AN ABSTRACT OF THE THESIS OF

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Title: OSMOTIC AND IONIC REGULATION IN THE DUNGENESS CRAB, CANCER MAGISTER (DANA)

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Osmotic and ionic regulation was studied in the Dungeness crab, Cancer magister, after acute exposure to a range of five salinities from 25% to 125% sea water (100% = 32‰). Blood, urine and muscle tissue were sampled at 24 and 48 hours after exposure. Analyses were made of blood and urine osmotic concentrations, as well as blood and urine sodium, potassium, calcium, and chloride ion concentrations. Data were also obtained for these ions and the total amino nitrogen (ninhydrin positive substances) in muscle tissue from crabs at the experimental salinities. Measurements of muscle extracellular space (interfiber water) and blood volume were determined with inulin-C^{14}.

In dilute sea water, blood is hyperosmotic to the medium but remains isosmotic at 100% sea water. Urine is slightly blood hypoosmotic over the salinity range. Hyperregulation is also demonstrated for the blood cations: sodium, potassium, and calcium.

Blood chloride, however, is weakly regulated and the blood:medium gradient is low. Urine cation concentrations are blood hypoionic in the salinities of 75% and 100% sea water compared to 25% and 50% sea water where they are isoionic. Urine chloride is isoionic in 75% and 100% sea water, but blood hyperionic in 25% and 50% sea water.

Increases in tissue water content as well as muscle extracellular (interfiber water) space occur when crabs are placed in dilute sea water. The inulin extracellular space is 7.3% in 100% sea water and this is lower than the chloride spaces (18%). Reductions in muscle ions concentrations and free amino nitrogen compounds occur. The blood:tissue gradient for potassium indicates intracellular regulation of this ion, while no regulation of sodium and chloride is found. Decreases in free amino nitrogen compounds in dilute sea water do not contribute significantly to decreases in cell volume through loss of osmotically active nonelectrolytes in the muscle.

In concentrated sea water (125%), there is some indication of blood hypoosmotic regulation while urine is regulated blood hypoosmotic. The pattern of blood sodium, potassium and chloride regulation suggests slight hypoionic regulation. Urine sodium and potassium are significantly blood hypoionic, while urine chloride is isoionic.

Muscle tissue in crabs at 125% sea water indicate decreases in

tissue water content, muscle sodium and muscle chloride in comparison to controls. Muscle potassium is regulated. Large increases in free amino nitrogen compounds are noted in muscle from crabs at this stress, and appear to indicate intracellular compensation for the cells to tolerate increased osmotic concentration.

Osmotic and Ionic Regulation in the Dungeness Crab, *Cancer magister* (Dana)

by

George Samuel Alspach, Jr.

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OSMOTIC AND IONIC REGULATION IN THE DUNGENESS CRAB, CANCER MAGISTER (DANA)

INTRODUCTION

Osmoregulatory patterns and mechanisms in decapod crustaceans have been extensively examined in an effort to understand the ability of many of these animals to tolerate salinity change in their natural habitats. Representatives of this order occupy a diversity of aquatic habitats varying in salinity from marine to fresh water. Certain species tolerate narrow ranges in salinity (Cancer pagurus, Maia, Cancer antennarius), while others are capable of withstanding much larger ranges in salinity (Eriocheir sinensis, Uca spp., Pachygrapsus crassipes). From numerous studies, it is apparent that a variety of patterns of osmotic and ionic regulation have evolved in decapods (see reviews by Krogh (1939), Prosser and Brown (1961), Lockwood (1962), Potts and Parry (1964), and Kinne (1964).

In osmoconformers, the osmotic concentration of the blood varies directly with that of the external medium. Several species of decapods employ this means of adjusting to environmental changes. Both Cancer antennarius (Jones, 1941) and Cancer pagurus (Krogh, 1939) for example remain isosmotic to the media throughout the salinity ranges tested.

Many species, however, show the capacity for osmoregulation,

i. e., they are capable of maintaining their internal osmotic concentration at levels different from those of their environment. This has a distinct homeostatic advantage where the species inhabits either brackish or concentrated sea water environments. In the former case, the blood osmotic concentration is maintained greater than the medium, i. e., hyperosmotic regulation. This type of response is exhibited by Uca, Pachygrapsus, and Hemigrapsus (Prosser, 1955; Gross, 1957, 1958, 1959; Dehnel, 1962, 1964, 1965). Some species inhabiting concentrated sea water employ hypoosmotic regulation, i. e., the maintenance of a blood osmotic pressure lower than the environment. Several decapods employ this means; e. g., Uca, Pachygrapsus, Gecarcinus, and Ocypode (Potts and Parry, 1964).

Osmoregulation involves control of the body fluid composition both in volume and concentration. A variety of means are available to accomplish this end. These include: (1) control of permeability of boundary membranes--integument, gill epithelium and gut lining; (2) transport of salt by active uptake between the medium and the body fluids; (3) excretion of salt and water, and (4) intracellular regulation of salt content, cell volume, or organic constituents.

Changes in the permeability of boundary membranes to water and salts can alter the body fluid composition and aid in the osmoregulatory process. Permeability studies of the integument of decapods indicate that strictly marine species are more permeable

to salt and water than are brackish water crabs (Gross, 1957). Recent investigations by Herreid (1969 a, b) have revealed that crab species exposed to the greatest osmotic stress have exoskeletons with low salt permeability. Gecarcinus, a land crab, has the least permeable "shell"; Ocypode and Uca pugilator, both intertidal crabs, show a greater water loss, but is less than that of Callinectes and Panopeus, which are strictly marine (Herreid, 1969 b). Further, Herreid (1969b) has attributed the water loss completely to the exoskeleton and not to arthropodal joint membranes. Other permeability studies show that the estuarine decapod, Carcinus, gains less weight with time in dilute sea water than do two marine forms, Maia and and Cancer pagurus (Huf, 1936). This suggests that Carcinus, a good hyperosmotic regulator, is less permeable than the two marine, osmoconforming crabs.

Active transport of salts from the surrounding water into the blood across body surfaces is another widely used mechanism of osmoregulation. By this method, ionic imbalance between the blood and the medium can be maintained. This ability to concentrate salt against a gradient was first shown in decapod crustaceans by Nagel (1934) working on Carcinus, and later elaborated in the same species by Shaw (1961). The list of decapods with this ability has been extended to other euryhaline forms: for example, Pachygrapsus (Gross, 1957; Prosser et al., 1955; Rudy, 1966), Ocypode

(Flemister, 1959), Eriochier (Shaw, 1961), Uca (Green et al., 1959) and Callinectes (Mantel, 1967). Such a mechanism can account for replenishment of ions lost both by renal and extra-renal routes.

The principal site for active salt absorption in decapods appears to reside in the gill epithelium (Potts and Parry, 1964; Gross, 1957; Flemister, 1959; Shaw, 1961; Green et al., 1959; Mantel, 1967; Rudy, 1966). In Gecarcinus Copeland (1968b) has observed the salt-absorbing cells to be highly concentrated in the three posterior lamellae (numbers 7, 8, 9). These specific lamellae cover the pericardial sac, which has been postulated as a storage organ assisting in molting (Copeland, 1968b; Bliss, 1963). Copeland (1968 a, b) has further noted from his histological examinations of both Callinectes and Gecarcinus that there is an association of mitochondria and highly folded membranes with gill lamellae, the principal osmoregulatory tissues.

Kidney function of marine decapods has been interpreted to be generally ineffective as a conservation mechanism in osmoregulation (Potts and Parry, 1964). Analyses of urine from several species of crabs under both hypo- and hyperosmotic stress indicate that the urine is always isosmotic to the blood (Potts and Parry, 1964). The renal organ or antennary gland, however, does play an important role in ionic regulation. In concentrated sea water several workers have shown selective secretion of magnesium ions by the kidney with a

simultaneous reabsorption of urine sodium: for example, Pachygrapsus (Prosser et al., 1955; Gross, 1966), Carcinus (Riegel and Lockwood, 1961), Uca (Green et al., 1959), Hemigrapsus (Gross, 1964; Dehnel and Carefoot, 1969; Alspach, MS thesis, OSU, 1967). In dilute sea water the urine remains isosmotic to the blood, but changes in urine volume flow have been observed. Carcinus, for example, loses water and salts in dilute sea water through increased urine production, but without an overall change in urine concentration; i.e., the urine remains isosmotic (Shaw, 1961).

Tissues must be able to tolerate any changes brought about through osmotic and ionic adaptations of the blood. Changes in the cellular environment may quantitatively affect any or all of the following: cell volume (intracellular water content) and concentrations of permeable ions and low molecular weight organic compounds, such as free amino acids. For example, when Carcinus is transferred from 100% to 40% sea water there is an increase in water content of muscle fibers as measured by wet-dry weight determinations (Shaw, 1955b). Similar increases in muscle hydration were found in Pachygrapsus (Gross and Marshall, 1960) and Hemigrapsus nudus (Alspach, MS thesis, OSU, 1967) using the same technique.

Regulation of cell volume involves changes in hydration and concomitant changes in the total number of moles of intracellular solutes. Both of these changes constitute the concept of "isosmotic

intracellular regulation" as described by Duchateau and Florkin (1955) and Lange (1968, 1970). After initial swelling in a hypotonic medium, successive changes return the cell to its original volume through elimination of an isosmotic solution from the cell. In the invertebrate groups studied most extensively so far (crustaceans, bivalve molluscs, echinoids) the osmotically active solutes eliminated from the cells are primarily free amino acids (Lange, 1968, 1970). Isosmotic intracellular regulation by cells has recently been reported for Callinectes, a euryhaline crab (Lang and Gainer, 1969). In vitro measurements of Callinectes muscle fiber diameters were related to volumetric changes in experimental salines. Muscle fibers in hypotonic saline returned to their original volume within a period of six hours. These changes in cell volume appear to result from the loss of osmotically active nonelectrolytes, perhaps free amino acids. Such responses have not been previously reported in decapods.

Through changes in the levels of certain intracellular osmotic particles, mainly permeating ions and free amino acids, the blood: tissue osmotic gradient is kept at a minimum. For example, when both Carcinus and Callinectes are transferred to hypoosmotic medium, there is a reduction in the concentration of intracellular sodium, potassium, and chloride (Shaw, 1955b, 1958a; Hays et al., 1968). Larger reductions are found in organic compounds, namely, free amino acids, trimethylamine oxide, and betaine (Shaw, 1958 a, b).

Similar findings have been reported by Camien et al. (1951) for Astacus; Duchateau and Florkin (1955) for Eriocheir; Kermack et al. (1955) for Homarus; and Robertson (1961) for Nephrops. Further studies show that specific amino acids--glutamic, proline, glycine, alanine, and arginine--apparently account for the major decreases in organic compounds in some decapods, and, thereby, a reduction in intracellular osmotically active particles (Duchateau-Bosson and Florkin, 1961; Lange, 1968). Such reductions are responsible for cell volume regulation (Lange, 1968), and play a role in maintaining intracellular osmotic activity; at least in Carcinus (Shaw, 1958b) and Nephrops (Robertson, 1961) muscle fibers.

Within the crab families Cancridae and Portunidae, work on Carcinus maenus, Cancer pagurus and Cancer antennarius has revealed a range of osmoregulatory patterns. Hyperosmotic regulation occurs in C. maenus in low salinity water and conformity in 100% sea water and above (Potts and Parry, 1964; Shaw, 1955). Both Cancer pagurus and Cancer antennarius are osmo-conformers over the entire salinity range (Jones, 1941; Krogh, 1939). These patterns may be related to the ecology of these species.

Cancer magister, the Pacific edible crab abundant in Oregon coastal waters, has received little attention since the preliminary work of Jones (1941) on its osmoregulatory ability. Because of the range of habitats tolerated by this species, from brackish water

estuaries to the subtidal open coast, it was felt that a comprehensive investigation of the osmoregulatory behavior of C. magister would prove rewarding. The present study includes measurement of osmotic and ionic concentrations of the blood, urine and muscle in response to salinity stresses ranging from 25% to 125% sea water. Measurements were also made of blood volume and muscle extracellular space. The latter measurements allow one to estimate the water held in the interfiber spaces of muscle, and thereby obtain a more accurate estimate of the intracellular ion concentrations. An investigation which covers all of these parameters of osmoregulation will hopefully lead to a greater understanding of the osmoregulatory process in this species.

MATERIALS AND METHODS

Collection and Handling

The Dungeness crab, Cancer magister, is distributed along the West Coast of North America from Alaska to Mexico. The crabs used in the present research were obtained from Yaquina Bay, an estuary, near Newport, Oregon. Crab pots and otter trawling in the channel of Yaquina Bay or in Sally's Slough (a small channel off the marked navigation channel) were the two means of catching experimental animals. Only male, intermolt (Stage C) crabs were used in order to reduce sex and molting as factors in the experiments.

Animals were brought to the Oregon State University Marine Science Center and placed in fiberglass holding tanks supplied with a continuous-flow sea water system. The water temperature in the laboratory during the summer months ranged from 10-13° C and the salinity ranged from 25-33‰. No mortalities were noted under these conditions. Animals were fed ad libitum in the holding tanks and acclimated for a period of at least 5 days.

Experimental salinities of 25, 50, 75, 100 and 125% sea water were used. A salinity of 32‰ was chosen as 100% sea water. Quantities of each salinity were mixed in a 20 gallon plastic garbage can and the salinity determined with a RS5-3 portable salinometer

(Industrial Instruments, Inc.). A chloride determination was made on a water sample of each salinity using the method of Schales and Schales (1941).

Following the equilibration period (at least 5 days), the crabs were placed singly in polyethylene refrigerator trays containing 2 liters of aerated sea water of the desired test salinity. In all cases, this transfer was directly from the holding tanks, and considered to be an acute stress. The trays were kept in a constant temperature bath at 12 ± 0.5° C.

Experimental Design

Four different experimental approaches were chosen to comprehensively analyze the osmotic responses of Cancer magister. The parameters studied included: blood and urine osmotic and ionic concentrations, muscle tissue ions and total amino nitrogen, extracellular volume and interfiber water.

One series of experiments was run to determine the long-term osmotic and ionic adjustments of blood and urine when the crabs were exposed to the five test salinities. Blood and urine were sampled at 12 hour intervals for four days (96 hours). When crabs survived beyond 4 days, samples were taken on the 5th, 7th and 10th days, and the experiment was then terminated. The time at which a crab became moribund in a test medium was noted.

Because crabs generally survived at least 48 hours in all test media, a second series of experiments was run to obtain a more detailed picture of the blood and urine osmotic regulation pattern, as well as the changes in the muscle tissue ions. During the 48 hour period of the test, the blood, urine and muscle were sampled twice--once at 24 hours, and again at the close of the experiment; but each crab was sampled only once and then discarded. Animals were run at all five test salinities.

A third series of experiments was carried out to estimate blood volume, employing the inulin isotope dilution technique. In order to measure the blood volume of crabs stressed to different salinities, a crab was first exposed to the test salinity for 24 hours, at which time a known amount of inulin-C^{14} was injected. The crab was returned to the test medium and blood samples were withdrawn at 10 minute intervals for the first hour, at 30 minute intervals for the second hour, and at 60 minute intervals for the following 2 hours. A total of four hours was allowed to determine the time concentration curve.

