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EFFECTS OF BIOGENIC AMINES, CYCLIC-AMP AND PERICARDIAL ORGAN ON OSMOREGULATORY PROCESSES IN THE GREEN CRAB, CARCINUS MAENAS (L.)

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Effects of Biogenic Amines, Cyclic AMP and Pericardial Organ on Osmoregulatory Processes in the Green Crab, *Carcinus maenas* (L).

by

Michael J. Sommer

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Effects of Biogenic Amines, Cyclic AMP and Pericardial Organ on Osmoregulatory Processes in the Green Crab, Carcinus maenas (L).

by

Michael J. Sommer

Abstract:

Various aspects of osmoregulation in crustaceans have been shown to be under neuroendocrine control. This study indicates that some neuroendocrine factors may mediate their effects through the second messenger cyclic AMP.

In vivo injection of 10^{-5} M dopamine or 10^{-6} M dibutyryl cAMP into Carcinus maenas causes a significant increase ($p<0.001$ and $p<0.01$, respectively) in uptake of sodium from the external medium. The pericardial organ, which is known to contain dopamine, was also tested to determine its effect on sodium uptake in intact Carcinus maenas. Uptake of sodium was significantly greater ($p<0.05$) than sodium uptake in control animals at the 3 hour sampling period. No statistical significance were observed at the 1 hour and 2 hour sampling periods.

The effects of dopamine and dibutyryl cAMP were tested on Na/K-ATPase activity of the gills. Dopamine was injected in adult intermolt green crabs at a concentration of 10^{-5} M. Three hours following injection, the gills were assayed for Na/K-ATPase activity. In the second experiment, gills from one side of the crab were incubated in 10^{-5} M dbcAMP for 2 hours, while control gills were incubated in Ringer's. Animals injected with dopamine or gills treated with dbcAMP showed a significant increase ($p<0.005$) in Na/K-ATPase activity of the microsomal fraction over controls. The effect of

pericardial organ (PO) extracts on Na/K-ATPase activity were tested. These studies show that PO is able to increase Na/K-ATPase activity in the microsomal fraction significantly ($p<0.01$) over that of the control.

Studies were carried out to see the effects of dopamine and octopamine on levels of cAMP in the anterior and posterior gills of C. maenas. These experiments showed that dopamine increased cAMP levels significantly ($p<0.01$) in the anterior and posterior gills of this crab. Octopamine was able to induce increases in cAMP levels in gill tissue but these increases were not significantly different from control values.

Experiments to determine the effects of long term transfer and acute transfer of C. maenas to 40% SW on cAMP levels in the gills were also undertaken. Long term acclimation of green crabs resulted in lower cAMP values in the anterior and posterior gills. Acute transfer of green crabs to 40% SW resulted in large increases in cAMP levels in both anterior and posterior gills. The increases observed in the posterior gills are significantly different ($p<0.05$) from control cAMP concentrations.

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Introduction:

Osmotic Regulatory Mechanisms in Marine Crustaceans

Marine decapods, when placed in 100% sea water, are usually isosmotic to the medium, and the proportions of sodium and chloride in hemolymph is similar in concentration to that in their surroundings. In general, concentrations of potassium and calcium are higher in the hemolymph than in 100% seawater, while internal magnesium is reduced to about half that present in the medium (Jeuniaux, 1971). The values for these ions in the hemolymph fluctuate seasonally in many animals and may also be influenced by the stage of the intermolt cycle (e.g. the content of calcium in the hemolymph often increases in premolt) (Mantel and Farmer, 1983).

On acclimation to dilute medium below 80% sea water (24 ppt), euryhaline decapods become hyperosmotic regulators to varying degrees. This has several physiological implications. Since the hemolymph is more concentrated than the medium, the organism will undergo an efflux of salts and an influx of water. This problem is compounded by the fact that marine crustaceans produce a urine that is isosmotic to the blood. Thus increased urine flow, to get rid of the water load, causes further loss of salts. To prevent fatal dilution of the hemolymph, crustaceans must decrease permeability to both water and salts. This has been shown to be the case in marine crustaceans. For example, in Sphaeroma serratum, water fluxes are 3 to 4 times greater in 100% SW than when this crustacean is in 50% SW. Further, if this isopod is transferred acutely from full strength sea water to dilute sea water, the decrease in permeability occurs within 30 seconds after

transfer (Thuet, 1978). In contrast, the crab Rhithropanopeus harrisii also reduces permeability to water when acutely challenged by a dilute medium. However, the reduction occurs much more slowly, requiring 6 hours for a decrease to occur (Mantel and Farmer 1983). This ability of euryhaline crustaceans to reduce permeability to water when in a dilute medium is believed to be related to changes in the fluidity of the membranes, perhaps caused by an increased concentration of saturated fatty acids in the gills (Morris et al., 1982). Other factors possibly involved in decreasing membrane permeability include changes in the blood flow or changes in ventilation rates of the gills, although no evidence for the latter has been obtained. Smith (1967) coined the term "apparent permeability" to indicate changes in the rate of influx and efflux of water or ions in the whole animal under different conditions, since the particular mechanism(s) are not yet completely known.

The ability to reduce "apparent permeability" to water is of prime importance to osmoregulation. For instance, Callinectes sapidus, a strong hyperosmotic regulator, is able to reduce surface permeability to a greater extent than is Carcinus maenas, a moderate hyperregulator. This fact allows the former to enter lower salinity water than the latter. However, even Callinectes sapidus cannot make itself totally impermeable. Epithelial surfaces, such as those used for absorption of oxygen, uptake of nutrients, and elimination of waste, must remain permeable in order to carry on their function. As a result, some mechanism of active transport must exist to reclaim salts lost over these epithelia and in the urine, if the crab is to survive in dilute media (Evans, 1975). The principal tissues involved in this

active transport process are the gills (King, 1965; Croghan, 1958; Mantel, 1967; Quinn and Lane, 1966; Cameron, 1978; Cameron and Batterton, 1978). Further regulation of osmotic and ionic composition of the hemolymph in crustaceans is accomplished by the gut and antennal glands (Bliss et al., 1966; Mantel, 1968; Mantel, 1975).

Implicated in this active transport process is the Na/K pump catalyzed by Na/K-ATPase enzyme. Na/K-ATPase was first described by Skou (1957), who reported that homogenized nerves of the shore crab Carcinus maenas possess an ATPase which is dependent upon the simultaneous presence of Na and K. Since that time this enzyme has been studied extensively in many tissues including rat kidney cortex (Kinne, 1971), turtle bladder, and many others (Towle, 1981).

In crustaceans, the presence of Na/K-ATPase has been reported in the gill and the antennal gland of the freshwater crayfish Procambarus clarkii (Horiuchi, 1980). Quinn and Lane (1966) reported the presence of Na/K-ATPase in the gill of the semiterrestrial crab, Cardisoma guanhumi, which is very tolerant of variable salinities. They found that Na/K-ATPase in crustaceans is similar to Na/K-ATPase found in other organisms, including its presence in a membrane fraction, its dependence on magnesium for activity, its activation by Na and K, and its inhibition by ouabain.

Studies correlating Na/K-ATPase activity with changes in environmental salinity have implicated its involvement in the osmoregulatory processes of crustaceans. For example, Na/K-ATPase enzyme in the pleopods of Sphaeroma serratum were studied by Philippot et al. (1972). This isopod resembles Carcinus maenas and Callinectes sapidus in its

osmoregulatory capabilities in that it is hyperosmotic in dilute sea water but is isosmotic at 100% sea water. S. serratum lives in tide pools that are subjected to wide fluctuations in environmental salinities. Phillipot and his coworkers found that ATPase activity fluctuates when external salinities are altered. In high sodium media, the activity is low, while it increases in low sodium media.

Pequeux and Gilles (1977) found higher Na/K-ATPase activities in the posterior gills than in the anterior gills of the crab Eriocheir sinensis. Fine structural studies of these gills indicate that higher Na/K-ATPase activity in the posterior gills coincides with a hypertrophy of salt transporting cells (Copeland, et al., 1968). Such a profile of higher enzyme activity in the posterior gills of other crabs has also been reported by Mantel and Olsen (1976), Mantel and Landesman (1977), Pequeux and Gilles (1977), and Spencer et al., (1979). Siebers et al., (1982) have also shown higher activities in posterior gills of Carcinus maenas, and that the posterior gills show increased activity when this crab is exposed to dilute salinities. In crabs which are osmoconformers such differences between anterior and posterior gills are lacking (Mantel and Landesman, 1977; Neufield and Pritchard, 1979).

In the fiddler crab Uca pugilator, maintained in 36 ppt sea water, Na/K-ATPase activity in the posterior gills 5 and 6 exceeded the specific activity in the anterior gills 1-4 by a factor of about three (Graszynski and Drews, 1981). Transfer to dilute sea water resulted in further increase in enzyme activity by 20-30% in gill 5 and 6. Among the anterior gills 1-4, enzyme activity was significantly elevated in gill 4 only. However, the authors state

that due to the low activity, this increase was regarded as unimportant. In another species of fiddler crab, Uca minax, similar results emerge. Wanson et al. (1984) found that this crab is able to control its sodium in media ranging from 1/8 SW to full strength SW. They state that the observed increases in Na/K-ATPase activity in the gills, when this crab encounters a dilute medium, is necessary to reabsorb Na lost over permeable surfaces. Enzyme activity, as has been demonstrated in other euryhaline crustaceans, is higher in the posterior gills as compared to anterior gills. Yet the enzyme present in the anterior gill shows significant discrepancies in Km, Ka, and Vmax values when compared with posterior gills. Wanson and coworkers state that these findings support the possible existence of two different Na/K-ATPase enzymes in the gills of Uca minax. They hypothesize that Na/K-ATPase activity in the anterior gills may be more specifically involved in intracellular regulation of Na, K and cell volume rather than in trans-epithelial movement of cations.

Siebers et al. (1982) studied the osmoregulatory importance of Na/K-ATPase in the gills of Carcinus maenas. They found that ouabain, a specific inhibitor of Na/K-ATPase activity, added to the external medium at a concentration of 10^{-4} M, did not affect serum osmolality. However, serum osmolality was effectively reduced after injection of ouabain (1 and 3 x 10^{-5} M) into the blood. These results support the hypothesis that Na/K-ATPase is located within the basolateral parts of the plasma membrane, i.e. in close contact to the blood, and that it serves an important function in hyperregulatory processes of this crab.

Towle et al., (1976) studied Na/K-ATPase in gills of Callinectes sapidus exposed to 100% and 15% sea water. They found that the enzyme resides in the microsomal fraction of the gills and the enzyme activity increases when exposed to dilute media. They further indicate that this increase took place 2 to 3 hours after transfer. This implies that Na/K-ATPase enzyme can be rapidly activated when this crab is challenged by a dilute environment. Results obtained by Neufield et al., (1980) do not agree with the results of Towle et al., (1976) and indicate that induced changes in Na/K-ATPase activity require 1-2 weeks for completion, a value more consistent with synthesis of new enzyme. More recently, Pequeux et al., (1984) report rapid modulation of Na/K-ATPase activity in a membrane fraction from the gills of Eriocheir sinensis in response to dilution in the environmental medium. Savage and Robinson (1983) found that by injecting the blue crab Callinectes sapidus acclimated to 100% sea water with hemolymph from crabs acclimated to 30% sea water they could induce an increase in gill Na/K-ATPase activity 20 minutes after injection. These results suggest that long term changes in enzyme activity, which are correlated with microscopic changes in gill morphology, may be superimposed upon short term changes in enzyme activity, brought about by activation of preexisting enzyme.

Nature of the Enzyme

Na/K-ATPase has been purified to 60% purity from the brine shrimp, Artemia salina and, like the enzyme from vertebrate tissues, is composed of two different subunits: the catalytic alpha subunit which binds ouabain (MW

103,000) and a smaller beta subunit whose function is unknown (MW 44,100) (Peterson and Hokin, 1980). Studies of isolated cells from various sources indicate that the enzyme appears to span the plasma membrane with the site of ATP hydrolysis facing the cytoplasm and the ouabain binding site facing the blood. A model to explain the vectorial exchange of Na for K suggests that binding of three sodium ions to the intracellular carrier site allows for phosphorylation of the enzyme. Once phosphorylated, the enzyme undergoes a conformational change that translocates sodium ions to the blood. Affinity for sodium is now reduced and affinity for K increases. Potassium ions now bind to the vacant site on the enzyme and subsequent dephosphorylation returns it to the original condition, bringing the bound potassium ions into the cell (Cantley, 1981). In many cells, hydrolysis of one ATP molecule energizes the exchange of three Na for two K ions, resulting in low sodium and high potassium inside the cell. Cantley (1981) suggested that NH_4 can substitute for K ions on the Na/K-ATPase enzyme thus allowing for nitrogen excretion as well as maintaining ion balance.

Localization of Na/K-ATPase and Implications for Transport

In the plasma membranes of most salt transporting epithelial cells, including those of crustacean and teleost gill, Na/K-ATPase molecules are not distributed randomly but rather seem to be restricted to the basolateral membrane. This enzyme is well placed in crabs that are hyperregulating. Under these circumstances, Na enters the gill epithelial cell passively, through amiloride sensitive channels (Towle, in press; Kirschner, 1983). Once the sodium is inside the cells, Na/K-ATPase enzyme acts to move

sodium from the cytosol into the extracellular fluid in exchange for potassium. When the environment becomes dilute this enzyme is believed to allow for maintenance of blood osmolalities higher than that of the medium. In teleosts and other hyporegulators the participation of Na/K-ATPase in ion secretion must be indirect. This stems from the basolateral location of this pump and its ability to tranport sodium to the blood and not to the external medium. Currently under investigation is the possibility that Na/K-ATPase may energize a Na-coupled Cl uptake from the blood to the cytosol across the basolateral membrane. According to this model, Na/K-ATPase exports Na across the basolateral membrane in exchange for K. The sodium expelled into the blood is then transported with chloride into the cytosol by a Na-coupled Cl transport system which would allow for transepithelial NaCl secretion.

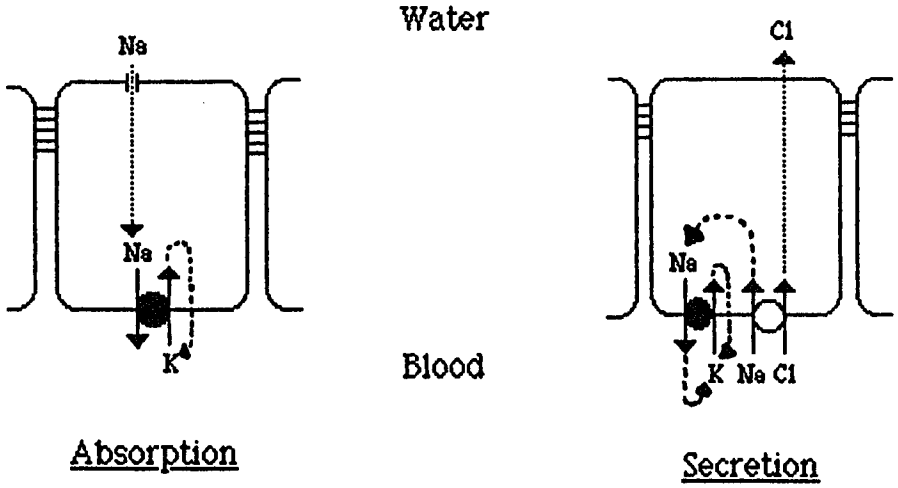


Figure 1 - Model of NaCl absorption and secretion by branchial epithelia of aquatic animals.

Neuroendocrine Control of Hydromineral Balance

Estuarine organisms are subject to widely varying salinities and they must actively regulate their content of salt and water in order to avoid fatal dilution of their tissues. Additionally, in crustaceans, hydromineral balance becomes particularly important because of the need to take up large amounts of water in order for successful ecdysis to take place. Experimental evidence suggests that this hydromineral balance is under the control of neuroendocrine factors. Scudamore (1947) reported that removal of the eyestalks or sinus glands results in increased weight with greater water content in the crayfish Cambarus immunis. Implantation of a single sinus gland prevents the increase in weight. Carlisle (1955) and Bliss et al., (1966) reported similar results in Carcinus maenas and Gecarcinus lateralis, respectively. These increases in weight were observed at the time of, or just prior to, molt. Mantel et al. (1985) have suggested the presence of factors in the brain and thoracic ganglia of Gecarcinus lateralis that regulate water movement at ecdysis. They suggest that neuroendocrine factors produced by the thoracic ganglia and released by the eyestalk sinus gland regulate water movement from the blood into the foregut for storage of water at ecdysis.

More recent studies (Bliss et al., 1966; Mantel, 1968; Kato and Kamemoto, 1968; Kamemoto and Ono, 1969) indicate the presence of factors, presumably neurosecretory in nature, produced by the central nervous system, which are involved in hydromineral regulation. Tullis and Kamemoto (1974) found at least two factors that can be separated from the brain and

thoracic ganglionic mass of Thalamita crenata. A water soluble fraction (MW between 800 and1000 daltons) causes a dramatic decrease in influx of tritiated water, while an acetone soluble fraction causes an increase in influx of tritiated water when injected into a crab or crayfish. Cantelmo (1977) has shown that extracts from the thoracic ganglion of Callinectes sapidus acclimated to 40% sea water can decrease permeability of isolated gills and gut of crabs to water. Similar results have also been reported by Berlind and Kamemoto (1977). In the freshwater crayfish Procambarus clarkii, removal of the eyestalks causes an increased uptake of water coupled with a greater urine flow (Kamemoto and Ono, 1969). These authors state that increased urinary flow acts to alleviate a build up in hydrostatic pressure within the crayfish. Muramoto (1981) found that uptake of water through the gut of this crayfish is under neuroendocrine control and may partially account for the greater urine flow seen by Kamemoto and Ono.

Ramamurthi and Sheer (1967) found that an extract obtained from the cephalothorax of the shrimp Pandalus jordani contains an active factor that retards outflux of Na ions from the body of the crab Hemigrapsus nudus. Kamemoto and Tullis (1972) showed that Na and Cl levels in the hemolymph decrease upon removal of the eyestalks from the freshwater crayfish, Procambarus clarkii. Injection of brain homogenates into normal crayfish resulted in an increased concentration of sodium and chloride in the blood. Homogenates of the thoracic cord of crayfish also caused an increase in the chloride concentration of the blood, although they were less effective than brain homogenates. Heit and Fingerman (1975) found that eyestalkless Uca pugilator maintained in hypoosmotic sea water, underwent a decrease in Na

concentration of blood; injection of eyestalk extract increased the Na concentration. Similar treatment of crabs in hyperosmotic sea water had no significant effect. Kamemoto and Oyama (in press) more recently demonstrated that extracts of the pericardial organ increased the influx of sodium into the isolated perfused gill of Callinectes sapidus. They have also shown that extracts from pericardial organ affect levels of the cyclic nucleotide cAMP. The components of the pericardial organ are known and include dopamine, octopamine, serotonin, and proctolin. Serotonin and the pentapeptide cardioexcitor, proctolin, had no effect upon nucleotide levels; however, dopamine and octopamine caused dramatic increases in cAMP but had no effect on cGMP (Kamemoto and Oyama, in press).

General Mechanism of Hormone Action

Apparently all hormones, with the exception of steroids, stimulate cells by interaction with a specific membrane receptor. This initial interaction results in transduction of the signal to the cytoplasmic side of the membrane where adenylate or guanylate cyclase enzymes are activated, resulting in the production of cAMP or cGMP, respectively (Hadley, 1984). These membrane receptors are visualized as macromolecules, probably glycoproteins, which display a unique affinity for the hormone ligand.

The question now is how do receptor-ligand complexes stimulate an increase in adenylate or guanylate cyclase activity? Receptors and enzymes were once thought to be structurally coupled, such that once a hormone became bound to the receptor a conformational change in the enzyme was induced, thus activating it. This hypothesis is no longer accepted; instead, it

is thought that the receptor is "free floating" in the fluidity of the membrane in an unoccupied state and may possess negligible affinity for the enzymes adenylate cyclase or guanylate cyclase (Kahn, 1976). Upon hormone binding, however, a conformational change in the receptor occurs, thus establishing a recognition site for the enzyme. This may allow the activated receptor to bind to the enzyme to form an active complex (Hadley, 1984). This two step process of hormone-receptor interaction with adenylate cyclase is consistent with many (if not all) known events on hormone action at the membrane level (Hollenberg and Cuatrecasas, 1976; Jacobs and Cuatrecasas, 1977). Evidence that both receptor and cyclyzing enzymes are discrete entities comes from numerous sources. Hadley (1984) suggests that hormone receptors and adenylate cyclase are the products of separate genes. The products of two separate genes suggests the production of two separate proteins and not one protein. Other evidence indicates that fusion of particular cells that lack receptors, but have adenylate cyclase, to cells that have receptors but lack the adenylate cyclase enzyme, produce a hybrid cell, which synthesizes cyclic AMP in response to hormonal stimulation (Schramm, et al., 1977; Schulster, 1976).

