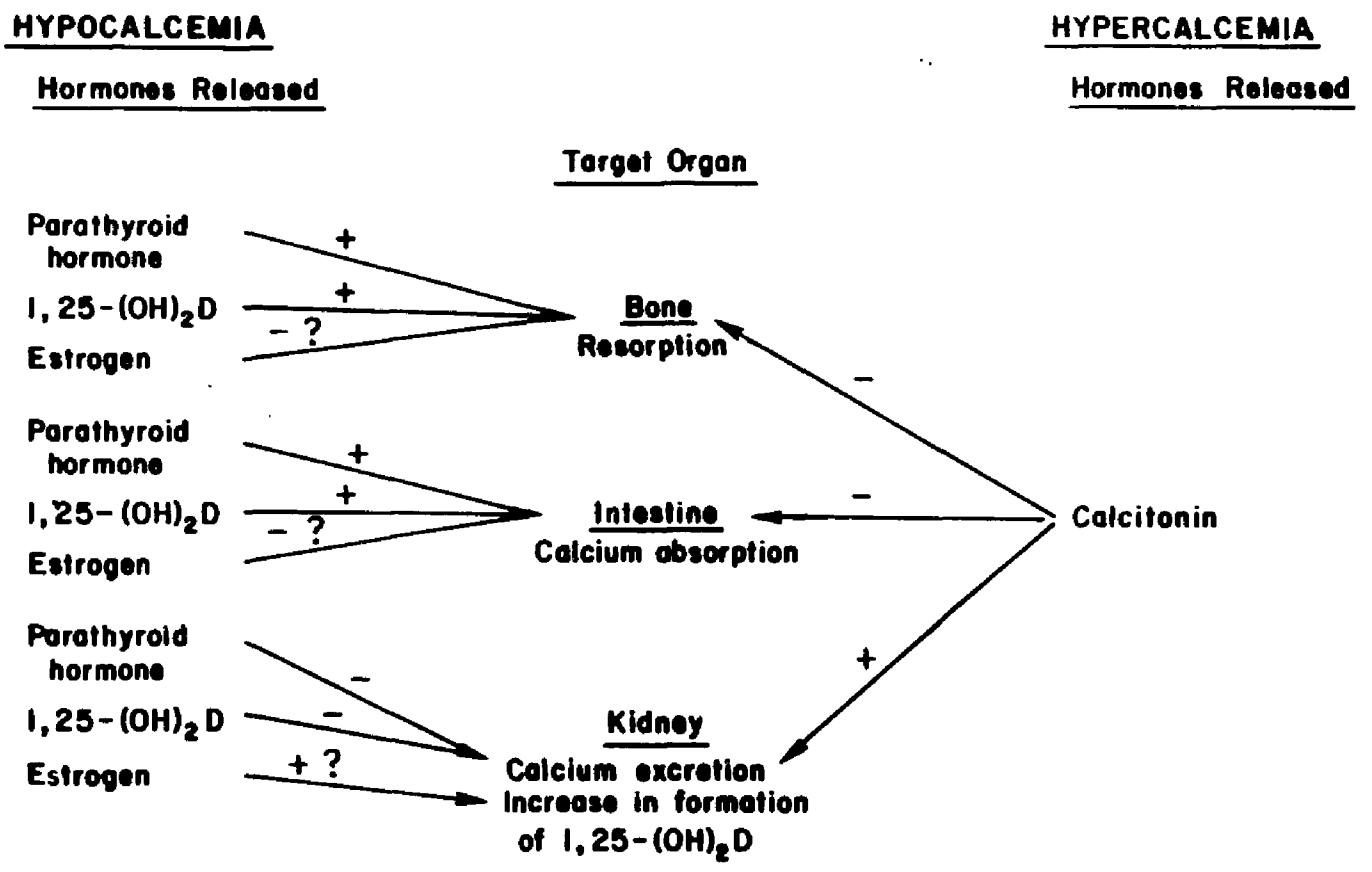

HYPOCALCEMIA
Hormones Released
HYPERCALCEMIA
Hormones Released
Target Organ
Parathyroid hormone
1,25-(OH)2D
Estrogen
+
+
- ?
Bone
Resorption
Parathyroid hormone
1,25-(OH)2D
Estrogen
+
+
- ?
Intestine
Calcium absorption
Parathyroid hormone
1,25-(OH)2D
Estrogen
-
-
+ ?
Kidney
Calcium excretion
Increase in formation of 1,25-(OH)2D
-
-
+
Calcitonin

Figure 2. Effect of Ca^{2+} regulating hormones on target organs in aquatic vertebrates.

? = uncertain

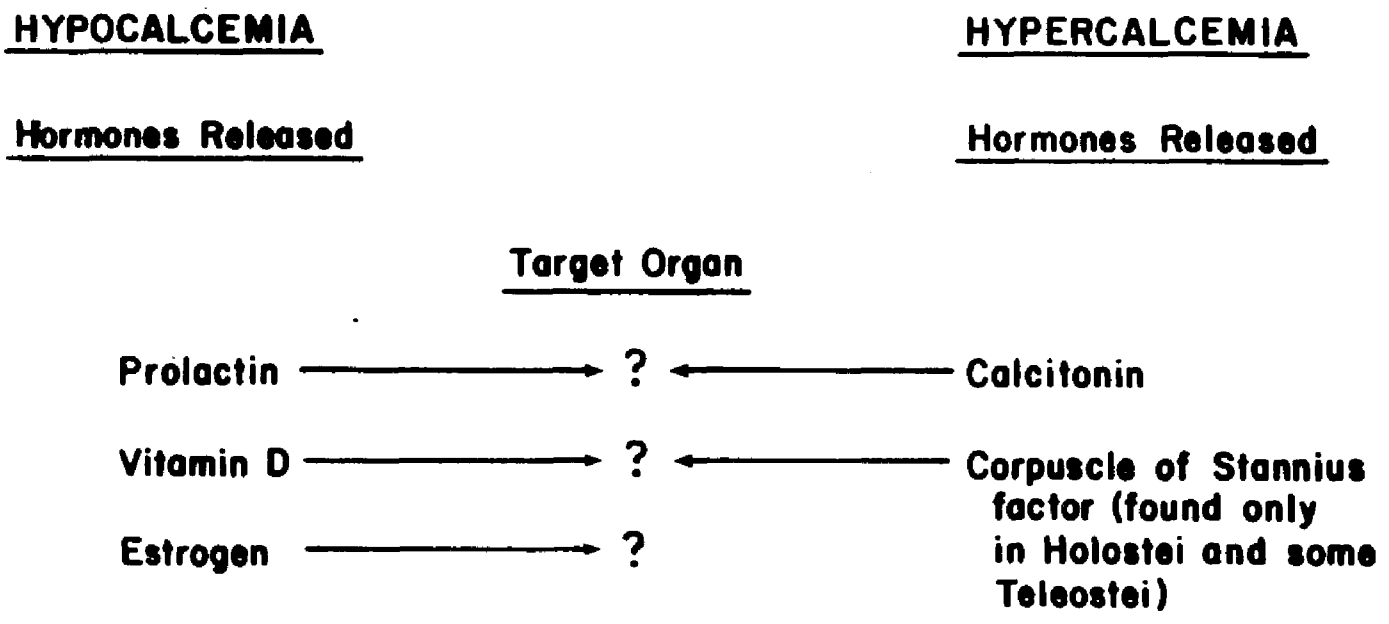

HYPOCALCEMIA
HYPERCALCEMIA
Hormones Released
Hormones Released
Target Organ
Prolactin
?
Calcitonin
Vitamin D
?
Corpuscle of Stannius factor (found only in Holostei and some Teleostei)
Estrogen
?

Figure 3. Effect of Mg^{2+} on $Ca^{2+}+Mg^{2+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the MISEM of 3 to 5 determinations.

Seawater fish:
Activity at 10mM Mg^{2+} concentration significantly different from the activities at other Mg^{2+} tested. ($P<0.05$).

Freshwater fish:
Activity at 10mM Mg^{2+} concentration significantly different from the activities at other Mg^{2+} tested. ($P<0.05$). Activity at 3mM Mg^{2+} significantly different from activities at 2mM, 4mM and 10mM Mg^{2+} ($P<0.02$).

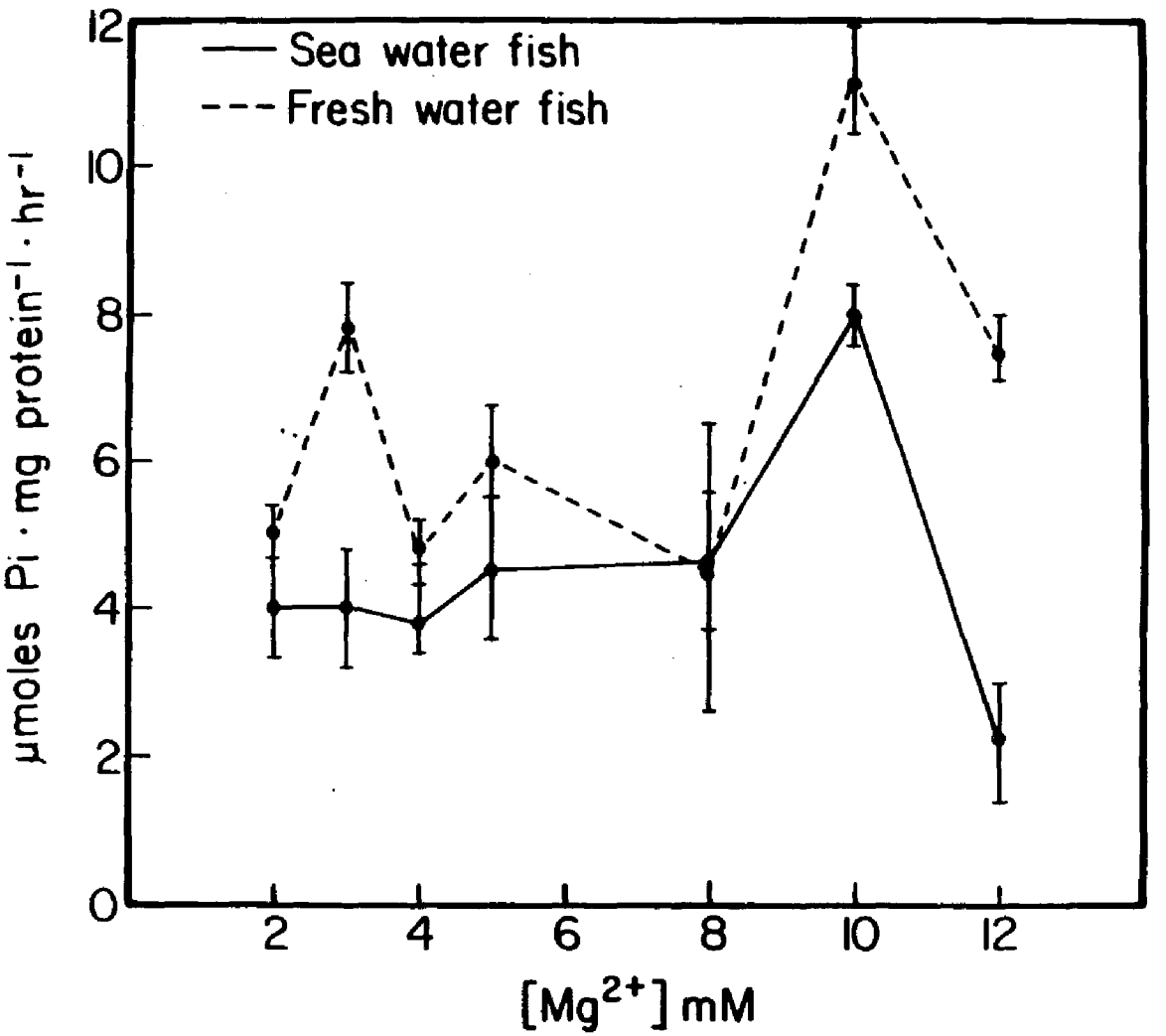

Sea water fish
Fresh water fish
µmoles Pi · mg protein⁻¹ · hr⁻¹
0
2
4
6
8
10
12
2
4
6
8
10
12
[Mg2+] mM