The fourth experimental series was designed to measure the in vivo muscle extracellular volume, using inulin-C^{14} as the probe molecule. Measurement of the muscle extracellular volume in a crab exposed to a test medium for 24 hours involved injection of the inulin-C^{14} into the whole crab 12-20 hours prior to sampling. This 12-20

hour equilibration period following the isotope injection was determined on the basis of other investigations on Callinectes and Nephrops (Hays et al., 1969; Robertson, 1961). Paired muscle samples from the first walking leg, plus blood samples, were obtained for measurement of the radioactivity, as described below.

Analytical Procedures

Blood and Urine Analysis

Before blood sampling, each crab was blotted and held in paper toweling to prevent contamination from sea water. Samples of blood were drawn by membrane puncture at the base of the coxopodite of the first walking leg. The membrane was punctured with a Pasteur pipet (Dispo) connected to a mouth tube for suction. The blood was transferred to a 2 ml. plastic snap-cap vial and frozen until analyzed.

Frozen blood was macerated with a glass rod to break the clot and then allowed to thaw completely. A 100 µl sample of the resulting "serum" was diluted 1:250 with deionized distilled water and used for analysis of calcium, sodium, potassium and chloride. Separate, undiluted samples were used for the determination of osmotic pressure.

Urine was obtained from crabs which had been blotted with paper toweling. To facilitate withdrawal of urine from the nephropore, it was necessary to move, and firmly hold, the third maxilliped away from the nephridial operculum. A Pasteur pipet drawn to a

fine tip and connected to a mouth tube was used for collection and transfer of the urine. The tip of the pipet was inserted beneath the operculum with the aid of a probe. This procedure usually caused the release of a volume of urine sufficient for analysis. The application of slight mouth suction was necessary to draw the urine into the pipet. From the pipet, urine was transferred into a 1 ml. dispo beaker and frozen for future analysis.

After thawing, a 100 µl sample of urine was diluted 1:250 with deionized water and analyzed for sodium, potassium and chloride. Separate, undiluted samples were used for determination of osmotic pressure.

For both blood and urine dilutions, the sodium, calcium and potassium ion concentrations were measured with a Coleman Flame Photometer. Chloride ion concentration was determined using a Buchler-Cotlove chloridometer. All ion results were expressed as mEq/liter. Osmotic concentrations of the undiluted blood and urine were measured with a Mechrolab Vapor Pressure Osmometer. Milliosmolar concentrations of sodium chloride were utilized as standards. The results are expressed as milliOsmoles/liter.

Tissue Sampling and Determination of Tissue Ions

Samples of muscle tissue were obtained from the first pair of walking legs. Paired muscle samples were removed, blotted on

wet paper to remove excess fluid and weighed to 0.1 milligram. Samples were dried at 60-70° C for at least 48 hours, the period necessary to achieve a constant dry weight; a dry weight was then recorded. From the wet and dry weights, the muscle water content was determined and expressed as a dry weight.

The dried muscle was ground in a mortar and pestle and a weighed subsample of approximately 150 mg was digested for ion analysis. Another weighed subsample of dried muscle (approximately 50 mg) was used for alpha-amino nitrogen determination.

Digestion of the dried muscle for ion analysis was carried out in 2.0 ml. of 30% hydrogen peroxide overnight in 25 ml. closed Erlenmeyer flasks; the digest was filtered and the filtrate diluted to 20 ml. with distilled water. An aliquot was analyzed for tissue chloride with the Buchler-Cotlove chloridometer. Preliminary tests showed that comparable results were obtained using either the method of Schales and Schales or the chloridometer. Other aliqouts of the digest filtrate were further diluted 1:10 for sodium and 1:50 for potassium analyses. These ion concentrations were measured with a Coleman flame photometer. Muscle ion concentrations are expressed in mEq/kg muscle water.

Free intracellular amino acids of the muscle were measured by a quantitative ninhydrin positive method (Rosen, 1957) on trichloroacetic acid homogenates. The powdered muscle sample was

homogenized in 20% trichloroacetic acid at 0-5° C preceding color development. Samples were read against a D-L leucine standard at 570 mμ on a Bausch and Lomb Spectronic 20. The results are expressed in mM/kg muscle water.

Blood Volume Determination

An estimate of blood volume in Cancer magister was obtained with the inulin isotope-dilution technique. On the dorsal surface of the crab carapace above the pericardial region, a small area of exoskeleton was scraped away with a sharp scalpel. Care was taken not to puncture the underlying membrane. A rubber dental dam was cemented over this area using the tissue cement Loctite 404 (Loctite Corp. Catalog #65-51). This provided a dry working area for injection of the inulin-C^{14} and for serial sampling of the blood.

For the blood volume determinations, a known amount of inulin-C^{14} was injected directly through the rubber dam into the pericardial region. The crabs were exposed to the test salinities and blood samples were removed anterior to the point of injection by insertion of an insect pin probe through the rubber damming. Samples were taken over a four hour period to determine the time concentration curve.

Blood samples (0.1 ml.) containing the inulin-C^{14} were placed directly in liquid scintillation vials containing 0.9 ml. distilled water.

Following addition of 10 ml of scintillation fluorer, the vials were counted in a Nuclear-Chicago model 6819 ambient temperature counter. The scintillation fluorer consisted of 1 liter Triton X-100, 2 liters reagent grade toluene, 100 mg/l POPOP, and 4 g l PPO. The time for 10,000 counts was recorded: corrections for an inulin-C^{14} standard and background were made. No correction was made for quenching by the blood. Blood volume was determined according to the following formula:

$$\text{Blood volume (ml)} = \frac{\text{cpm injected}}{\text{cpm blood/ml}}$$

The denominator (cpm blood/ml) was determined by extrapolation of the time course dilution to zero time. The time course--dilution showed little deviation after the first 10 minute sample to the sample at 240 minutes.

Muscle Extracellular Space Determination

In vivo muscle extracellular space measurements were made using inulin-C^{14} (New England Nuclear Co.) as the probe molecule. A known amount of the isotope was injected into the whole crab through a rubber dam on the dorsal surface of the carapace. After an equilibration period of 12-20 hours (as described earlier, P. 11) a blood sample was withdrawn. The extensor and flexor muscles of the meropodite of the first walking legs were dissected out, blotted on

paper, and weighed. One pair (extensor and flexor) was dried to obtain a percent dry weight; the other pair was digested in 2 ml. of 30% hydrogen peroxide. After digestion for four hours, the volume was adjusted to 10 ml. using distilled water, and a 1.0 ml. aliquot placed in liquid scintillation vials. The scintillation fluorer was added and the vials were counted in a Nuclear Chicago model 6819 ambient temperature counter. Quench correction for the tissue was not determined. The fluorer used was the same as that described on page 16.

Radioactivity in the blood sample was compared to that of the muscle tissue because blood is the primary external medium bathing the muscle tissue. After corrections for background activity, the muscle extracellular space (MEXS) was computed according to the formula below:

$$\text{MEXS (E) in milliliters} = \frac{\text{total tissue cpm}}{\text{blood cpm/ml}}$$

The percentage of MEXS may then be expressed in one of the following ways:

A. $$\%\text{MEXS} = \frac{\text{ml MEXS}}{\text{100 g tissue } H_2O} = 100 \times \frac{\text{ml MEXS}}{\text{g tissue water}}$$

B. $$\%\text{MEXS} = \frac{\text{ml MEXS}}{\text{100 g tissue wet wt.}} = 100 \times \frac{\text{ml MEXS}}{\text{g tissue wet wt.}}$$

In order to clarify the equations above, we can partition a 100 g whole muscle into the following compartments:

$$C + E + D = 100$$

Where: C = cell water

E = extracellular space (interfiber water)

D = dry weight

The applicable units can be any of the following: grams, milliliters, or they can be expressed as percentage (%) of one compartment to another. From the above, the following considerations were made:

(1) C + E + D = 100 = total wet tissue

(2) C + E = T = total tissue water

(3) C + D = total cellular mass and its volume

Thus, the muscle extracellular space (E) represents the amount of volume or weight which surrounds the outside of the muscle cells, i. i. e., interfiber water. This quantity (E) can be expressed as (a) the % of the total tissue water (C+E=T) as in A above, or (b) the % of the total wet tissue (100) as in B above.

The extracellular space calculations were used to correct the muscle ion concentrations and the corrected ion data were expressed as mEq/kg cell water, i. e., they are expressed in terms of cell water (C) minus MEXS(E). This correction was calculated from the following equation:

$$[X]_{\text{kg cell water}} = \frac{[X]_{\text{muscle (mEq/kg muscle water)}} - [X]_{\text{Blood(mEq/l)}} \cdot E}{1 - E}$$

where E was expressed as a % of 100 g tissue wet weight (equation B). The use of this equation assumes that the blood concentration of any ion is in equilibrium with the concentration in the extracellular space of the muscle, and further assumes the ions are free of solution, not bound.

An alternative correction factor used by Hays et al. (1969) expresses the muscle ion data in terms of the % fiber water. The % fiber water subtracts the extracellular space (E) from the total wet weight, and is based on the % of cell water (C) to the total cellular mass and its volume (C+D). This equation is:

$$\% \text{ Fiber water} = 100 - \frac{\% \text{ dry weight}}{1 - \% E} \times 100$$

or:

$$\frac{C}{C + D} = 100 - \frac{D}{C + D} \times 100$$

The following is an example of this calculation based on data from Hays et al. (1969) for Callinectes muscle:

$D = 22.5$

$E = 13.9$

$C = 63.6$

$C + E + D = 100$

$C + D = 86.1$ = total cellular mass and its volume

$C + E = 77.5$ = total tissue water

$$\% \text{ Fiber water} = 100 - \frac{22.5}{100 - 13.9} \times 100$$

$$\simeq 73.9$$

or

$$\% \text{ Fiber water} = \frac{C}{C + D} \times 100$$

$$\simeq \frac{63.6}{86.1} \times 100$$

$$\simeq 73.9$$

Muscle ion values corrected to % fiber water are expressed as mEq/kg fiber water. In the present study muscle ion data was corrected to mEq/kg cell water (see above).

RESULTS

Blood and Urine Osmotic Regulation

The blood and urine osmotic concentrations of Cancer magister, measured after exposure to a range of experimental salinities, are shown in Figures 1 through 3. The blood and urine osmotic concentrations at time zero in Figure 3 represent a mean of 40 animals. This figure plots the results of long-term osmotic stresses on crabs kept in the sea water dilutions, and shows the time-course adjustments to the salinities imposed. Figures 1 and 2 show only osmotic concentrations after 24 and 48 hours in the test salinities.

The blood osmotic concentration of crabs in 75% sea water and below is hyperosmotic to the medium, i.e., a higher osmotic concentration is found in the blood compared to sea water (Figure 1 and 2). With time, however, the blood: medium osmotic gradient for animals in dilute sea water is reduced as shown by the greater blood: medium gradient at 24 hours (Figure 1) compared to 48 hours (Figure 2).

Crabs in normal sea water (100% sea water = 32‰ salinity) maintain their blood isosmotic to the medium (Figures 1 and 2). No changes in this blood osmotic pattern occur in the time course plotted in Figure 3 (a 10-day period).

The results of the measurements on animals kept in hypersaline sea water (125%) show some hypoosmotic regulation, i.e., a lower

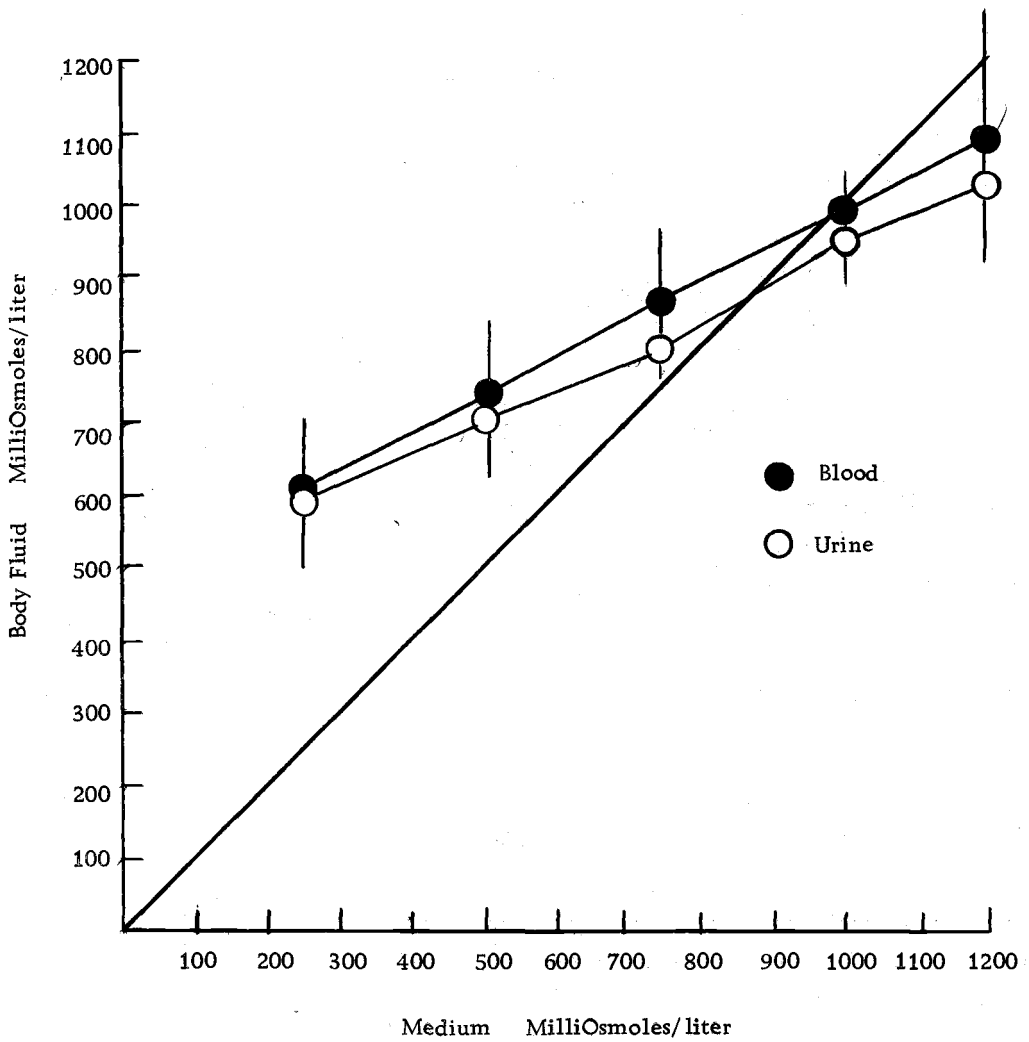

Figure 1. Osmotic concentrations in the blood and urine after 24 hours exposure to the experimental salinity. Each point represents the mean value of ten animals ±2 S. E., and is expressed in milliOsmoles per liter. Temperature 12°C.