It should be pointed out that maximal production of cAMP by one hormone is not augmented by the addition of other hormones. That is, the action of hormones on production of cAMP is not additive. Thus, although many hormones mediate their effects through separate membrane receptors, transduction of their signals is funneled through one adenylate cyclase enzyme and not through different cyclases linked to each receptor (Hadley, 1984).

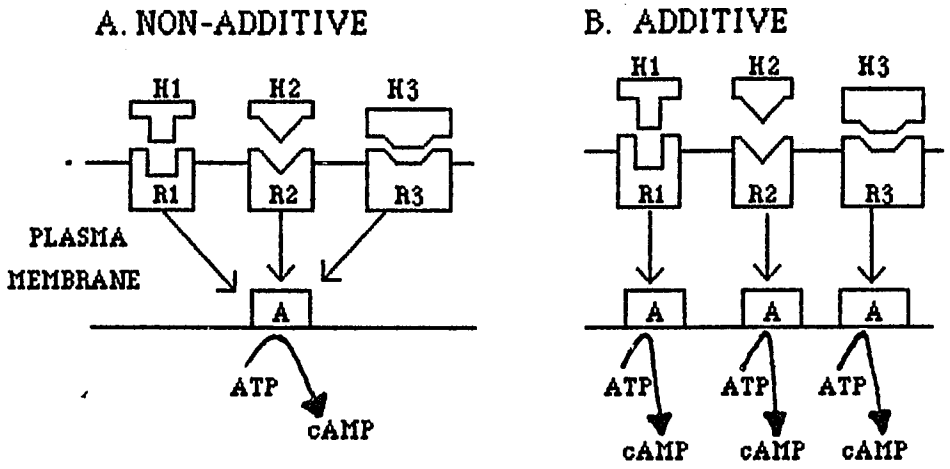


Figure 2 - Although many hormones (H1, H2, H3) mediate their effects through separate membrane receptors, transduction of their signal is funneled through only one adenylate cyclase (A.) and not through different cyclases linked separately to each receptor (B.) (Hadley, 1984).

Once the adenylate cyclase enzyme is activated, ATP is converted to the secondary messenger 3',5'-cyclic adenosine monophosphate, cyclic AMP (cAMP). Some hormones activate guanylate cyclase, which is responsible for the production of the secondary messenger cGMP from GTP. These secondary messengers then combine intracellularly with specific cyclic nucleotide-dependent protein kinases. For example, cyclic AMP-dependent protein kinase consists of a regulatory unit and a catalytic unit. When cAMP binds to the regulatory site of the protein kinase it causes the release of the catalytic unit, thus activating the protein kinase so that it can now act as a phosphorylating enzyme. Enzyme phosphorylation leads to a cascading phenomenon, in which phosphorylation of the first enzyme allows this enzyme to act as kinase to phosphorylate a second enzyme which in turn acts as a kinase as well. This system can then act as an amplifier in which a few

first messengers can produce a physiological response which is several orders of magnitude larger (Hadley, 1984).

Sutherland (1972) was the first to demonstrate that extracellular first messengers could cause the production of the intracellular secondary messenger cAMP. This first messenger- second messenger hypothesis of hormone action must now be expanded to include the secondary messengers cGMP (Goldberg, 1974), and possibly other cyclic nucleotides such as cUMP (cyclic uridine monophosphate) and cCMP (cyclic cytidine monophosphate). In some instances, different cyclic nucleotides may function in opposition to each other in what has now come to be known as the Yin-Yang hypothesis for hormone action (Goldberg, 1974). For example, cGMP is correlated with smooth muscle contraction while increases in cAMP level have been correlated with smooth muscle relaxation. In excitable tissues, higher levels of cGMP are related to depolarization of muscle and nerve while higher cAMP concentrations are correlated with with hyperpolarization of these tissues (Hadley, 1984).

The actions of cyclic nucleotides are halted by a cyclic nucleotide dependent phosphodiesterase which converts 3',5'-cyclic adenosine monophosphate, for example, to its inactive form, 5' adenosine monophosphate. Methylxanthines inhibit cAMP- and cGMP-dependent phosphodiesterase activities. Caffeine, theophylline, and theobromide are methylxanthines derived from coffee, tea, and coco respectively (Hadley, 1984). Theophylline is the most potent phosphodiesterase inhibitor of the three, and, when present in relatively low concentrations, potentiates the effects of cAMP. This occurs because the cAMP generated by the the hormone is not

degraded by phosphodiesterase. At high concentrations (10^{-3} M) theophylline or caffeine alone may induce a response in a particular tissue. This suggests that adenylate cyclase is constantly producing cAMP but this cyclic nucleotide is continually being destroyed (Hadley, 1984).

Because many hormones mediate their effects through the activation of adenylate cyclase and subsequent production of cAMP, Sutherland (1972) and his colleagues suggested that certain criteria should be satisfied before concluding that a specific hormone mediates its effects through the secondary messenger cAMP: 1) the hormone should stimulate adenylate cyclase in broken cell preparations; 2) physiological levels of cAMP should be elevated before or concurrently with the physiological response; 3) methylxanthines, which inhibit phosphodiesterase activity, should potentiate and at high levels mimic the activity of the particular hormone in question; 4) there should be a correlation between the activity of the hormone analogs that elevate cAMP and their ability to activate that particular response, and 5) exogenous cAMP or its analog should mimic the effects of the hormone.

Cyclic AMP added to the extracellular environment may cause a physiological response, but only at high levels. This observed phenomenon is apparently due to the poor ability of cAMP to penetrate into the target cells through the plasmalemma. To satisfy the fifth criterion, certain analogs of cAMP (e.g. dibutyryl cAMP) are used, because their less polar nature makes them more membrane permeable (Stryer, 1975). Also, these analogs tend to be considerably more resistant to inactivation by phosphodiesterase.

Studies done on invertebrates have indicated that cAMP and cGMP may play an important role in mediating the action of many hormones. For

example, the action of crustacean hyperglycemic hormone, which is located in the eyestalk, is mediated by cyclic nucleotides. Whether this is cAMP or cGMP has not yet been resolved. It is thought that the crustacean hyperglycemic hormone exerts its effects either by activation of the phosphorylase system or by inactivating the the glycogen sythetase system (Kleinholtz and Keller, 1979; Sedlmeier, 1985; Keller et al., 1985). Cyclic AMP has been shown to activate phosphorylase in the leg muscle of Eriocheir sinesis (Bauchau et al., 1968). Both cAMP and cGMP are able to mimic the effects of crustacean hyperglycemic hormone, although cGMP has the greater effect of the two (Keller and Andrews, 1973; Spindler et al., 1976, Sedlmeier, 1985; Keller et al., 1985). Theophylline, a phosphodiesterase inhibitor, is able to elevate levels of glucose in the blood of the crayfish Orconectes sp. with a time course paralleling that of cGMP-induced hyperglycemia (Spindler et al., 1976). Sedlmeier and Keller (1981) studied the effect of crustacean hyperglycemic hormone on cAMP and cGMP levels in various tissue (heart, integument, antennal gland, hepatopancreas, abdominal muscle, and hemolymph) in Orconectes sp. They found that all tissues, with the exception of the hemolymph, showed far greater levels of cGMP than cAMP. They further point out that cGMP levels were elevated within 30 minutes after administration of the crustacean hyperglycemic hormone, whereas maximal induced hyperglycemia occurs 2 hours after injection of either the crustacean hyperglycemic hormone or cGMP. This satisfies the second criterion of Sutherland and his colleagues (1976), in that intracellular levels of cGMP are increased well before hyperglycemia occurs. Crayfish antennal glands showed elevated levels of cGMP 2 minutes

after incubation with eyestalk extract. Such results led Sedimier and Keller (1981) to conclude that cGMP mediates the effects of the crustacean hyperglycemic hormone. Kamemoto and Oyama (in press) also noted this type of response in the crayfish Procambarus.

Ahearn and Kullama (1984) state that dibutyryl cAMP or theophylline abolished active transmural chloride transport across the intestine of the freshwater prawn Macrobrachium rosenbergii. In addition, transmural membrane potentials resulting from the movement of both chloride and sodium are abolished in the presence of 10 mM theophylline or 1 mM dibutyryl cyclic AMP. Cyclic AMP appears to have its effect on the epithelial apical membrane, where increased intracellular cAMP or some other cyclic nucleotide (i.e. cGMP) may interact directly or together with calcium ions to eliminate coupled Na^{+}-Cl^{-} influx into the cell from the gut lumen. Nwoga and Bittar (1983) state that injection of 10^{-3}M cAMP into the barnacle Balanus nubilis was able to stimulate ouabain insensitive sodium efflux in the muscle fibers of this barnacle. They further state that even with the use of analogs of cAMP such as 8 bromo-cAMP, which is reported as being resistant to hydrolysis by phosphodiesterase, the response to this analog is transitory. However, the response to this cAMP analog decays more slowly than cAMP ($t_{1/2}$ 48 $\pm$ 2 min, with 8-bromo-cAMP vs $t_{1/2}$ 22 $\pm$ 2 min, with cAMP). They reason that the short lived effects of cAMP occur because these muscle fibers possess a powerful cAMP phosphodiesterase system capable of degrading even the resistant analogs of cAMP.

In decapod crustaceans, the eyestalks, composed of the medulla terminalis X-organ and sinus gland, are a source of the ovary inhibiting

hormone (OIH). Panouse (1943) demonstrated that extracts from the sinus gland were able to inhibit ovarian development in the the prawn Palaemon serratus. Cyclic nucleotides have been shown to affect ovarian development in crustaceans. Studies by Eastman-Reks and Fingerman (1984) indicate that in Uca pugilator, the ovarian inhibitory hormone (OIH) may mediate its effects through cAMP. Their studies show that cyclic AMP at a concentration of 10^{-6} M inhibited the rate of protein synthesis in previtellogenic ovaries but not in mature ovaries.

In insects, cyclic nucleotides have been shown to play a prominent role in many physiological processes. Injection of partially purified (Spencer and Candy, 1976) or synthetic (Gade, 1979) preparations of the adipokinetic hormone results in a rise in cAMP concentrations in the fat bodies of locust. Aston (1975) found that stimulation of isolated malpighian tubules of the bug Rhodnius prolixus with partially purified diuretic hormone causes levels of cAMP to rise within minutes of stimulation. Adenylate cyclase activity has also been detected in the rectal tissue of the locust Schistocerca gregaria. Isolated rectal preparations that have been perfused with extracts of the corpus cardiacum, known to contain the peptidic chloride transporting stimulating hormone (CTSH), showed 2- to 3-fold increase in the levels of cAMP (Spring and Phillips, 1980).

Certain biogenic amines, such as octopamine and dopamine, have also been found to increase levels of cAMP in insects. Nathanson and Greengard (1973) found that micromolar concentration of octopamine was able to increase adenylate cyclase activity in the thoracic ganglia of the cockroach Periplanata americana. Subsequently, octopamine sensitive adenylate

cyclase activity has been detected in other insects and invertebrates such as the mollusc Aplysia (Levitan and Barondes, 1974), the horseshoe crab (Atkinson et al., 1977), and the earthworm (Robertson and Osborne, 1979). The nature of the role of octopamine-stimulated adenylate cyclase activity is not at present understood. However, octopamine has been shown to stimulate phosphorylation of a protein (or proteins) in the abdominal ganglia of Aplysia, suggesting that the effects of octopamine may result in relatively long-lasting changes in a phosphoprotein in the ganglion, perhaps in postsynaptic cells (Levitan and Barondes, 1974).

Dopamine has also been shown to affect levels of cAMP in the thoracic ganglia and the brain of P. americana (Nathanson and Greengard, 1973; Harmar and Horn, 1977).

Purpose of the Study

The purpose of this study is an attempt to elucidate some of the mechanisms involved in the ability of Carcinus maenas to hyperregulate in dilute media. Based on the background information given above, I have investigated the following: (a) the effects of dopamine, dbcAMP and heated pericardial extracts on sodium uptake in vivo, (b) the effects of dopamine, dbcAMP, and heated pericardial extracts on Na/K-ATPase activity in the gills of Carcinus maenas (L) and (c) the effects of dopamine, octopamine, long term acclimation to 40% SW and acute transfer to 40% SW on levels of cAMP in the anterior and posterior gills of C. maenas, in an attempt to elucidate some of the mechanisms involved in the processes by which C. maenas can hyperregulate in dilute media.

MATERIALS AND METHODS:

Maintenance of Animals:

Carcinus maenas (Linnaeus), the green crab, is an abundant species which ranges from south Cape Cod to Wedgeport, Nova Scotia, and Canada (Ropes, 1968). It is easily obtained from bait wholesalers during the months of April to November. It is a moderate hyperregulator and is able to tolerate dilution to 40% SW. As a result, it makes a good subject on which to study osmoregulatory processes.

Eight to ten animals were kept in 10 to 15 liter containers inside an environmental chamber (Scherer Co.). The temperature inside the chamber was 13 to 14°C and a 12 hour light : 12 hour dark illumination cycle was used. All animals were acclimated to 100% (34 ppt: 1025 milliosmoles) Instant Ocean sea water for a period of at least two weeks. Aeration was maintained continously throughout their acclimation period. The animals were fed frozen fish once a week and the tank water was changed after feeding.

Molting:

Molting is the process by which crustaceans grow, through periodic shedding of their shell. During this time changes in physiological processes in marine decapod crustaceans occur. As a result it is important to determine what stage of the molt cycle marine crustaceans are in prior to experimentation. The following is a description of the molt cycle and the method employed to determine the stage of ecdysis.

Before the shell is shed, much synthetic activity takes place. The outer layers of a new exoskeleton are laid down, new limbs are regenerated to replace those that have been lost, and calcium salts are withdrawn from the old shell and in some species are deposited as gastroliths attached to the lining of the foregut. Following ecdysis, further synthetic activity is necessary for the completion of the new exoskeleton and subsequent hardening.

There are four principal stages of growth in crustaceans. The first stage immediately precedes the shedding of the old shell and is known as proecdysis. The second stage, ecdysis, involves the actual shedding process. The third stage is known as metecdysis: during this stage the newly formed shell hardens and the animal returns to its normal physiological condition. Finally, the fourth stage, called intermolt, occurs and may be either of two types: 1. Diecdysis, which is a short period that occurs in continually molting forms. In this type of intermolt, metecdysis passes imperceptibly into proecdysis. 2. Anecdysis, which is a long period of rest that takes place between successive molts. This occurs in crabs that molt seasonally such as Carcinus maenas. It is during this stage that our experiments were performed (Bliss and Boyer, 1964).

The stage of intermolt was determined by removal of the third right walking leg of each crab. The ratio of the size of the newly formed limb bud to the width of the carapace x 100 allows for determination of the stage of molt (Bliss and Boyer, 1964). This calculation is called the R-value and it represents a precise relationship to the stage of molt. Individual green crabs having R-values less than 5 were considered to be intermolt.

Experimental protocols:

1) The Effects of Dopamine on Sodium uptake in Vivo

Male crabs, of the same size were injected with 0.5 ml of 10^{-5} M dopamine. Control animals were injected with 0.5 ml Carcinus Ringer's alone. In each case the Carcinus Ringer's (Table 1) was isosmotic to the recipient crab. Osmolality of crab blood and Ringer's solutions were determined by freezing point depression using a Precision μ Osmette. Injections were carried out using 1 ml syringes fitted with a 26 gauge needle. This type of syringe was used in all experiments unless otherwise noted. The animals were left alone for a period of 3 hours in 100% SW and then ^{22}Na (specific activity = 859.89 mCi/mg) was added to their medium at an activity of 40uCi/L. The specific activity of the sea water medium (423.0 mM NaCl) was therefore calculated to be 0.094 uCi/mM. At time intervals of 1, 2, 3 hours, 0.2 ml hemolymph was removed and placed in scintillation vials together with 15 ml Aquasol. These were counted in a Beckman L.S. 250 and quench was determined by internal standards method. In this method, the samples were first counted for a period of 20 minutes in order to maximize counting efficiency. Whatever quenching agent exists in the vial has exerted its effect in reducing the the counting rate. Then an aliquot of a known radioactivity was added to the vial and the samples were counted a second time. By subtracting the counts of the sample from the counts obtained from the standard + sample, the counts of the standard were obtained. The efficiency was then determined from the following equation:

$$\text{Counting Efficiency} = \frac{\text{CPM - Background}}{\text{DPM}} \times 100\%$$

The efficiency calculated for each sample was then used to correct for quench (Long, 1976). The standard was prepared from $^{22}NaCl$ used in this study after appropriate dilution with Aquasol. Sodium uptake in μM ^{22}Na was calculated by the following formulas:

$$\frac{\text{DPM}}{2.22 \times 10^6 \text{ DPM/uCi}} \text{ uCi Sodium 22}$$

$$\frac{\text{uCi Sodium 22}}{0.094 \text{ μCi/mM in medium}} = \text{μCi Sodium 22/mM}$$

Rate constants of influx K were calculated for this experiment and for subsequent ^{22}Na uptake experiments from the following equation:

$$K = 1/t \log_e \frac{A_\infty}{A_\infty - A_t}$$

where t is the time in hours of influx, A_∞ is the activity in the medium before the influx has begun and A_t is the activity in the animal at time t (Subramanian, 1976). K was calculated for each time interval of all sodium uptake experiments.

Table 1 - Carcinus Ringer's Formula: (Prosser, 1973)

NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$MgCl \cdot 6H_2O$	$NaHCO_3$
585 mM/l	14 mM/l	12.7 mM/l	24.4 mM/l	1 M to pH=7

2) The Effect of Dibutyryl cAMP on in vivo Sodium Uptake by C. maenas

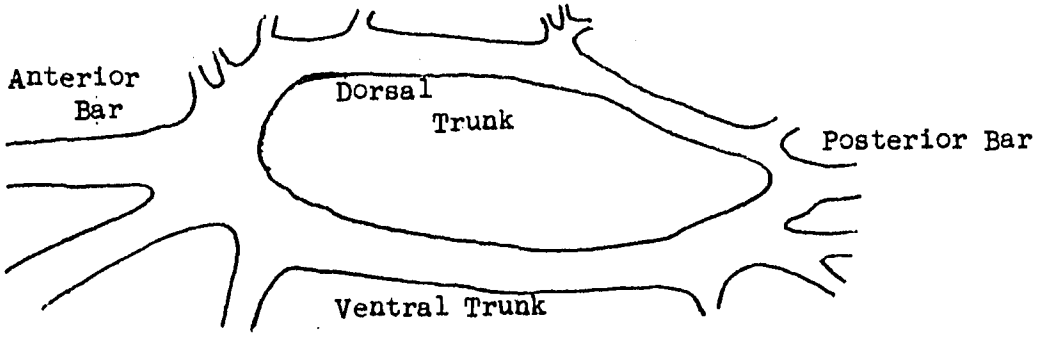
Adult Carcinus maenas of the same sex (preferably male) and approximately the same size were acclimated to 100% seawater prior to use for these experiments. Experimental animals were injected with 0.5 ml of 10^{-6} M dibutyryl cAMP in Carcinus Ringer's and control animals were injected with 0.5 ml Carcinus Ringer's alone. The dibutyryl derivative of cAMP was used because its less polar nature makes it more membrane permeable (Stryer, 1981). Each crab was placed in a separate chamber containing 100% SW. These were left alone for a period of 3 hours, after which ^{22}Na (Specific activity=859.89 mCi/mg) at an activity of 40 uCi/L or a specific activity (sea water medium) of 0.094 uCi/M Atoms was added . At intervals of 1, 2, and 3 hour intervals 0.2 ml hemolymph was removed from each crab and placed in liquid scintillation vials together with 15 ml Aquasol and counted in a Beckman L.S. 250. Quench was corrected for by internal standards method as previously described.

3) The Effects of Pericardial Extracts on Uptake of Sodium in Vivo.

Four pericardial organs were removed from two donor crabs acclimated to100% SW and homogenized in 0.5 ml Carcinus Ringer's. The dissection (performed on ice) was begun by first removing all the walking legs and chelipeds from the animal. Then a portion of the upper carapace was excised in order to expose the heart, which lies posteriorly and dorsally in the body cavity. The heart was raised by its posterior end with a pair of fine forceps and was cut on either side with a pair of microscissors under a

dissecting microscope, in order to detach the heart so it could be removed carefully from the body cavity. The dissected animal was then wedged on its side into a layer of clay placed on the bottom of a finger bowl filled with ice cold Carcinus Ringer's adjusted to the blood osmolality of the dissected crab. With the use of fine forceps, microscissors, and a dissecting microscope, the pericardial organs were excised from the lateral chambers on either side of the heart. The diagram given below shows the morphology of the pericardial organ as it appears in Carcinus maenas.

Figure 3 - Morphology of Pericardial Organ in C. maenas.



These organs were then homogenized in a glass micro-homogenizer. The extract was then heated in a boiling water bath for 2 minutes and centrifuged for 5 minutes on a table top centrifuge. Experimental animals were injected with the extract and control animals were injected with heated Carcinus Ringer's alone. These animals were placed in 100% SW and left alone for a period of 3 hours, after which ^{22}Na at an activity of 40 uCi/L was added to their medium. Samples of the hemolymph were drawn at 1, 2, and 3 hour intervals and counted as previously described. Quench was corrected by the internal standards method.