Figure 4. Determination of K_m and V_{max} values for Mg^{2+} in $Ca^{2+}+Mg^{2+}$-ATPase in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

The graphs were plotted from data shown in Figure 3 by the least squares method.

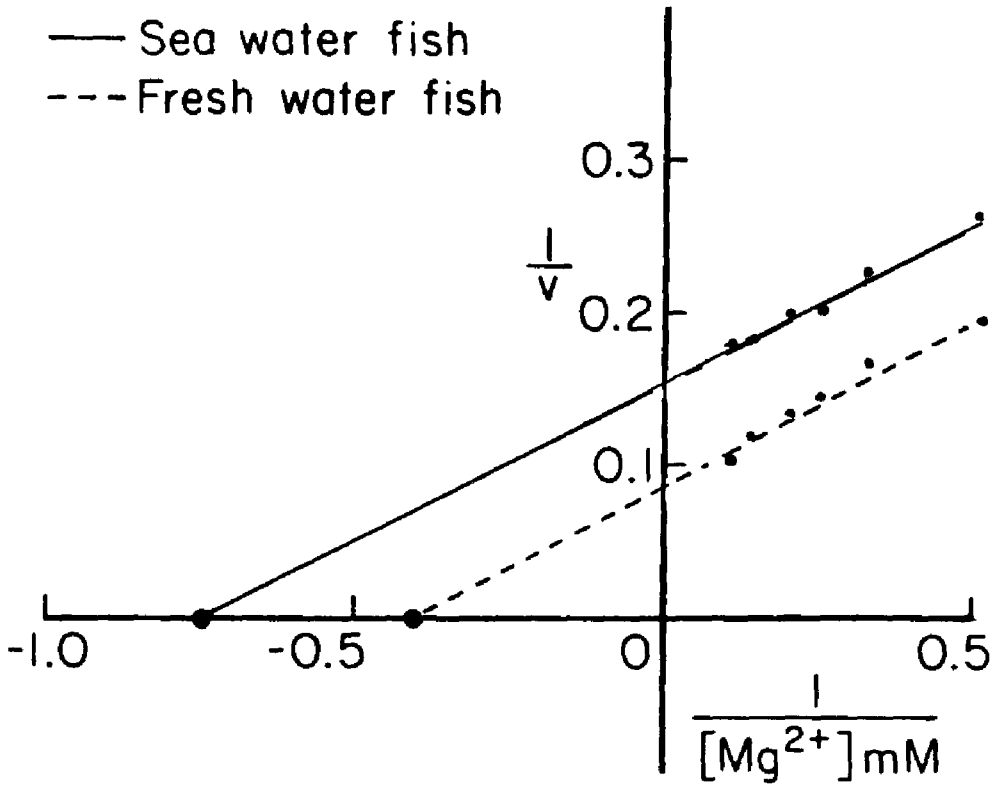

Sea water fish
Fresh water fish
0.3
0.2
0.1
1/V
-1.0
-0.5
0
0.5
1/[Mg2+]mM

Figure 5 Effect of Ca^{2+} on $Ca^{2+}+Mg^{2+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 2 to 11 determinations.

Seawater fish:
No significant difference in activities from 0.0025mM to 5mM Ca^{2+} ($P>0.9$).

Freshwater fish:
No significant difference in activities from 0.1mM to 1mM Ca^{2+} ($P>0.1$).

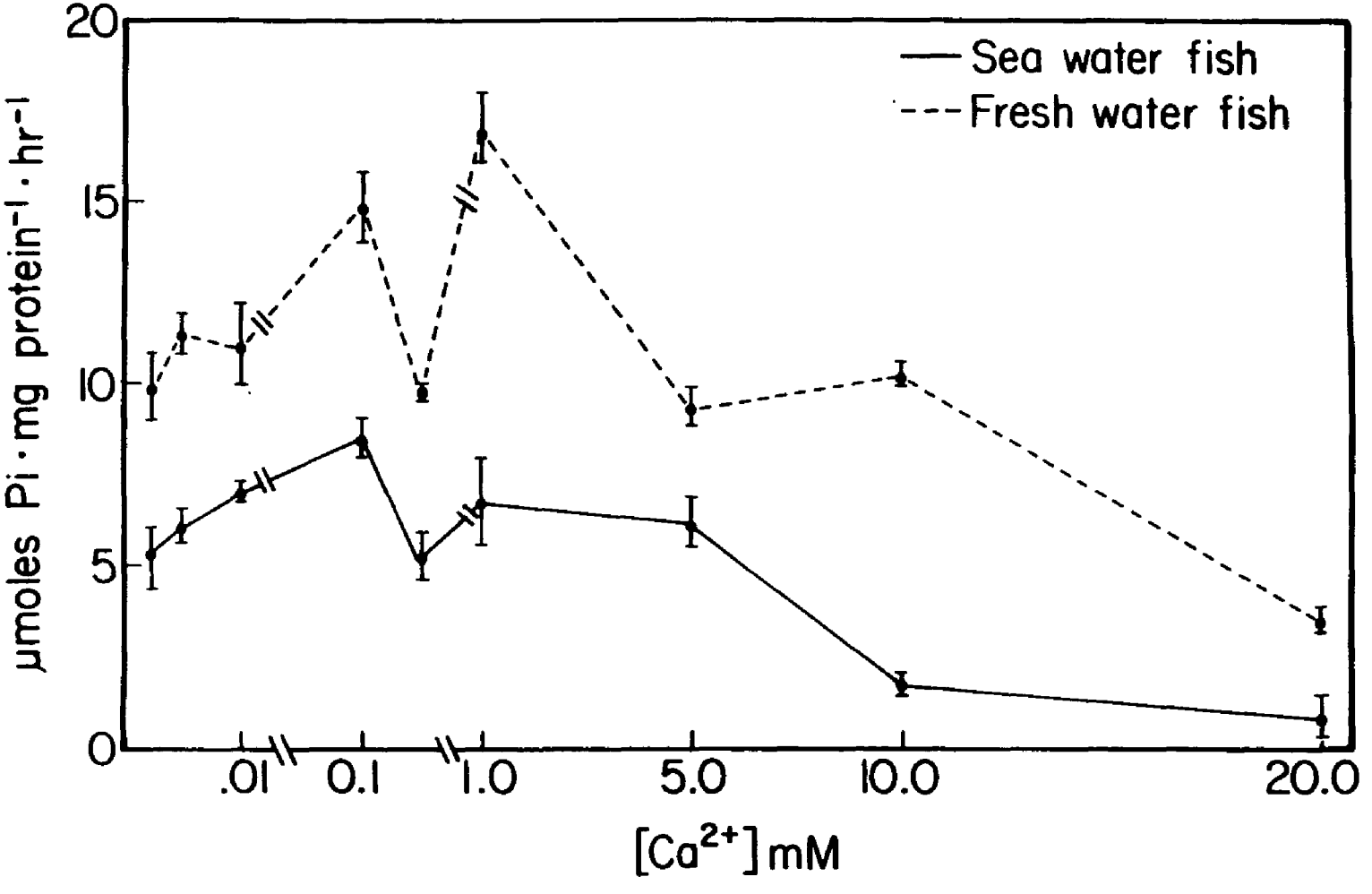

20
15
10
5
0
μmoles Pi · mg protein⁻¹ · hr⁻¹
Sea water fish
Fresh water fish
.01
0.1
1.0
5.0
10.0
20.0
[Ca2+] mM

Figure 6 Determination of K_m and V_{max} values for Ca^{2+} in $Ca^{2+}+Mg^{2+}$-ATPase in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

The graphs were plotted from data shown in Figure 6 by the least squares method.