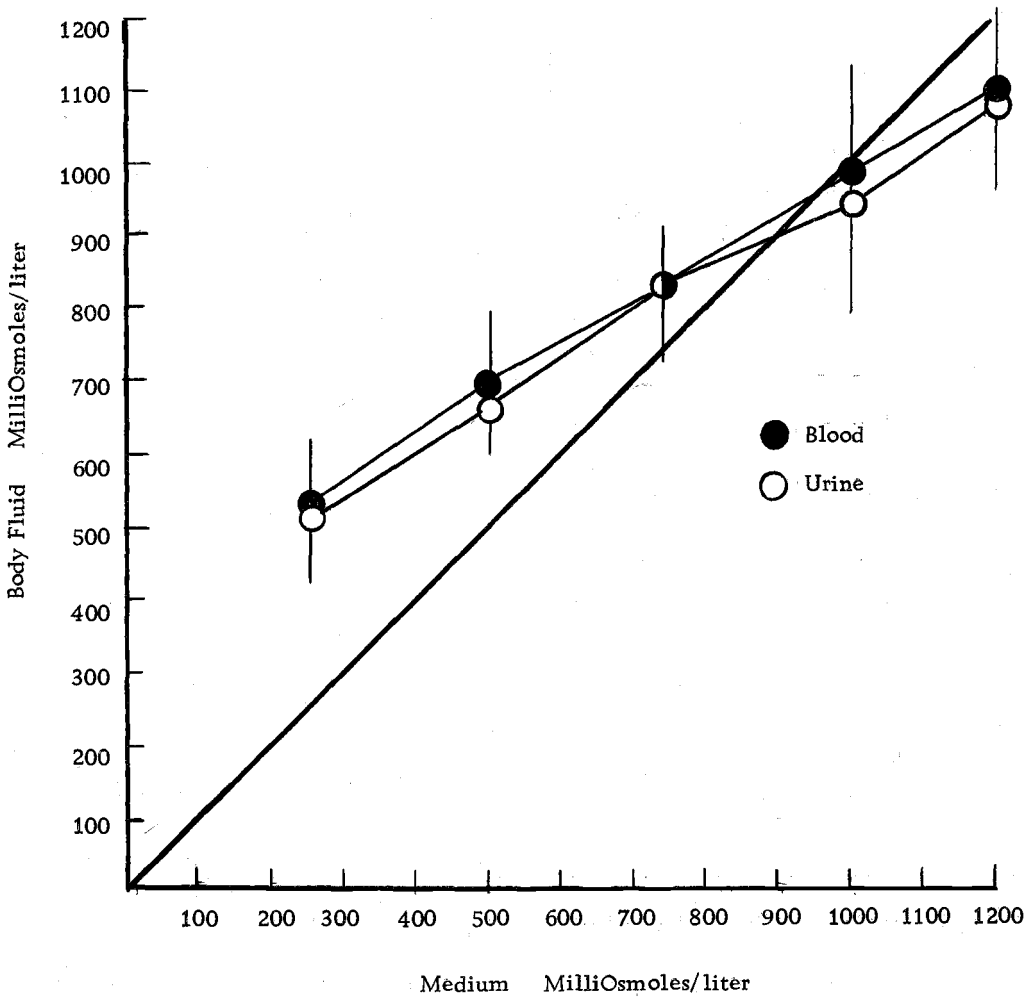

Figure 2. Osmotic concentrations in the blood and urine after 48 hours exposure to the experimental salinity. Each point represents the mean value of ten animals ± 2 S.E., and is expressed in milliOsmoles per liter. Temperature 12°C.

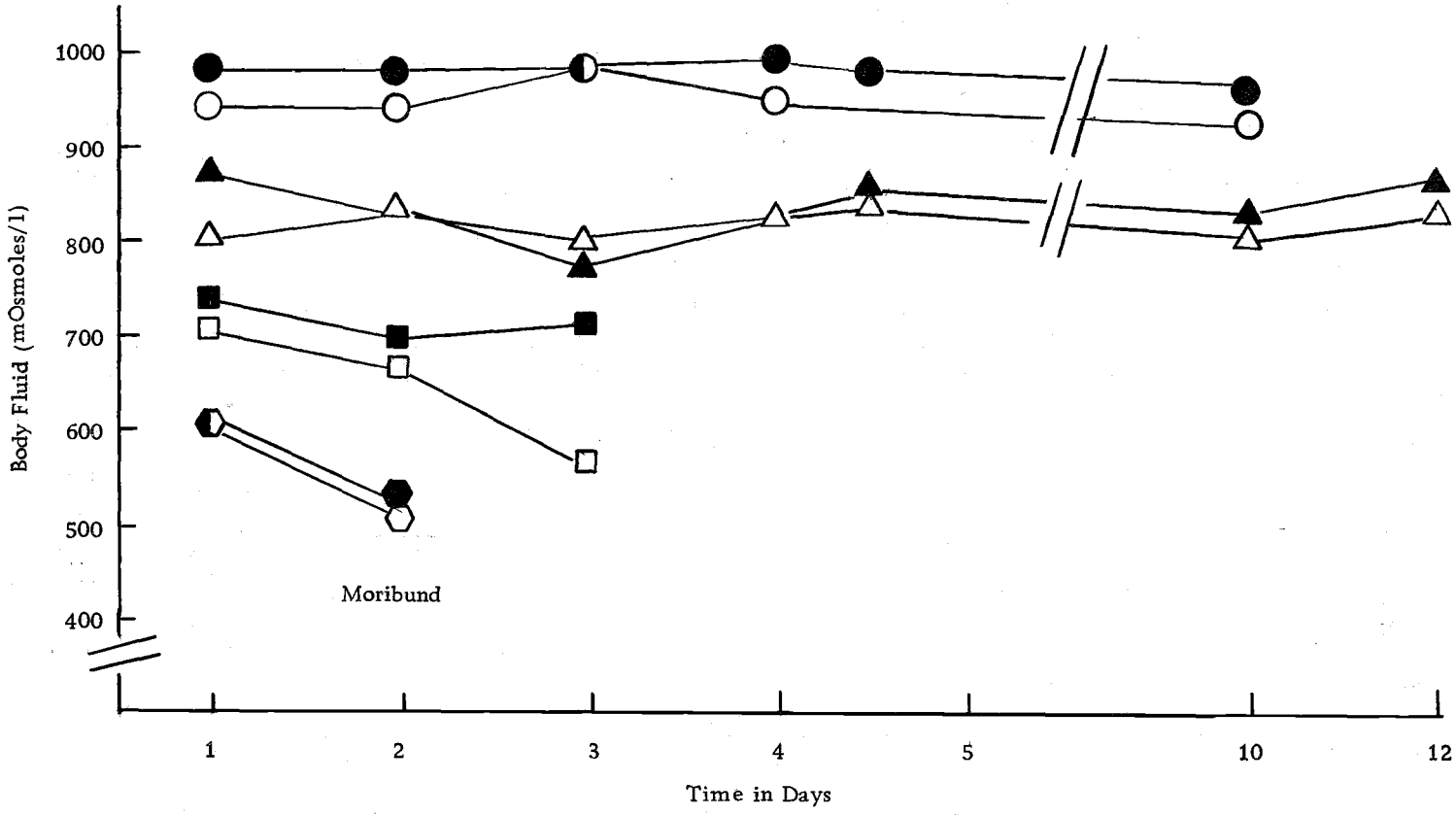

Figure 3. Osmotic concentrations in the blood (closed symbols) and urine (open symbols) as a function of time of exposure to each experimental salinity (○ = 100 % s.w. = 32‰; △ = 75% s.w.; □ = 50% s.w.; ⬡ = 25% s.w.). Values are expressed as milliOsmoles per liter. Animals were placed directly into each test salinity from the holding tanks.

osmotic concentration is maintained in the blood compared to the medium (Figures 1 and 2). The blood:medium osmotic gradient is increased slightly from 24 hours to 48 hours, but is not statistically significant. Unfortunately, crabs at 125% sea water were not followed for a longer time.

The urine osmotic concentrations is slightly blood hypoosmotic at all experimental salinities (Figures 1, 2, and 3). Further, the urine:blood ratios (Table 1) confirm this result with the U/B ratio almost consistently being less than 1.0; but the differences are not statistically significant. Thus, the urine is blood isosmotic.

The long-term osmotic adjustments plotted in Figure 3 clearly show that crabs in 75% and 100% sea water maintain an osmotic steady state over a 10-12 day sampling period. Osmotic equilibrium in crabs exposed to 75% and 100% sea water appears to be achieved within the first 24 hours of exposure. Thus, major changes in osmotic responses have occurred within this time. Crabs in 25% and 50% sea water, however, showed limited survival. C. magister was unable to withstand the former stress for longer than 48 hours. Generally, crabs exposed to 50% sea water survived for 60 to 72 hours (3 days) before becoming moribund. Moreover, the blood osmotic concentrations for crabs in 25% and 50% sea water show a continuous decline. A steady state level in blood osmotic concentration with respect to these media is thereby not established. The acute nature of the

Table 1. Urine to blood osmotic ratios of Cancer magister over a range of salinities.

Medium	Days 1	2	3	4	5	10
100%	0.96	0.96	1.06	0.96	0.91	0.96
75%	0.92	1.00	0.96	0.89	0.97	0.96
50%	0.96	0.95	0.79			
25%	0.99	0.98				

salinity stresses may affect this result. For example, an "acclimated state" might be reached if crabs were serially transferred in stepwise dilutions of sea water over a longer period of time.

Blood and Urine Ionic Regulation

The results of blood and urine measurements of sodium, potassium, calcium, and chloride in Cancer magister are shown in Figures 4 through 11. In Figures 4 through 7 the results of the blood ion concentrations for each ion are plotted over several days and are based on crabs acutely exposed to the experimental salinities. Figures 8 through 11 show only the results for each of the ions measured after 24 and 48 hours. The ion concentrations at time zero in Figures 4 to 7 represent the mean of 10 animals.

Sodium

The blood sodium ion concentration is generally hyperionic to the medium in salinities of 75% sea water and below (Figure 8). Crabs in dilute sea water show a greater blood sodium:medium gradient after 24 hours than after 48 (Figure 8). In normal sea water (100%) and in 125% sea water crabs maintain the blood sodium isoionic (Figure 8).

The pattern of blood sodium regulation, when followed over several days (Figure 4) is roughly similar to that shown for blood

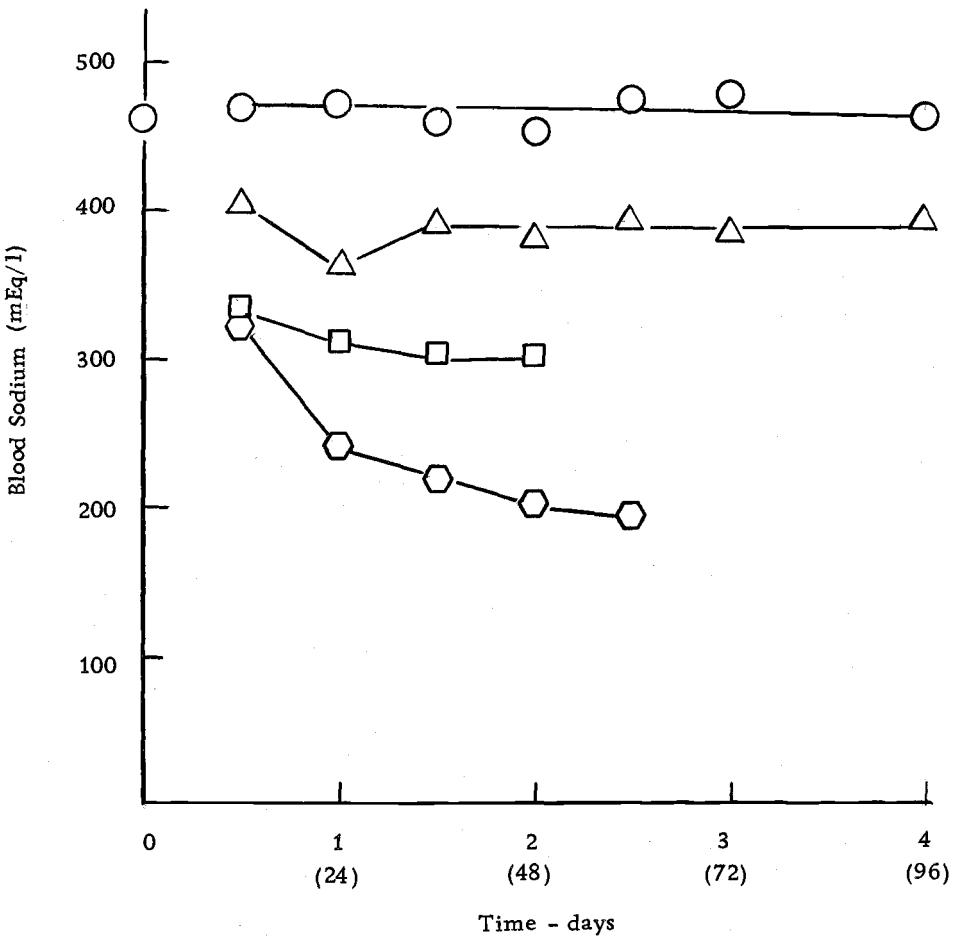

Figure 4. Time course adjustments of blood sodium concentration (mEq/l) of Cancer magister in various salinities (0 = 100% sea water = 32‰; △ = 75% sea water; □ = 50% sea water; ⬡ = 25% sea water) followed over several days (hours in parentheses). Temperature 12°C.

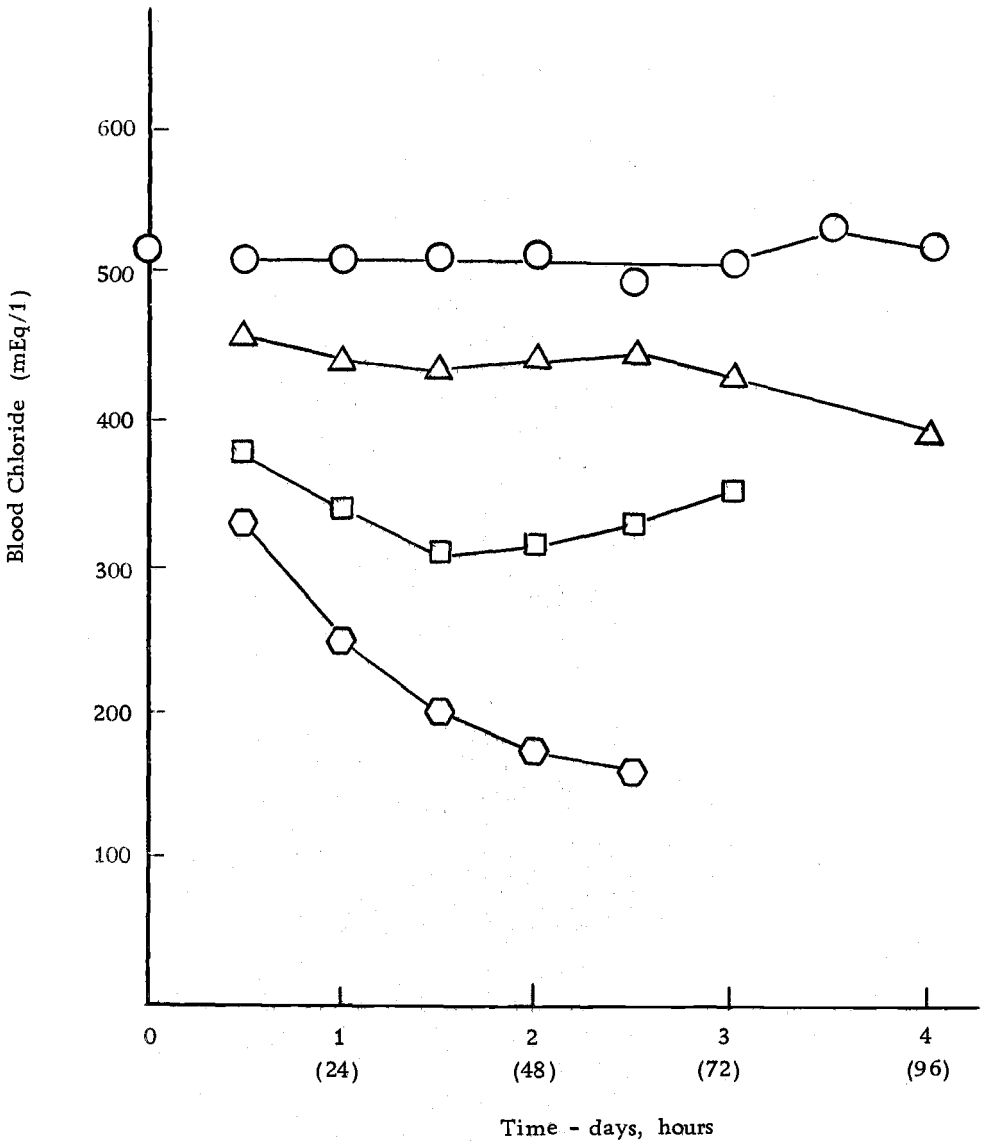

Figure 5. Time course adjustments of blood chloride concentration (mEq/l) of Cancer magister in various salinities (○ = 100% sea water = 32 ‰; △ = 75% sea water; □ = 50% sea water; ⬡ = 25% sea water) followed over several days (hours in parentheses). Temperature 12°C.