4) The Effects of Dopamine in vivo on Na/K-ATPase Activity.

Adult green crabs of the same sex (preferably male) acclimated to 100% seawater were used for this experiment. Experimental animals were injected with 0.5 ml dopamine in Carcinus Ringer's isosmotic to the recipient crab at concentration of 10^{-5} M and control animals were injected with 0.5 ml Carcinus Ringer's alone. The animals were kept in100% SW for 3 hours after which anterior and posterior gills were excised. The gills were homogenized and assayed for Na/K-ATPase activity according to a method modified from Spencer et al. (1979)(see p 27).

5) The Effects of dibutyryl cAMP in vitro on Na/K-ATPase activity in the gills.

Adult green crabs of the same sex (usually males) and acclimated to 100% seawater (34 ppt: 1025 milliosmoles) were used for this study. Experimental and control animals were dissected and the anterior (gills 1-5) and posterior gills (gills 6-8) were removed. Gills from the experimental animal were placed in dbcAMP in Carcinus Ringer's at a concentration of 10^{-5} M. The dibutyryl derivative of cAMP was used because its less polar nature makes it more membrane permeable. Control gills were placed in Carcinus Ringer's alone. The Carcinus Ringer's used was isosmotic to animals from which the gills were dissected. These gills were allowed to incubate for 2 hours, after which they were homogenized and assayed for Na/K-ATPase activity according to a method modified from Spencer et al. (1979)(see p 27).

6) The Effects of Pericardial Extract in vivo on Na/K-ATPase Activity in C. maenas.

A crude extract of pericardial organs was prepared by removing two pairs of these neuroendocrine organs from two green crabs, of the same sex (preferably male) acclimated to100% SW . These were homogenized in 0.5 ml Carcinus Ringer's, isosmotic to the recipient crabs, and heated for 2 minutes in a boiling water bath. The extract was then spun in a table top centrifuge for 5 minutes and the supernatant was drawn up in a 1 ml syringe and injected into the experimental crab at the junction between the abdomen and the dorsal carapace. Control crabs were injected in the same manner with a same volume of heated Carcinus Ringer's alone. These animals were kept in 100% SW for three hours and then anterior and posterior gills from each crab were excised and assayed for Na/K-ATPase activity.

Preparation of Mitochondrial and Microsomal Fractions for ATPase Assay.

All operations were performed on ice unless otherwise noted. After injection and incubation at the appropriate times, the gills were excised and separated into anterior gills (1-5) and posterior gills (6-8). These were then blotted to remove excess water. The gills were weighed and placed in homogenizing solution (0.25 M sucrose, 5 mM EDTA, 20 mM imidazole) equivalent to 29 times their weight followed by homogenization in a Teflon-glass Potter-Elvehjem apparatus until all tissues were disrupted. The homogenates were centrifuged at 1000x g at 0°C for 20 minutes and the supernatants were then poured into preweighed centrifuge tubes. These were spun at 14,000x g at 0°C for 30 minutes and the remaining pellets were

weighed and resuspended in homogenizing solution equivalent to 29 times the weight of the pellet. The tubes were vortex mixed and this mixture (mitochondrial fraction) together with supernatant (microsomal) were retained for subsequent ATPase assay.

Assay of Na/K-ATPase Activity

Duplicates of each sample (0.3 ml) were used for this assay. The reaction was started upon addition of the enzyme to the total assay mixture (2.7 ml) which contained 62.5 mM imidazol, 75 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 30 mM KCl, 5 mM ATP (adjusted to a pH of 7.5 with 1 N HCl). An additional set of samples were prepared containing the above assay mixture, 1 mM ouabain and 0.3 ml enzyme. These samples were incubated for1 hour at 37°C upon which the reaction was stopped by addition of 1.2 mls of ice cold 10% trichloroacetic acid (v/v). Each tube was then spun in a table top centrifuge for 5 minutes. Two ml aliquots of each sample were removed and mixed with 2 mls of ammonium molybdate reagent (0.14 M ferrous sulfate 7 hydrate and 1% ammonium molybdate in 1.15 N sulfuric acid). Blanks received 2 ml distilled water and 2 mls of ammonium molybdate reagent. Both the blanks and the samples were vortex mixed and the optical densities of the sample were determined after 2 minutes at 700 nm using a Beckman DBG spectrophotometer. These optical densities were recorded and compared with standards of known concentrations prepared with potassium phosphate monobasic. The portion of the total ATPase activity that was dependent on the simultaneous presence of Na/K-ATPase together with other ATPase enzymes were calculated as the difference between the total activity and the activity in

the presence of 1 mM ouabain. Na/K-ATPase activity is expressed as µM Pi released per hour per milligram of protein.

Protein determination was made by the method of Lowry et al. (1951) using an assay mixture consisting of 100 ml 2% Na_2CO_3 in 0.1 N NaOH, 1.0 ml of 1% $CuSO_4 \cdot 5 H_2O$, 1.0 ml of 2% Na-K tartrate. Samples were first mixed with 3 ml of the above assay mixture, vortex mixed and allowed to stand for 10 minutes, after which 0.3 ml of diluted (1:2) Folin phenol reagent was added. These samples were immediately vortex mixed and allowed to stand for 20 minutes. Spectrophotometric determinations made at 650 nm were recorded and compared with those solutions of known protein concentrations.

7) Effects of Dopamine on the Concentration of cAMP in the Gill.

Adult, intermolt, green crabs acclimated to 100% seawater were injected with 0.5 ml dopamine, at a concentration of 1×10^{-5} M. Control animals were injected with 0.5 ml Carcinus Ringer's alone. These animals were maintained in 100% SW for a period of 3 hours at which time they were sacrificed and the anterior 3rd, 4th and 5th gills and the posterior 6th and 7th gills from one side of the crab were excised.

Anterior and posterior gills were immediately homogenized separately in 2.0 ml of 3% perchloric acid. The pH of the supernatants were adjusted to 6.0 with 30% potassium bicarbonate and centrifuged again at 1000 x g for 20 minutes (0°C). All supernatant was frozen at this time and saved for subsequent analysis by radioimmunoassay. The homogenate obtained from the above procedure was then thawed on the following day and measured for

cyclic AMP concentration using a ^{125}I labelled radio-immunoassay kit obtained from New England Nuclear Corporation.

8) Effects of Octopamine on the Concentration of cAMP in the Gill.

Adult, intermolt, green crabs acclimated to 100% seawater were injected with 0.5 ml octopamine, at a concentration of 1×10^{-5} M. Control animals were injected with 0.5 ml Carcinus Ringer's alone. These animals were maintained in 100% SW for a period of 30 minutes at which time they were sacrificed and the anterior 3rd, 4th and 5th gills and the 6th and 7th gills from one side of the crab were excised. Anterior and posterior gills were homogenized separately as decribed previously and frozen for subsequent cAMP assay using a ^{125}I labelled radio-immunoassay kit.

9) Effects of Long Term Acclimation to 40% SW on the Concentration of cAMP in the Gill.

Adult, intermolt, green crabs were acclimated to either 100% or 40% seawater for at least 2 weeks after which they were sacrificed and the anterior 3rd, 4th and 5th gills and the posterior 6th and 7th gills from one side of the crab were excised. Anterior and posterior gills were homogenized separately as described previously and frozen for subsequent cAMP assay using a ^{125}I labelled radio-immunoassay kit. The concentrations of cAMP from the anterior and posterior gills of 100% SW acclimated crabs were compared with cAMP concentrations of green crabs acclimated to 40% SW.

10) Effects of acute transfer from 100% SW to 40% SW on cAMP Concentrations in the Anterior and Posterior Gills.

Adult, intermolt, green crabs acclimated to 100% SW were acutely transferred to 40% SW for a period of 24 hours after which they were sacrificed and the anterior 3rd, 4th and 5th gills and the posterior 6th and 7th gills from one side of the crab were excised. Gills from 100% SW acclimated green crabs which had not undergone this acute transfer were used as controls. Anterior and posterior gills were homogenized separately as previously described and frozen for subsequent assay of cAMP concentrations.

Theory of Radioimmunoassay

The basic principle of radioimmunoassay is the competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. When unlabeled antigen from standards or samples and a fixed amount of the labeled antigen are allowed to react with a constant and limiting amount of antibody, decreasing amounts of the labeled antigen are bound to the antibody as the amount of unlabeled antigen is increased. Thus fewer counts represent greater concentrations of cAMP in the samples. Both standards and samples were acetylated prior to addition of the antiserum. The acetylation reagent consisted of 100 ul triethylamine and 50 ul acetic anhydride. Acetylation allows for more efficient binding of antiserum with cAMP. Separation of bound from free antigen is achieved using a prereacted primary antibody/ secondary antibody complex.

Sample Preparation for Liquid Scintillation Counting

Note: duplicates of standards, unknowns and blanks were prepared for each analysis. After incubation and centrifugation (1200x g at 0°C for 15 minutes) the supernatant was discarded and the remaining pellet was solubilized by adding 200 µl Protosol to each tube. The samples were then vortex mixed and 1 ml Biofluor followed by 50 µl of glacial acetic acid (to reduce alkaline quench) were added to each tube and vortex mixed again. These samples were then poured into scintillation vials and the tubes washed twice with 2 ml aliquots of biofluor which were then added to the sample. The contents of each sample was then brought to a volume of 15 ml with additional biofluor and counted in a Beckman LS 250 counter for 5 minutes. Counts per minute were read on an open window and the average counts for each set of duplicates was determined. The average net counts were calculated by subtracting average blank counts from the average counts of all standards and unknowns. The average net counts for all samples were converted to "normalized" percent bound or % B/Bo. This was done using the following equation:

$$\% B/Bo = \frac{\text{Average Net Counts of Standard or Unknown}}{\text{Average Net Counts of Zero Standard}} \times 100$$

Results from standards were used to construct a standard curve (plotted on semi-logarithmic graph paper) from which the values of the unknowns were obtained by interpolation. A typical cAMP standard curve is given at the end of the material and methods section (see Figure 4). Since

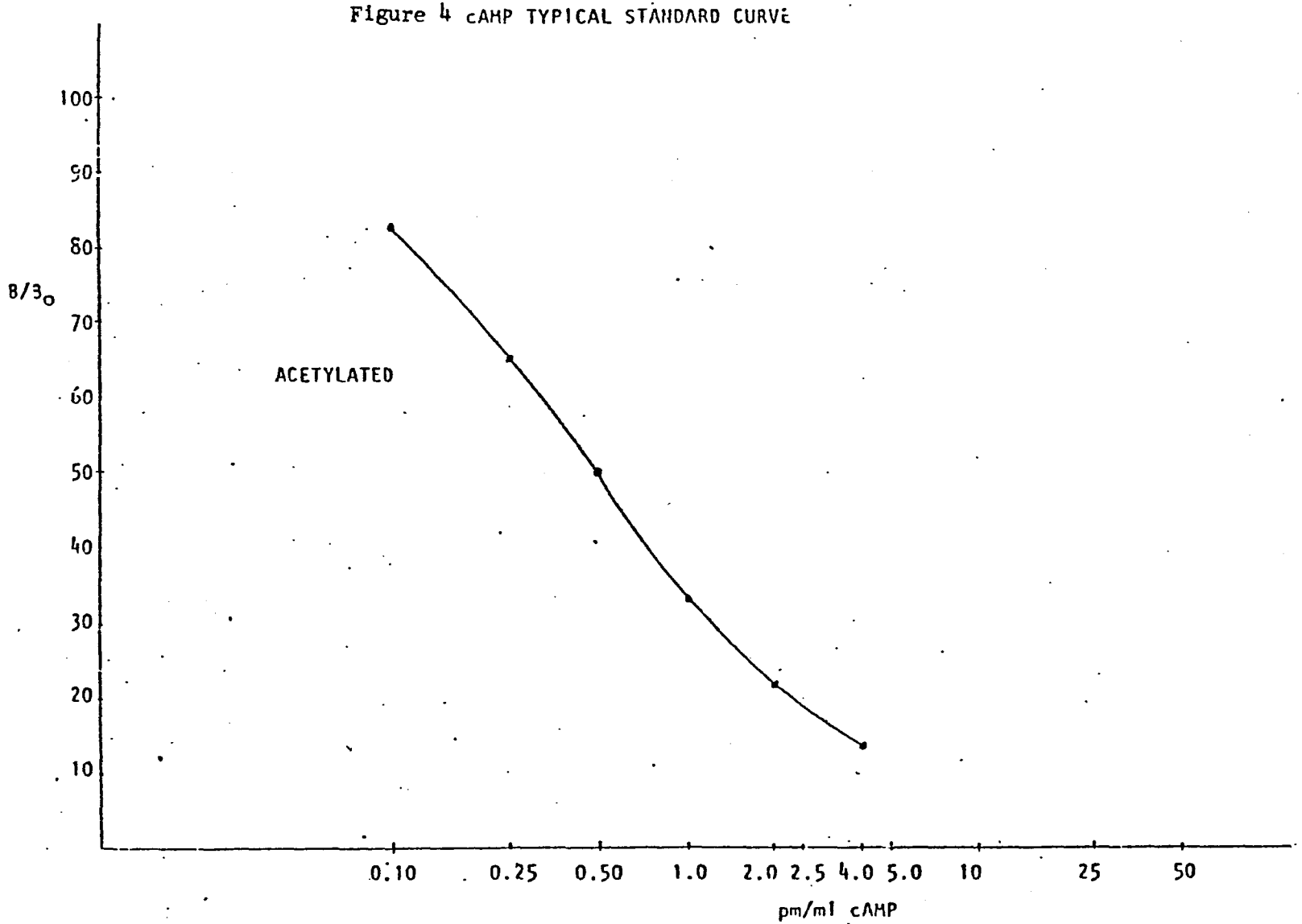
identical volumes are used for both standards and unknowns, and the standard curve is expressed as pmol/ml of cAMP, unknowns can be read as pmol/ml.

The homogenate was assayed for protein by a modified procedure after Lowry et al. (1951). This modification was used because the standard method used in our laboratory produced a cloudy solution with the prepared homogenate samples. This method yields a straight and consistent standard curve. An assay mixture consisted of 100 ml 2% Na_2CO_3 in 0.1 N NaOH, 1 ml 0.5 % $CuSO_4$, 1 ml 1.0% $Na_3C_6H_5O_7 \cdot 2H_2O$ (sodium citrate). Samples were first added to 5 ml of the above assay mixture, vortex mixed and allowed to stand for 10 minutes, upon which 0.5 ml of diluted (1:2) Folin phenol reagent was added. These samples were immediately mixed and allowed to stand for 30 minutes. Spectrophotometric determinations made at 750 nm were recorded and compared with those solutions of known protein concentrations. Results are given as picomoles of cAMP/mg protein.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. In certain instances the Arcsine Transformation was used to normalize the data prior to statistical anaysis. Paired t-test was utilized to compare means for statistical significance and a $p \leq 0.05$ was interpreted as being significant.

Figure 4 cAMP TYPICAL STANDARD CURVE



Results:

Effects of Acute Transfer from 100% SW to 40% SW on Osmolality of the Hemolymph in Carcinus maenas.

The results in Figure 5 show changes in blood osmolality of Carcinus maenas at various time intervals after transfer to 40% sea water (400 mOsm) from 100% SW (1030 mOsm.), to which they had been acclimated. Comparison of blood osmolalities at time zero (1024 mOsm) to that of the100% SW (1030 mOsm) indicates these crabs were all isosmotic to the medium prior to transfer. The dashed horizontal line represents the osmolality of the 40% SW medium throughout the experiment.

During the first 2 hours (n = 6) after transfer the osmolality of the hemolymph decreases rapidly, resulting in a 17.9% change. However, the drop in osmolality begins to taper off thereafter, resulting in a 8.9% decrease from 2-7 hours after transfer. Figure 5 indicates that osmolality of the blood in C. maenas stabilizes between 7 and 26 hours after transfer to dilute medium. This is shown by the plateau reached in the curve. During this time minimal change in blood osmolality occurs (2.8% difference). At this point the crabs are maintaining their blood osmolality substantially above that of their medium (329-350 mOsm. above the medium) and are therefore hyperregulating effectively. This is also seen to be true 48 hours after transfer. Variability in the data can be determined from the standard error of the mean values. This variability is small at time zero but begins to increase shortly after transfer to 40% SW, ranging from 22.1-32.3 mOsm. At 48 hours the standard error of the mean values has decreased

considerably (4.36 mOsm.). These data seem to indicate that while blood osmolality stabilizes at 7-26 hours after transfer to 40% SW, a decrease in individual variability was seen 48 hours after transfer to dilute medium. Table 2 shows all numeric data.

Table 2 - Changes in blood osmolalities vs time after acute transfer of Carcinus maenas from 100% SW to 40% SW. Results given in mOsm ± SEM.

Time (hours)	Osmolality (mOsm)	n
0	1024 ± 1.24	6
2	841 ± 32.3	6
7	750 ± 20.4	6
26	729 ± 22.1	5
48	754 ± 4.36	4

Effects of Dopamine and Dibutyryl cAMP on Uptake of Sodium 22 In Vivo.

Injection of 10^{-5} M dopamine or 10^{-6} M dbcAMP into intact green crabs caused a significant increase ($p<0.001$ and $p<0.01$ repectively) in ^{22}Na uptake (see Figures 6 and 7). These increases are readily seen at the one hour blood sampling period and become more pronounced at the 2 hour and the 3 hour blood sampling periods for both dopamine and dibutyryl cAMP. The findings further indicate that the effect of these injections in all situations lasted for at least 6 hours (3 hour incubation time + 3 hours to the end of the experiment = 6 hours in total). Even 6 hours after injection, the uptake of sodium 22 was not diminished. Tables 3 and 4 show the numerical results of these experiments.

Table 3 - Effects of 10^{-5} M dopamine on ^{22}Na uptake. Values have been corrected for quench and background radiation and are expressed as disintegrations per minute (DPM) ± SEM and µM ^{22}Na ± 1SE. Data was normalized by the arcsine transformation prior to statistical analysis.

	Time (hours)		
	1	2	3
Control (DPM)	2493.5 ± 155.5	5228.3 ± 183.2	7942.5 ± 172.8
Exp. (DPM)	3831.4 ± 110.9*	7261.3 ± 271.6*	9810.3 ± 201.0*

*p<0.001 for n=6

	Time (hours)		
	1	2	3
Control (µM ^{22}Na)	11.9 ± 0.7	25.1 ± 0.9	38.1 ± 0.8
Exp. (µM ^{22}Na)	18.4 ± 0.5*	34.8 ± 1.3*	47.0 ± 1.0*

*p<0.001 for n=6

Table 4 - Effects of 10^{-6}M dibutyryl cAMP on ^{22}Na uptake. Values have been corrected for quench and background radiation and are expressed as disintegrations per minute (DPM) ± 1 SE. Data was normalized by the arcsine transformation prior to statistical analysis.

	Time (hours)		
	1	2	3
Control (DPM)	1946.7 ± 161.5	4733.1 ± 511.6	7078.8 ± 467.0
Exp. (DPM)	4054.9 ± 645.5*	7637.1 ± 885.4*	10175.9 ± 927.7*

*p<0.01 for n=6

	Time (hours)		
	1	2	3
Control (µM ^{22}Na)	9.3 ± 0.88	26.7 ± 2.5	33.9 ± 2.2
Exp. (µM ^{22}Na)	19.4 ± 3.1*	36.6 ± 4.2*	48.8 ± 4.4*

*p<0.01 for n=6

The rate constants of influx, K, at 1, 2 and 3 hours were 0.243, 0.264 and 0.269 respectively for the sodium uptake in experimental animals injected with 10^{-5} M dopamine and are considerably higher than calculated rate constants for sodium uptake in control animals at 1, 2 and 3 hours (0.152, 0.174, and 0.197 respectively).

As with dopamine, dibutyryl cAMP shows similar results. The rate constant of influx, K for 1, 2 and 3 hours for experimental animals injected with 10^{-6} dbcAMP are 0.265, 0.291 and 0.296 respectively and is significantly higher than the K value for control animals; 0.118, 0.157 and 0.171, respectively.

The Effects of Pericardial Organ Extracts on Sodium Uptake In Vivo.

Extracts of P.O. caused an increase in ^{22}Na uptake (Figure 8). The increases in sodium uptake do not occur as rapidly as seen with dopamine or dbcAMP. Examination of the one hour sampling time shows only slight increases in sodium uptake compared with control animals and the differences are not statistically significant ($p>0.05$), but at the 2 hour sampling period the increase in sodium uptake in pericardial organ injected animals is higher. However, due to the variability in the data the results are not significantly different from control sodium uptake. The variability in this data can be readily seen in Figure 8 in which the line representing sodium uptake in animals injected with heated pericardial extract are less linear than those graphs obtained for dopamine, dbcAMP or even control situations. At

the 3 hour sampling period sodium uptake has decreased somewhat compared with sodium uptake at the 2 hour interval but is still greater in green crabs injected with pericardial organ extract compared with control crabs. In this case the differences seen are statistically significant ($p<0.02$) and this is due to decreased variability in the data at this time period. Table 5 shows the numerical data ± 1 SE.