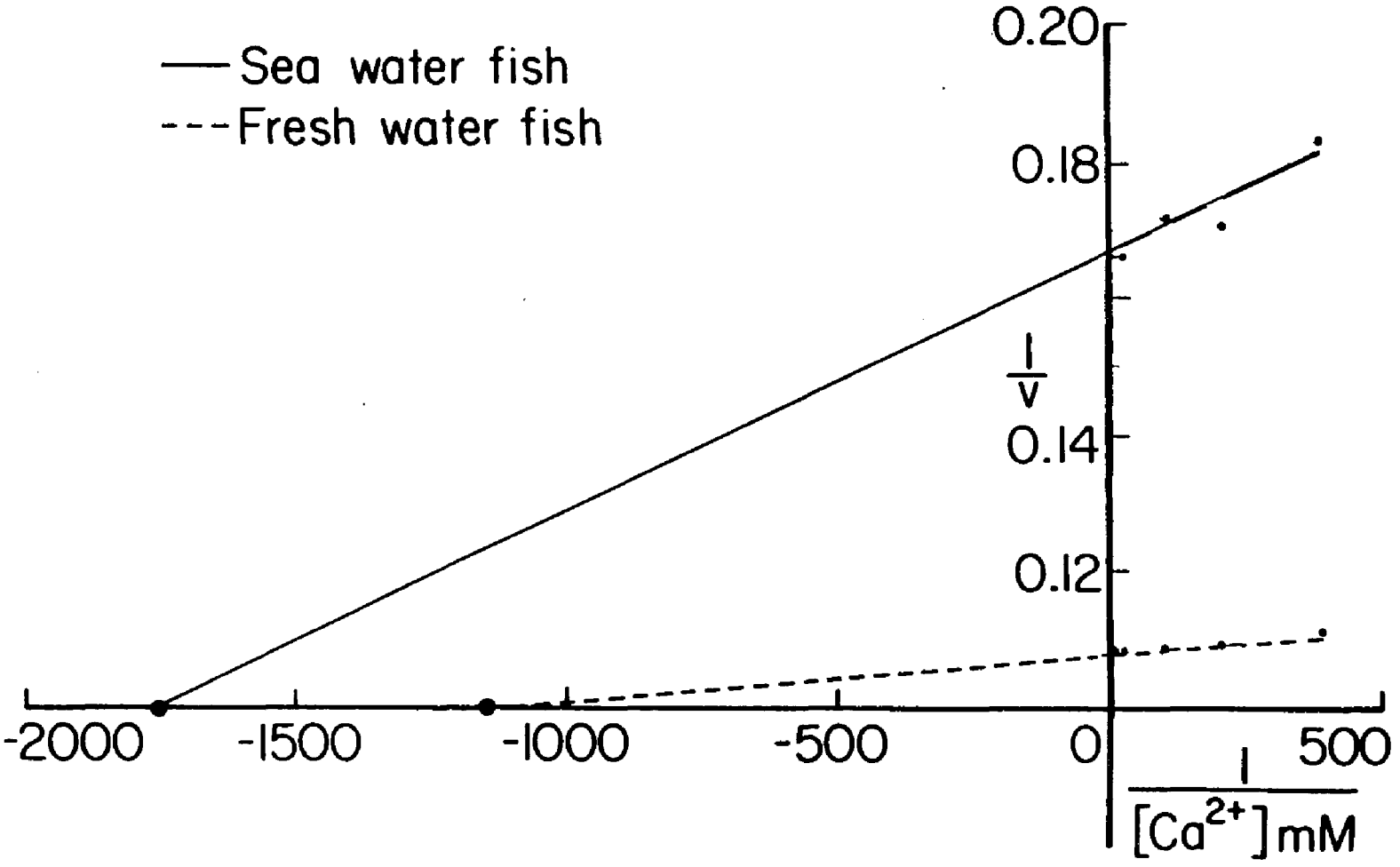

Sea water fish
Fresh water fish
0.20
0.18
1/V
0.14
0.12
-2000
-1500
-1000
-500
0
500
1/[Ca2+]mM

Figure 7 Effect of ATP on $Ca^{2+}+Mg^{2+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 to 15 determinations.

Seawater fish:
No significant difference in activities at other ATP concentrations ($P>0.7$).

Freshwater fish:
No significant difference from 4mM to 7mM ATP concentration ($P>0.5$).

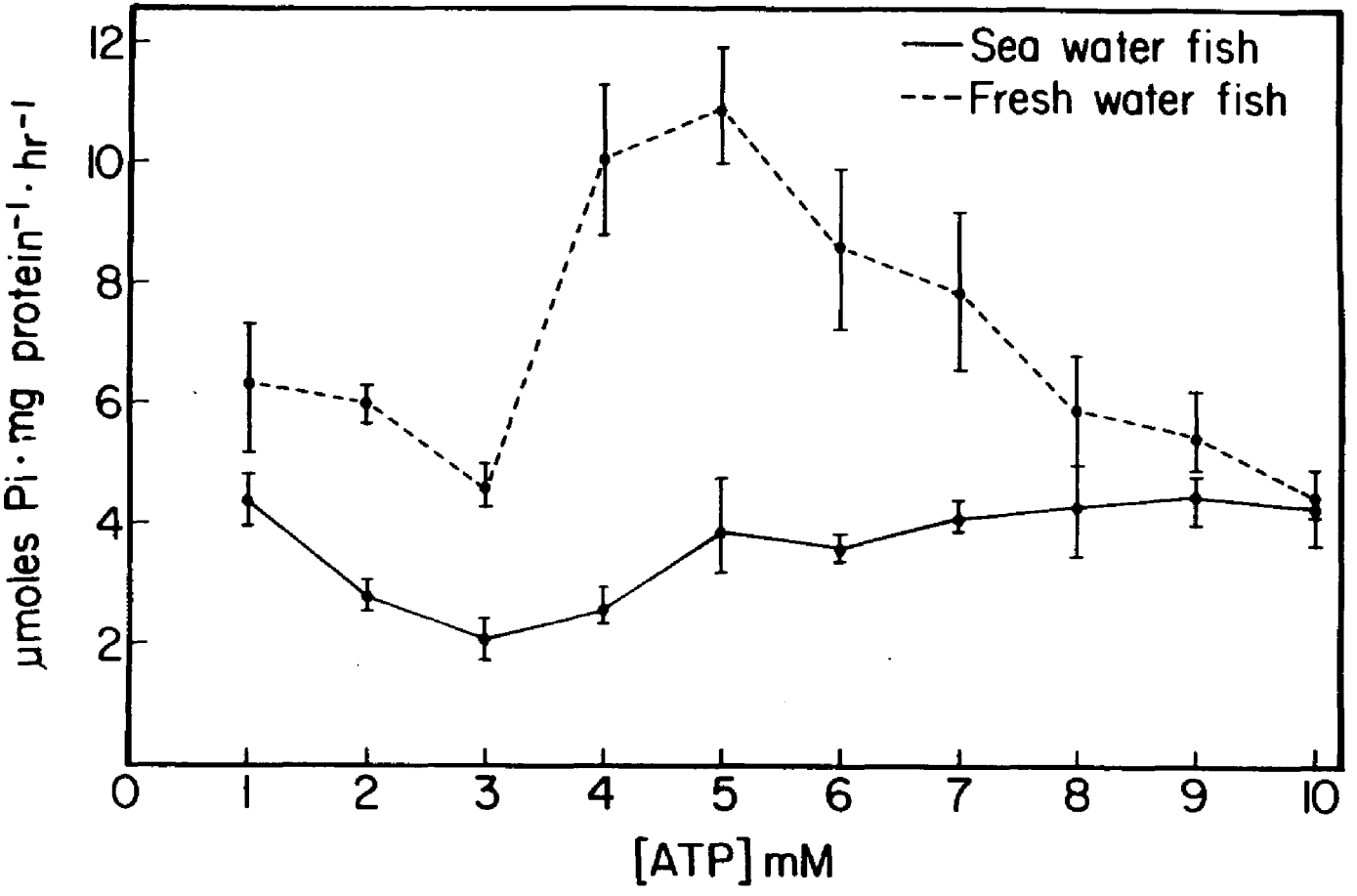

Sea water fish
Fresh water fish
µmoles Pi · mg protein⁻¹ · hr⁻¹
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
[ATP] mM

Figure 8 Determination of the K_m and V_{max} values for ATP in $Ca^{2+}+Mg^{2+}$-ATPase in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

The graphs were plotted from data shown in Figure 9 by the least squares method.

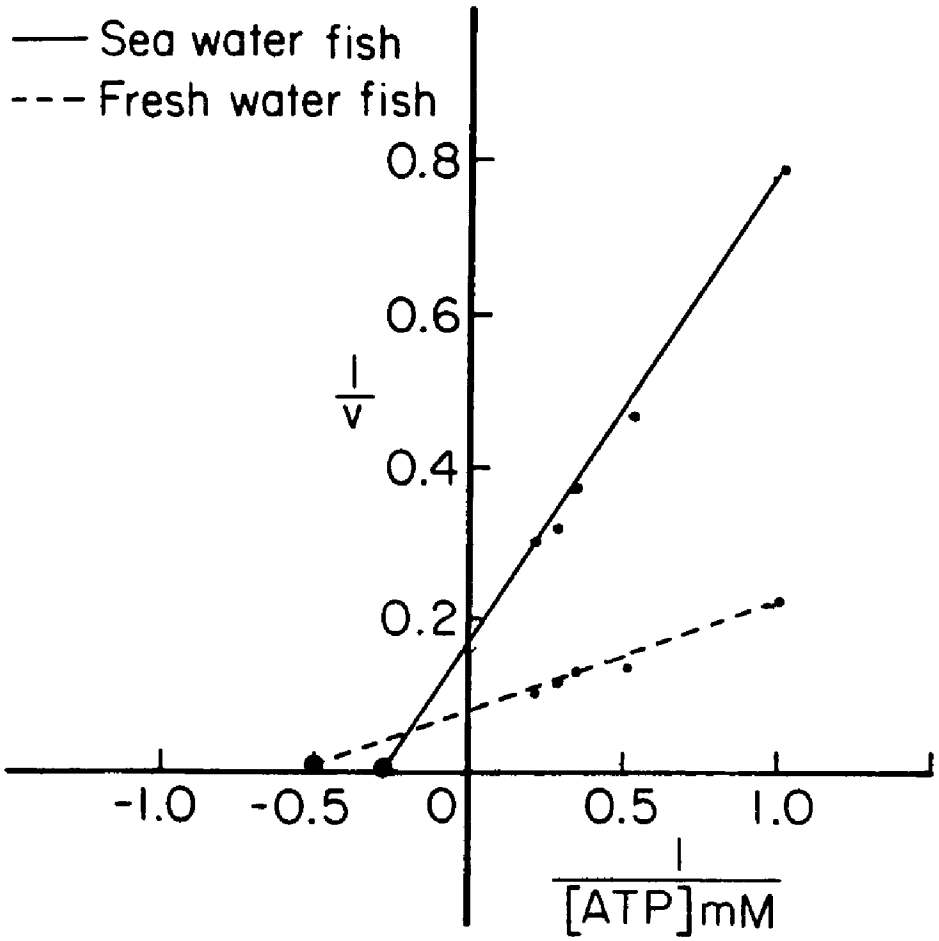

Sea water fish
Fresh water fish
0.8
0.6
1/v
0.4
0.2
-1.0
-0.5
0
0.5
1.0
1/[ATP]mM

Figure 9 Effect of pH on $Ca^{2+}+Mg^{2+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 to 8 determinations.

Seawater fish:
No significant difference in activities from pH 7.5 to pH 8.0 ($p>0.2$).

Freshwater fish:
Activity at pH 7.5 significantly different from activities at other pHs ($p<0.001$).