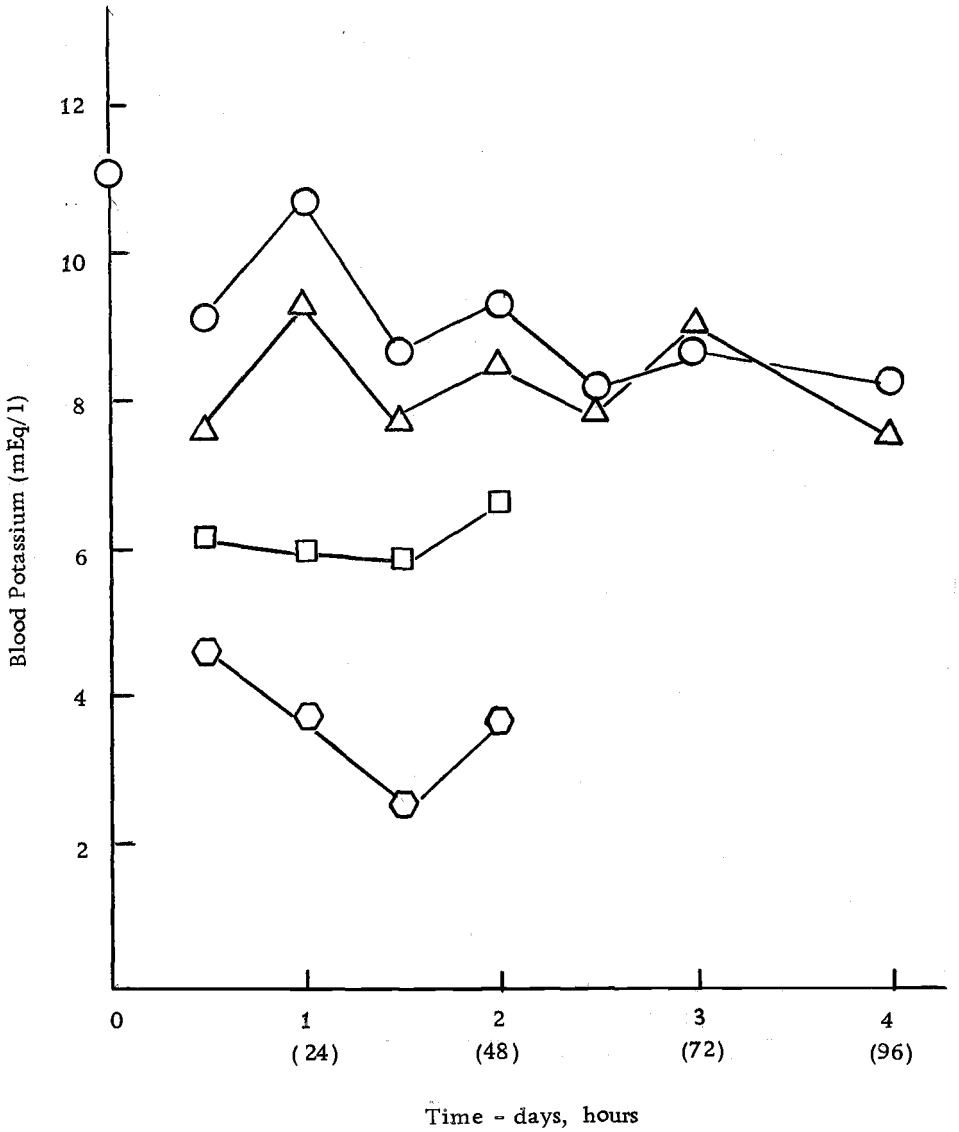

Figure 6. Time course adjustments of blood potassium concentration (mEq/1) of Cancer magister in various salinities (○= 100% sea water = 32‰; △= 75% sea water; □ = 50% sea water; ⬡ = 25% sea water) followed over several days (hours in parentheses). Temperature 12°C.

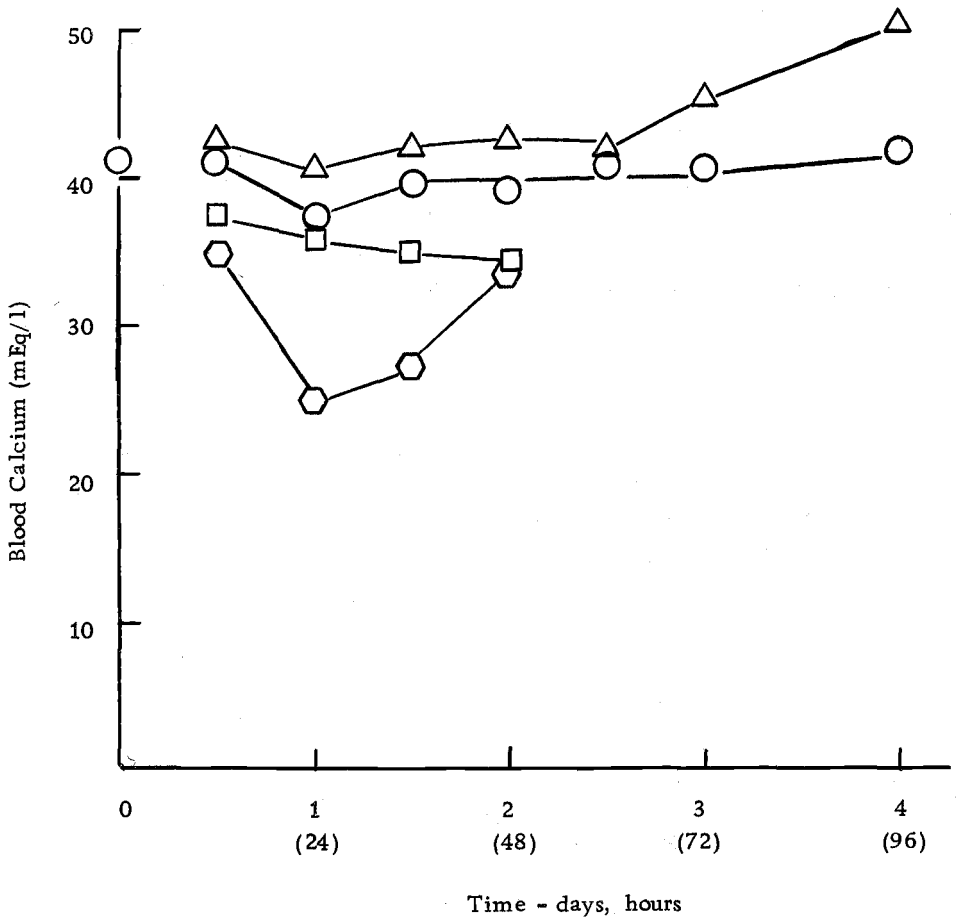

Figure 7. Time course adjustments of blood calcium concentration (mEq/l) of Cancer magister in various salinities (○ = 100% sea water = 32‰; △ = 75% sea water; □ = 50% sea water; ⬡ = 25% sea water) followed over several days (hours in parentheses). Temperature 12°C.

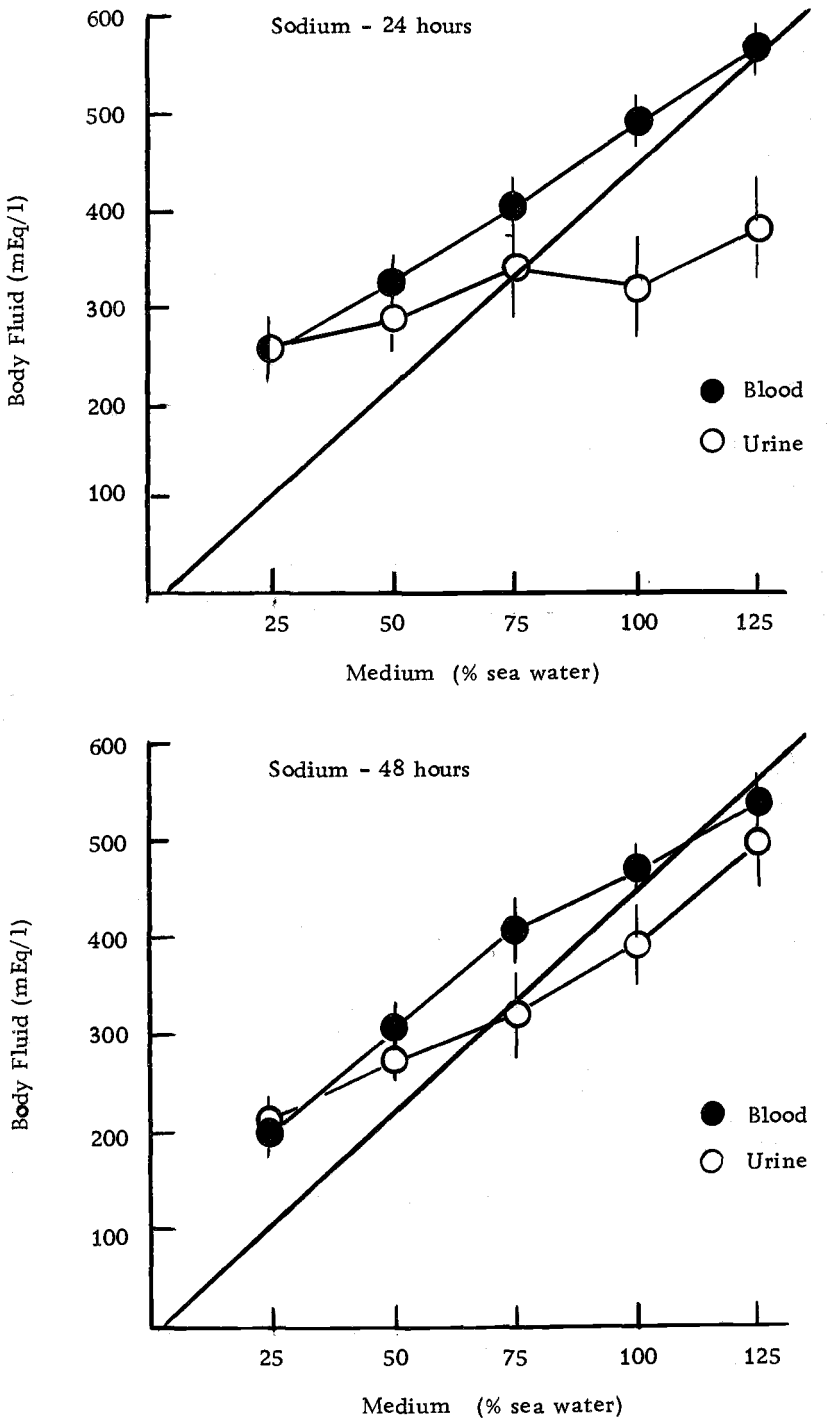

Figure 8. Sodium ion concentrations (mEq/l) in the blood and urine of Cancer magister as a function of salinity. Measurements were made after 24 hours (top) exposure and 48 hours (bottom) exposure to each experimental salinity. Temperature was 12°C. Each point represents a mean value ± 2. S.E. of 10 animals.

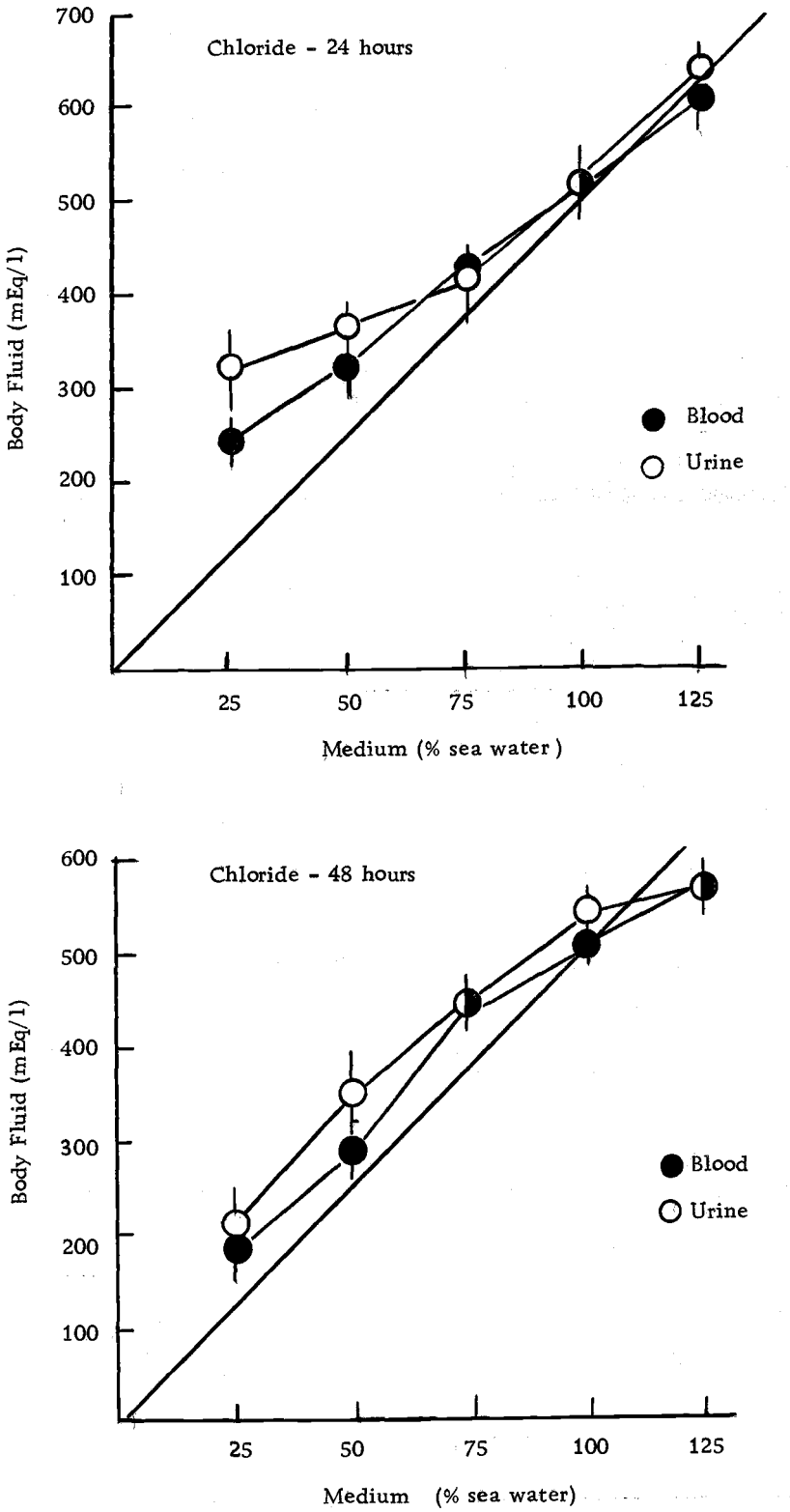

Figure 9. Chloride ion concentrations (mEq/l) in the blood and urine of Cancer magister as a function of salinity. Measurements were made after 24 hours (top) exposure and 48 hours (bottom) exposure to each experimental salinity. Temperature 12°C. Each point represents a mean value ±2 S. E. of 10 animals.