Table 5 - Effects of Pericardial Extract on Uptake of ^{22}Na In Vivo in C. maenas. Values have been corrected for quench and background radiation and are expressed as disintegrations per minute (DPM) ± 1SE and as μM ^{22}Na ± 1 SE. Data transformed by the arcsine transformation prior to statistical analysis.

	Time (hour)		
	1	2	3
Control (DPM)	2273.2 ± 175	4832.9 ± 332	6756.1 ± 581
Exp. (DPM)	2674.7 ± 302[b]	7504.2 ± 1193[b]	8534.1 ± 527[a]

a = $p<0.02$ b = $p>0.05$; n=4

	Time (hour)		
	1	2	3
Control (μM ^{22}Na)	10.9 ± 0.8	23.2 ± 1.6	32.4 ± 2.8
Exp. (μM ^{22}Na)	12.8 ± 1.4[b]	36.0 ± 5.7[b]	40.9 ± 2.5[a]

a=$p<0.02$ b=$p>0.05$; n=4

The rate constant of influx at 1 hour is, K = 0.138 for control animals injected with heated Carcinus ringers and K = 0.164 for experimental animals

injected with heated pericardial organ extract and show no significant difference in rates of uptake of sodium ($p > 0.05$). The rate constants for influx at 2 hours are K = 0.159 and K = 0.286 for control and experimental crabs respectively and show an increase in influx of sodium but are not statistically significant ($p > 0.05$). At 3 hours the rate constants of influx of sodium are K = 0.161 and K = 0.220 for control and experimental crabs, respectively. The rate constants for experimental crabs at 3 hours is statistically higher than control crabs ($p < 0.02$).

These values indicate for the most part that influx of sodium into intact green crabs is considerably higher when dopamine, dibutyryl cAMP, or heated pericardial organ extracts are injected as compared to controls injected with Carcinus ringers alone. Table 6 shows the rate constants for influx, K.

Table 6 - The rate constants for influx of ^{22}Na at each hour (K) in the green crab Carcinus maenas after injection with Carcinus Ringers, heated Carcinus Ringers, 10^{-5} M dopamine, 10^{-6} M dibutyryl cAMP or 4 heated pericardial organs/0.5 ml Carcinus Ringers. Results are given as K ± 1 SE.

	K ± 1 SE		
Material injected	1 hour	2 hours	3 hours
Carcinus Ringers	0.152 ± 0.010	0.174 ± 0.007	0.197 ± 0.006
Dopamine	0.243 ± 0.008[a]	0.264 ± 0.010[a]	0.269 ± 0.008[a]
Carcinus Ringers	0.118 ± 0.010	0.157 ± 0.020	0.171 ± 0.010
Dibutyryl cAMP	0.265 ± 0.050[b]	0.291 ± 0.050[c]	0.296 ± 0.040[b]
Heated Ringers	0.136 ± 0.010	0.159 ± 0.010	0.161 ± 0.020
Pericardial Organ	0.164 ± 0.020[d]	0.286 ± 0.060[d]	0.220 ± 0.020[b]

a = $p < 0.001$; b = $p < 0.02$; c = $p < 0.01$; d = $p > 0.05$ (compared to values above)

Effects of Dopamine on Na/K-ATPase Activity In Vivo.

Comparison of fractions indicate the mitochondrial component contains approximately 10 times more activity than the microsomal component (Table 7) in both control and experimental situations. The differences between the control mitochondrial fraction and control microsomal fraction are statistically significant ($p << 0.001$) (See Figure 9). In addition, comparison of anterior and posterior gills shows greater Na/K-ATPase activity in posterior gills of both mitochondrial and microsomal fractions. The last two observations hold true in all assay situations.

Results in Figure 9 show that injection of 10^{-5} M dopamine into intact green crabs results in substantial increases ($P<0.005$) in enzyme activity of the microsomal fraction in both anterior and posterior experimental gills compared with controls. Anterior and posterior experimental gills showed 58% and 49% increase over control gills respectively. As in previous experiments anterior gills show less activity than posterior gills.

In the mitochondrial fraction, anterior experimental gills showed a slight increase in activity over anterior control gills. Posterior control gills, however, showed greater activity than the posterior experimental gills (Figure 11). These results, however, were too variable to obtain statistical significance ($p>0.05$).

Table 7 - Effects of Dopamine on Na/K-ATPase activity in vivo on both microsomal fractions and mitochondrial fractions. Activities expressed as uM Pi/mg protein/hr ± 1SE.

Microsomal Fraction	Activity	n
Ant. Gill Control	108 ± 14	6
Ant. Gill Exp.	170 ± 10*	6
Post. Gill Control	194 ± 17	6
Post. Gill Exp.	289 ± 18*	6

*p<0.005

Mitochondial Fraction	Activity	n
Ant. Gill Control	2754 ± 142	6
Ant. Gill Exp.	2900 ± 226	6
Post. Gill Control	5521 ± 359	6
Post. Gill Exp.	4632 ± 316	6

*p>0.05

Effects of Dibutyryl cAMP on Na/K-ATPase Activity in Isolated Gills.

Table 8 shows the effects of in vitro incubation of anterior and posterior gills in 10^{-5} M dbcAMP on Na^+/K^+-ATPase activity. Microsomal fractions of experimental anterior (219 ± 11) and posterior gills (472 ± 27) showed significant ($p<0.005$) increases in enzyme activity of 42% and 43% over controls (154 ± 14 and 331 ± 24) respectively when treated with dibutyryl cAMP (Figure 12). The dibutyryl derivative of cAMP was used because its less polar nature makes it more membrane permeable. Increases in the Na/K-ATPase activity of the microsomal fraction correlate well with increases seen in our lab in the microsomal fraction after acclimation to 40% SW.

The mitochondrial fraction, however, showed no real difference

between control anterior gills and experimental anterior gills. The posterior control gills show ATPase values higher than those of the experimental posterior gills but these differences are not great (Figure 13) and no statistical differences were obtained ($p>0.05$). The mitochondrial fraction appeared to be highly variable and no consistent pattern was observed. Table 8 shows mean activities of both fractions ± 1 SE.

Table 8 - Effect of dbcAMP on Na/K-ATPase activity *in vitro* in *C. maenas*. Activity of Na/K-ATPase expressed as uM Pi/mg protein/hr. ± 1 SE.

Microsomal Fraction	Activity	n
Ant. Gill Control	154 ± 14	6
Ant. Gill Exp.	219 ± 11*	6
Post. Gill Control	331 ± 24	6
Post. Gill Exp.	472 ± 27*	6

*$p<0.005$

Mitochondrial Fraction	Activity	n
Ant. Gill Control	2803 ± 333	6
Ant. Gill Exp.	2723 ± 243	6
Post. Gill Control	5659 ± 355	6
Post. Gill Exp.	5191 ± 176	6

*$p>0.05$

The Effects of Pericardial Organ Extract on Na/K-ATPase Activity In Vivo.

The above data, together with information from the literature that dopamine is contained in the pericardial organ (PO) of decapod crustaceans

(Alexandrowicz, 1953; Berlind and Cooke, 1970; Berlind and Goldstone, 1970) led me to try the effects of crude homogenates of pericardial organ extract on Na/K-ATPase activity. Kamemoto and Oyama (in press) found that perfusion of isolated gills of Callinectes sapidus with heated extracts of PO caused a significant increase in ^{22}Na uptake. I have shown that this increased influx of sodium may be the result of activation of Na/K-ATPase. Results in Figure 14 show a significant ($p<0.01$) increase in Na/K-ATPase activity in the microsomal fraction over controls after injection of heated pericardial organ and heated Carcinus Ringers, respectively. Anterior experimental gills showed a 63% increase over controls and posterior experimental gills showed a 52% increase over controls.

In the mitochondrial fraction anterior control gills showed higher activities than anterior experimental gills. Posterior control gills, however, showed lower enzyme activities than posterior experimental gills. Due to the variability in this data no significant difference ($p>0.05$) was observed in the mitochondrial fraction (Figure 15). Results are presented in Table 9.

Table 9 - Effects of pericardial organ extract on Na/K-ATPase activity. Activities are expressed as µM Pi/Mg protein/hr ± SEM.

Microsomal Fraction	Activities	n
Ant. Gill Control	94 ± 14	7
Ant. Gill Exp.	153 ± 13*	7
Post. Gill Control	188 ± 14	7
Post. Gill Exp.	285 ± 26*	7

*$p<0.01$

Mitochondrial Fraction	Activities	n
Ant. Gill Control	2475 ± 223	6
Ant. Gill Exp.	2201 ± 155	6
Post. Gill Control	3961 ± 233	6
Post. Gill Exp.	4544 ± 274	6

*p>0.05

From the data presented thus far it appears that both dopamine and cAMP increase Na/K-ATPase activities in the gills of C. maenas. The increases seen in sodium uptake after treatment with these substances may thus be brought about by increases in Na/K-ATPase.

The In Vivo Effects of Dopamine on Levels of cAMP in the Gills of Carcinus maenas.

Figure 16 indicates the effects of in vivo injections of 10^{-5} M dopamine on cAMP concentrations. Results show significant ($p < 0.01$) increases in anterior and posterior gills of 94% and 95% respectively, that correlate well with the observed increases noted in Na/K-ATPase activity in the anterior and posterior gill after injection of dopamine. In addition, posterior gills show greater concentrations of cAMP than do anterior gills in both control and experimental situations, however, these differences are not statistically significant ($p>0.05$). Table 10 depicts the data together with the standard error of the mean.

Table 10 - Effects of 10^{-5} M dopamine on cAMP concentrations in the gills of C. maenas. Cyclic AMP concentration is given in pmol/mg protein ± 1 SE.

Tissue	cAMP concentration	n
Anterior Gill Control	96.1 ± 11.20	5
Anterior Gill Exp.	186.0 ± 14.50*	5
Posterior Gill Control	124.6 ± 7.50	5
Posterior Gill Exp.	243.1 ± 27.1*	5

*$p < 0.01$

Effects of Octopamine in Vivo on cAMP concentration in the gills.

Octopamine is a known component of the pericardial organ in crustaceans (Berlind and Cooke, 1970). In insects it has been shown to increase adenylate cyclase activity in various tissues including the thoracic ganglia and brain of the cockroach and the locust (Bodnaryk, 1983). Kamemoto (personal communication) has shown that in vivo injection of 10^{-5} M dopamine or octopamine is able to increase cAMP levels in the gills of the decapod crustacean Thalamita crenata, which is an excellent osmoregulator. This increase took place 10 minutes after injection of octopamine.

This study was done to see if such increases could be obtained in Carcinus maenas, a moderate regulator, 30 minutes after injection of 10^{-5} M octopamine. My studies show that 10^{-5} M octopamine had no significant effect on cAMP levels in either the anterior gills or posterior gills of C. maenas (Figure 17). As in previous the experiment, cAMP levels in the anterior gills are lower than cAMP concentrations in the posterior gills of C. maenas under both control and experimental conditions, however, these differences are not statistically significant ($p > 0.05$). The numerical data is presented in Table 11.

Table 11 - Effects of octopamine on *in vivo* on cAMP levels in the anterior and posterior gills of *Carcinus maenas*. Gills 3-5 and 6-7 represent anterior and posterior gills respectively. Results are presented as pmol cAMP/mg protein ± 1 SE.

Tissue	cAMP Concentration	n
Ant. Control Gills	171.7 ± 18.6	5
Ant. Experimental Gills	225.8 ± 30.1	5
Post. Control Gills	210.6 ± 41.5	5
Post. Experimental Gills	231.9 ± 27.0	5

*p > 0.05

Effects of Long Term Acclimation to 40% SW on cAMP Levels in the Gills.

Long term acclimation to dilute salinities has been linked to increased Na/K-ATPase activity in the gills of osmoregulating crustaceans. Since I have shown that dbcAMP is able to increase Na/K-ATPase activity in isolated gills, this study was undertaken to examine the effects of acclimation to 40% SW (400 mOsm.) for more than 2 weeks on cAMP levels in the anterior and posterior gills of *Carcinus maenas*. The results of this study indicate that levels of cAMP decreased in both anterior and posterior gills of 40% SW acclimated crabs by 24% and 31% respectively as compared to 100% SW acclimated green crabs (Table 12; Figure 18). However, these differences are not statistically significant ($p>0.05$). The differences in the concentration of cAMP between anterior and posterior gills are small and there is no statistical difference between anterior and posterior gills in the 100% SW acclimated crabs or the 40% SW acclimated crabs ($p>0.05$) (Figure 18).

Table 12 - Effects of Long Term Acclimation to 40% SW on cAMP levels in the anterior and posterior gills of Carcinus maenas. Gills 3-5 and 6-7 represent the anterior and posterior gills respectively. Data given in pmol. cAMP/mg protein ± SEM.

Tissue	cAMP concentration	n
Ant. Cont. Gill (100% SW)	178.2 ± 26.5	3
Ant. Exp. Gill (40% SW)	135.7 ± 25.9	3
Post. Cont. Gill (100% SW)	183.8 ± 4.90	3
Post. Exp. Gill (40% SW)	127.0 ± 10.0	3

*$p > 0.05$

Effects of Acute Transfer of 100% SW Acclimated Green Crabs to 40% SW on cAMP Concentrations in the Anterior and Posterior Gills.

Acute transfer experiments were performed in which 100% SW acclimated green crabs were transferred to 40% SW. Twenty four hours after transfer, the gills of these crabs were excised and assayed for cAMP concentrations. These concentrations were compared to cAMP concentration of crabs which were not transferred to 40% SW. These experiments are preliminary and this is reflected in the low number of replicates performed. However, the results suggest an increase in cAMP levels occurs 24 hours after acute transfer to 40% SW compared to crabs kept in 100% SW (Figure 19; Table 13). Differences between anterior gills of crabs kept in 100% SW compared to the anterior gills of those crabs acutely tranferred to 40% SW are not statistically significant ($p > 0.05$). Comparison of posterior gills of crabs kept in 100% SW compared with posterior gills of crabs acutely transferred to 40% SW are statistically significant ($p < 0.05$). Examination of cAMP concentration of anterior and posterior gills of 100% SW acclimated crabs shows higher activities in the anterior gills than in the posterior gills

while differences between anterior and posterior gills 24 hours after acute transfer to 40% SW shows slightly higher cAMP levels in the posterior gills compared to the anterior gills.

Table 13 - Cyclic AMP concentrations in the anterior and posterior gills of green crabs 24 hours after acute transfer to 40% SW as compared to 100% SW acclimated green crabs. Gills 3-5 and 6-7 represent anterior and posterior gills respectively. Data is given in pmol cAMP/mg protein ± 1 SE.

Tissues	cAMP Concentrations	n
Ant. Gills (100% SW)	66.9 ± 8.03	3
Ant. Gills (40% SW)	116.9 ± 18.6[a]	3
Post. Gills (100% SW)	52.9 ± 6.23	3
Post. Gills (40% SW)	125.8 ± 9.16[b]	3

a=p> 0.05; b=p< 0.05

Figure 5 - The effects of acute transfer from 100% SW to 40% SW on the osmolality of the hemolymph of Carcinus maenas. Osmolality was measured by freezing point depression and values are given in milli-osmoles. The horizontal line represents the osmolality of 40% SW to which the animals were transferred. The experiment was carried out over a 48 hour period.

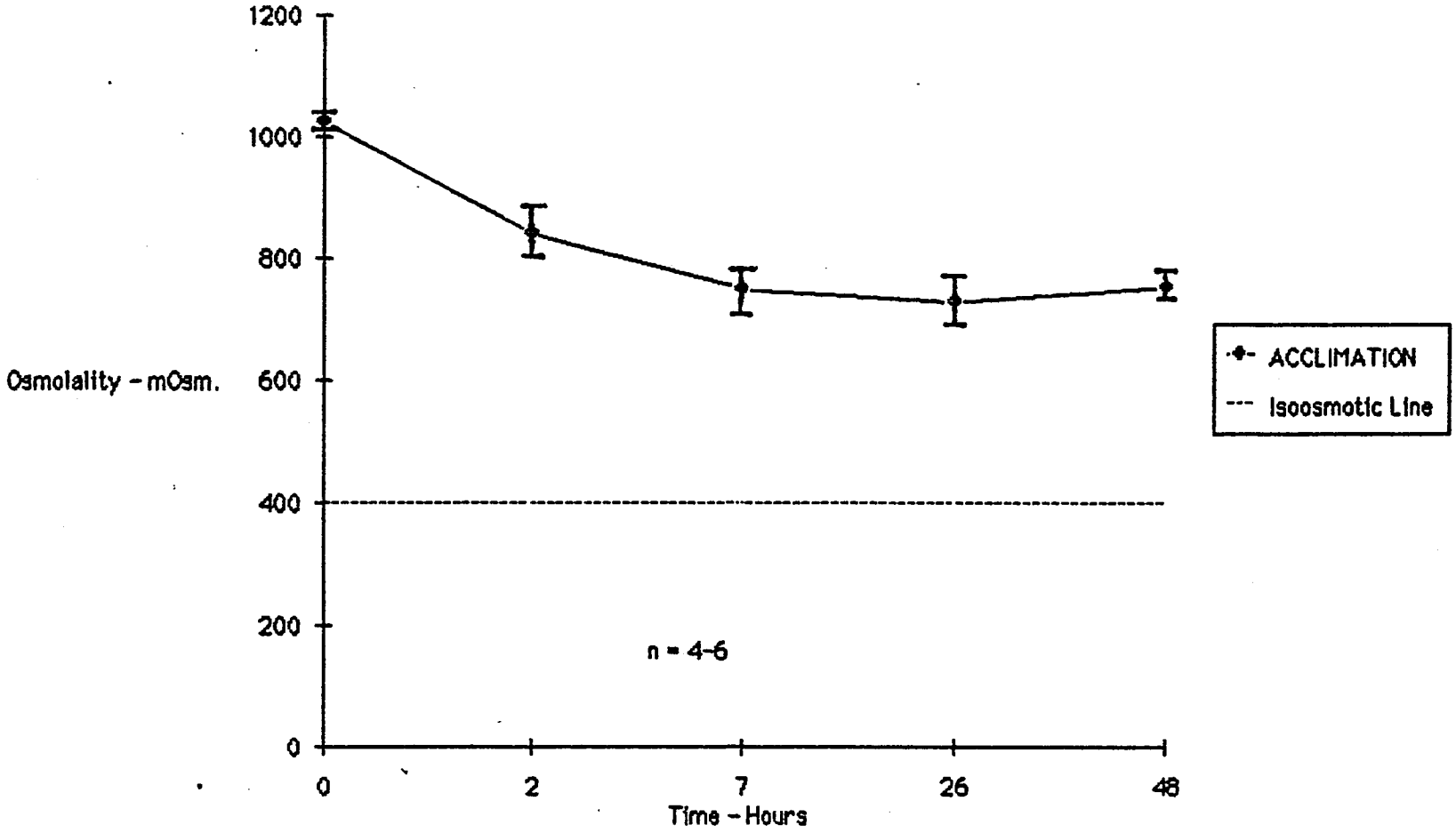

1200
1000
800
600
400
200
0
Osmolality - mOsm.
n = 4-6
0
2
7
26
48
Time - Hours
ACCLIMATION
Isoosmotic Line

Figure 6 - The effects of 10^{-5} M dopamine on ^{22}Na uptake *in vivo* on *C. maenas*. Values have been corrected for quench by internal standards method and are given as disintergrations per minute.