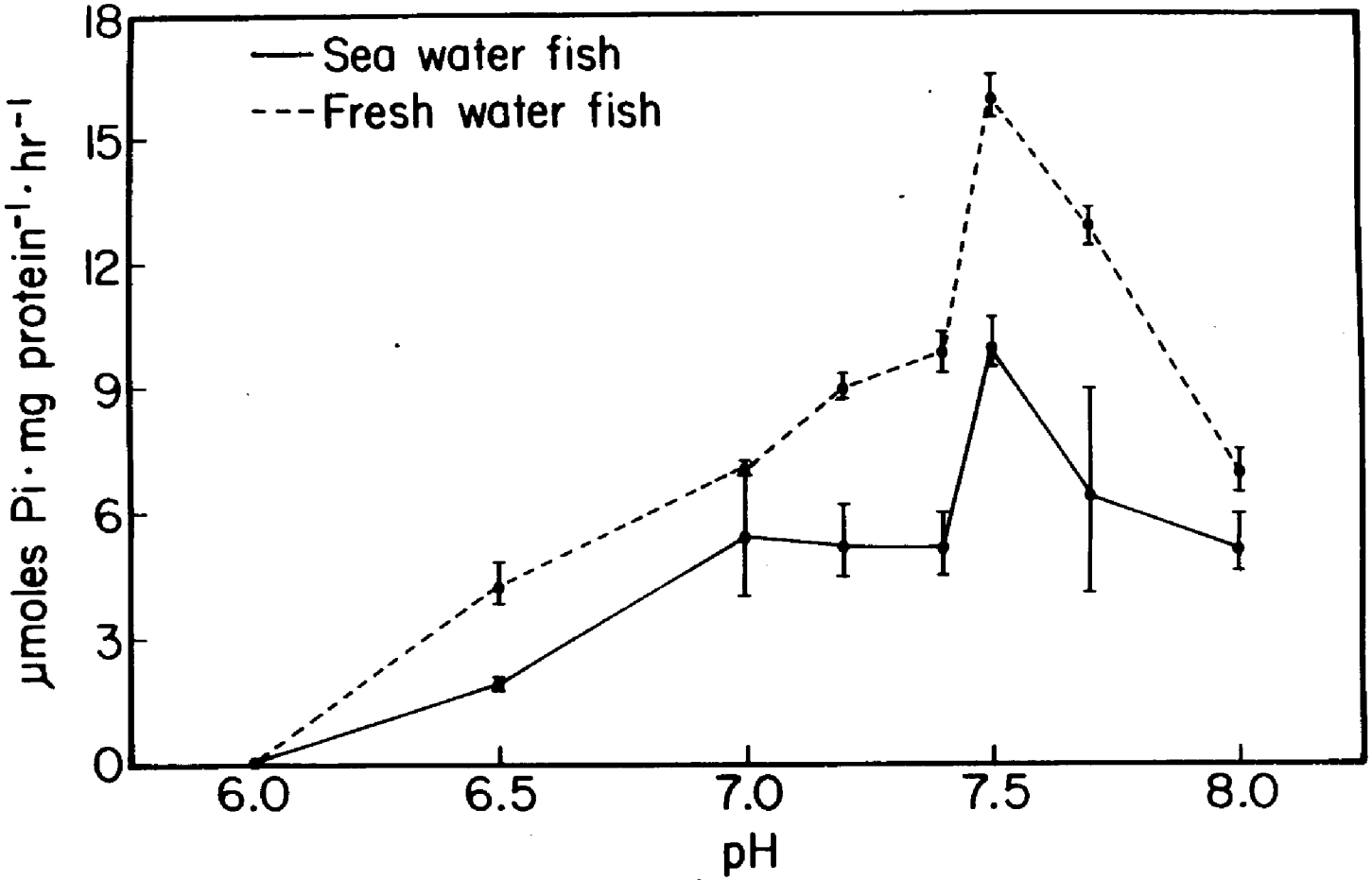

Sea water fish
Fresh water fish
µmoles Pi · mg protein⁻¹ · hr⁻¹
0
3
6
9
12
15
18
6.0
6.5
7.0
7.5
8.0
pH

Figure 10. Effect of temperature on $Ca^{2+}+Mg^{2+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 to 8 determinations.

Seawater fish:
No significant difference in activities from 15^0C to 50^0C ($P>0.05$).

Freshwater fish:
No significant difference in activities between 30^0C and 37^0C ($P>0.5$).

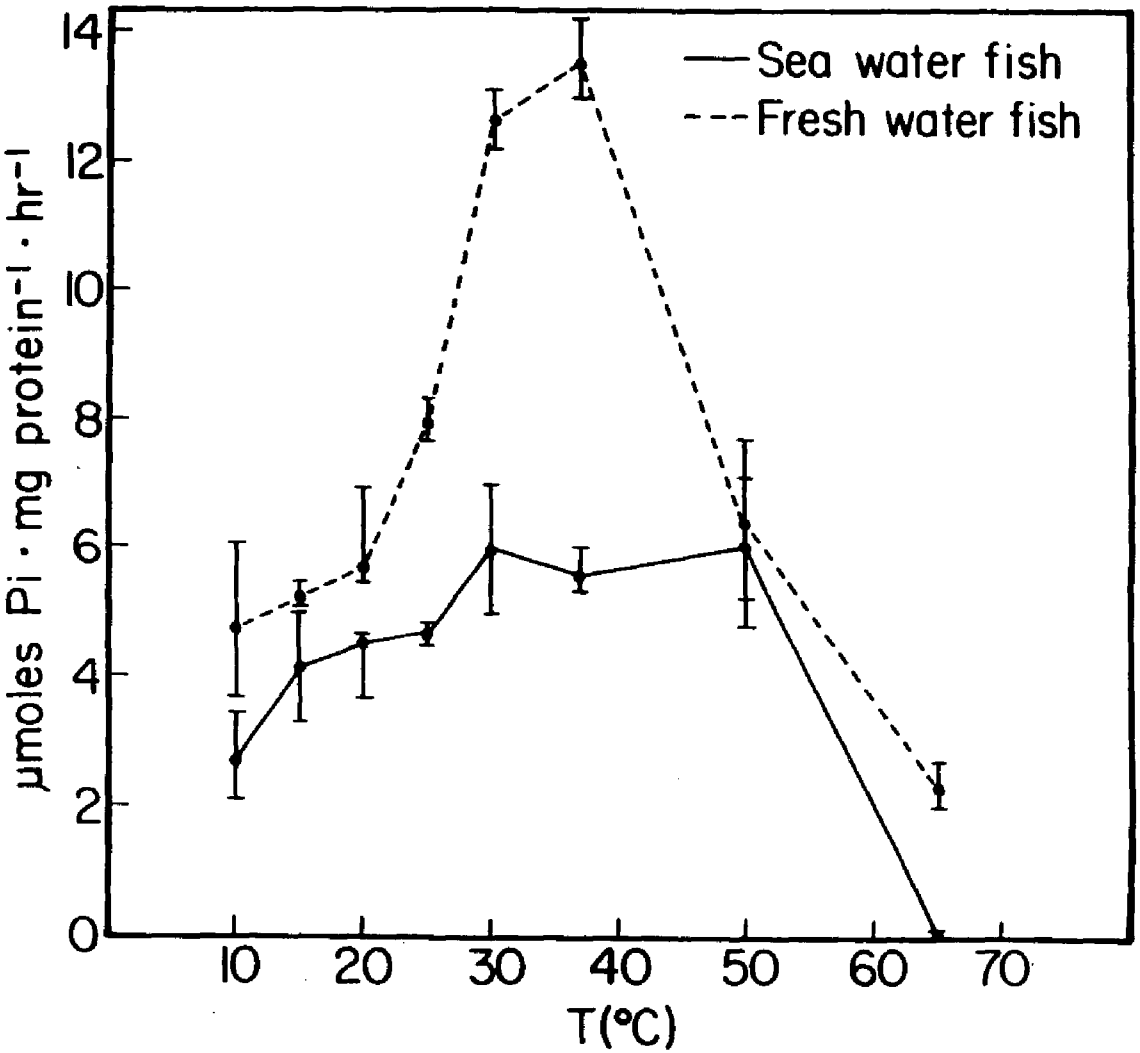

Sea water fish
Fresh water fish
μmoles Pi · mg protein⁻¹ · hr⁻¹
0
2
4
6
8
10
12
14
10
20
30
40
50
60
70
T(°C)

Figure 11 Effect of Na^+ on $Ca^{2+}+Na^+$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 to 12 determinations.

Seawater fish:
No significant difference in activities from 10mM to 60mM Na^+ ($p>0.9$).

Freshwater fish:
No significant difference from 10mM to 60mM Na^+ ($p>0.7$).

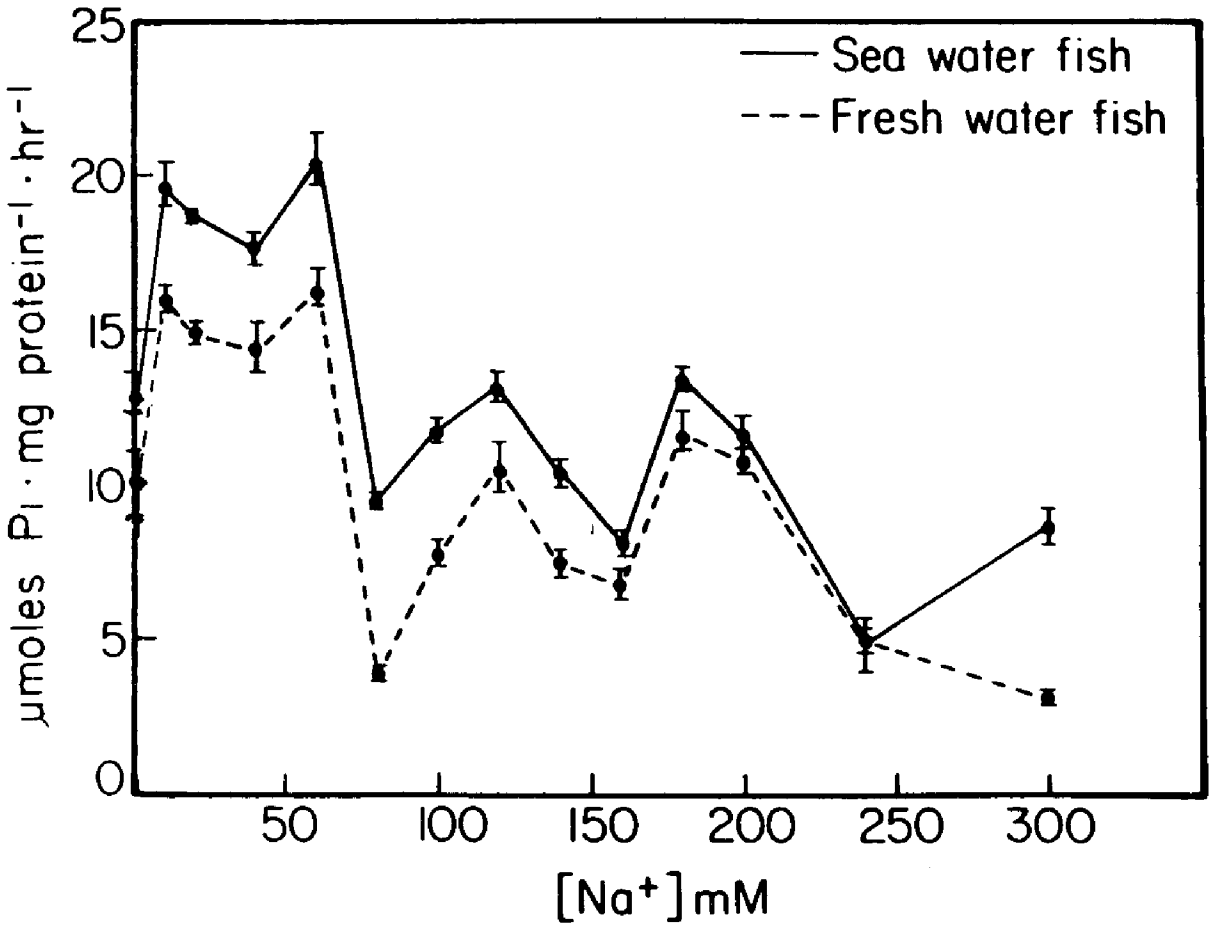

Sea water fish
Fresh water fish
μmoles Pi · mg protein⁻¹ · hr⁻¹
0
5
10
15
20
25
50
100
150
200
250
300
[Na⁺] mM

Figure 12 Determination of K_m and V_{max} values for Na^+ in Ca^{2+}+Na^+-ATPase in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

The graphs were plotted from data shown in Figure 13 by the least squares method.