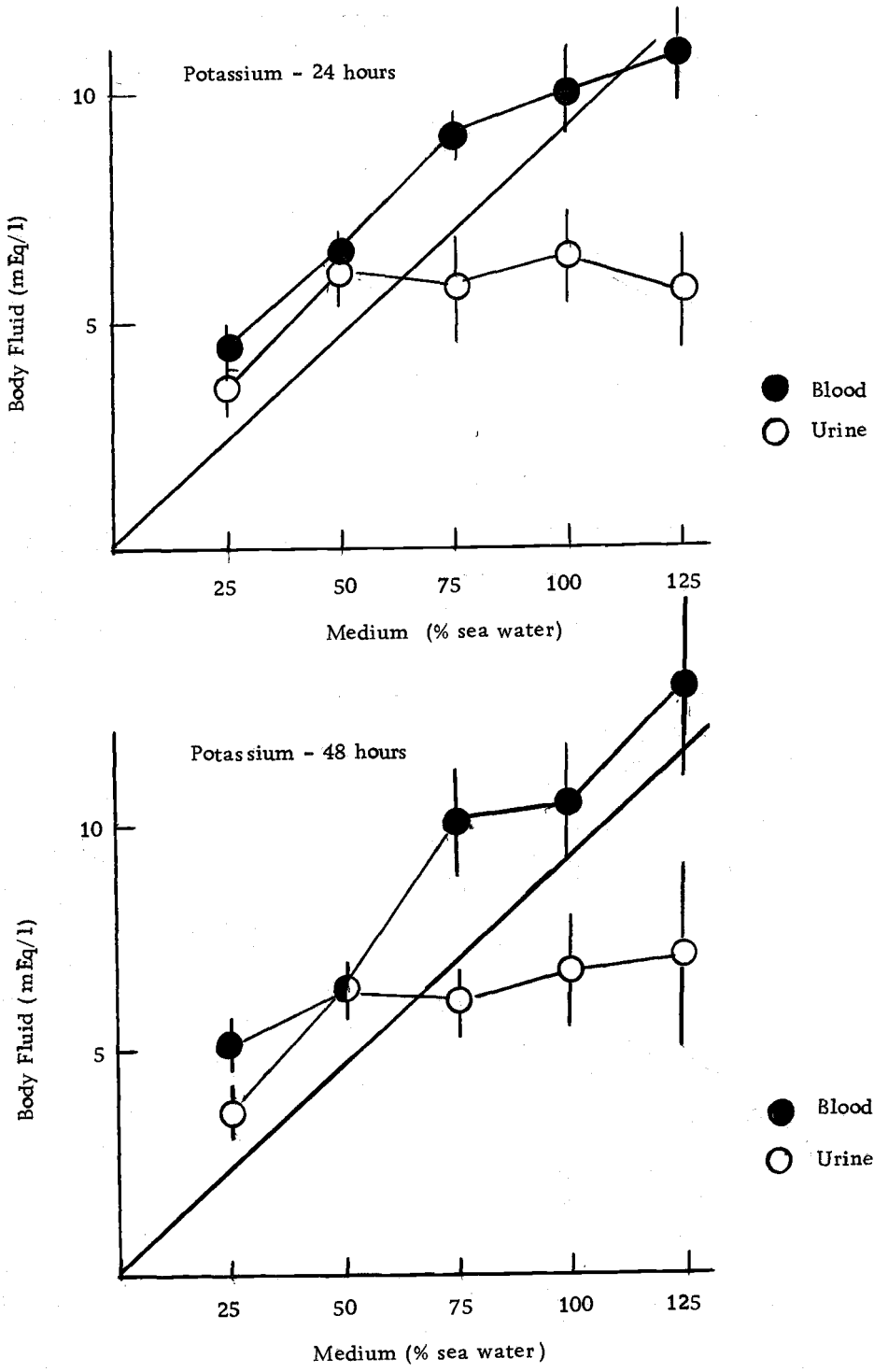

Figure 10. Potassium ion concentrations (mEq/l) in the blood and urine of Cancer magister as a function of salinity. Measurements were made after 24 hours (top) exposure and 48 hours (bottom) exposure to each experimental salinity. Temperature 12°C. Each point represents a mean value ±2 S. E. of 10 animals.

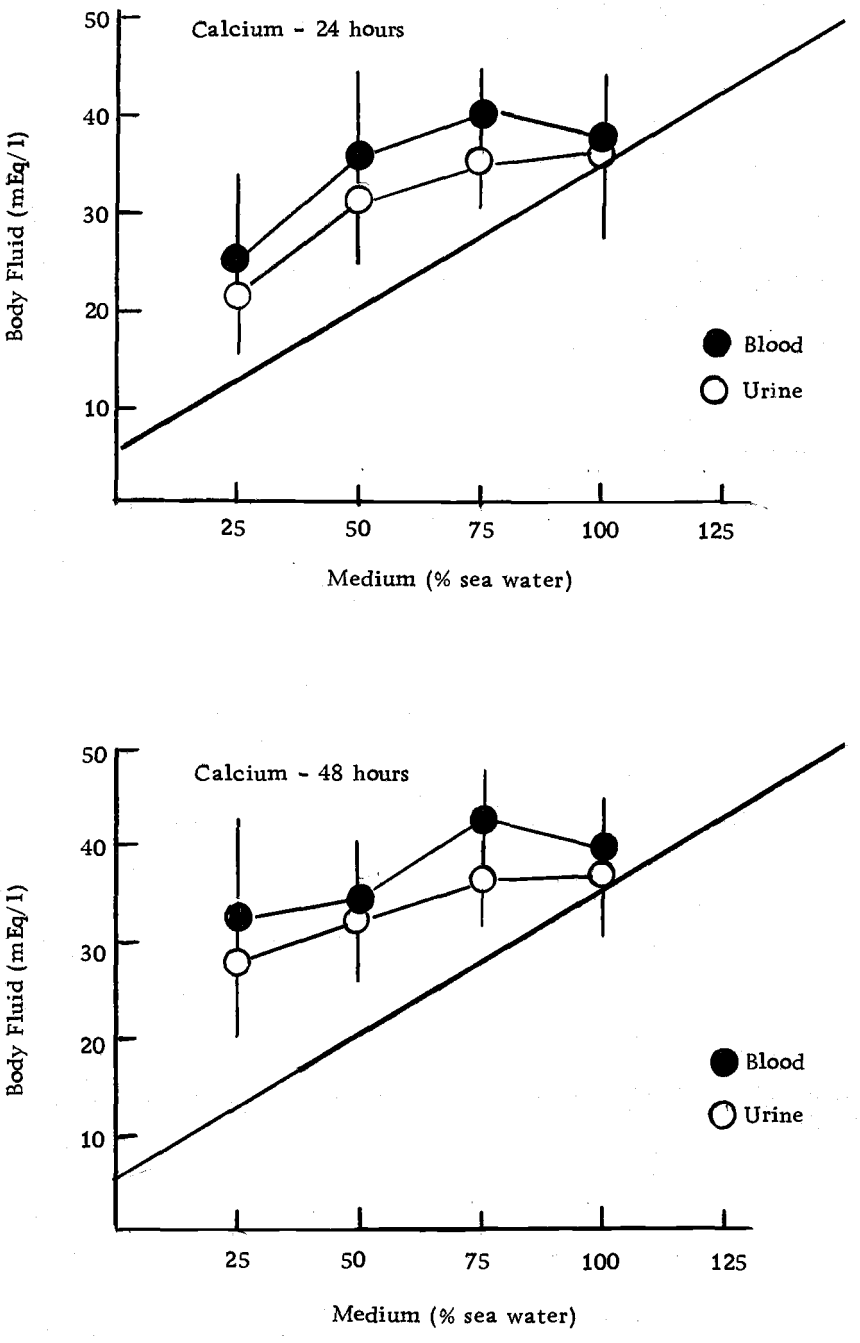

Figure 11. Calcium ion concentrations (mEq/l) in blood and urine of Cancer magister as a function of salinity. Measurements were made after 24 hours (top) exposure and 48 hours (bottom) exposure to each experimental salinity. Temperature 12°C. Each point represents a mean value ±2 S. E. of 10 animals.

Table 2. Urine to blood ratios for ion concentrations in Cancer magister measured after 24 and 48 hours exposure to each experimental salinity. Mean values were used to calculate the ratio.

Medium	Hours	Cl^-	Na^+	K^+	Ca^{++}
125%	24	1.06	.67	.46	N. M.
	48	.99	.92	.53	N. M.
100%	24	1.01	.65	.56	0.95
	48	1.08	.84	.75	.95
75%	24	.96	.85	.62	.88
	48	1.02	.76	.57	.86
50%	24	1.14	.89	.93	.86
	48	1.23	.89	.98	.94
25%	24	1.31	1.01	.78	.84
	48	1.18	1.03	1.42	.85

osmotic concentration (Figure 3). Crabs at 75% and 100% sea water maintain a steady state level of blood sodium. In addition, the blood sodium level in crabs at 25% and 50% sea water appears to be approaching a steady state by the end of the time period. This is in contrast to the apparent lack of attainment of a steady state for blood osmotic concentration.

Urine sodium is blood hypoionic under all stresses imposed except 25% sea water (Figure 8 and Table 2). There is a significant difference between urine:blood sodium in 75% sea water ($P < .05$), 100% sea water ($P < .01$), and 125% sea water ($P < .01$) which is clearly shown in the U/B ratios after 24 hours of exposure (Table 2).

Chloride

Blood chloride is regulated hyperionic to the medium and follows a pattern roughly similar to blood sodium (Figure 9). However, a lower blood:medium gradient is maintained for chloride than for sodium; i.e., the blood chloride concentration is parallel, and slightly hyperionic, to the medium at all salinities (Figure 9).

Long-term blood chloride regulation (Figure 5) does not attain the steady state shown for blood sodium (Figure 4). However, blood chloride in 50% sea water appears to be approaching a new and different steady state level after 48 hours compared to 75% and 25% sea water. Crabs in 25% sea water show a continuous decrease in

the blood chloride concentration with time of exposure.

In contrast to urine sodium, the urine chloride is blood isoionic in salinities of 75% and greater, while remaining slightly hyperionic in the lower salinities (Figure 9 and Table 2).

Potassium

On the whole, blood potassium is regulated in a similar fashion to blood chloride and sodium (Figure 10). Cancer magister regulated blood potassium hyperionic to the medium in salinities of 75% sea water and below, while remaining isoionic in the higher salinities (100% and 125%).

Some maintenance of steady state conditions in blood potassium is shown in Figure 6. The blood potassium in low salinities (25% and 50% sea water) appears to approach an equilibrium with respect to these media. At higher salinities (75% and 100% sea water) there is wide oscillation of blood potassium concentrations over a 4-day period.

Urine potassium is regulated in a similar manner to urine sodium. The urine is consistently hypoionic to the blood in 75%, 100% and 125% sea water ($P < .01$) after both 24 and 48 hours (Figure 10 and Table 2). The U/B ratios (Table 2) confirm this observation.

Calcium

Blood calcium is observed to be hyperionic to the medium (Figure 11). In contrast to other ion data, the blood:medium gradient for calcium is greater at 48 hours than at 24 hours. This suggests better regulation of this ion. At all test salinities, C. magister maintains urine calcium hypoionic to the blood ($P < .05$).

Measurements over several days show the blood calcium values to be maintained at a fairly constant level in 50%, 75% and 100% sea water. The blood calcium levels fall sharply in 25% sea water at 24 hours, but are regained to what might be new levels at 48 hours. However, a high mortality results about this time and prevented further measurements.

Blood Volume

Blood volume measurements are presented in Tables 4 and 5, and Figures 12 and 13. A representative time course curve showing the dilution of inulin-C^{14} for blood volume determinations is shown in Figure 12. Measurements are based on the equation given in the Materials and Methods, p. 16.