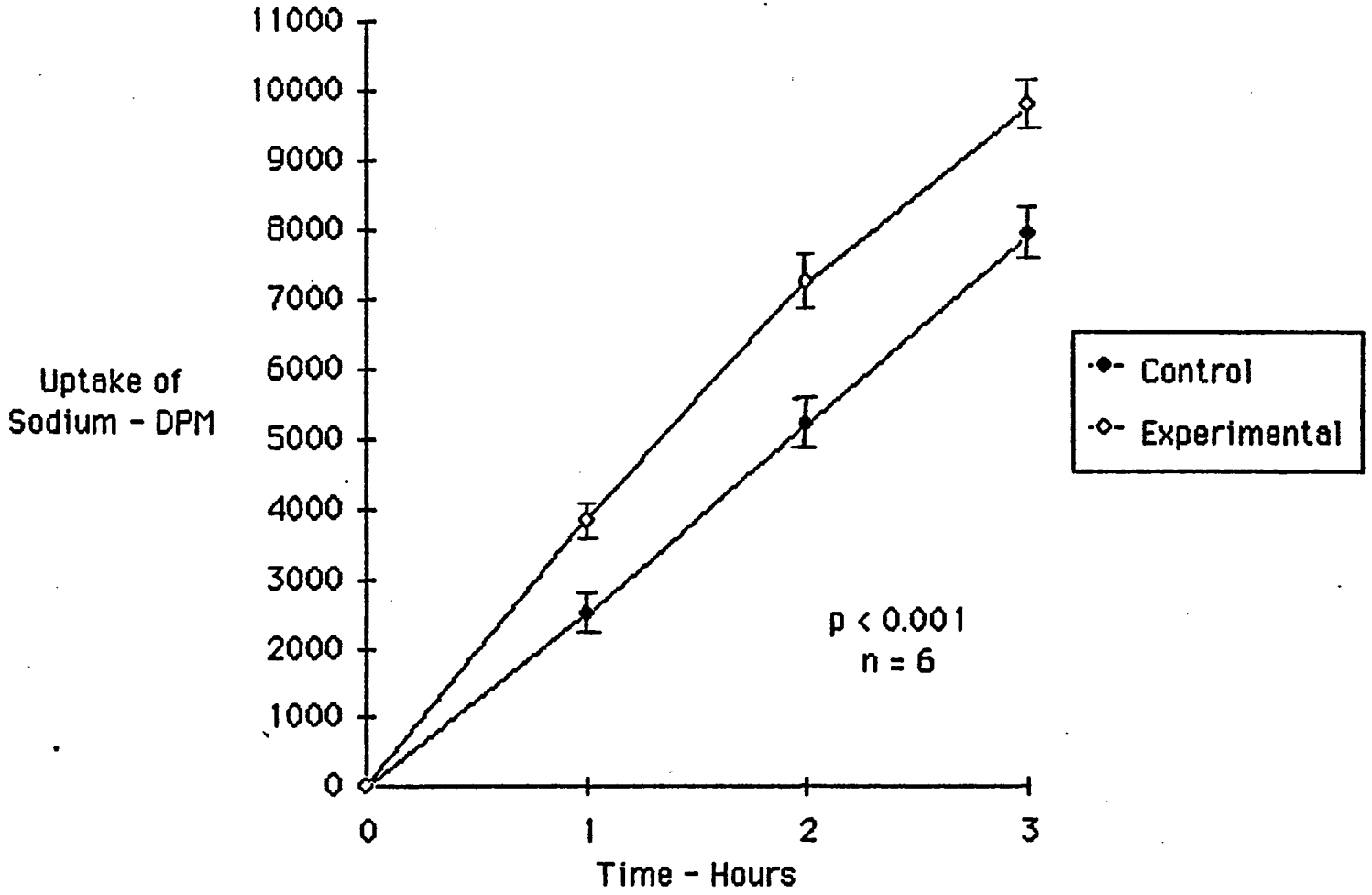

11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Uptake of
Sodium - DPM
0
1
2
3
Time - Hours
p < 0.001
n = 6
Control
Experimental

Figure 7 - The effects of 10^{-6} M dibutyryl cAMP on ^{22}Na uptake. Values have been corrected for quench and background radiation and are expressed as disintegrations per minute (DPM).

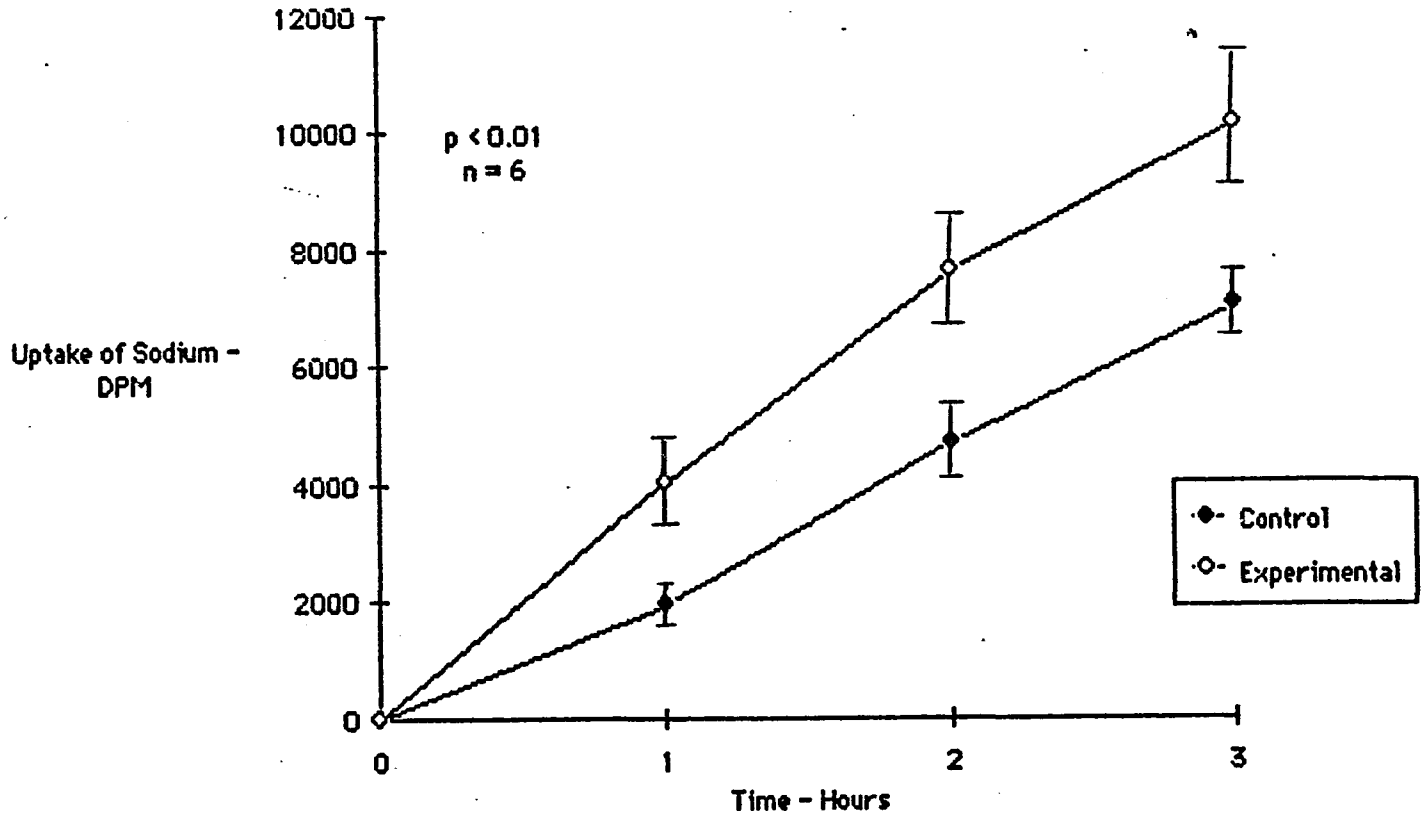

12000
10000
8000
6000
4000
2000
0
p < 0.01
n = 6
Uptake of Sodium - DPM
Control
Experimental
0
1
2
3
Time - Hours

Figure 8 - The effects of pericardial organ extract on uptake of ^{22}Na *in vivo* in *C. maenas*. Values have been corrected for quench and background radiation and are expressed as disintegrations per minute (DPM).

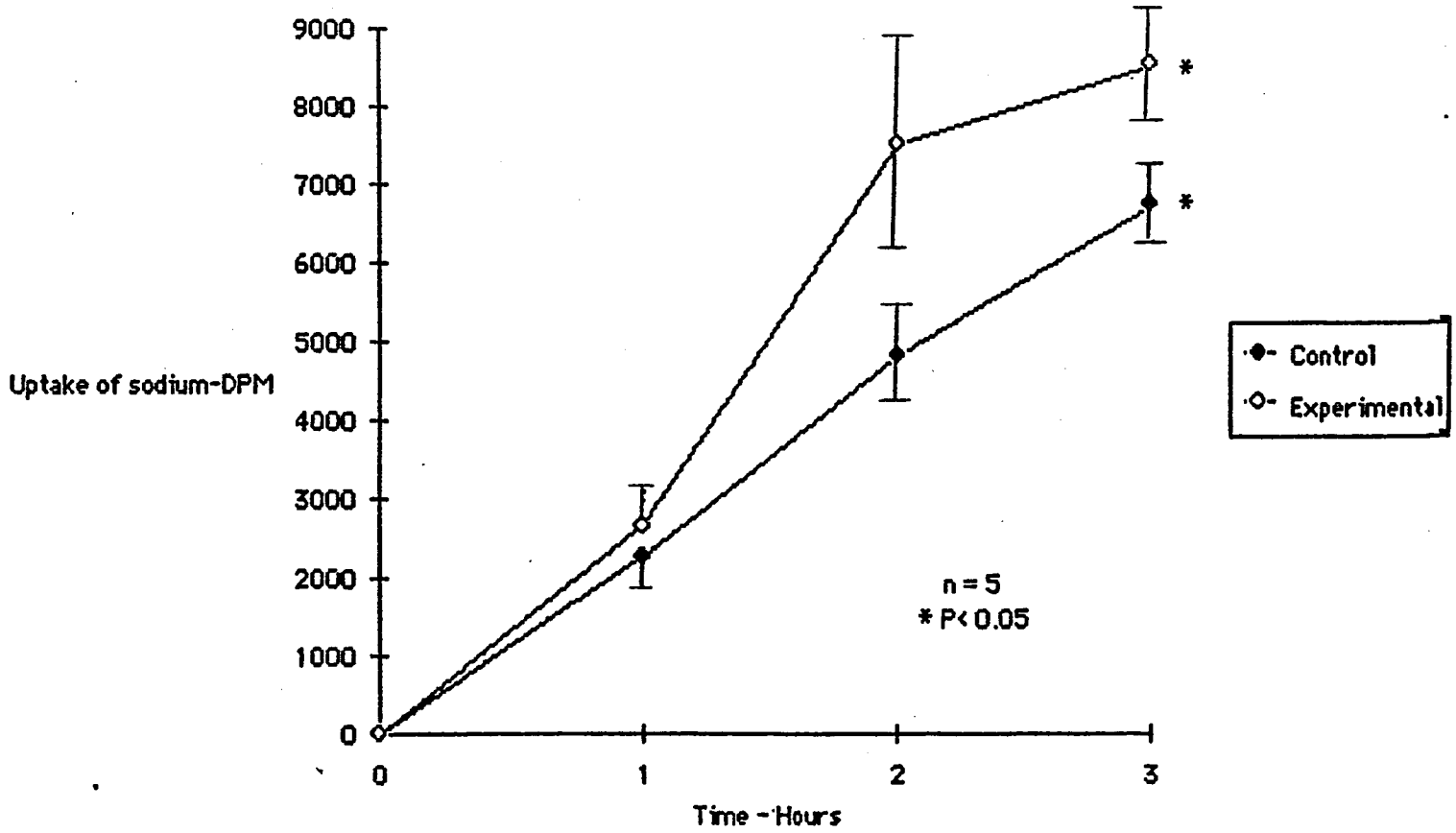

Uptake of sodium-DPM
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
Time - Hours
*
*
n = 5
* P< 0.05
Control
Experimental

Figure 9 - Comparison of Na/K-ATPase activity in the microsomal fraction and the mitochondrial fraction of the anterior and posterior gills of Carcinus maenas. Gills 1-5 and 6-8 represent the anterior and posterior gills respectively. Data is given in uM Pi/mg protein/hr. Differences between the fractions in both anterior and posterior gills are statistically significant ($p \ll 0.001$).

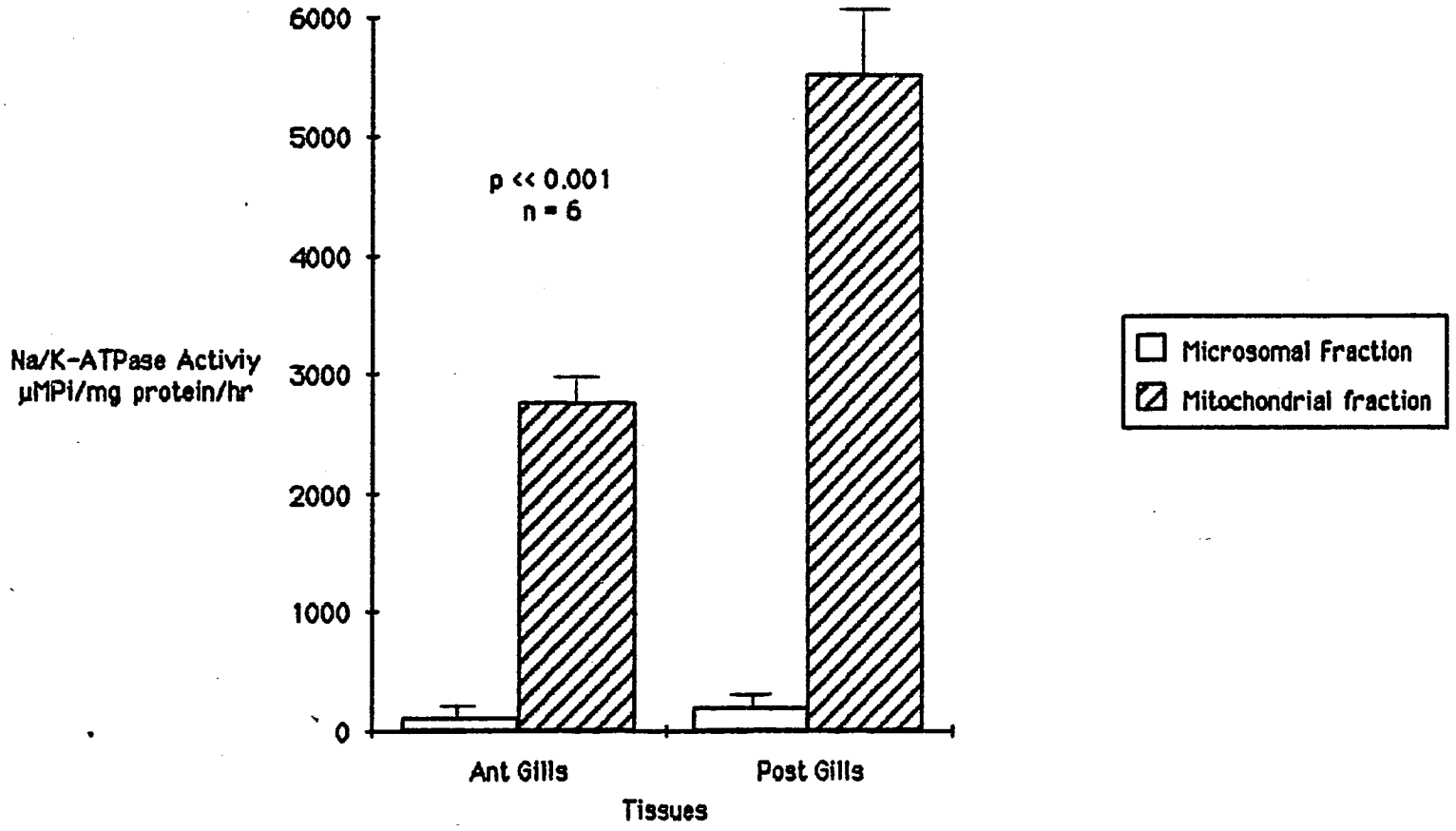

6000
5000
4000
3000
2000
1000
0
Na/K-ATPase Activiy
µMPi/mg protein/hr
p << 0.001
n = 6
Ant Gills
Post Gills
Tissues
Microsomal Fraction
Mitochondrial fraction

Figure 10 - The effects of dopamine at a concentration of 10^{-5} M on Na/K-ATPase activities in the microsomal fraction of the anterior and posterior gills of Carcinus maenas. Activities are given in uM Pi/mg protein/hour. Gills 1-5 represent the anterior gills and gills 6-8 represent the posterior gills.

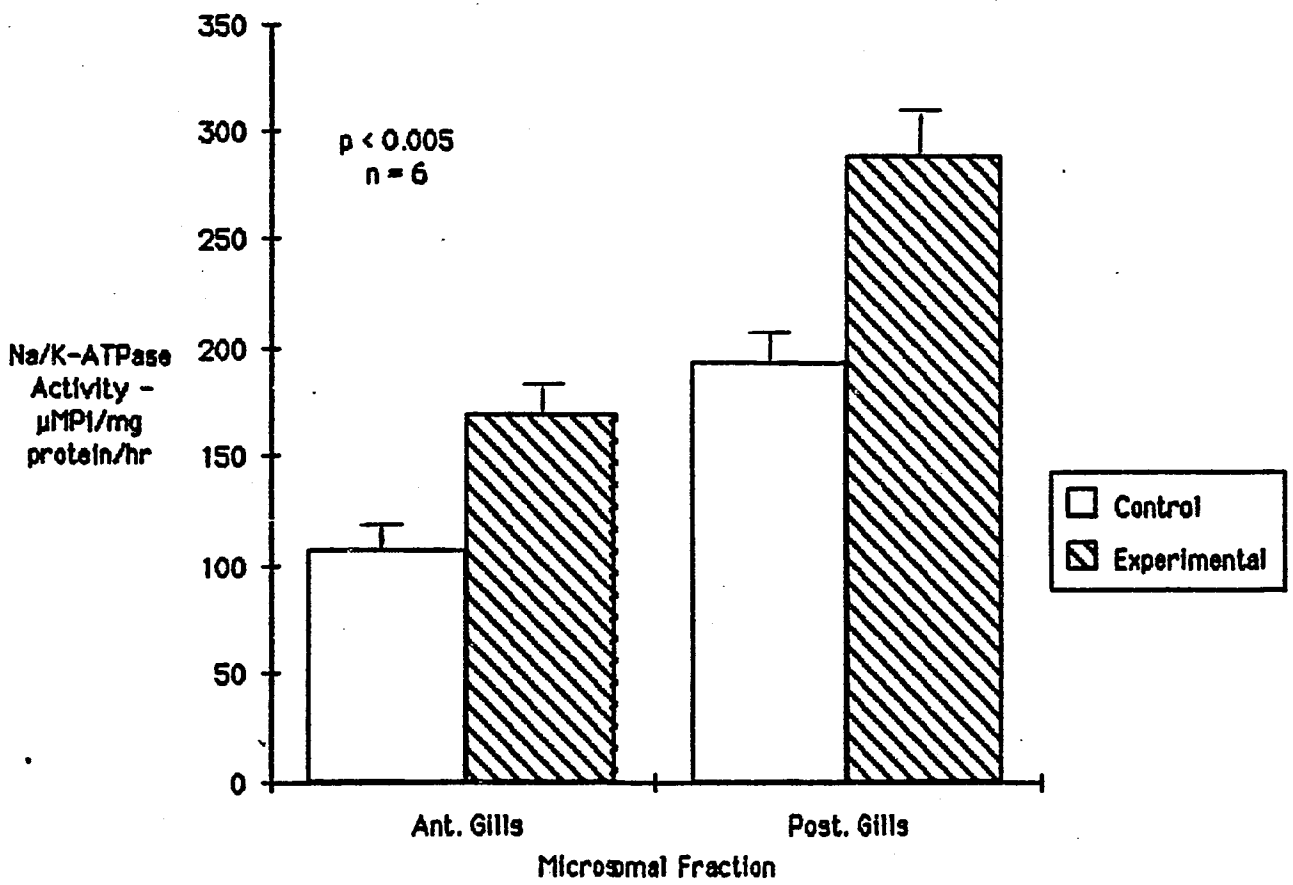

350
300
250
200
150
100
50
0
p < 0.005
n = 6
Na/K-ATPase Activity - µMPi/mg protein/hr
Control
Experimental
Ant. Gills
Post. Gills
Microsomal Fraction

Figure 11 - The effects of 10^{-5} M dopamine on Na/K-ATPase activities in the mitochondrial fraction of the anterior and posterior gills of Carcinus maenas. Activities are given in uM Pi/mg protein/hour. Gills 1-5 represent the anterior gills and gills 6-8 represent the posterior gills in this study.