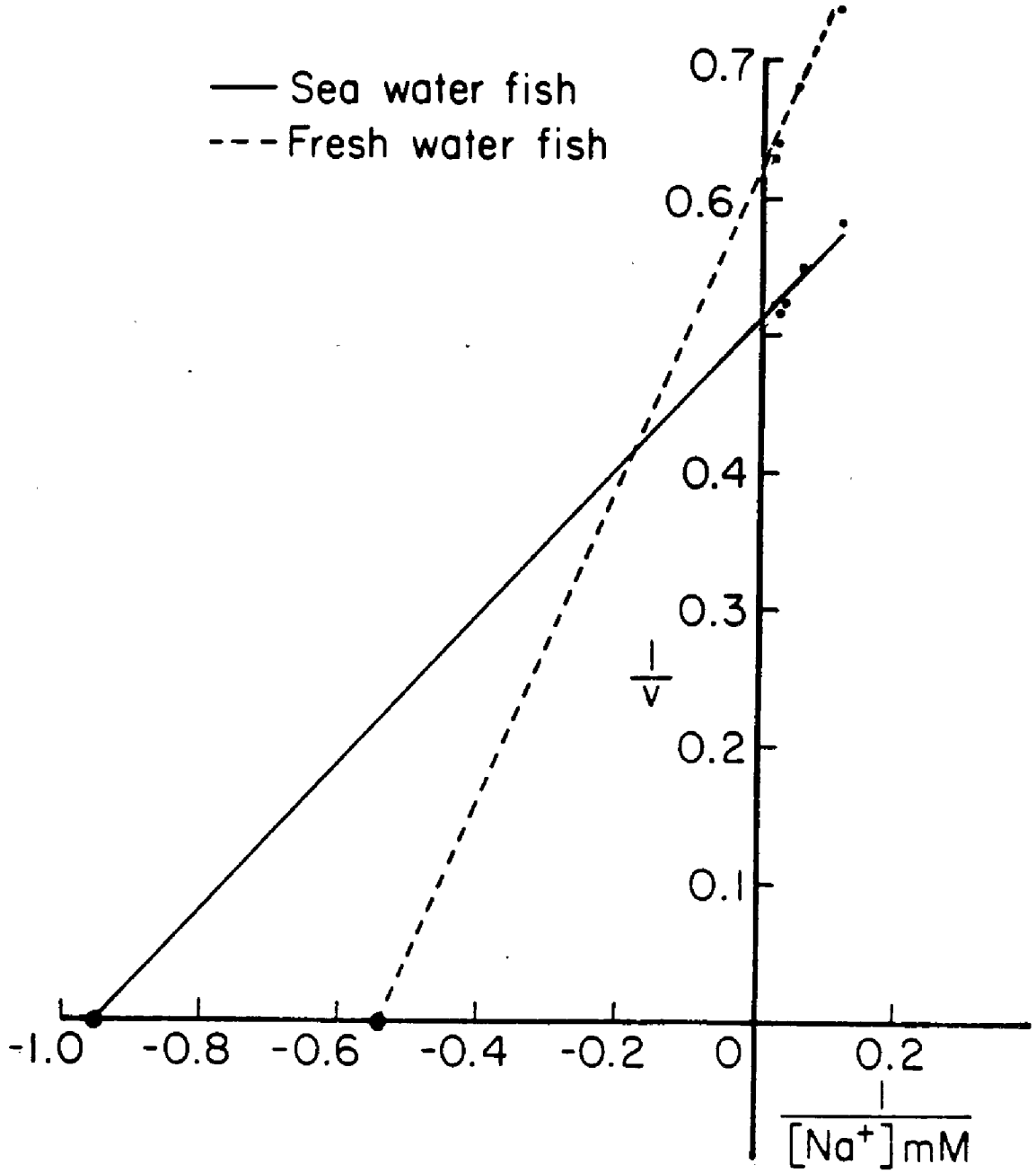

Sea water fish
Fresh water fish
0.7
0.6
0.4
0.3
1/V
0.2
0.1
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
1/[Na+]mM

Figure 13 Effect of Ca^{2+} on $Ca^{2+}+Na^{+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 determinations.

Seawater fish:
Activity at 20mM Ca^{2+} concentration significantly different from activities at other Ca^{2+} ($p<0.01$).

Freshwater fish:
No significant difference in activities from 10mM to 20mM Ca^{2+} ($p>0.3$).

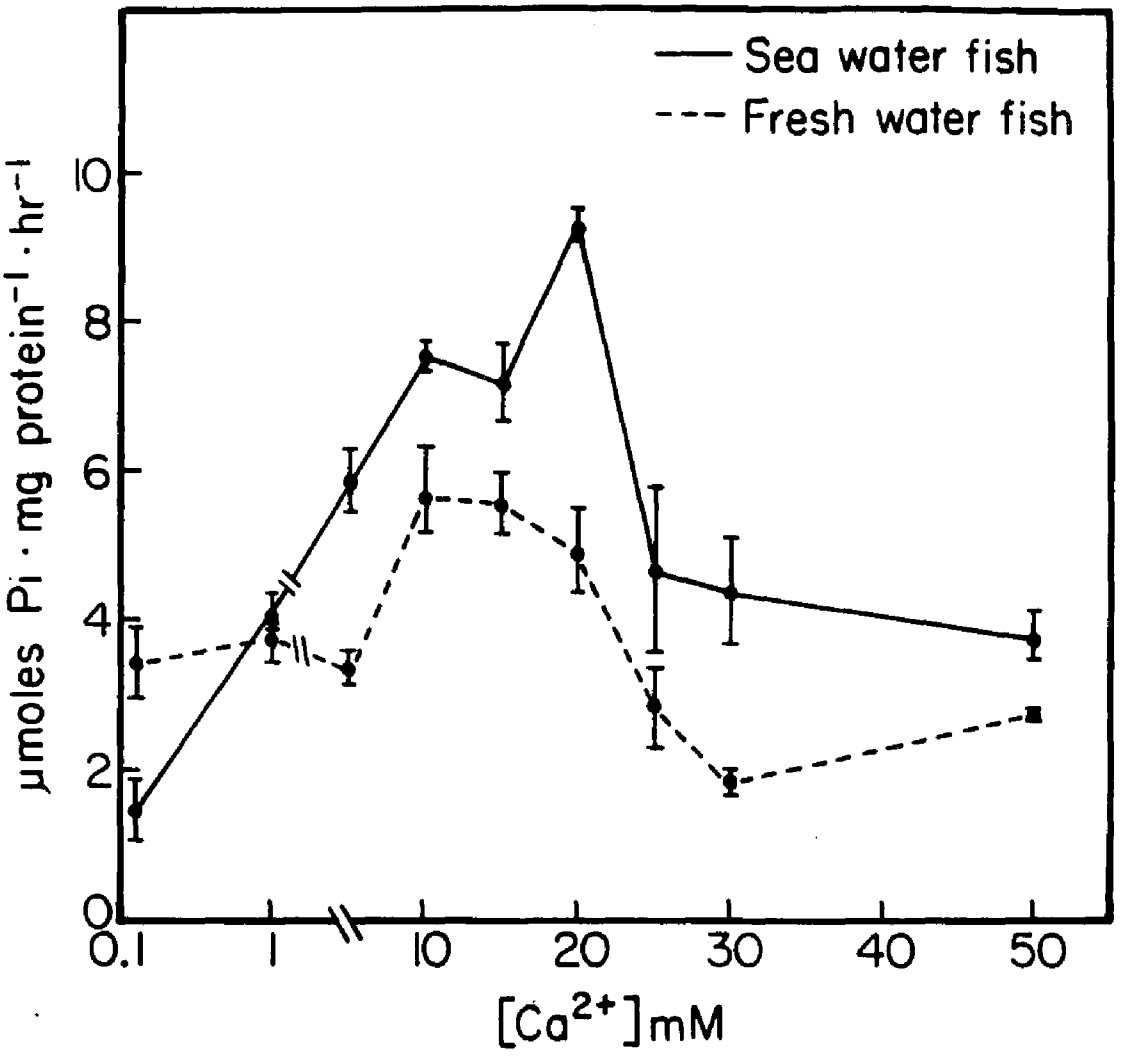

Sea water fish
Fresh water fish
μmoles Pi · mg protein⁻¹ · hr⁻¹
0
2
4
6
8
10
0.1
1
10
20
30
40
50
[Ca2+]mM

Figure 14 Determination of K_m and V_{max} values for Ca^{2+} in Ca^{2+}+Na^{+}-ATPase in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

The graphs were plotted from data shown in Figure 16 by the least squares method.