A linear relationship is shown between the blood volume (ml) and the body weight (g) of crabs exposed to 100%, 75% and 25% sea water. This relationship is described by the three regression

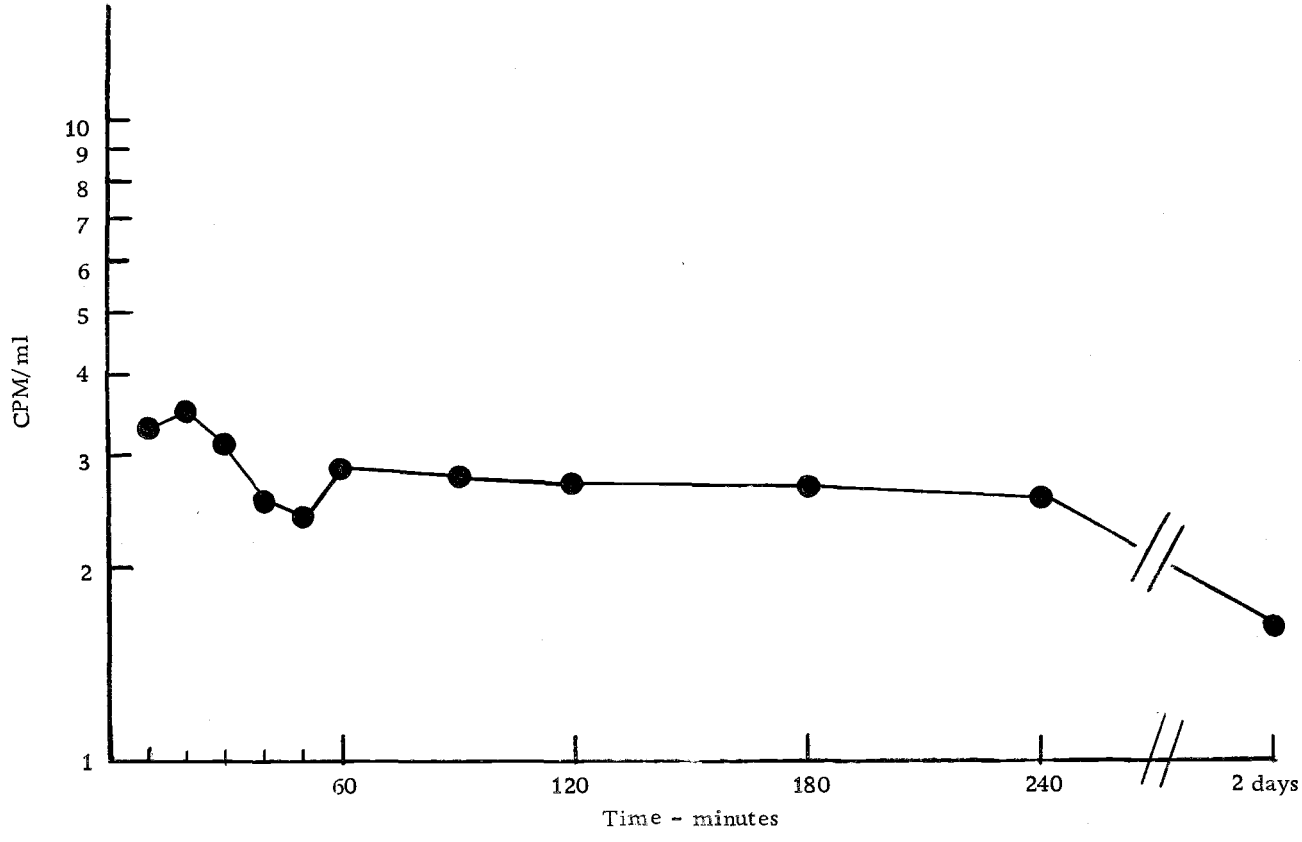

Figure 12. Representative time course dilution curve of inulin-C^{14} for blood volume determination. A semi-logarithmic plot of CPM/ml of inulin-C^{14} with time (minutes) for Cancer magister. The curve is extrapolated to zero time to obtain the CPM/ml for instantaneous dilution.

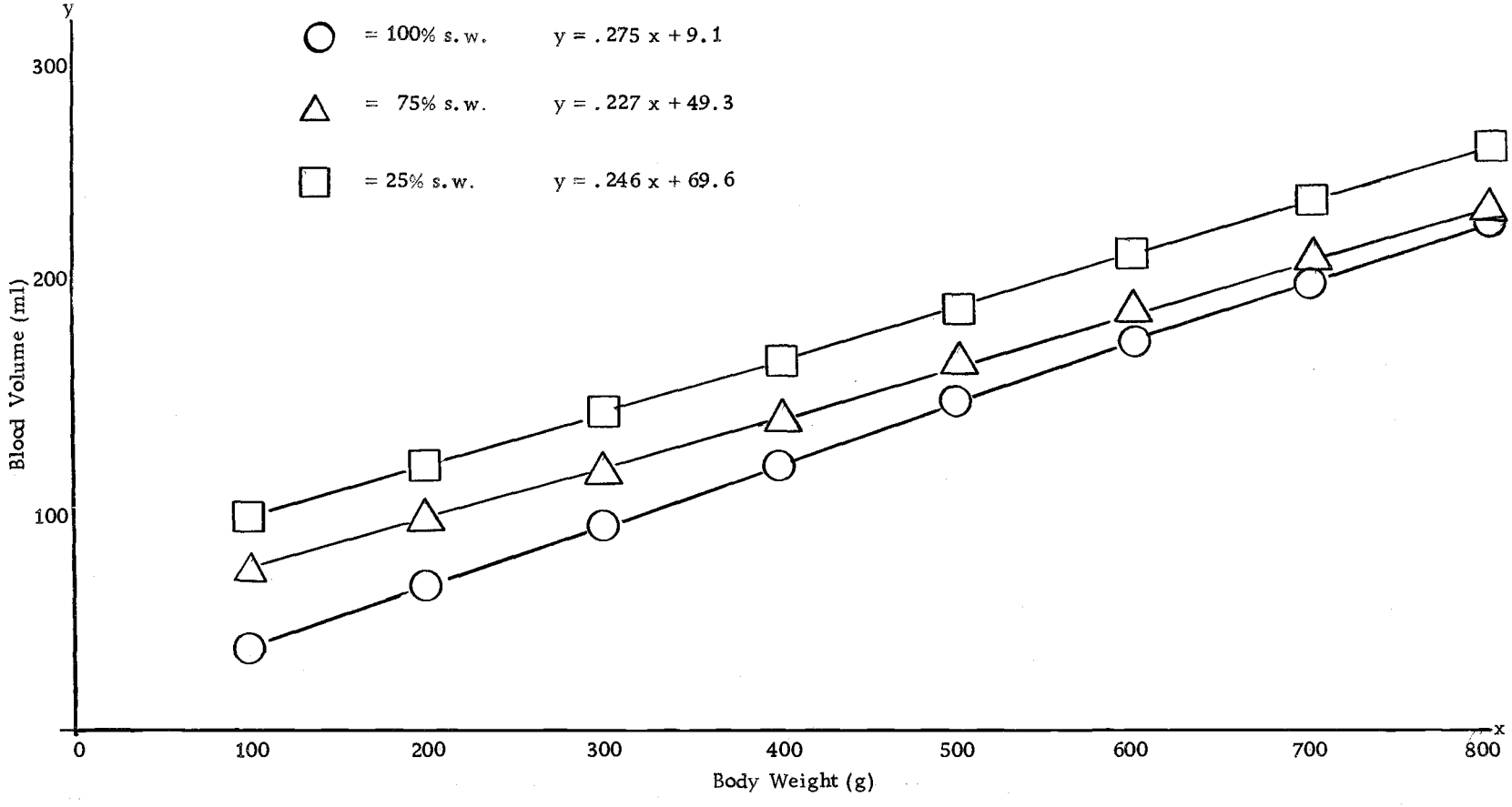

Figure 13. Regression line of blood volume (ml) on body weight (g) in Cancer magister at various salinities.

Table 3. Blood volume measurements of Cancer magister over a range of salinities.

Medium	n	weight range (g)	mean blood volume (% body weight)
100%	11	146-834	27.61 ± 1.16
75%	11	259-679	35.01 ± 4.24*
50%	10	266-559	35.36 ± 6.19 n.s.
25%	12	264-565	39.87 ± 2.08**

*P <.05

**P <.01

Blood volume expressed as % body weight $= \frac{\text{ml}}{\text{body weight}} \times 100$

Values are means ± S.E.

Table 4. Regression of blood volume (ml.) on body weight (g) in Cancer magister at various salinities.

Medium	regression equation	r	F value	degrees of freedom
100%	y = .275x + 9.1	.76	12.4	1.9
75%	y = .227x + 49.3	.56	4.0	1.9
50%	**********	.38	--	--
25%	y = .246x + 69.6	.58	117.0	1.21

equations presented in Table 4. The analysis of covariance of these linear regression equations shows a significant difference ($P < .01$) between measurements in 100% and 75% sea water, and a significant difference ($P < .01$) between measurements in 75% and 25% sea water. Animals stressed to 25% sea water increase their blood volume over the control 100% sea water value. The difference between mean blood volumes for crabs at 100% and 25% sea water is significant ($P < .01$). The average values obtained for Cancer magister are similar to those reported for Carcinus, namely 32.6 ml/100 g animal (Robertson, 1960).

Because the variation among the blood volume data collected from crabs at 50% sea water is large and the range of body weights narrow (Table 4), a low correlation coefficient was obtained for crabs exposed to this salinity (Table 5), and a regression analysis was not done.

Muscle Analysis

The measurements of muscle ions, water content, total alpha amino nitrogen and extracellular space were performed on Cancer magister at each of the experimental salinities. The flexor and extensor muscles of the meriopodite of the first pair of walking legs were used. Samples were obtained after 24 and 48 hours exposure to each salinity stress.

Water Content

Dry-wet weight determinations were made at each salinity in order to ascertain the amount of water in the muscle tissue under each condition. The 24 hour values reported in Table 3A show a significant increase ($P < .05$) in hydration of muscle fibers at reduced salinities (50% and 25% sea water) - values of 79.7% to 79.5% muscle water- compared with 77.6% in 100% sea water. The 48 hour muscle samples from crabs at 75% and 25% sea water clearly show an increased hydration (Table 3B).

In 125% sea water, mean muscle water values show a slight decrease from the normal (100% sea water) values 77.6% (24 hours) and 75.4% (48 hours) to 76.7% and 74.2% respectively. The differences are not statistically significant.

Muscle Ions and Total Alpha Amino Nitrogen

The mean values of muscle ions, expressed as mEq/kg muscle water, and the corresponding blood values, expressed as mEq/l, are presented in Tables 6 and 7. Table 6 gives the results obtained from crabs exposed for 24 hours at each salinity, while Table 7 shows corresponding results for 48 hours.

The ion concentrations in muscle appear to decrease in dilute sea water (75% and below) and to increase above 100% sea water values at the higher salinities (125% sea water). Both muscle sodium

Table 5. Water content of Cancer magister muscle tissue after exposure to each experimental salinity for 24 hours (A - top) and 48 hours (B - bottom).

A 24 hours

Medium	n	% Dry Weight	% Muscle H_2O*
125%	4	23.32 ± .19	76.68
100%	10	22.39 ± .47	77.61
75%	10	21.56 ± .39	78.44
50%	14	20.25 ± .44**	79.75
25%	14	20.46 ± .16**	79.54

B 48 hours

Medium	n	% Dry Weight	% Muscle H_2O*
125%	4	25.77 ± .18	74.23
100%	12	24.65 ± .77	75.35
75%	12	22.29 ± .30**	77.71
50%	4	23.69 ± .19	76.31
25%	8	16.78 ± .76**	83.22

*% Muscle water is obtained by difference

** $P < .05$.

Table 6. Blood and muscle tissue ion concentrations after 24 hours exposure of Cancer magister to various salinities.

Medium		Cl^-	Na^+	K^+	Ca^{++}
125%	Blood	605 ± 12	563 ± 10	10.8 ± 1.0	---
	Muscle	149.9 ± 5.9	134.2 ± 15.2	148.5 ± 2.1	---
	Blood:tissue ratio	4.03	4.20	0.074	
100%	Blood	510 ± 7.2	492 ± 12	10.1 ± 0.9	37.9
	Muscle	93.2 ± 4.1	71.9 ± 3.5	146.8 ± 2.5	27.3 ± 2.6
	Blood:tissue ratio	5.48	6.83	0.068	1.39
75%	Blood	430.3	402.6	9.1 ± 0.4	40.6
	Muscle	75.3	54.5 ± 1.9	131.0 ± 2.9	20.4 ± 0.8
	Blood:tissue ratio	5.73	7.27	0.069	1.99
50%	Blood	320 ± 13	323 ± 10	6.6 ± 0.4	36.1
	Muscle	47.3 ± 2.2	44.6 ± 3.4	113.3 ± 1.8	15.8 ± 1.5
	Blood:tissue ratio	6.81	7.18	0.058	2.28
25%	Blood	244.4 ± 12.4	254.0 ± 12.5	4.4 ± 0.5	25.8
	Muscle	41.2 ± 1.6	44.6 ± 4.6	113.9 ± 15.2	18.3 ± 1.2
	Blood:tissue ratio	5.81	5.64	0.039	1.41

Mean blood in concentrations expressed as mEq/l ±S. E. Muscle ion concentrations expressed as mEq/kg muscle water g ± S. E. Each value represents the mean of 10 animals.

Table 7. Blood and muscle tissue ion concentrations after 48 hours exposure of Cancer magister to various salinities.

Medium		Cl^-	Na^+	K^+	Ca^{++}
125%	Blood	567 ± 15	533 ± 12	13.0 ± 0.9	---
	Muscle	133.6 ± 4.5	102.5 ± 8.5	124.9 ± 9.2	---
	Blood:tissue ratio	4.23	5.17	0.104	
100%	Blood	503 ± 11	461.7 ± 9.9	10.5 ± 0.3	39.2
	Muscle	109.2 ± 5.0	77.1 ± 6.9	156.4 ± 1.2	26.8 ± 4.6
	Blood:tissue ratio	4.61	5.98	0.067	1.46
75%	Blood	441.8 ± 10.4	405.0 ± 16	8.9 ± 0.4	42.1
	Muscle	69.9 ± 5.4	59.4 ± 4.4	125.7 ± 7.7	24.7 ± 3.4
	Blood:tissue ratio	6.39	6.82	0.071	1.70
50%	Blood	285.5 ± 14	302.2 ± 10	6.4 ± 0.6	36.1
	Muscle	47.5 ± 2.8	46.5 ± 4.4	112.2 ± 1.3	17.3 ± 2.6
	Blood:tissue ratio	6.01	6.49	0.057	2.09
25%	Blood	181 ± 14.9	199.5 ± 8.7	3.6 ± 0.5	34.4
	Muscle	45.7 ± 2.3	63.3 ± 3.7	90.2 ± 7.9	20.4 ± 2.6
	Blood:tissue ratio	3.96	3.15	0.039	1.69

Mean blood in concentrations expressed as mEq/l ± S. E. Muscle ion concentrations expressed as mEq/kg muscle water g ± S. E. Each value represents the mean of 10 animals.

and chloride, for example, decrease in dilute sea water from their values in 100% sea water. The increase in blood:tissue ratios for these ions in both 75% and 50% sea water suggest that the muscle ions do not decrease in proportion to the decrease in blood ion concentrations. On the other hand, in 25% sea water the blood:tissue ratios for sodium and chloride decrease. The decrease in blood:tissue ratio after 48 hours exposure in 25% sea water falls below the control values, and suggests a greater proportional decrease in both blood and muscle ion concentrations.

A decrease in muscle potassium is found with a reduction in salinity. The blood:tissue ratio for potassium decreases in both 50% and 25% sea water, and suggests that the blood concentration of potassium falls faster than its tissue concentration. Some regulation of muscle potassium therefore is apparent in dilute sea water (50% and 25%) (Figure 14). Although some reduction in muscle potassium is evident in 75% sea water, the blood:tissue ratio shows little change from that in the control medium.

The blood:tissue ratio for muscle potassium increases in crabs stressed in 125% sea water for both 24 and 48 hours. This increase might indicate some leakage of potassium out of the cells, as shown by the decrease in potassium concentration from crab muscle obtained after 48 hours; crabs exposed for 24 hours, on the other hand, show a slight increase in muscle potassium.