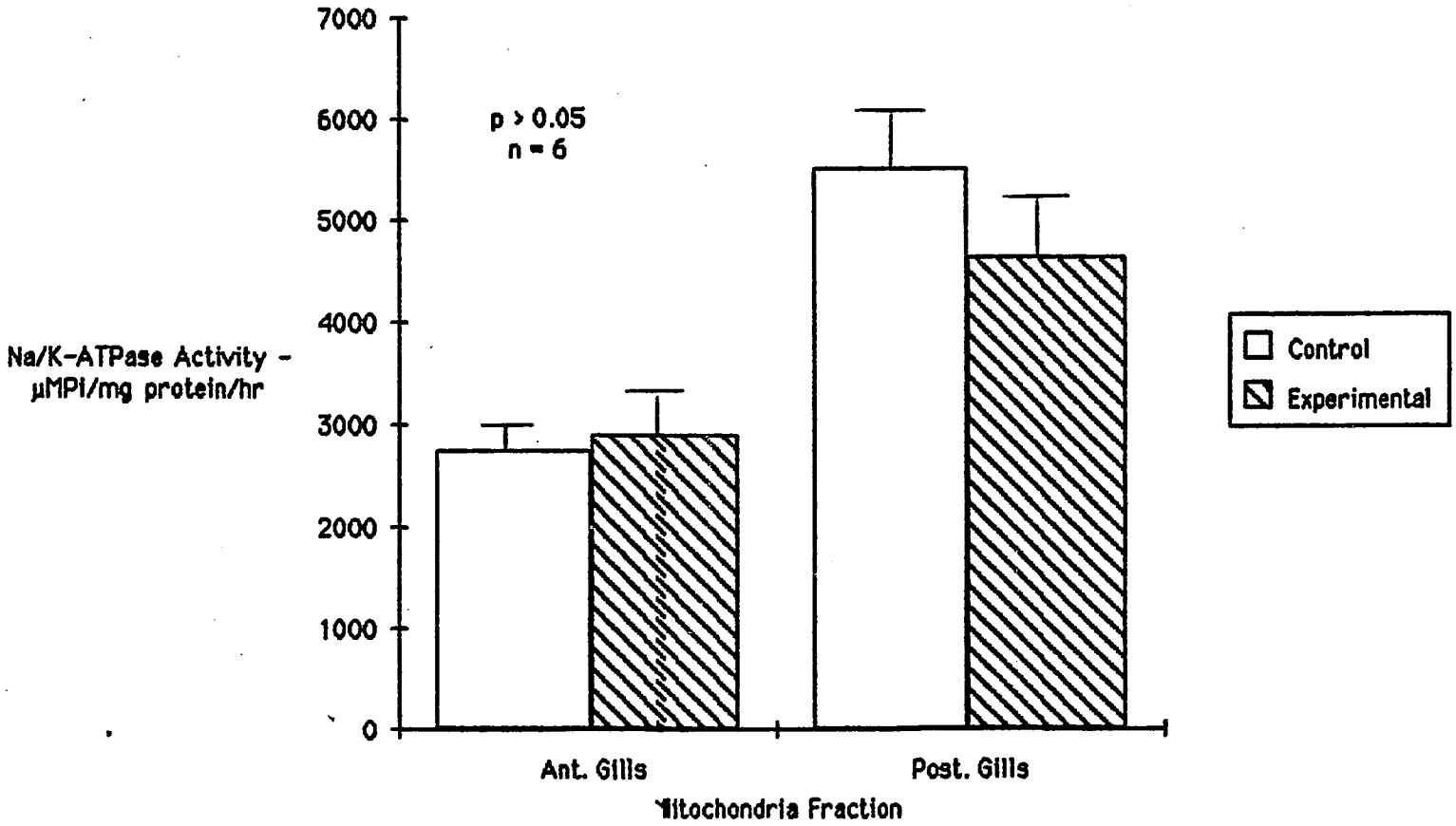

7000
6000
5000
4000
3000
2000
1000
0
p > 0.05
n = 6
Na/K-ATPase Activity -
µMPi/mg protein/hr
Control
Experimental
Ant. Gills
Post. Gills
Mitochondria Fraction

Figure 12 - The effects of 10^{-5} M dibutyryl cAMP on Na/K-ATPase activity in the microsomal fraction in isolated anterior and posterior gills of Carcinus maenas. Enzyme activity is given in uM Pi/mg protein/hour. Gills 1-5 represent the anterior gills and gills 6-8 represent the posterior gills.

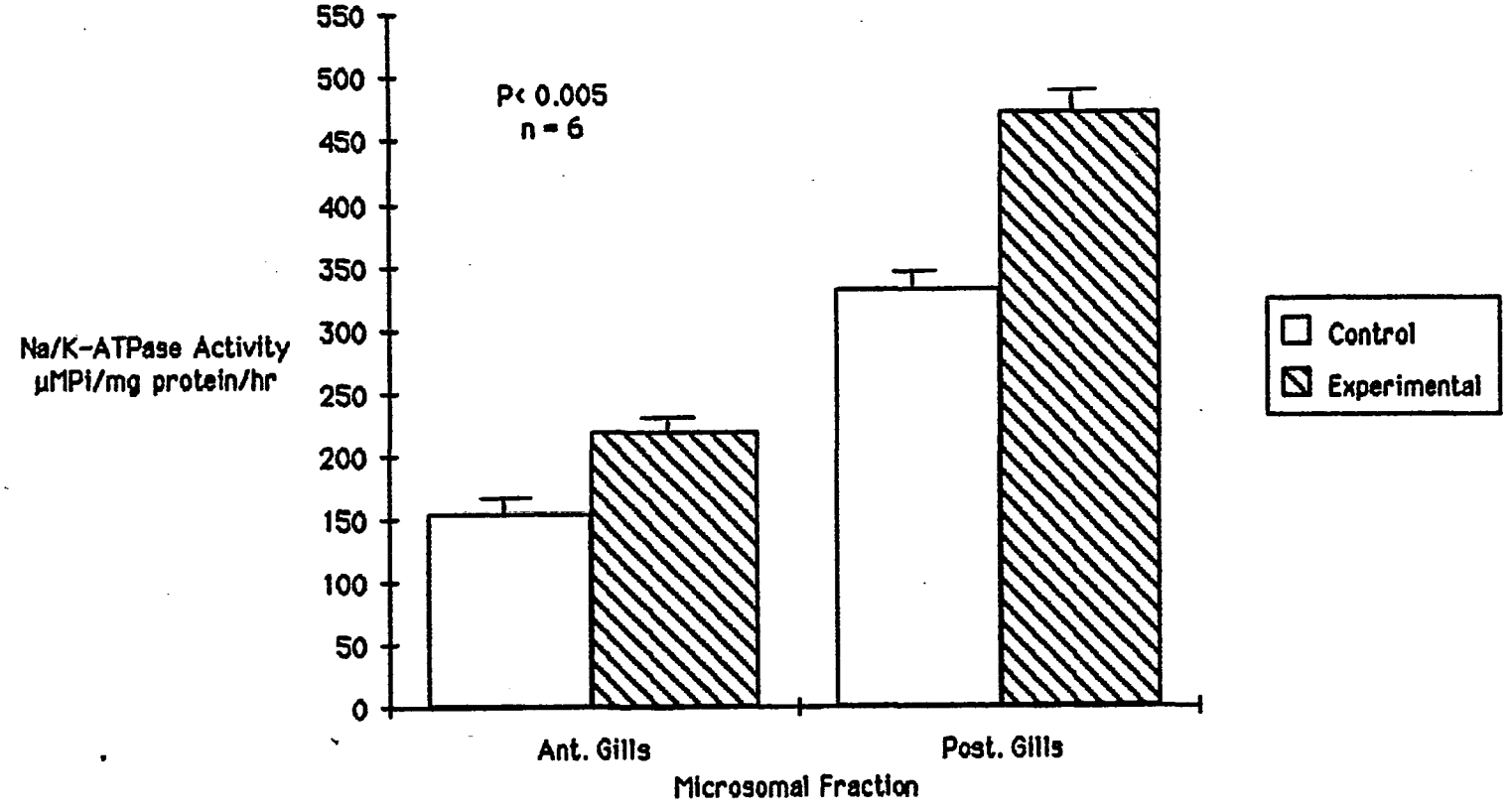

P< 0.005
n = 6
Na/K-ATPase Activity
µMPi/mg protein/hr
550
500
450
400
350
300
250
200
150
100
50
0
Ant. Gills
Post. Gills
Microsomal Fraction
Control
Experimental

Figure 13 - The effects of 10^{-5} M dibutyryl cAMP in the mitochondrial fraction of isolated anterior and posterior gills. Gills 1-5 represent the anterior gills and gills 6-8 represent the posterior gills in this study. Enzyme activities are given as uM Pi/mg protein/hour.

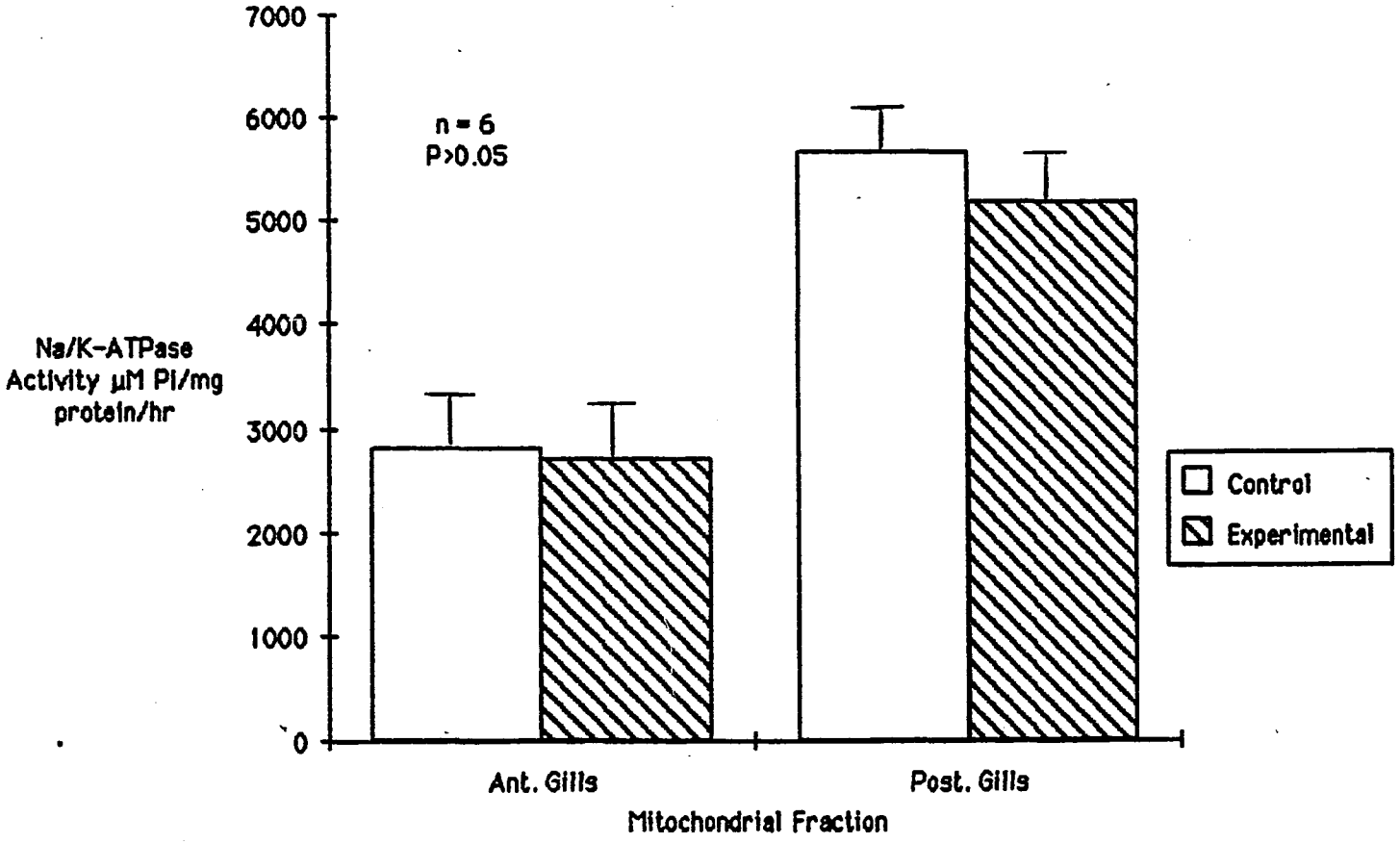

7000
6000
5000
4000
3000
2000
1000
0
n = 6
P>0.05
Na/K-ATPase
Activity µM Pi/mg
protein/hr
Control
Experimental
Ant. Gills
Post. Gills
Mitochondrial Fraction

Figure 14 - The effects of heated pericardial organ extracts on Na/K-ATPase activity in microsomal fraction of the anterior and posterior gills of C. maenas. Enzyme activity is given as uM Pi/mg protein/hour. Gills 1-5 represent the anterior gills and gills 6-8 represent the posterior gills in this study.

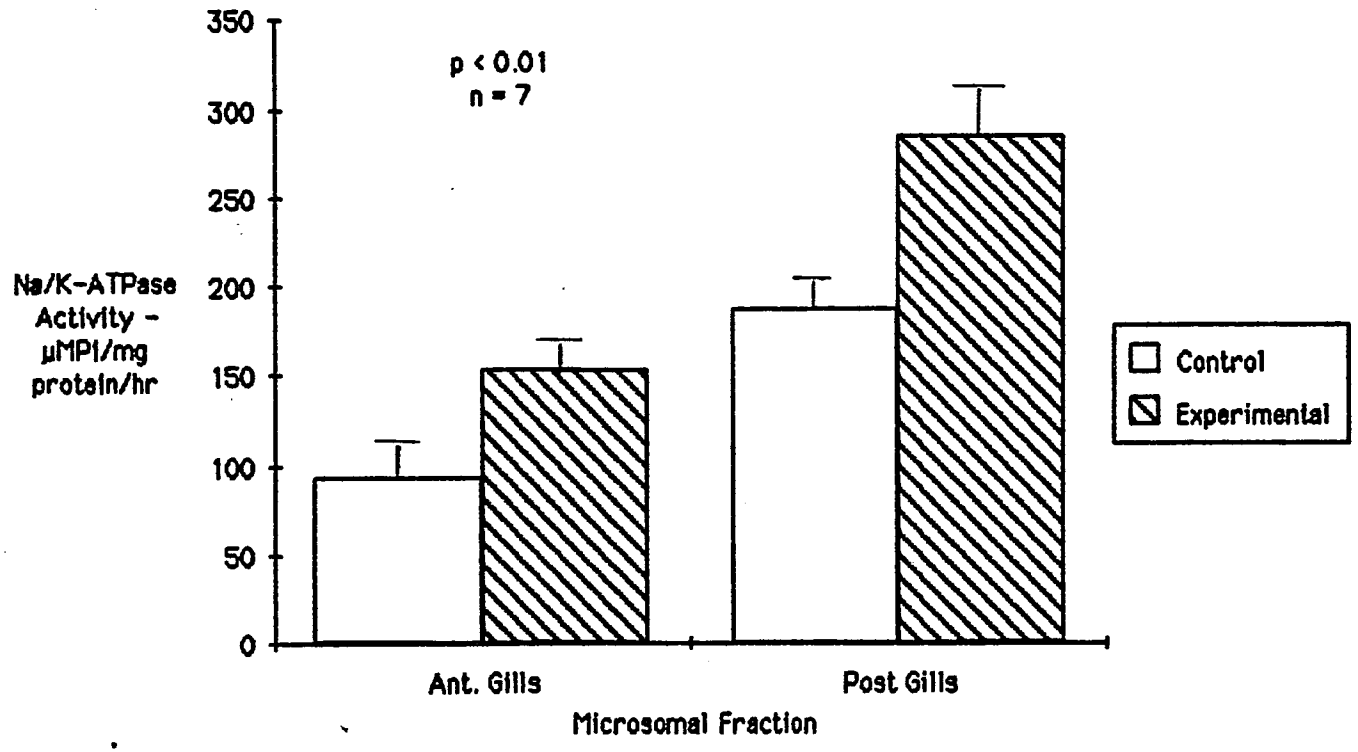

p < 0.01
n = 7
Na/K-ATPase Activity - µMPI/mg protein/hr
350
300
250
200
150
100
50
0
Ant. Gills
Post Gills
Microsomal Fraction
Control
Experimental

Figure 15 - The effects of heated pericardial organ extract on Na/K-ATPase activity in the mitochondrial fraction of anterior and posterior gills of C. maenas. Gills 1-5 represent the anterior gills and gills 6-8 represent the posterior gills. Enzyme activities are given in uM Pi/mg protein/hour.

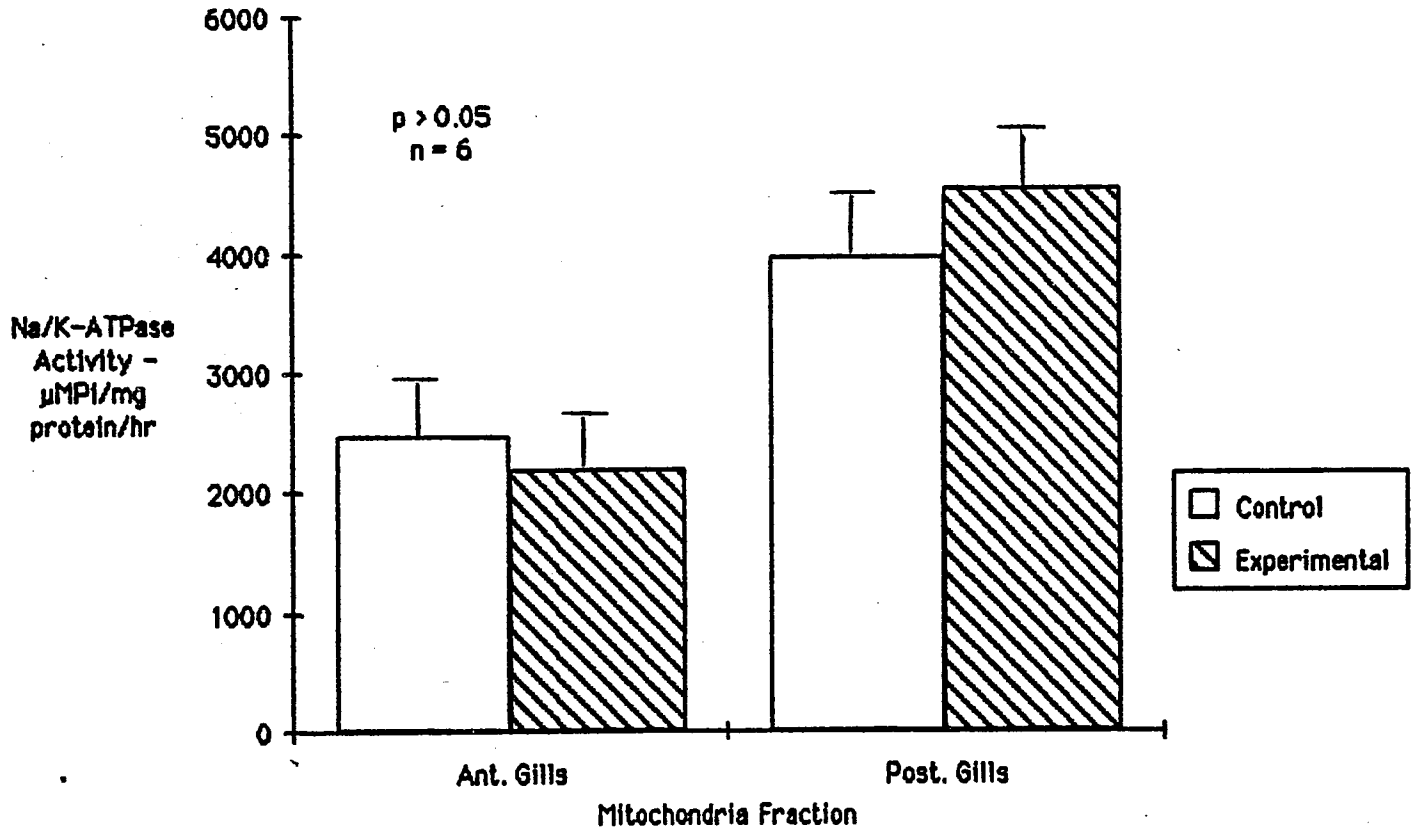

6000
5000
4000
3000
2000
1000
0
p > 0.05
n = 6
Na/K-ATPase Activity - µMPi/mg protein/hr
Control
Experimental
Ant. Gills
Post. Gills
Mitochondria Fraction

Figure 16 - The effects of 10^{-5} M dopamine on cAMP concentrations in the anterior and posterior gills of C. maenas. Gills 3-5 represent the anterior gills and gills 6-7 represent the posterior gills. Cyclic AMP concentrations are given in pmol/mg protein.