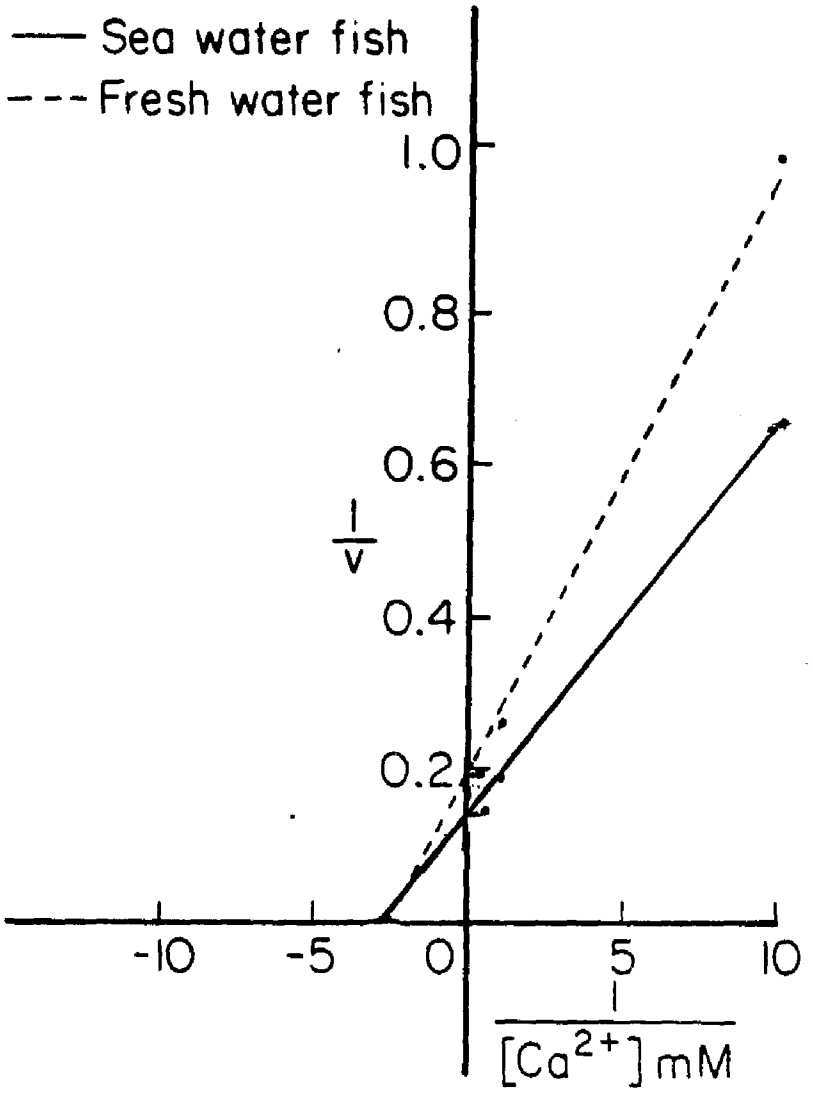

Sea water fish
Fresh water fish
1.0
0.8
0.6
0.4
0.2
1/V
-10
-5
0
5
10
1/[Ca2+] mM

Figure 15 Effect of ATP on $Ca^{2+}+Na^{+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 determinations.

Seawater fish:
No significant difference in activities between 5mM to 6mM ATP concentration ($p>0.5$).

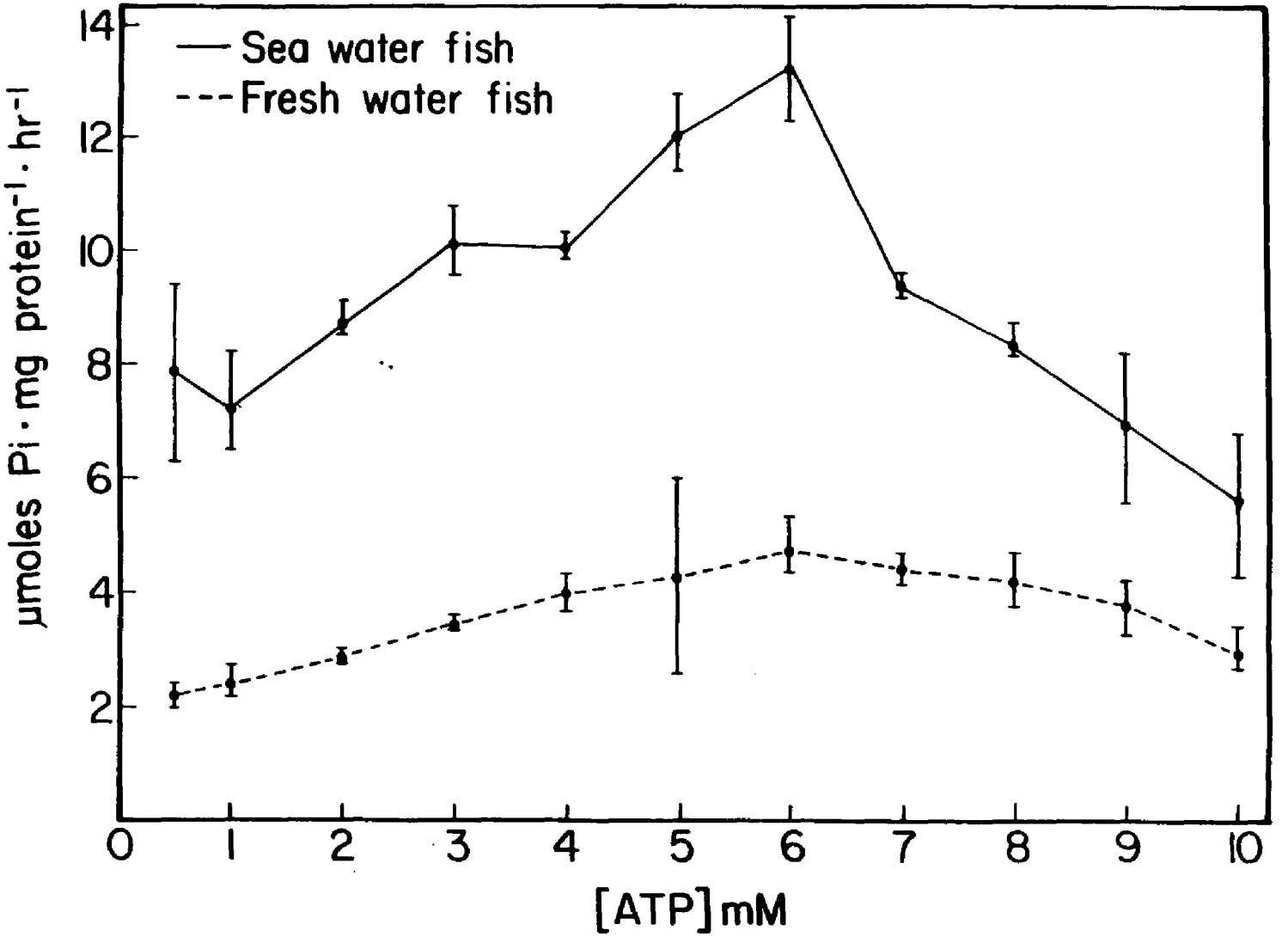

Sea water fish
Fresh water fish
μmoles Pi · mg protein⁻¹ · hr⁻¹
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
[ATP] mM

Figure 16 Determination of K_m and V_{max} values for ATP in $Ca^{2+}+Na^{+}$-ATPase in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

The grpahs were plotted from data shown in Figure 19 by the least squares method.

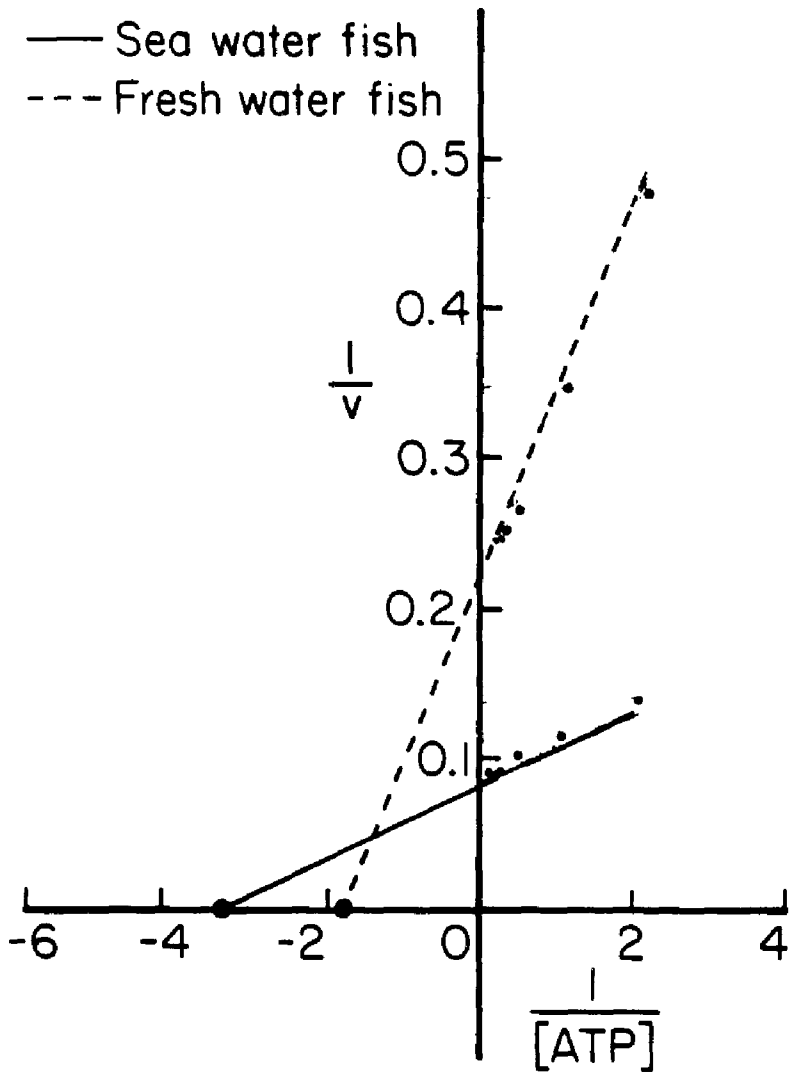

Sea water fish
Fresh water fish
0.5
0.4
0.3
0.2
0.1
1/v
-6
-4
-2
0
2
4
1/[ATP]

Figure 17 Effect of pH on $Ca^{2+}+Na^{+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents M±SEM of 4 determinations.

Seawater fish:
No significant difference in activities from pH 7.0 to pH 7.5 ($p>0.9$).

Freshwater fish:
No significant difference in activities from pH 6.6 to pH 7.4 ($p>0.5$).