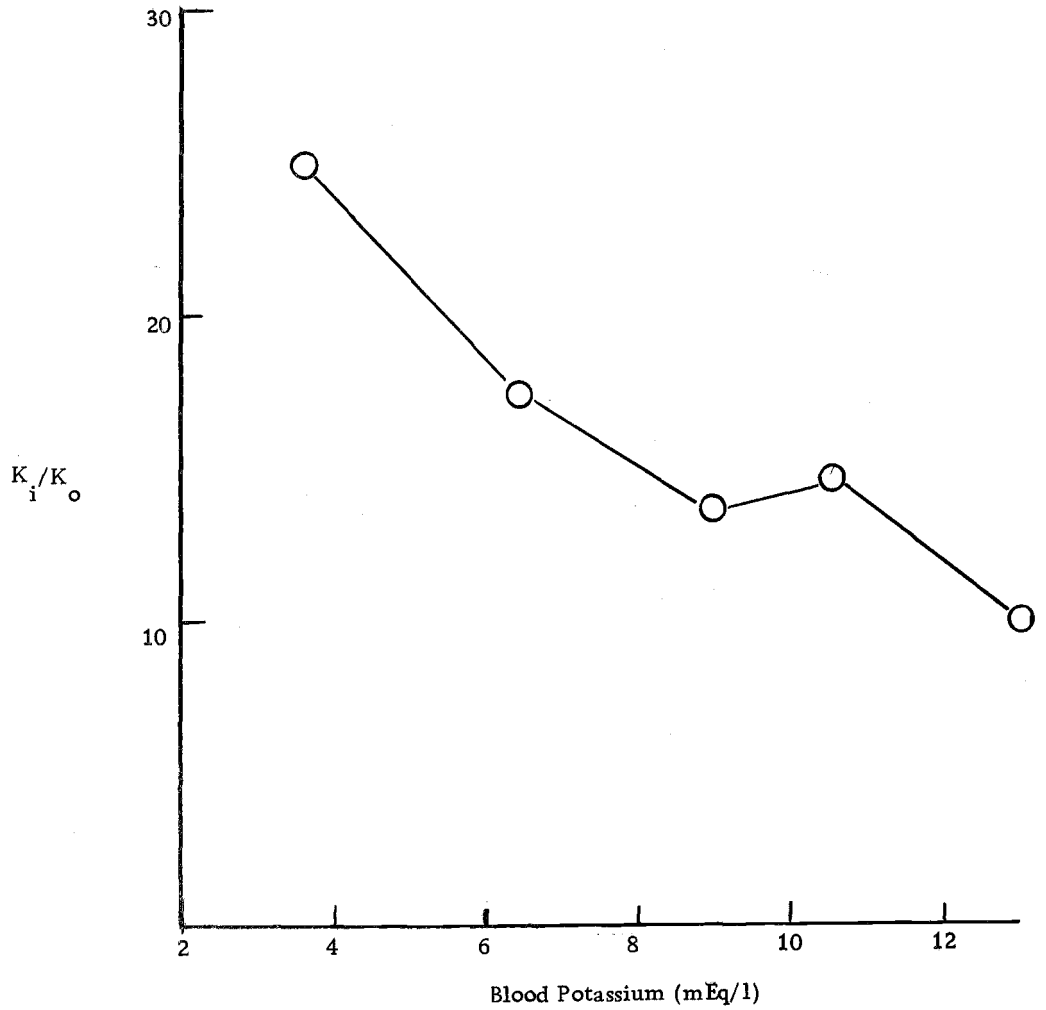

Figure 14. Ratio of muscle potassium concentration (K_i) to the blood potassium concentration (K_o) plotted against blood potassium concentration (mEq/l). Values used for this calculation are from Table 7.

At all salinities, the changes in muscle calcium are similar to those of muscle sodium and chloride. In both 75% and 50% sea water a reduction in muscle calcium is shown with a concomitant increase in the blood:tissue ratio. Muscle calcium from crabs in 25% sea water shows a decrease in the blood:tissue ratio which is similar to the pattern exhibited by muscle sodium and chloride.

The results for muscle total alpha amino nitrogen are presented in Table 8 where the values are expressed as mM/kg muscle water. These values for total alpha amino nitrogen also include compounds which react with ninhydrin such as ammonia, urea, and taurine.

The total alpha amino nitrogen (ninhydrin-positive substances) of muscle does not differ in animals from 100% and 75% sea water after 48 hours. There is, however, a significant decrease after 24 hours ($P < .05$). Significantly lower values were obtained in crabs in 25% and 50% sea water ($P < .01$) compared to 100% sea water. A substantial increase in muscle amino nitrogen is found in crabs in 125% sea water ($P < .01$). This amounts to a 45% increase over the 100% sea water value after 24 hours and is twice the value of 100% sea water after 48 hours exposure.

The results of the addition of muscle osmotically active components (total muscle ions plus amino nitrogen) are shown in Table 9 at each salinity. The sum of muscle components is not comparable to the blood osmotic concentration and accounts for approximately

Table 8. Total free alpha amino nitrogen concentrations (mM/kg muscle water) in muscle tissue of C. magister at various salinities.

Medium	24 hours	48 hours
125%	414 ± 15**	600 ± 14**
100%	284 ± 21	290 ± 9
75%	243 ± 13*	290 ± 11 n.s.
50%	186 ± 12**	218 ± 13**
25%	132 ± 15**	179 ± 11**

*P < .05

**P < .01

n.s. not significant

Table 9. Addition of osmotically active components.

A Medium (% sea water)	(A) Muscle ions + amino nitrogen (mM/kg muscle water)	(B) Blood osmotic concentration (mOsmoles/l)	Difference B-A
125	846.6	1090	243.4
100	623.2	980	357
75	524.2	865	340.8
50	406.0	735	329
25	350.0	610	260

B 48 hours Medium (% sea water)	A Muscle ions + amino nitrogen (mM/kg muscle water)	B Blood osmotic concentration (mOsmoles/l)	Difference B-A
125	961.0	1080	119.0
100	659.32	978	318.7
75	568.9	830	261.1
50	441.5	695	253.5
25	398.6	525	126.4

Table 10. Ratio of the sum of the muscle ions and amino nitrogen to the blood osmotic concentrations in *C. magister* at various salinities.

Medium	24 hours	48 hours
125%	0.78	0.89
100%	0.64	0.67
75%	0.61	0.69
50%	0.55	0.64
25%	0.57	0.76

50% of the osmotic concentration. Some osmotically active particles however were not accounted for in this study. Muscle magnesium, phosphate compounds and total nitrogen were not determined, for example, and these might at least partially account for the discrepancy. This is discussed later.

The deviation between the blood and muscle tissue decreases with dilution of sea water, and is less in crabs exposed for a longer time (48 hours), which suggests an attempt to maintain a blood:tissue osmotic equilibrium.

Muscle Extracellular Space

Measurements of the muscle extracellular space were made using two methods: the inulin-C^{14} distribution between the whole muscle and blood (Table 11) and the chloride space (Table 12). The inulin-C^{14} calculations are outlined in the Materials and Methods section. In calculating the chloride space, it was assumed that all the chloride ions were extracellular. Since a small amount of chloride is found intracellularly, this assumption will lead to an over estimation of the calculated chloride extracellular space. The chloride space is obtained by the following expression:

$$\text{Chloride space} = \frac{(Cl^- \text{ muscle})}{(Cl^- \text{ blood})} \times 100$$

Table 11. Inulin muscle extracellular space.

Medium	n	Hours incubated	$\frac{ml}{100\ g\ tissue\ H_2O}$	$\frac{ml}{100\ g\ tissue\ wet\ wt.}$
100% s.w.		12	7.34	5.54
		20	4.51	3.43
75% s.w.		12	11.25	8.93
		20	7.25	5.84
50% s.w.		12	8.29	6.56
		20	6.85	5.43
25% s.w.		12	16.79	13.26
		20	15.67	12.37

Inulin space is defined as:

$$\text{MEXS (ml)} = \frac{\text{CPM Total Tissue}}{\text{CPM/ml Blood}}$$

$$\%\ \text{MEXS (ml/100 g } H_2O) = \frac{\text{ml}}{\text{total tissue water}} \times 100$$

Total tissue water is determined by the wet weights of the muscle times the $\%\ H_2O$ calculated from the difference of wet and dry weights.

Table 12. Chloride muscle extracellular spaces.

Medium	Chloride Spaces* 24 hrs.	48 hrs.
25%	16.84	25.24
40%	14.75	16.63
75%	17.49	15.64
100%	18.27	21.70
125%	24.77	23.56

*Chloride Space is defined as:

$$\% \text{ Chloride Space} = \frac{(Cl^- \text{ muscle})}{(Cl^- \text{ blood})} \times 100$$

where Cl^- muscle = mEq/kg muscle H_2O

and Cl^- blood = mEq/L.

The Cl^- values are from the averages in Tables 6 and 7.

The chloride space is based on the assumption that all the chloride is extracellular.

Table 13. Muscle ion concentrations corrected for interfiber water.*

A. 24 hours exposure to each experimental salinity

Medium	Cl^-	Na^+	K^+	Ca^{++}
125% s.w.	114	100	158.5	--
100% s.w.	60.2	38.6	157.5	26.3
75% s.w.	30.2	10.13	146.5	17.8
50% s.w.	22.5	19.4	122.5	13.9
25% s.w.	3.56	5.45	136.0	16.9

B. 48 hours exposure to each experimental salinity

Medium	Cl^-	Na^+	K^+	Ca^{++}
125% s.w.	99.0	68.3	133	--
100% s.w.	76.5	46.4	167	25.6
75% s.w.	22.4	15.5	141.5	22.6
50% s.w.	25.8	23.4	127	15.5
25% s.w.	20.5	37.9	106.5	17.8

*Values expressed as mEq/kg cell water.

The blood and muscle chloride values from Tables 6 and 7 were used for this calculation.

The chloride space values are given for comparison to the inulin space values. Robertson (1961) also compared these values and observed the chloride space to be about twice the inulin space in lobster muscle (Nephrops). If there were no chloride in the cells, this value would represent the % volume of muscle water which is extracellular (i.e. interfiber space). The chloride space values (18%-21%) for C. magister in 100% sea water are similar to those reported by Robertson (1961) in Nephrops muscle (20%). The inulin extracellular space in C. magister of 7.3% in 100% sea water is lower than for the lobster, Nephrops (12%); and considerably lower than that for the horseshoe crab, Limulus (22.8%); and for the blue crab, Callinectes (17.4%) (Robertson, 1961, 1970; Hays et al., 1969). The reason for the discrepancy between the inulin space values for C. magister and those for Nephrops (in contrast to the similar chloride space values) is not clear. It should be noted, however, that radioactive inulin-C^{14} was used in the present study, while Robertson (1961, 1970) used non-radioactive inulin and a chemical method of determining the inulin concentration.

Extracellular (inulin) space measurements were made on C. magister muscle at all salinities except 125% sea water. These values (Table 11) show a general increase in the inulin space from

100% to 25% sea water with the exception of 50% sea water, in which the value decreased. Therefore, the practice of some workers who measure the space at one salinity condition and use that value for all experimental conditions is questionable.

DISCUSSION

The results of this study show that the Dungeness crab is capable of hyperosmotic regulating in dilute sea water and, shows degree of hypoosmotic regulation in concentrated sea water. Crabs in normal sea water maintain a blood concentration isoosmotic to the medium.

In addition to analyzing osmotic and ionic regulation in blood and urine, this study also provides data on ion concentrations in muscle tissue in crabs stressed to various salinities. The muscle measurements show that increases occur in tissue water content as well as interfiber water when crabs are placed in dilute sea water. Concomitant reductions in muscle ion concentrations occur in response to decreases in blood ion concentrations. Muscle tissue thus appears to tolerate some fluctuations in its intracellular osmotic concentrations.

Hyperosmotic Regulation

Hyperosmotic regulation has been shown to occur widely in decapods occupying brackish water (Lockwood, 1962; Potts and Parry, 1964). Animals placed in a dilute medium (hypoosmotic medium) may gain water and lose salt to osmotically adjust. Any maintenance of a steady state level greater than the medium would

involve elimination of excess water, and either salt retention or active uptake from the medium against a concentration gradient.

The data from this study (Figures 1-3) and those of Jones (1941) indicate that Cancer magister is capable of hyperosmotic regulation in dilute media. The values obtained by Jones are limited to three measurements of blood osmotic concentration in dilute sea water, and are presented below for comparison with data from the present study:

% sea water	Jones' study		Present study	
	medium (mOsm)	blood (mOsm)	medium (mOsm)	blood (mOsm)
40	419	615	--	--
50	522	662	500	735
75	775	873	750	865
100	1005	--	1000	980

From limited data Jones concluded that C. magister can maintain its blood hyperosmotic in brackish water, and further assumed that this species in oceanic (100%) water would be isosmotic to the medium. The present study confirms both of these conclusions and further extends the limits of hyperosmotic regulation to include salinities as low as 25% sea water (Figures 1 and 2). The limited survival of crabs in 25% and 50% sea water, however, indicates that they are not able to tolerate these conditions for an extended period

of time when acutely stressed to the salinities (Figure 3). The acute nature of the stress may impose too large a burden on the osmoregulatory mechanisms, whereas a step-wise dilution might permit gradual adjustments to occur without overtaxing the regulatory mechanisms. A step-wise dilution would also approach more closely the normal sea water fluctuations. Recent evidence (Ballard, 1969) suggests that exposure of Callinectes to gradual changes in salinity results in the crab being able to acclimate and maintain higher levels of blood osmotic concentration. This trend was more pronounced when a crab was transferred from a higher salinity to a lower one than in the reverse transfer.

Measurements of the blood osmotic concentrations in species closely related to Cancer magister show a variety of regulatory patterns. Cancer antennarius and Cancer gracilis, for example, are found to be isosmotic at all concentrations of sea water tested and are classified as non-regulators or osmoconformers (Jones, 1941; Gross, 1957b, 1964). This pattern probably reflects the nature of its habitat. This subtidal species is rarely exposed to changes in salinity (Jones, 1941; Gross, 1964). In contrast to the above osmoconformers, good hyperosmotic regulation is shown by Cancer pagurus (Krogh, 1939), Carcinus maenus (Krogh, 1939; Potts and Parry, 1964), and Callinectes sapidus (Ballard, 1969). All three species regulate

their blood hyperosmotic in dilute media. Callinectes can regulate hyperosmotically in 5-10% sea water and inhabits a more estuarine environment than the other species (Ballard, 1969; Krogh, 1939; Prosser and Brown, 1961).

The urine osmotic concentration in Cancer magister is slightly hypoosmotic to the blood in the range of salinities imposed (Figures 1 and 2). Statistical analysis, however, shows it is not significantly different from the blood. Thus there is, some indication of a weak salt conservation role attributed to the kidney. Urine isosmotic to the blood has been demonstrated in several decapod crustaceans; for example, Carcinus maenus (Nagel, 1934) and Hemigrapsus nudus (Dehnel and Stone, 1964; Alspach, 1967, MS thesis, OSU). In addition, Shaw (1961) reports that Carcinus increases urine production by a factor of two when crabs are transferred from 100% to 50% sea water. This indicates the kidney is capable of excreting large amounts of water through the urine while remaining blood isosmotic and thus not conserving salts. It would be interesting to determine urine production rates in Cancer magister at various salinities.

The results for blood and urine ion concentrations are presented in Figures 8 to 11. The data show that the blood sodium, potassium, chloride and calcium are regulated hyperionic to the medium in dilute sea water. The blood:medium gradients rise during the first 24 hour period of stress, but decline over the next 24 hours. The ion

concentrations continue to remain higher than the media. Further, the patterns of blood sodium, potassium and calcium, when followed over several days, suggest the crabs are approaching a steady state with respect to these ions (Figures 4, 6 and 7). This contrasts to the apparent lack of attainment of a steady state for blood osmotic concentration. On this basis, it appears that C. magister regulates its blood ion concentrations better over a long term than its blood osmotic concentration. Cancer magister maintains a lower blood: medium gradient for sodium and chloride compared to Uca, Pachygrapsus, and Eriochier (Prosser et al., 1955; Gross, 1958; Shaw, 1961). Results for C. magister, however, are similar to those obtained by Shaw (1961) for Carcinus, by Nagel (1934) for Cancer pagurus, and by Robertson (1970) for Limulus. The blood ionic concentrations of C. magister would suggest that this species is a weak ionic regulator.