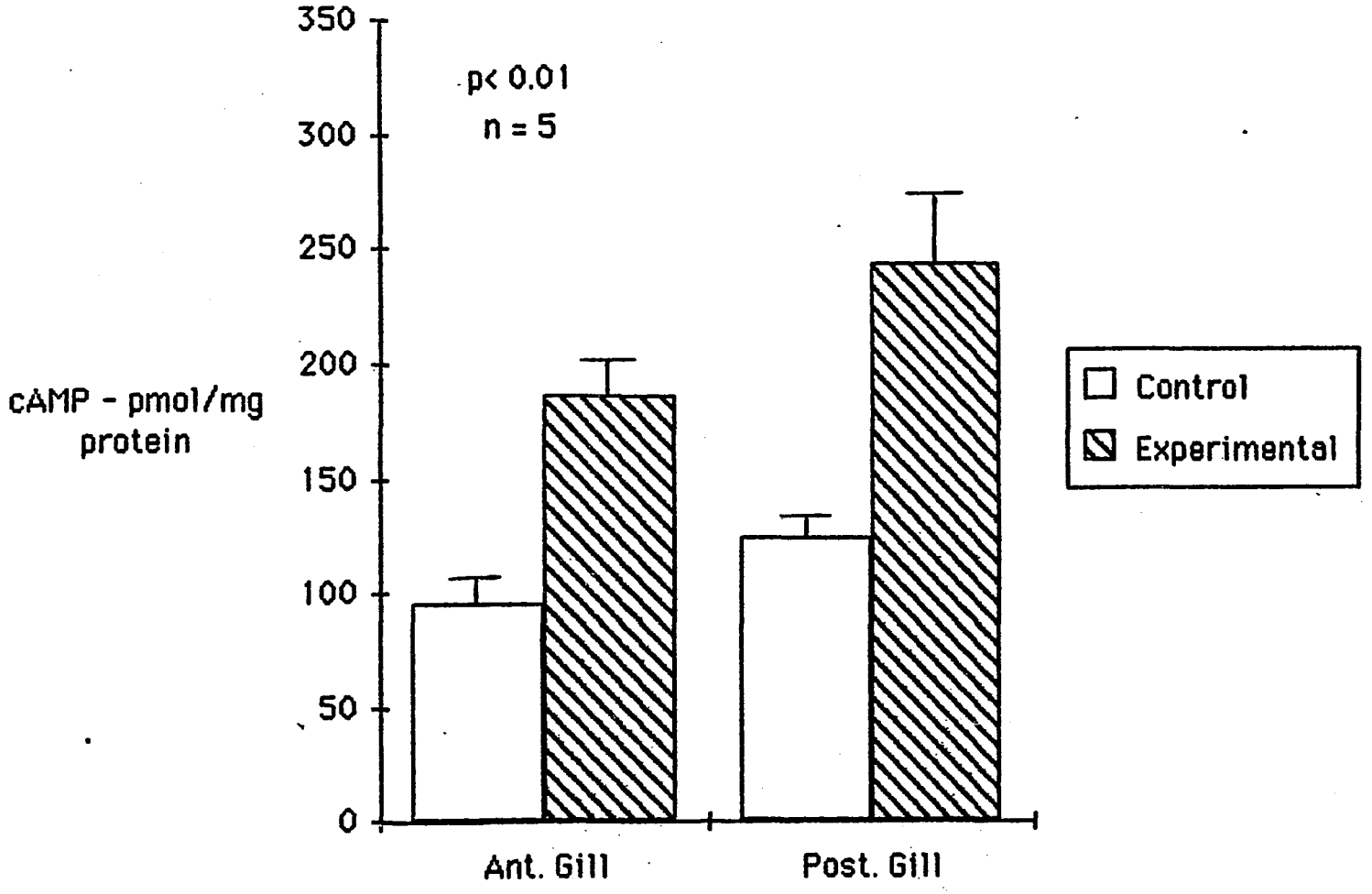

350
300
250
200
150
100
50
0
p< 0.01
n = 5
cAMP - pmol/mg protein
Control
Experimental
Ant. Gill
Post. Gill

Figure 17 - The effects of 10^{-5} M octopamine on cAMP levels in the anterior and posterior gills of Carcinus maenas. Gills 3-5 and 6-7 represent the anterior and posterior gills respectively. Cyclic AMP concentrations are given in pmol./mg protein.

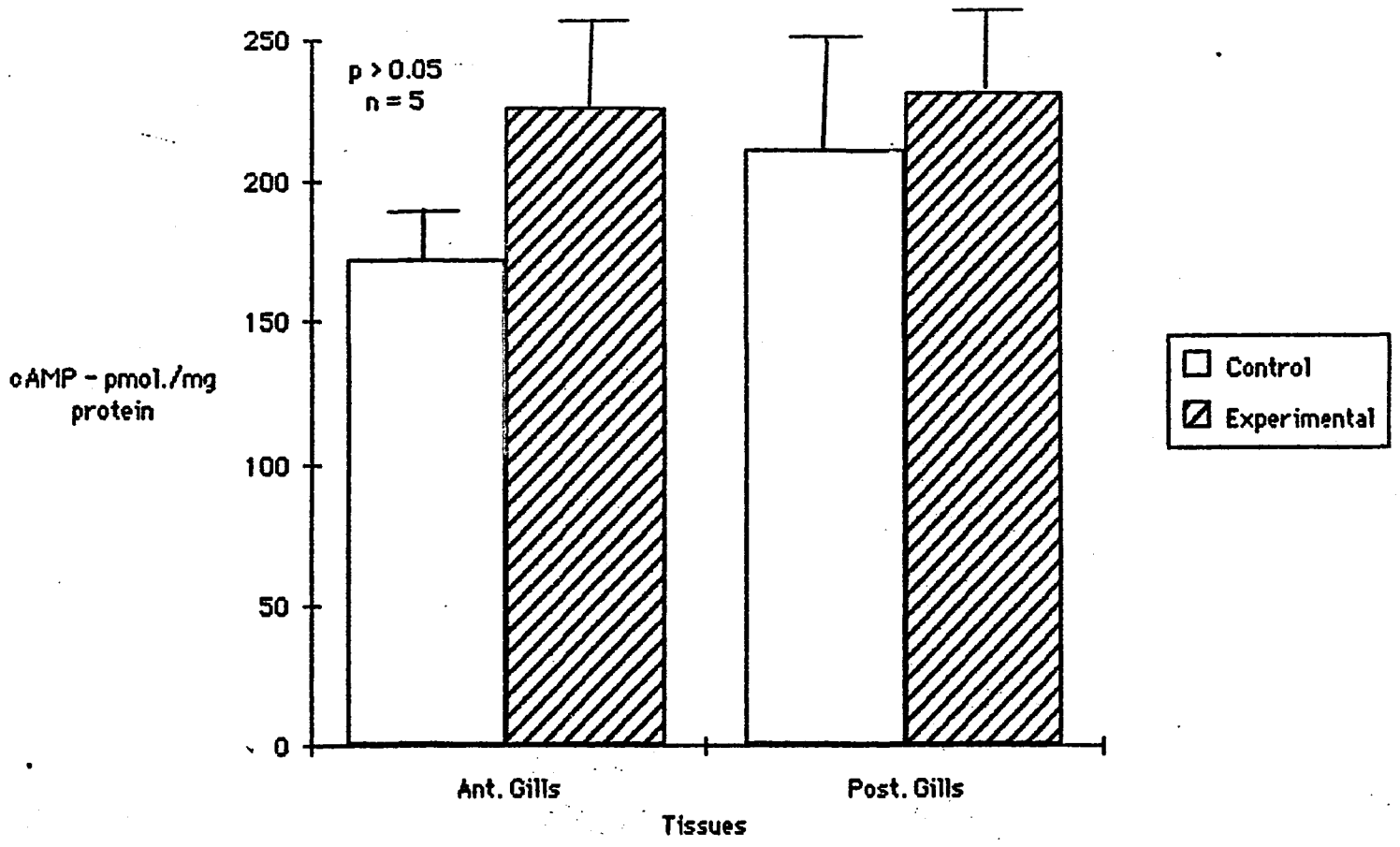

250
200
150
100
50
0
p > 0.05
n = 5
cAMP - pmol./mg protein
Ant. Gills
Post. Gills
Tissues
Control
Experimental

Figure 18 - The effects of long term acclimation on cAMP levels in the anterior and posterior gills of Carcinus maenas. Gills 3-5 and 6-7 represent anterior and posterior gills respectively. Cyclic AMP concentrations are given in pmol.cAMP/mg protein. Differences between cAMP concentrations in the gills of 100% SW acclimated animals and 40% SW acclimated animals are not statistically significant ($p>0.05$).

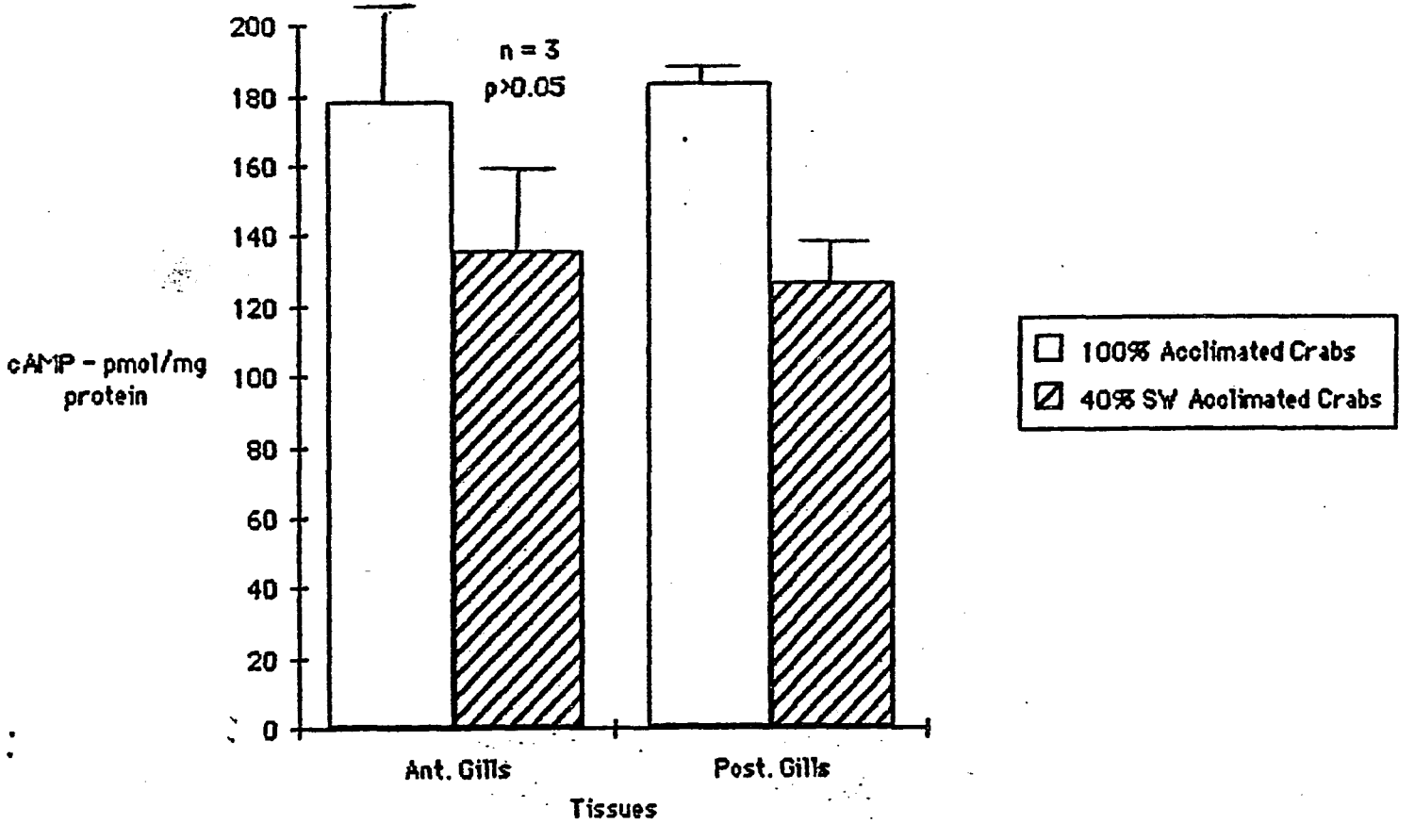
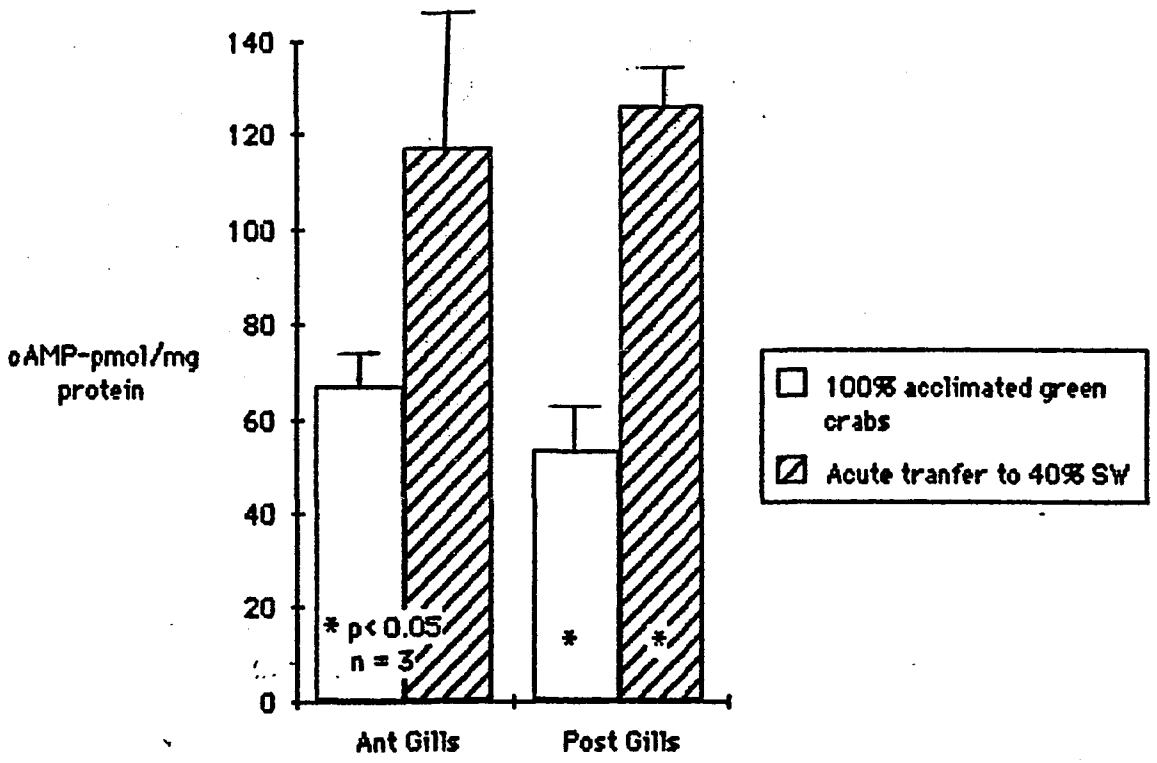

200
180
160
140
120
100
80
60
40
20
0
n = 3
p>0.05
cAMP - pmol/mg protein
Ant. Gills
Post. Gills
Tissues
100% Acclimated Crabs
40% SW Acclimated Crabs

Figure 19 - Effects of acute transfer from 100% SW to 40% SW for 24 hours on cAMP concentrations in the anterior and posterior gills of green crabs Carcinus maenas. Gills 3-5 and 6-7 represent anterior and posterior gills respectively. Data is given as pmol cAMP/mg protein. Differences between cAMP concentrations in the posterior gills of 100% SW acclimated crabs and green crabs acutely transfered to 40% SW for 24 hours are statistically significant ($p<0.05$). Differences between anterior gills of both groups are not statistically significant.


cAMP-pmol/mg protein
140
120
100
80
60
40
20
0
* p< 0.05
n = 3
*
*
Ant Gills
Post Gills
100% acclimated green crabs
Acute tranfer to 40% SW

Discussion:

Effects of Acute Transfer from 100% SW to 40% SW on Osmolality of the Hemolymph.

The data in Figure 5 indicate that the osmolality of the hemolymph of Carcinus maenas reaches a new stable value within 24 hours after acute transfer to a dilute medium. At this point the animals are effectively hyperregulating the osmolality of their internal fluids above that of their medium. In order to accomplish this rapid equilibration, mechanisms must operate to help the animal hyperregulate shortly after transfer to dilute salinities. One of these mechanisms appears to include active uptake of sodium ions, primarily across the gill epithelium (Mantel, 1967; Schoffeniels and Gilles, 1970; Smith and Linton, 1970; Engel et al., 1974). The membrane-localized enzyme Na/K-dependent adenosine triphosphatase (Na/K-ATPase), is believed to play an essential part in active exchange of Na for K across the plasma membrane of many animals (Skou, 1965; Glynn and Karlish, 1975; Siebers et al., 1982). The ability of C. maenas to hyperregulate effectively after acute transfer to dilute salinities lends good support for rapid activation of transport processes in gills, particularly those of Na/K-ATPase. This becomes especially important since short term decreases in sodium permeability do not occur in Carcinus maenas when the external medium is diluted acutely (Shaw, 1961). My study suggests that uptake of sodium must occur if a constant blood osmolality is to be maintained in the face of dilution of the medium.

Effects of Dopamine and Dibutyryl cAMP on Uptake of Sodium 22 In Vivo.

Kamemoto and Oyama (in press) have indicated that dopamine is able to stimulate sodium uptake in the isolated gill of Callinectes sapidus. Lohrmann and Kamemoto (personal communication) were able to show that dbcAMP increased sodium uptake in the isolated gills of this crab, thus mimicking the effects of dopamine. My study was undertaken to see if similar results could be obtained in vivo in Carcinus maenas. My experiments indicate that injection of either 10^{-5} M dopamine or 10^{-6} M dbcAMP causes a substantial increase in ^{22}Na uptake. My results further indicate that, at least in Carcinus , the effects of dopamine or dbcAMP are long lasting. Sodium uptake is not diminished 6 hours after injection. This observation is consistent with activation of protein kinases which in turn may activate Na/K-ATPase through subsequent phosphorylation (Figure 20).

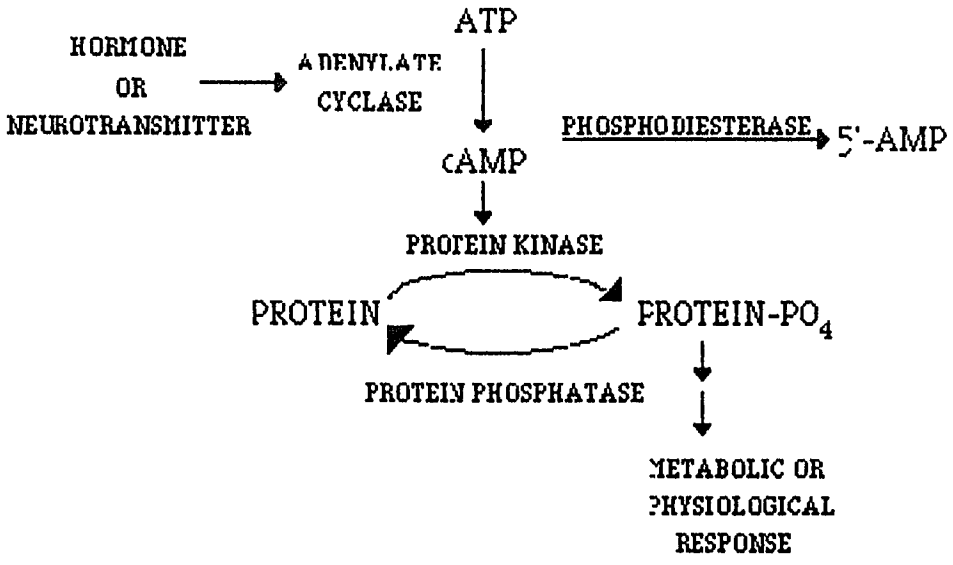


Figure 20 - Schematic diagram of the proposed role of protein kinase in mediating the biological effects of those hormones, neuromodulators, and neurotransmitters acting through cAMP (Greengard, 1978).

Studies carried out in other marine animals are in agreement with my results. Dietz and Scheide (1982) were able to induce an increase in sodium influx in the freshwater mussels Ligumia subrostrata and Carunculina texasensis 30 minutes after injection of dopamine or dibutyryl cAMP into the blood of these bivalves. In elasmobranchs, the secretion rate of sodium chloride in the rectal gland has been shown to be greatly increased by the addition of either cAMP or theophylline, a cyclic nucleotide phosphodiesterase antagonist (Silva et al., 1977). In addition, Shuttleworth and Thompson (1978) have stated that treatment of elasmobranch rectal glands with dbcAMP for 2 hours caused an increase in ouabain sensitive oxygen consumption and ouabain binding. These results correlate well with the results obtained in this study, and I suggest that this sodium uptake is mediated through the enzyme Na/K-ATPase.

At time 1, 2 and 3 hours the rate constants are higher for crabs injected with dopamine or dbcAMP compared to those crabs injected with Carcinus Ringer's alone. What is particularly interesting, however, is that the rate constants within the control or experimental groups increase at each time interval during the 3 hour sampling period. This is attributed to handling and sampling of blood from the animals which could induce the neuroendocrine system to release small amounts of hormones thus elevating sodium uptake slightly at each time interval. This type of response is not uncommon. Spindler et al. (1976) in a study of hyperglycemic hormone effects, noted a distinct increase in hemolymph glucose levels in intact

Orconectes controls. They attribute this to a stress effect caused by injection of saline and repetitive sampling of the blood. Hanaoka and Takahashi (1977) observed a major rise in the levels of hemolymph trehalose in water injected Periplaneta controls. This may be due to release of hyperglycemic hormone during injection.

The Effects of Pericardial Organ Extract on Sodium Uptake in Vivo.

Previous studies have indicated that dopamine is contained in the pericardial organ (Cooke and Goldstone, 1970). My study was undertaken to determine if this neuroendocrine organ has any effect on sodium uptake in Carcinus. My results suggest that pericardial extracts heated for 2 minutes in a boiling water bath increase sodium uptake in C. maenas, with the increases becoming prominent at 2 and 3 hours. The response of sodium uptake to this crude extract is more variable than the results obtained with dopamine or dbcAMP. The variable nature of the response to pericardial organ extracts is not surprising, since this organ contains 5 known components; which include proctolin, serotonin, octopamine, and dopamine (Cooke and Goldstone, 1970). It is not known what the mixed effect of all these substances may be on sodium 22 uptake. In support of my data are the results of Kamemoto and Oyama (in press) which show that pericardial organ extracts heated in a similar fashion caused increased uptake of sodium in isolated gill of C. sapidus. It should be noted, however, that the method employed in preparation of the pericardial organ extract inactivates dopamine. It is possible that some other factor(s) is (are) present in the

pericardial organ which may stimulate sodium uptake in Carcinus. Dietz et al. (1982) state that, in addition to dopamine, noradrenalin, adrenalin, and serotonin stimulate unidirectional influx and net flux of sodium in freshwater mussels. Clearly, further work on the effects of various catecholamines and serotonin on sodium uptake needs to be done since these substances are present within the neuroendocrine systems.