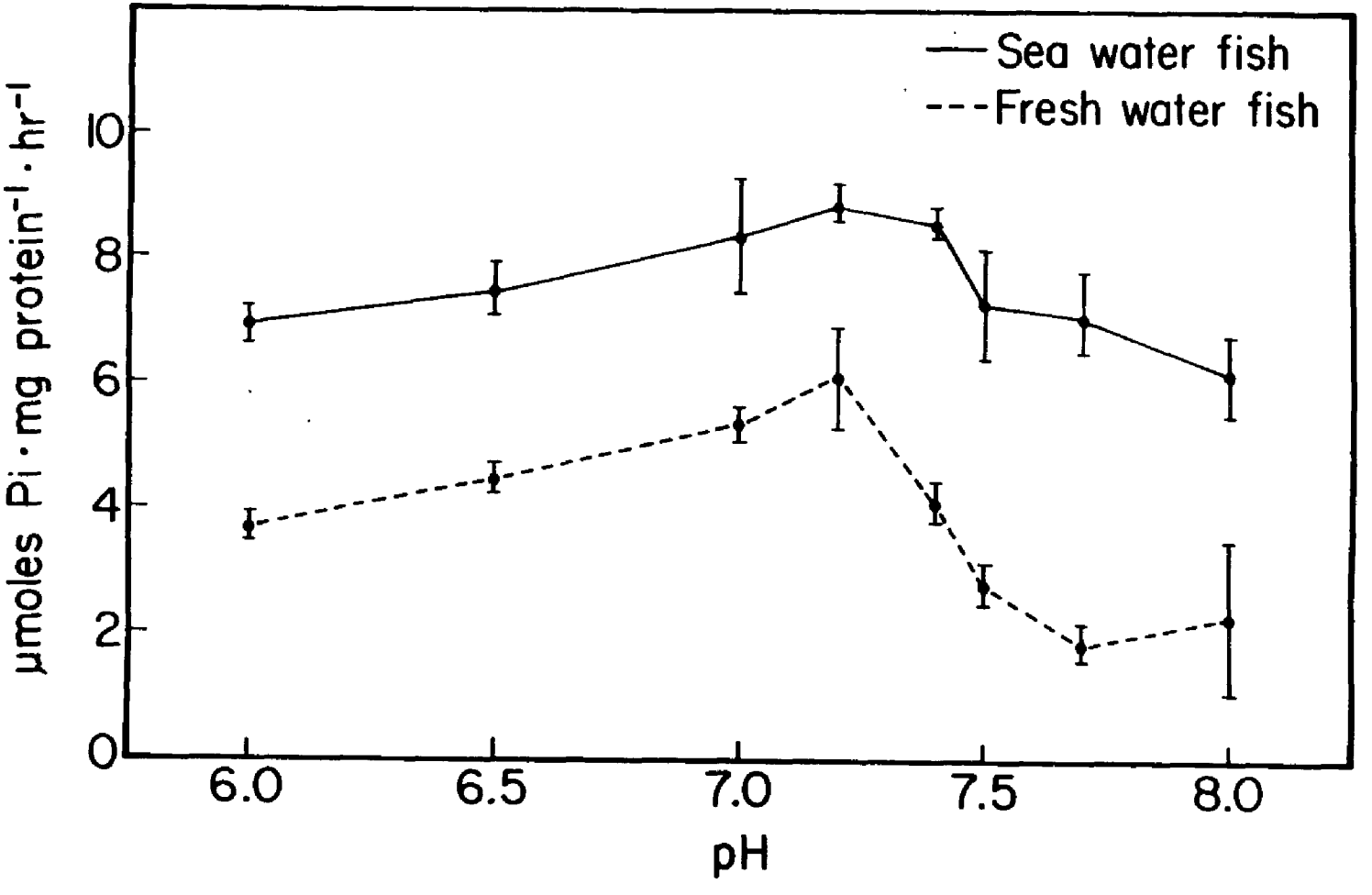

Sea water fish
Fresh water fish
µmoles Pi · mg protein⁻¹ · hr⁻¹
0
2
4
6
8
10
6.0
6.5
7.0
7.5
8.0
pH

Figure 18 Effect of temperature on $Ca^{2+}+Na^{+}$-ATPase activity in gill epithelium from seawater and freshwater-adapted Fundulus heteroclitus.

Each point represents the M±SEM of 4 determinations.

Seawater fish:
No significant difference in activities from $30^{0}C$ to $50^{0}C$ ($p>0.7$).

Freshwater fish:
No significant difference in activities between $37^{0}C$ and $50^{0}C$ ($p>0.1$).

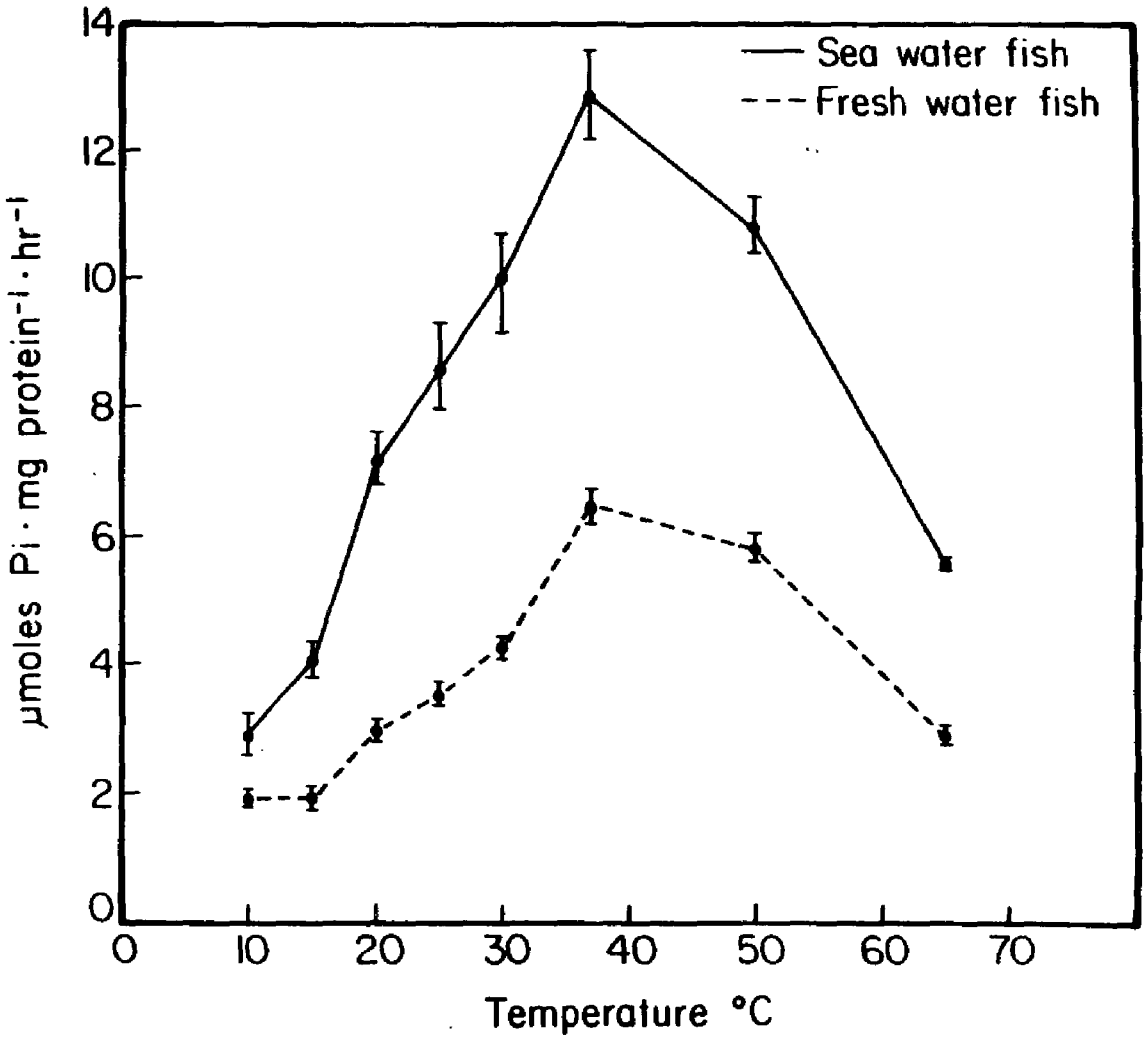

Sea water fish
Fresh water fish
µmoles Pi · mg protein⁻¹ · hr⁻¹
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
Temperature °C

Figure 19 Effect of transfer from freshwater to seawater on blood Ca^{2+} levels and activities of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in gill epithelium

Each point represents the M±SEM of 4 to 7 determinations.

$Ca^{2+}+Mg^{2+}$-ATPase:
Activity at day 13 is significantly different from activities at days 0 to 10 ($p<0.001$), but not significantly different from activities at days 15 to 21 ($p>0.3$).

$Ca^{2+}+Na^{+}$-ATPase:
Activity at day 15 is significantly different from activities at days 0 to 13 ($p<0.05$), but not significantly different from activities at days 17 adn 21 ($p>0.7$).

