ASPECTS OF ANTENNAL GLAND FUNCTION IN THE DUNGENESS CRAB, *CANCER MAGISTER* (DECAPODA, BRACHYURA)

by

CHARLES WALTER HOLLIDAY, JR.

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VITA

NAME OF AUTHOR: Charles Walter Holliday, Jr.

PLACE OF BIRTH: Alexandria, Virginia

DATE OF BIRTH: September 19, 1946

UNDERGRADUATE AND GRADUATE SCHOOLS ATTENDED:

Marietta College, Marietta, Ohio
University of Oregon, Eugene, Oregon

DEGREES AWARDED:

Bachelor of Science, 1968, Marietta College

AREAS OF SPECIAL INTEREST:

Comparative Physiology of Salt and Water Balance in Animals
Invertebrate Zoology
Marine Biology

PROFESSIONAL EXPERIENCE:

Instructor, Department of Biology, Southwestern Oregon Community College, 1978

Graduate Research Fellow, Department of Biology, University of Oregon, 1977

Graduate Teaching Fellow, Department of Biology, University of Oregon, 1973-1976

Graduate Teaching Fellow, Oregon Institute of Marine Biology, Summers of 1974-1976

Marine Science Technician 1st Class, U.S. Coast Guard, 1969-1973

AWARDS AND HONORS:

Beta Beta Beta Freshman Biology Award, Marietta College, 1965
Biology Honors Award, Marietta College, 1968
Teaching Fellowship, University of Oregon, 1973-1976
Research Fellowship, University of Oregon, 1977
Best Paper Award, Western Society of Naturalists, 58th Annual Meeting, 1977

PUBLICATIONS:

"U.S. Coast Guard Sea Surface Temperature Survey", 1970-1973, Commander, Coast Guard Pacific Area, San Francisco, California
"A New Method for Measuring the Urinary Rate of a Brachyuran Crab." Comp. Biochem. Physiol., 1977, 58A: 119-120

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CHAPTER 1

INTRODUCTION

Crustaceans are a diverse, successful group inhabiting marine, estuarine, freshwater and terrestrial environments. The volume and ionic composition of the internal milieu in these invertebrates are, in part, regulated by the antennal gland (Robertson, 1960a; Potts and Parry, 1964; Lockwood, 1967; Riegel, 1972). In marine crustaceans the antennal gland has been implicated in control of hemolymph volume, hyporegulation of magnesium and sulfate ions in the hemolymph, excretion of organic compounds and reabsorption of glucose and amino acids from the urine (reviewed by Riegel, 1972).

Studies of the structure of the crustacean antennal gland date from the middle of the 19th century and are widely scattered through the literature. Marchal (1892) published the first review of decapod antennal gland structure, and several reviews have followed (Burian and Muth, 1924; Fischer, 1925; Balss, 1944; Goodrich, 1944). In recent years the ultrastructure of various parts of the antennal glands of several decapods has been studied (Anderson and Beams, 1956; Kümmel, 1964; Miyawaki and Ukeshima, 1967; Schmidt-Nielsen, et al., 1967; Peterson and Liozzi, 1973).

A general model of invertebrate segmental organs, based on physiological and ultrastructural studies, has been published by Berridge and Oschman (1972) and is shown in modified form in Fig. 1. It is thought that urine is formed at the coelomosac by ultrafiltration of the hemolymph under arterial pressure. This primary urine flows into a highly divided chamber or labyrinth and then into a terminal bladder. In some crustacean species there is an additional structure, the tubule, interposed between the labyrinth and the bladder. There is evidence that the urine is modified by reabsorption and/or secretion by the labyrinth, tubule and bladder of various decapod crustaceans (reviewed by Riegel, 1972).

The role of the antennal gland of crustaceans in hemolymph volume control is well established (Lockwood, 1967). The urinary rates of many crustaceans have been measured with a variety of techniques and under numerous experimental conditions (reviewed by Parry, 1960; Potts and Parry, 1964; Lockwood, 1967). Urinary rates of marine and brackish water crustaceans increase when the salinity of the medium is decreased, and a few workers have shown that there is a direct relationship between the urinary rate and the osmotic differential across the body surface (Werntz, 1963; Hunter, 1973). Although a growing literature exists on the hormonal control of epithelial water permeability in crustaceans