Effects of Dopamine and Dibutyryl cAMP on Na/K-ATPase Activity in Vivo.

In general, my results suggest that short term activation of Na/K-ATPase activity occurs in the microsomal fraction and is likely to be brought about by dopamine, a factor released by the pericardial organ. Further, the effect of dopamine appears to be mediated by the secondary messenger cAMP. These conclusions are derived from several lines of evidence from my study: 1. dopamine at a concentration of 10^{-5}M is able to increase uptake of ^{22}Na in vivo in intact adult green crabs; 2. Dibutyryl cAMP at a concentration of 10^{-6} M is able to increase uptake of ^{22}Na in vivo thus mimicking the effects of dopamine and providing a link between the first hormone (dopamine) and the secondary messenger cAMP; 3. Dopamine at a concentration of 10^{-5}M is able to increase Na/K-ATPase activity in the gills of C. maenas; 4. cAMP also causes increased Na/K- ATPase activity, again mimicking the effects of dopamine; and 5. Dopamine actually increases levels of cAMP in gill tissue (Sutherland, 1972).

Rapid activation of Na/K-ATPase has been controversial. Towle et al. (1976) have shown that rapid modulation of this enzyme occurs in the blue

crab, Callinectes sapidus. They found that Na/K-ATPase activity in the microsomal fraction increases 2-3 hours after transfer of the crab from 30 ppt to 5 ppt salinity. In addition, Towle has shown that rapid modulation of this enzyme also occurs in the killifish, Fundulus heteroclitus, and the euryhaline clam, Rangia cuneata (Towle et al., 1976; Towle et al., 1977; Saintsing and Towle, 1978). Neufeld et al. (1980) also studied rates of activation of Na/K-ATPase in C. sapidus and their results are not in agreement with those of Towle (1976). Neufeld et al. (1980) states that activation requires 1-2 weeks for completion, a value much more consistent with synthesis of new enzyme. The results I have obtained are in agreement with those of Towle.

My results suggest that rapid enzyme modulation in C. maenas occurs in the microsomal fraction of gill homogenate. The discrepancy between Towle and Neufeld is explainable in terms of the gill fractions examined. Towle looked at the microsomal fraction specifically, which is known to contain membrane fragments. Neufeld et al., (1980) looked at a crude homogenate which includes both microsomal and mitochondrial fractions. My studies examined both fractions separately. I have found that, at least for Carcinus, it is necessary to separate the fractions in order to determine long and short term activation of Na/K-ATPase. This is so because the larger and extremely variable enzyme activity of the mitochondria would tend to mask any differences that potentially exist in the microsomal fraction. Results that I have obtained indicate that the mitochondrial fraction contains approximately 10 times more activity than the microsomal fraction. These

results do not correlate perfectly with those of Pequeux et al, (1984). They indicate higher activities in the mitochondrial fraction, but this is only about 2 times higher than the microsomal fraction. These differences may be related to the manner of preparation of the various fractions.

Support of short term activation of Na/K-ATPase has been given by Pequeux et al., (1984). They have shown short term activation of Na/K-ATPase in a membrane fraction of both Eriocheir sinensis and Carcinus maenas and state these results agree in general with those results obtained by Towle et al., (1976). Results of Savage and Robinson (1983) lend further support for short term neuroendocrine activation of Na/K-ATPase in C. sapidus. Blue crabs acclimated to 100% SW, but given injections of serum from animals exposed to 30% SW, show higher Na/K-ATPase activities than are observed in crabs acclimated to 100% SW and given serum from 100% SW acclimated crabs. These results are also correlated with an increase in sodium uptake. In unpublished results, Lohrmann and Kamemoto showed an increase in uptake of ^{22}Na in isolated gills of Callinectes sapidus after treatment with hemolymph from blue crabs acclimated to 25% SW. Both these effects have been shown to occur 20 to 30 minutes after treatment with the blood extract. They state their results suggest the presence of factors in the circulation which affect salt movement through the gills.

Sabourin and Saintsing (1980) have investigated the time course of low salinity induced changes in gill Na/K-ATPase activity in the hermit crab, Clibanarius vittatus. Their results showed that increases in the microsomal

fraction occur 3 hours after transfer to dilute medium and thus are in agreement with Towle et al. (1976). Studies by Holliday (1985) also indicated rapid modulation of Na/K-ATPase occurs in the crab Uca pugnax. His results show, however, that this increase in enzyme activity occurred 24 hours after transfer.

Mantel and Farmer (1983) indicate that short term exposure to more dilute media results in activation or elaboration of sodium carrier sites, reflected by an increase in J max. J max in this instance is analogous to V max used in enzyme kinetic studies. The maximal rate in which the enzyme converts the substrate to product is called V max and is attained when the enzyme sites are saturated with substrate. Sutcliffe (1968) found that J max in Gammarus zaddachi exposed to low salinity (0.3 mM NaCl) was approximately three times that of individuals acclimated to 10 mM NaCl. Shaw (1959) showed, that in Austropotamobius pallipes, J max for sodium increased in a similar manner, except that no change in carrier affinity was noted. These observations are consistent with activation of Na/K-ATPase. Short term increases in J max would preclude an increase in the amount of active Na/K-ATPase. In addition, Mantel and Farmer (1983) indicate that isolated gills show increased metabolic rates in animals that are hyperregulating, compared with animals which are isosomotic to the medium. They further state that metabolic rates are especially elevated when the animal has been acutely stressed by low salinity. They also state it is likely that a part of this increase is due to activity of Na/K-ATPase.

Rapid activation of Na/K-ATPase has also been shown to occur in

vertebrates. Mernissi and Doucet (1984) were able to increase Na/K-ATPase activity significantly in the medullary loop of Henle and the distal convoluted tubule of the kidney in adrenalectomized rabbits within 3 hours after administration of dexamethasone. Their studies further show that dexamethasone has a two pronged effect: First, it quickly activates the existing enzyme units, and later it induces the synthesis of new catalytic units.

This mechanism is also consistent with the data available on crustacean gills. There appears to be short term activation of Na/K-ATPase, which is superimposed upon a long term mechanism, resulting in dramatic structural changes in the gill epithelia. These long term changes probably involve an increase in the number of Na/K-ATPase catalytic units. Barra et al. (1983) have shown that gills of crabs acclimated to dilute medium for several weeks are characterized by a complex and well-developed deep laterobasal folds which come in close contact with the membrane of mitochondria. The mitochondria become very abundant, eventually completely filling the cytoplasmic spaces that surround these folds. Such extensive elaboration of the plasma membrane in the form of tubules, crypts, infoldings and interdigitations opening to the extracellular space and in close association with mitochondria seems to be common to all the "salt transporting tissues" including the salt gland of turtles, the anal papillae of insect larvae, elasmobranch rectal glands and mammalian kidney tubules (Barra et al., 1983). Presumably the large number of mitochondria provide energy for the operation of the Na/K-pump in the microsomal fraction of the

gill.

Cyclic nucleotides have also been shown to affect Na/K-ATPase in other marine animals. The secondary messenger cAMP and the phosphodiesterase inhibitor theophylline are able to stimulate an increase in ouabain binding and ouabain-sensitive oxygen consumption in the rectal gland of the elasmobranchs Scyliorhinus canicula and Squalus acanthias (Shuttleworth and Thompson, 1978; Shuttleworth, 1983). Silva et al. (1983) showed that isolated rectal gland from the elasmobranch Squalus acanthias perfused with cAMP and theophylline together was able to increase ouabain-sensitive oxygen consumption and ouabain binding sites. Ouabain is a cardiac glycoside that specifically inhibits Na/K-ATPase. Thus it appears that cAMP can increase Na/K-ATPase activity in rectal glands of elasmobranchs, and this seems to correlate well with my results which show substantial increases in Na/K-ATPase activity obtained in crab gills after exposure to dbcAMP.

The Effects of Pericardial Organ Extract on Na/K-ATPase Activity in Vivo

Extracts of heated pericardial organs (PO) injected in vivo are able to increase Na/K-ATPase activity. These results correlate well with my studies on sodium uptake after injection of heated PO and those of Kamemoto and Oyama which show that heated pericardial extracts are able to increase sodium uptake in the isolated gill of Callinectes sapidus. Futher studies by Kamemoto and Oyama suggest that PO is able to elevate levels of cAMP in the gills of the blue crab, C. sapidus. This correlates well with my work with Carcinus that shows increases in Na/K-ATPase activity after treatment with

dbcAMP.

The In Vivo Effects of Dopamine on Levels of cAMP in the Gills of Carcinus maenas.

Studies that I have done suggest that dopamine is able to increase levels of cAMP in the anterior and posterior gills of C. maenas in vivo. This lends further support for the effects of dopamine on salt balance via the secondary messenger cAMP. Kamemoto and Oyama (in press) have also shown that isolated posterior gills of Callinectes sapidus perfused with dopamine show significantly increased levels of cAMP 10 minutes after injection of dopamine. My results show that these higher cAMP concentrations remain elevated up to 3 hours after injection of dopamine in C. maenas. This suggests a rather slow acting phosphodiesterase enzyme. Phosphodiesterase functions to convert 3':5' cAMP to the inactive form 5' AMP. This is in contrast to phosphodiesterase enzyme in barnacle muscle, which has been shown to be rapidly activated when increases in cAMP occur (Nwoga and Bittar, 1983). My results further show that cyclic AMP levels are slightly higher in the posterior gill than in the anterior gill although these differences are not statistically significant ($p > 0.05$).

Studies by Scheide and Dietz (1983) support my results. They state that injection of dopamine into the freshwater mussel Ligumia subrostrata was able to increase adenylate cyclase activity in gill homogenate. Adenylate cyclase is the enzyme responsible for the production of cAMP and

increased activity in this enzyme is well correlated with increased concentrations of cAMP.

Work done on insects in relation to cAMP is extensive and provides further support for my results on Carcinus. For example, dopamine has been found to increase cAMP levels in the thoracic ganglia, the corpus cardiacum and the brain of Periplaneta americana (Nathanson and Greengard, 1973; Harmar and Horn, 1977). Cyclic AMP levels in insects and other animals are much lower than in the gills of euryhaline crustaceans. Bodnaryk (1983) has stated that levels of cAMP in insect tissue fall in the range of 1 to 40 pmoles per mg protein. For example, the concentration in the brain of Leucophaea maderae was found to range from 12.6 to16.8 pmoles cAMP per mg protein. In the accessory gland of adult Mantis religiosa cAMP levels were 7.3 pmoles per mg protein and in the gonads of Leucophaea maderae, levels of cAMP were 9.8 and 8.4 pmole cAMP per mg protein for male and female respectively. In addition, Bodnaryk (1983) states that the concentration of cAMP is comparable to those found in typical vertebrate tissues. However, these values are considerably less than those concentrations indicated by my data in gill tissue of C. maenas. In fact cAMP concentrations for control green crabs are almost ten times more concentrated then those levels found in various insect tissues. Kamemoto and Oyama (in press) found concentrations of cAMP in the posterior gill of Callinectes sapidus that are in the same range as the results for C. maenas. Their results show that cAMP levels in control animals range from 116.5 to 297.5 pmoles per mg protein. My results by comparison are 96.1 pmoles

per mg protein for anterior gills 3, 4, and 5 and 124.6 pmoles per mg protein for posterior gills 6, and 7.

Effects of Octopamine on cAMP Levels in the Anterior and Posterior Gills.

Kamemoto and Oyama (in press) state that 10^{-5}M octopamine is able to increase levels of cAMP in the isolated gills of Callinectes sapidus 30 minutes after treatment. My studies indicate that octopamine elevates cAMP levels in the gills of intact green crabs slightly, 30 minutes after injection. However these increases are not statistically significant. The differences in results could be related to the period of time allowed between injection of octopamine and removal of the gills. Kamemoto and Oyama (in press) allowed 20 minutes before assaying for cAMP. In my studies with dopamine, I allowed a 3 hour time period between in vivo injection of dopamine and excision of the gills. It may be that more time is required for octopamine to cause larger increases of cAMP in C. maenas. Clearly more work needs to be done in order to further elucidate the effects of octopamine on cAMP levels in the gills of C. maenas.

Effects of acclimation to 40% SW on cAMP levels in the anterior and posterior gills.

This study was designed to see if long term acclimation to 40% SW would affect cAMP levels in the anterior and posterior gills of C. maenas. I thought that since 40% SW acclimated green crabs are hyperregulating effectively at this point, changes in cAMP levels should occur. The results show that concentrations of this nucleotide decreased in both anterior and

posterior gills compared to crabs acclimated to 100% SW. However, it should be noted that these differences are not statistically significant. These results seem reasonable since short term osmoregulatory mechanisms would have already been activated and long term mechanisms would have developed as well. Maintenance of high cAMP levels in the gills would then be counter productive and cAMP levels must then be lowered by increased phosphodiesterase activity.

Effects of acute transfer to 40% SW from 100% SW on cAMP levels in the anterior and posterior gills.

If mechanisms involved in active uptake of sodium from the medium can be activated rapidly by dopamine or cAMP in C. maenas, then it would seem reasonable to expect that acute transfer to 40% SW would be able to raise cAMP concentrations in the gills of this crab in a similar manner. My studies show that transfer of C. maenas to 40% SW for 24 hours resulted in increased levels of cAMP in both anterior and posterior gills of this crab. Although many osmoregulatory mechanisms could be activated by this acute transfer, it is possible that one of these mechanisms would be active uptake of sodium from the medium. The results I have obtained suggest this to be true since elevated concentrations of cAMP in the gills have been correlated with increases in sodium uptake and increased activity of Na/K-ATPase. Further evidence given by Holliday (1985) indicates that a significant increases in Na/K-ATPase activity occurs in the gills of Uca pugnax 24 hours after acute transfer to dilute medium. This time course correlates well with my results in which elevated levels of cAMP were found in the anterior and

posterior gills of C. maenas 24 hours after acute transfer from 100% SW to 40% SW. The long time involved in activation of sodium uptake mechanisms is not unexpected since I do not know what may trigger the release of neuroendocrine factors related to this process. It may be that the osmolality of the blood must decrease to certain level after transfer to a dilute medium before dopamine is released from the pericardial organ. It could also be hypothesized that it takes time for enough dopamine to build up in the blood before an effect is detected.

Studies performed thus far lend good support for the regulation of salt balance by the pericardial organ, first by the action of cardioexcitor hormones (Hume and Berlind, 1976) which increase the flow of blood through the gills, and secondly by increasing the movement of sodium into the gills.

Mechanistically, the results obtained are consistent with existing models on transport of sodium in the gills. Apical transfer of sodium is believed to be passive, through amilioride sensitive membrane channels. This movement is favored by both electrical and diffusive gradients (Kirschner, 1983). Epithelial cell membranes are polarized, being positively charged outside and negatively charged inside. This allows for electrical attraction of sodium from the medium to the inside of the cell. Second, the intracellular concentration of sodium is much less than that of the medium and this favors the transfer of sodium down a chemical gradient. Once inside the cell, movement of sodium into the hemolymph is active, mediated by Na/K-ATPase enzyme. Kirschner (1983) has stated that there is no evidence for any sodium transport system in the basolateral membrane of

gill tissue with the exception of the Na/K-pump. Thus if sodium is to enter the hemolymph at an increased rate after injection of dopamine or cAMP, as my results show, this would necessitate an increase in Na/K-ATPase enzyme activity. Thus the results I have obtained are in agreement with accepted facts known about sodium movement across epithelial cells.

Activation of Na/K-ATPase by cAMP in crustacean gill may be similar to that of other epithelia. Diamond (1982) states that the apical and basolateral membrane of an epithelia cell may interact with each other in what is now called "transcellular cross-talk". This means that factors affecting the apical membrane may indirectly affect the basolateral membrane, and vice versa. Diamond (1982), using frog skin, found that inhibition of the basolateral pump resulted in decreased ionic permeabilty of both apical and basolateral membranes. It appears that stimulation of the basolateral Na/K-pump with CO_2 increases apical Na permeability, while inhibiting the pump by metabolic poisons such as ouabain reduces apical membrane permeability (Diamond, 1982). Changes in the apical membrane also appear to affect the basolateral membrane. Recent studies show that blocking the apical Na channels with amiloride decreases basolateral Na/K-exchange. In the leaky epithelium of the small intestine of Necturus, addition of alanine or glucose (Na transport partners) to the luminal surface increases apical sodium conductance, which in turn increases basolateral Na conductance.

Transcellular cross-talk has also been recently demonstrated in the

shark rectal gland by Shuttleworth (1983). He states that increases in ouabain-sensitive oxygen consumption and ouabain binding sites by cAMP is an indirect effect secondary to stimulation of sodium entry via a furosemide-sensitive, chloride-coupled transport system. He provides several lines of evidence for this conclusion. For example, all effects of cAMP on sodium pump activity are blocked by furosemide, or by the absence of extracellular chloride. Secondly, amphotericin B stimulates basolateral sodium pump activity in a similar way to cAMP, except that amphotericin-induced increases are not sensitive to furosemide. Amphotericin has its effect presumably by increasing passive sodium permeability. Thirdly, in the presence of verapamil, cAMP-stimulated, but not amphotericin-B stimulated, basolateral Na transport is inhibited. Thus stimulation of the basolateral sodium pump is a two step process: 1. the cAMP-mediated increase in sodium entry, and 2. the stimulation of sodium pump activity by the increased rate of apical Na entry (Shuttleworth, 1983).

It should be pointed out that the conclusions proposed by Shuttleworth (1983) are not in agreement with the results of Silva et al., (1983). They state that neither incubation of the rectal gland of Squalus acanthias with furosemide or bumetanide, nor exposure to an incubation medium low in sodium or chloride is able to alter ouabain binding produced by dibutyryl cAMP and theophylline. Therefore they conclude that changes in ouabain binding result directly from the cascade of cellular events initiated by cAMP and not from increased ion movement over the apical membrane. The question of whether crustacean gill Na/K-ATPase activity is stimulated by

cAMP through transcellular cross-talk or through direct stimulation has yet to be answered. Studies utilizing furosemide, amphotericin B, and bumetanide would greatly add to our understanding of first messenger-second messenger function.

Conclusions:

1. Acute transfer of the green crab Carcinus maenas to 40% SW indicates that blood osmolality of this animal stabilizes 24 hours after transfer. This lends good support for rapid activation of sodium transport mechanisms to reclaim sodium lost over permeable surfaces.

2. Injection of dopamine into intact green crabs Carcinus maenas results in increased uptake of ^{22}Na three hours after injection. This hormone may be responsible for short term activation of sodium uptake.

3. Injection of the dibutyryl derivative of cAMP results in increased uptake of ^{22}Na in vivo, thereby lending support for a first messenger-second messenger hormone system.

4. The effects of both dopamine and dibutyryl cAMP on sodium uptake are long lasting and this may be consistent with activation of a protein kinase which in turn would result in rapid activation of a sodium transport system.

5. The pericardial organ which has been shown to contain dopamine also increases sodium uptake in the green crab Carcinus maenas . This experiment suggests that extracts of the pericardial organ, which has been shown to contain dopamine, is a source of hormones.

6. Comparison of Na/K-ATPase activity in both microsomal and mitochondrial fractions of the gills indicates that the latter has far greater enzyme activity than the former.

7. Injection of dopamine results in rapid activation of Na/K-ATPase in the microsomal fraction of the gills of Carcinus maenas. This study suggests that the sodium uptake observed after injection of dopamine may be mediated by Na/K-ATPase activity in the gill. No significant change was noted in mitochondrial enzyme activity in any of the experiments performed.

8. Treatment of isolated gills of C. maenas with dibutyryl cAMP mimics the effects of dopamine by increasing Na/K-ATPase activity in the microsomal fraction.

9. Pericardial organs which have been shown to contain dopamine are able to induce an increase in Na/K-ATPase activity in the gills of C. maenas. However, it should be noted that the extract preparations used in this procedure inactivates dopamine. It is concluded that perhaps some other factor may also be involved in hydromineral balance.

10. Injection of dopamine into intact green crabs results in a significant rise in cAMP levels in the gill tissue. This conclusively links dopamine as a first messenger to the second messenger cAMP.

11. Slight increases in cAMP levels in the gills of C. maenas were noted 30 minutes after injection of octopamine. However, the differences were not significantly different from control values.

12. Long term acclimation to 40% SW resulted in a decrease in cAMP levels in the gills of the green crab Carcinus maenas when compared to 100% SW acclimated animals. It appears that short term mechanism have long been activated and the elevated concentrations of cAMP in the gills may have been reduced by the enzyme phosphodiesterase.

13. Acute transfer of green crabs to 40% SW for a period of 24 hours results in a significant rise in cAMP levels in the gill tissues. This lends further support for short term activation of sodium transport processes in the gills of Carcinus maenas.

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