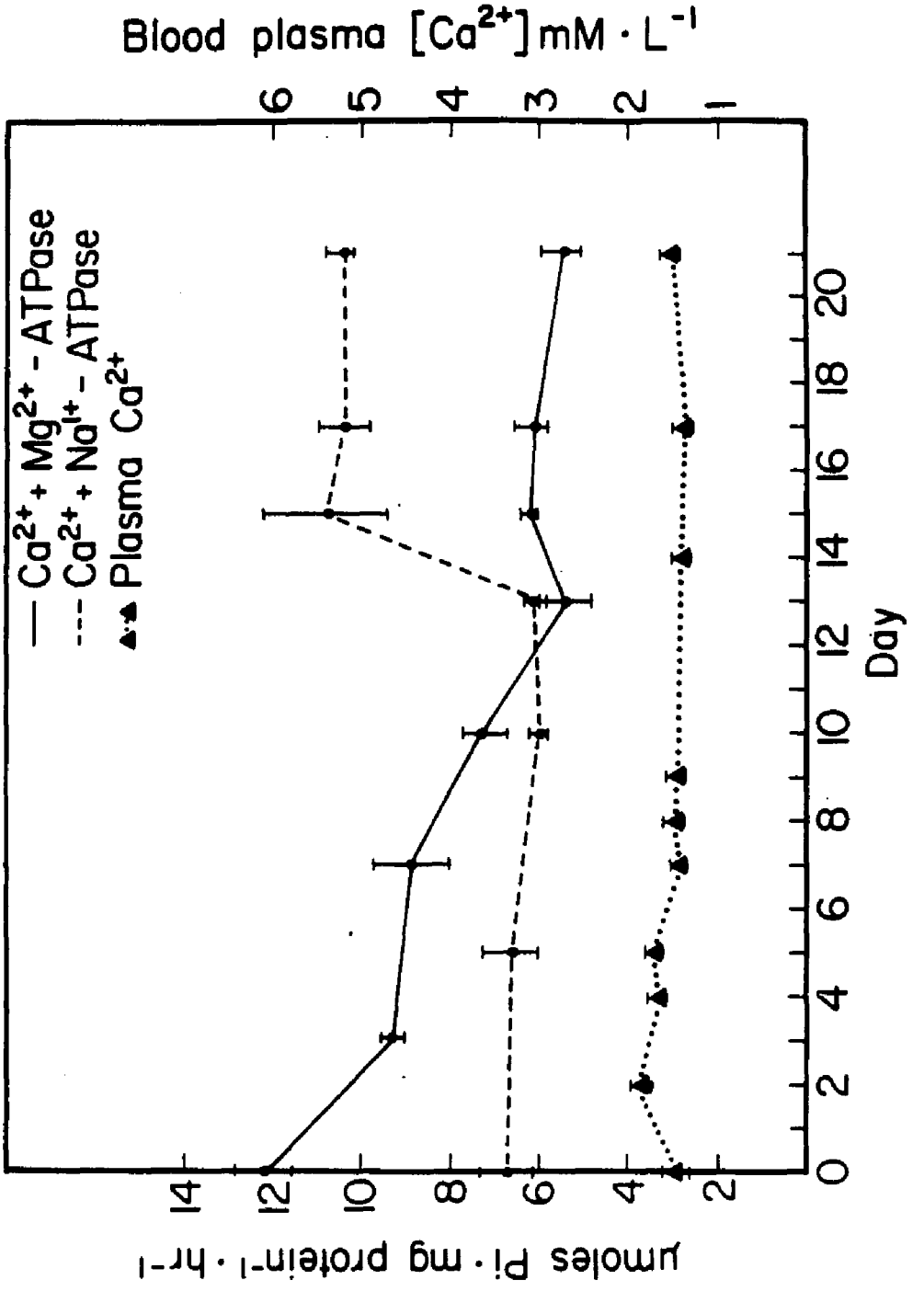

Blood plasma [Ca2+] mM · L-1
6
5
4
3
2
1
— Ca2+ + Mg2+ - ATPase
--- Ca2+ + Na1+ - ATPase
▲▲ Plasma Ca2+
0
2
4
6
8
10
12
14
16
18
20
Day
14
12
10
8
6
4
2
μmoles Pi · mg protein-1 · hr-1

Figure 20 Effect of transfer from seawater to freshwater on blood Ca^{2+} levels and activities of $Ca^{2+}+Mg^{2+}$-ATPase and $Ca^{2+}+Na^{+}$-ATPase in gill epithelium.

Each point represents the M±SEM of 2 to 7 determinations.

$Ca^{2+}+Mg^{2+}$-ATPase:
Activity at day 13 is significantly different from activities at day 0 to 10 ($p<0.001$), but not significantly different from activities at days 15 to 21 ($p>0.3$).

$Ca^{2+}+Na^{+}$-ATPase:
Activity at day 13 is significantly different from activities at day 0 to 10 ($p<0.05$), but not significantly different from activities at days 15 to 21 ($p>0.9$).

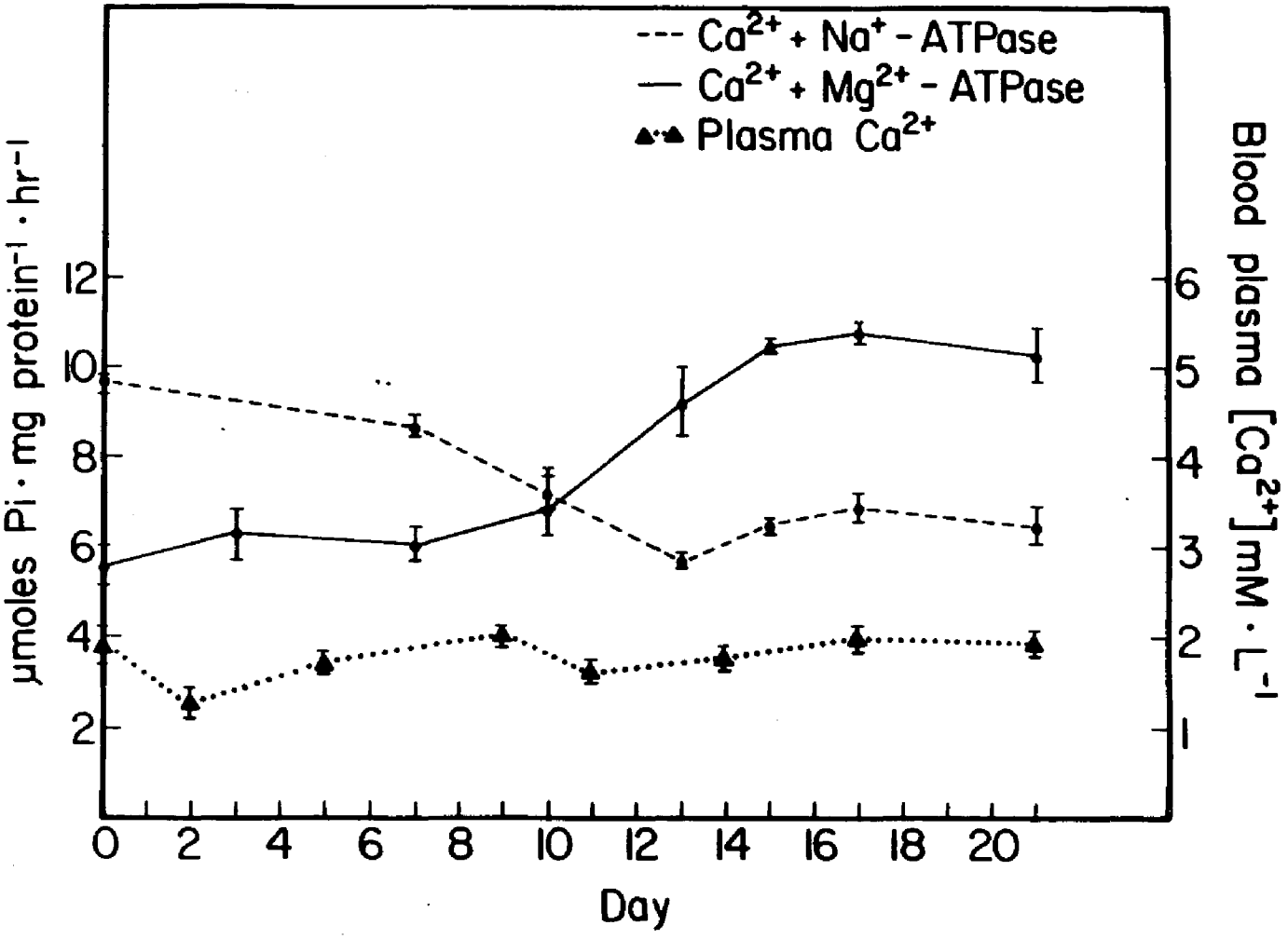
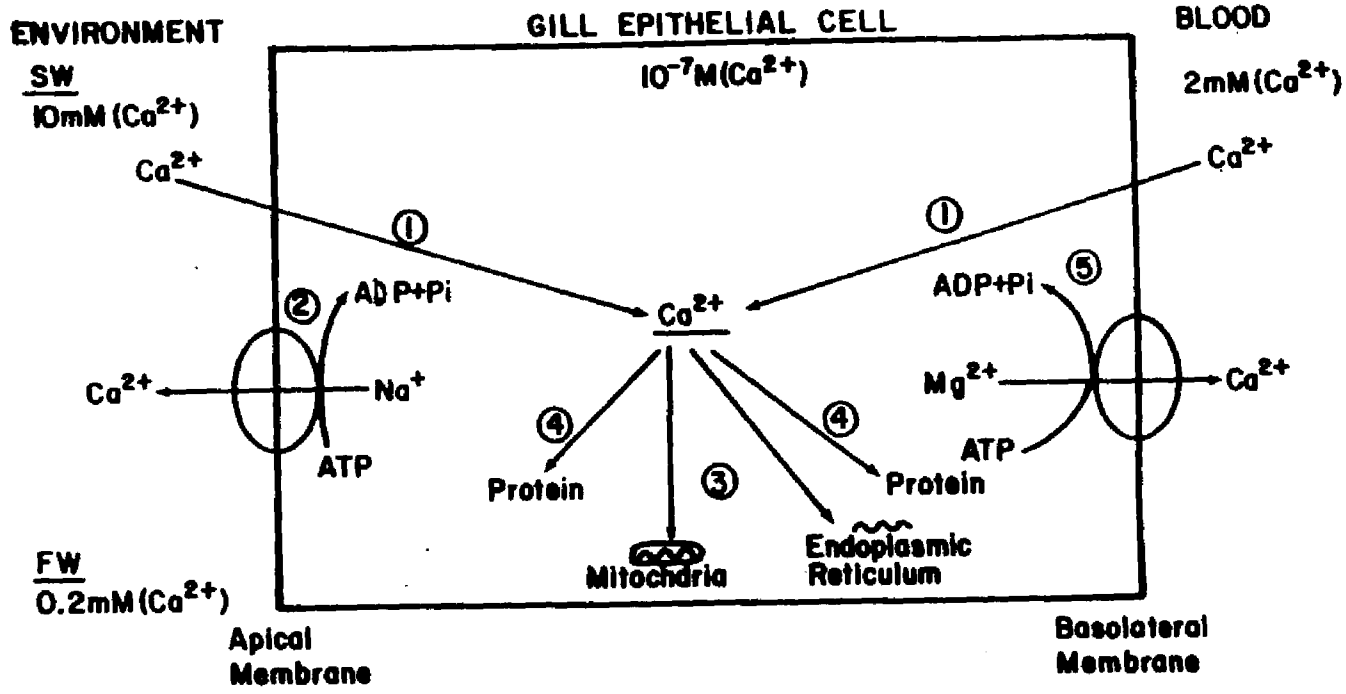

--- Ca^{2+} + Na^{+} - ATPase
— Ca^{2+} + Mg^{2+} - ATPase
Plasma Ca^{2+}
µmoles Pi · mg protein^{-1} · hr^{-1}
Blood plasma [Ca^{2+}] mM · L^{-1}
Day
0 2 4 6 8 10 12 14 16 18 20
2 4 6 8 10 12
1 2 3 4 5 6

Figure 21. Hypothetical model for slow adaptation mechanism for Ca^{2+} regulation in a gill epithelium cell from Fundulus heteroclitus.


ENVIRONMENT
SW
10mM (Ca^{2+})
FW
0.2mM (Ca^{2+})
Apical Membrane
GILL EPITHELIAL CELL
10^{-7}M (Ca^{2+})
BLOOD
2mM (Ca^{2+})
Basolateral Membrane
Ca^{2+}
Ca^{2+}
①
①
②
ADP+Pi
Ca^{2+}
Na^{+}
ATP
Ca^{2+}
④
④
Protein
③
Protein
Mitochondria
Endoplasmic Reticulum
ADP+Pi
⑤
Mg^{2+}
Ca^{2+}
ATP

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