Urine ion concentration measurements suggest the antennary gland plays a major role in preventing salt loss in C. magister. After 48 hours, urine sodium, potassium, and calcium are all blood hypoionic in salinities of 75% and 100% sea water. Urine sodium is also hypoionic in 50% sea water, while urine calcium is hypoionic in both 25% and 50% sea water. Renal reabsorption of some ions is thus indicated. The urine chloride does not show the same pattern, however, being blood hyperionic in salinities of 25% and 50% and

isoionic in 100% and 75% sea water. In contrast to urine sodium, urine chloride appears to be eliminated by the kidney in dilute sea water, suggesting, perhaps, an attempt to maintain some internal sodium:chloride balance.

The weak blood hyperionic regulation in C. magister suggests an inadequate uptake mechanism which cannot maintain a large concentration gradient between the animal and its medium. Thus, the burden of ionic adjustment appears to be handled by the reabsorption of ions in the kidney which, in turn, aids in the maintenance of some blood homeostasis.

Hypoosmotic Regulation

The maintenance of a blood hypoosmotic to the external medium has been shown in a limited number of decapod crustaceans which inhabit either a semi-terrestrial or hypersaline environment (Jones, 1941; Gross, 1964). Hypoosmotic regulation in a concentrated medium might prevent excessive concentration of salt in the blood and thereby reduce the osmotic burden imposed. This would be of adaptive significance.

The results of the present study indicate there is some blood hypoosmotic regulation by Cancer magister in 125% sea water (Figures 1 and 2). This appears to be the first account of hyporegulation by this species and any member of the family Cancridae. Reports on

related species (Cancer pagurus and Cancer antennarius) show the the blood to be isosmotic in hypersaline media (Gross, 1964). Any adaptive significance of hypoosmotic regulation in C. magister is unclear. The upper limit of the salinity range to which this crab is naturally exposed is 100% sea water.

Urine osmotic concentration in C. magister is regulated blood hypoosmotic for crabs exposed to 125% sea water (Figures 1 and 2). Again, the significance of a blood hypoosmotic urine in a hypersaline environment is unclear since a hyperosmotic medium would present the problem of salt gain and water loss.

In 125% sea water, the pattern of blood sodium, potassium and chloride regulation suggests slight hypoionic regulation (Figures 8, 9 and 10). It is of interest, however, to observe the urine ion concentrations. Urine sodium and potassium are significantly blood hypoionic in both 100% and 125% sea water after 24 hours, but only urine potassium remains significantly hypoionic ($P < .01$) after 48 hours in 125% sea water (Figures 8 and 10). The pattern for urine sodium and potassium suggest some reabsorption of these ions in concentrated sea water. Urine chloride, however, is isoionic to the blood in 100% and 125% sea water (Figure 9).

Crabs were exposed to 125% sea water for a total of 48 hours. It is possible that longer exposure, as used in the time-course studies in diluted sea water (Figures 4-7) would show the attainment of a

steady-state at different osmotic and ionic concentration levels.

In salinities more concentrated than normal sea water, reduced sodium concentrations in the urine have been shown to be accompanied by large increases in urine magnesium concentration. This has been well-documented in several grapsid crabs; namely, Pachygrapsus (Prosser et al., 1955; Gross and Marshall, 1960), Uca (Green et al., 1959) and Hemigrapsus nudus (Dehnel and Carefoot, 1965; Alspach, 1967, MS thesis, OSU). It has been suggested that magnesium excretion may compensate osmotically for sodium retention (Gross and Marshall, 1960). Unfortunately, magnesium was not determined in the present study and any interaction between sodium and magnesium changes in the urine cannot be discussed.

Some animals which are capable of hypoosmotic regulation have been shown to drink sea water and remove water from it. Dall (1967) has established that water drinking occurs in such marine crustacea as: Metapanaeus, a penaeid shrimp; Metapograpsus, a grapsoid crab; and Macropthalmus, an ocypodid crab. Further, he showed that salt is removed via the gut of these hypoosmotically regulating crustaceans. This route of water intake and salt loss could possibly explain the present findings, namely, a hypoosmotic blood in C. magister exposed to 125% sea water. In 125% sea water, the excess salt would be removed and the water retained. Future experiments must be performed to test this conclusion.

Tissue Regulation

In discussing the adaptation of muscle cells to various blood concentrations, it is assumed that osmotic equilibrium exists between the intracellular and extracellular (blood) compartments. With a decrease in blood osmotic concentration, as previously discussed, changes in muscle water content, as well as reductions in the intracellular ion concentrations, could be made which would maintain the osmotic equilibrium between the cells and the blood. An alternative mechanism would be the reduction of non-permeating but osmotically active components in the muscle, thereby reducing the intracellular osmotic concentration and maintaining an osmotic balance between the intracellular and extracellular compartments. In fact, a combination of both probably is used to achieve the balance.

In Cancer magister some hydration of muscle tissue occurs in dilute sea water (Table 5). This hydration is significant ($P < .05$) for crabs in 25% sea water after 48 hours exposure. Similar results have been shown for Carcinus muscle fibers in 20% sea water (Shaw, 1955b). Shaw (1955b) attributed this change in water content to two causes: (1) water entering the fibers causing them to swell or (2) intracellular reduction in diffusible substances or reduction in non-permeating, but osmotically active, particles. Lange (1968) suggests that volume regulation involves increased hydration in muscle tissue (when

measured as gH_2O/g wet wt.) accompanied by decreasing intracellular osmolarity. Furthermore, Lange (1968) states that volume regulation requires varying lengths of time dependent upon the salinity stress imposed. C. magister does not survive long enough in 25% sea water to regulate its tissue volume.

An alternative experimental approach to measuring muscle water content would be to observe directly any volume changes in the muscle fibers which result from increased hydration. This would involve calculating the fiber volume by measuring changes in fiber diameters (Lang, 1969). With this technique, Lang (1969) has observed in vitro volume readjustment of muscle fibers from Callinectes in hypotonic saline. These adjustments are dependent upon the level of external sodium. Volume readjustment is suggestive of isosmotic intracellular regulation in which cellular hydration is kept constant as the cells adjust to changes in the blood osmotic pressure. This phenomenon has been observed in many cases of euryhaline marine invertebrates (Lange, 1968; Florkin and Schoffeniels, 1970).

Water movements can also be assessed by examining changes in the total extracellular space or blood volume of an animal. The present study clearly indicates an increase in mean blood volume in C. magister transferred from 100% to 25% sea water (Table 4). This increase might be explained in part by exoskeletal permeability,

since this is one avenue through which crabs may increase their water content. Although this parameter was not measured in C. magister, it is interesting to examine results for closely related decapod crustaceans. Gross (1957) has measured the relative permeability of the exoskeleton in various decapods which exhibit different regulatory capacities. In Cancer gracilis and Cancer antennarius, both non-regulators, the permeability of the exoskeleton was greater than in Cambarus spp. and Pachygrapsus, both good hyperosmotic regulators. If the exoskeleton of Cancer magister were to possess permeability properties similar to its close relatives C. gracilis and C. antennarius, then one might expect an increased permeability in dilute salinities. Cancer magister in 75%, 50% and 25% sea water shows an increase in blood volume over control animals. Those animals in 25% sea water had a 42.8% increase in blood volume, but there is a high mortality at this salinity. This probably is of little adaptive value.

Changes in the intracellular ion concentrations may occur when the blood ion concentrations decrease. All muscle ions in C. magister were found to decrease in crabs exposed to dilute media. There is, however, an indication that this crab is capable of regulating some muscle ions (Tables 6 and 7). Thus, a decrease in the blood:tissue ratio would suggest some degree of regulation by the muscle fibers. Changes in the blood:tissue ratio of ions may be associated with

changes in cell permeability. The present results show muscle sodium and chloride are not regulated as indicated by the increased blood:tissue ratios of crabs in 75% and 50% sea water. Some regulation of muscle sodium and chloride by crabs in 25% sea water is apparent; the blood:tissue ratio decreases for both of these ions. On the other hand, the blood:tissue gradient for potassium decreases in the lower salinities (Table 7, Figure 14). This indicates that the muscle tissue maintains a fairly consistent level of intracellular potassium in dilute sea water. Possible explanations for this occurrence might involve a Donnan equilibrium or a potassium transport system. From the present study, it is difficult to fully assess which system, if either, operates. However, a rough estimate of a Donnan equilibrium can be made through examination of the following ratio:

$$\frac{[K_i]}{[K_o]} = \frac{[Cl_o]}{[Cl_i]}$$

Equality in this ratio is predicted in a Donnan equilibrium system; but the present data do not support this conclusion: The ion ratios are:

$$\frac{K_i}{K_o} = 15.8\,; \quad \frac{Cl_o}{Cl_i} = 8.5.$$

An alternative suggestion involves transport of potassium into the muscle fibers, an idea also postulated by Shaw (1955b) for muscle fibers at very low external potassium solutions, i.e., 10% and 20% sea water.

Measurements of the membrane potential (E_m) of muscle fibers was 70 mv for Callinectes (Hays et al., 1969) and 58 mv for Carcinus (Shaw, 1955b). With these values, muscle ion concentrations can be predicted for muscle in Cancer magister from the following equation: $E_m = 58 \log \frac{C_o}{C_i}$. Using the 70 mv value, the calculated chloride (C_i) is 32 mEq/kg cell water and the potassium calculated to be 160 mEq/kg cell water. A 58 mv potential gives 51 mEq/kg cell water for chloride and 100 mEq/kg cell water for potassium. Comparisons between these data and the present data show agreement for potassium with 70 mv, and for chloride with 58 mv. In either case, one of the ions is not in equilibrium. For this reason a Donnan equilibrium is not verified. It is of interest to calculate a potential with the present data for comparison. A value of 54 mv is obtained using the chloride ion concentrations in blood and muscle for 100% sea water. This agrees well with Shaw's value of 58 mv, but is lower than the 70 mv measured by Hays et al. (1969).

In concentrated sea water, 125%, the blood:tissue ratio for sodium and chloride decreases compared to normal, while an increase is noted in the ratio for potassium. The data suggest, therefore, that

sodium and chloride are not regulated in 125% sea water, while potassium is regulated.

Amino acids in the tissue have been implicated as a major contributor to the total intracellular osmotic pressure in crustaceans (Duchateau and Florkin, 1955, 1961). In Carcinus, Shaw (1958b) has shown the participation of muscle amino nitrogen compounds in intracellular regulation in dilute salinities. He observed reductions in the concentrations of amino nitrogen compounds in crabs transferred from 100% to 40% sea water. These reductions were greater than could be accounted for by a change in muscle water content only, and thus could account for the reductions in osmotic activity measured in the muscle fibers of Carcinus in 40% sea water.

The data from the present study on C. magister show a decrease in the concentration of muscle amino nitrogen compounds in crabs in dilute salinities. Analysis of the present data using the computations developed by Shaw (1955b, 1958b) can indicate whether the changes observed can be accounted for by a change in muscle water content alone. The calculated values are computed using the % change in water content noted between normal (100% sea water) and any lower salinity. For example, the % increase in muscle water content from 100% to 25% sea water in the present study was 31.9%; the muscle amino nitrogen concentration in 100% sea water was 290 mM/kg muscle water; and the computed amino nitrogen concentration in 25%

sea water was 31.9% less of 290 mM/kg muscle water, or 198 mM/kg muscle water. This computed value represent the expected change in amino nitrogen concentration in 25% sea water based on the change in water content. The actual measured amount was 179 mM/kg muscle water. The difference between the measured and the calculated amount was 19 mM/kg muscle water which represents the loss of amino nitrogen not accounted for by a change in water content. For C. magister, this is a loss of about 6% in the amino nitrogen compounds from the tissue, compared to a loss of 43% in Carcinus as measured by Shaw (1958b). The large reductions in the amino nitrogen compounds shown by Shaw could account for the reduction in the osmotic activity measured in the muscle fibers of Carcinus. However, the 6% reduction noted in C. magister probably does not alter significantly the osmotic activity of muscle cells. Any role of the amino acids as osmotic particles and possibly in Donnan ratios should not be neglected, however.

Direct measurement of intracellular osmotic pressure is extremely difficult and this parameter is almost always estimated indirectly (Lange, 1964). Generally it has been assumed that there is osmotic equilibrium between the intracellular and extracellular compartments. If we accept this assumption, it can be practically used to calculate the change in intracellular osmotic pressure which would be expected from a change in muscle water content alone. For

example, if the measured blood osmotic concentration in crabs at 25% sea water is 525 mOsmoles (Table 9), the muscle would be expected to have a similar value assuming an equilibrium. The new osmotic pressure expected to occur as a result of the change in water content alone however is 672 mOsmoles. A difference of 147 mOsmoles exists between the calculated and measured values. The small reduction (19mM) in amino nitrogen (Table 8) obviously does not contribute significantly toward making up the difference. This is in contrast to Shaw's work on Carcinus where the 43% loss (173 mM) in amino nitrogen did account for the 150 mOsmolar difference in intracellular osmotic pressure not due to the water content alone.

Another means of assessing the intracellular osmotic pressure would be to compare the blood osmotic concentration with the sum of the osmotically active components measured in the muscle. The difference between the two measurements gives some indication of the amounts of osmotically active compounds not measured in the cells. The differences suggest that shifts in some unmeasured components may contribute to the intracellular osmotic concentration (Table 9). Compounds contributing to this change which were not measured in the present study include: magnesium, phosphorus, phosphates, betaine, taurine, trimethylamine oxide, and other nitrogen compounds. Some of these compounds have been shown to

contribute to osmotic changes in the muscle fibers. For example, Robertson (1961) has reported a 10% change in phosphorus in Nephrops muscle; Shaw (1958b) has shown a 15 mMolar change in trimethylamine oxide, a 10 mMolar change in other nitrogen compounds and a change in betaine in Carcinus muscle when in dilute media.

CONCLUSION

When Cancer magister is exposed to a range of experimental salinities (25% to 125% sea water) hyperosmotic regulation and some hypoosmotic regulation is observed. This pattern is similar to the observations made on Cancer pagurus and Carcinus maenus, but unlike that of closely related species, e.g., Cancer antennarius and Cancer gracilis, both osmoconformers. Moreover, C. antennarius is largely found subtidally off shore and not found, at least, within the Yaquina Bay estuary. Further, it would be of interest to study the osmoregulatory ability in the larval forms of C. magister for comparison to the adults.

The ability of C. magister to regulate in dilute salinities is advantageous in an estuarine habitat. Included in this regulatory pattern are changes in the intracellular components. The present results indicate a reduction in most intracellular constituents with a reduction in blood osmotic pressure, an exception being muscle potassium. Thus, changes in the intracellular permeability might allow maintenance of an equilibrium between intracellular osmotic pressure and blood osmotic pressure.

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