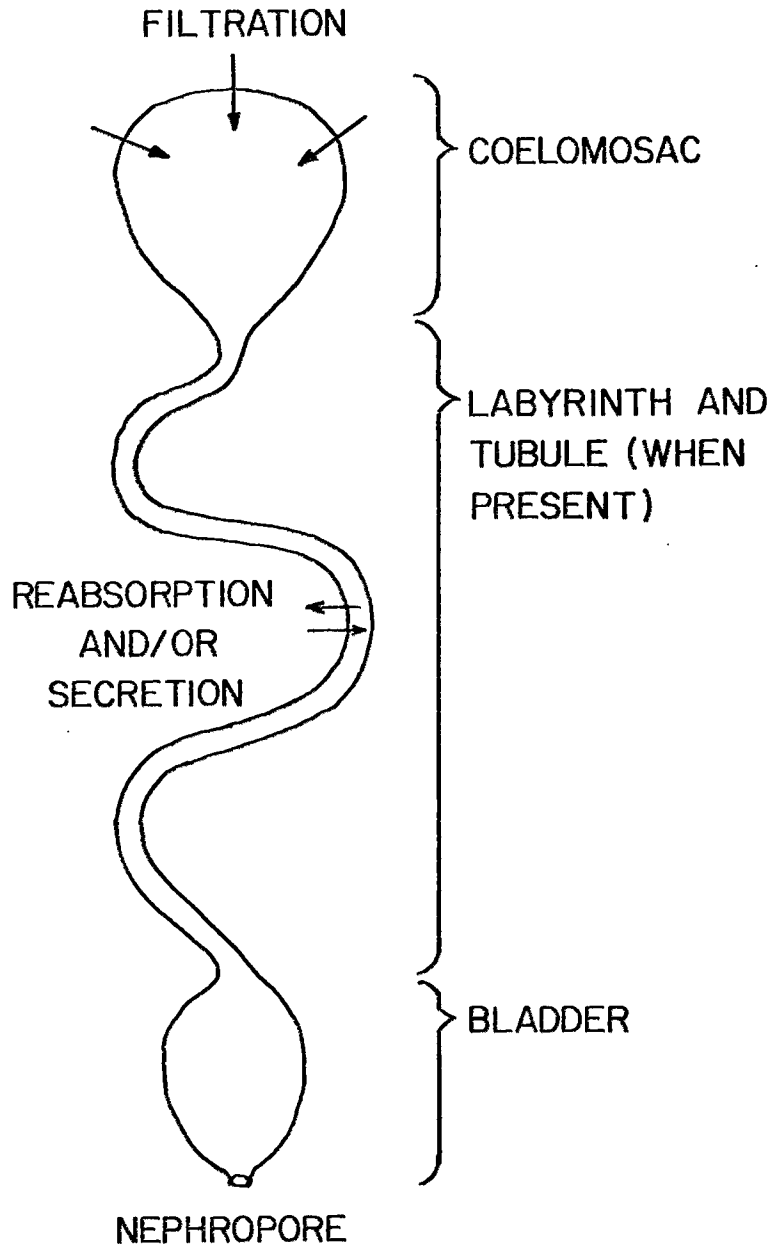


Figure 1. Generalized invertebrate segmental organ. Modified after Berridge & Oschman, 1972.

(reviewed by Fingerman, 1970; Kamemoto, 1976), little work has been done on the mechanism of control of urinary rate per se. Several workers have noted the fact that injection of water or other fluids causes diuresis in macrurans (Burger, 1957; Riegel, 1961; Kamemoto and Ono, 1969) but only one study on the mechanism of control of urinary rate is available (Norfolk, 1976). The crustacean gut has also been implicated as an organ of volume control (Lockwood, 1967, 1970; Dall, 1967; Hannan and Evans, 1973).

Most marine crustaceans regulate hemolymph levels of magnesium (Mg^{++}) and sulfate ($SO_4^{=}$) ions below those of the medium. Urine/serum ratios for these ions are usually greater than one and are further increased in media containing elevated levels of Mg^{++} or $SO_4^{=}$, indicating that the antennal gland is responsible for the observed hyporegulation of Mg^{++} and $SO_4^{=}$ (Robertson, 1960a; Potts and Parry, 1964; Lockwood, 1967; Riegel, 1972). There is evidence that the bladder is responsible for the elevated levels of Mg^{++} in the urine (Gross and Capen, 1966), and Mg^{++} excretion is apparently under physiological control, as crabs depleted of Mg^{++} stop excreting it (Lockwood and Riegel, 1969).

There is an extensive body of evidence for the formation of urine in decapod crustaceans by a process of ultrafiltration (reviewed by Riegel, 1972). Glucose is normally absent from or present in low levels in crustacean

urine (Riegel, 1972) and thus it must be reabsorbed from the primary urine somewhere in the antennal gland. Several workers have shown that phlorizin causes glucosuria in crustaceans (Burger, 1957; Riegel and Kirschner, 1960; Gross, 1967; Binns, 1969) and these observations are consistent with the hypothesis that glucose is filtered in the antennal gland and later reabsorbed. The only study on the site of glucose reabsorption in the antennal gland was carried out by Gross (1967), who showed that the bladder of the crab, Pachygrapsus crassipes, absorbs glucose from perfusion fluid introduced into it via the nephropore. The results of this study indicate that the bladder may be the site of reabsorption of glucose from the urine in crustaceans.

The purpose of the present study was to investigate the structure of the antennal gland, its role in the maintenance of hemolymph volume and hemolymph levels of magnesium and sulfate ions and in reabsorption of glucose from the urine of the Dungeness crab, Cancer magister. Experiments were also conducted to determine the nature of the control mechanisms of these functions. C. magister, the Pacific coast market crab was chosen for these studies because of its large size, availability and the fact that its osmotic and ionic regulatory abilities have been well studied (Hunter, 1973; Englehardt & Dehnel, 1973; Hunter & Rudy, 1975)

CHAPTER 2

GENERAL METHODS

Capture and Maintenance of Animals

Cancer magister (Dana) was collected near the mouth of Coos Bay, Oregon (Lat. 43° 20.5', Long. 124° 19.3') from June, 1975 to August, 1977. Crab rings (mesh size = 3 in) baited with fish were used to capture the crabs. Only male crabs between 200 and 800 g were used in this study. Newly molted crabs and those which appeared about to molt were not used.

Immediately after capture the crabs' chelipeds were immobilized with rubber bands to prevent the animals from damaging each other. Crabs were transported to the Oregon Institute of Marine Biology, Charleston, Oregon, and kept in large (1000 l) fiberglass holding tanks supplied with running Coos Bay sea water from the laboratory sea water system. During the period of this investigation the salinity of the laboratory sea water varied between 30 and 34% and the temperature varied between 10 and 14 °C. Crabs were usually used in experiments within one week of capture and were not fed. In a few cases, crabs were held for longer periods and were fed fish scraps at intervals of 2-3 days. In all cases

crabs were allowed 2 days after capture or feeding to clear the gut and to assure that they were in a post-absorptive state.

Acclimation to Experimental Media

Crabs were acclimated for 48-72 hr in sea water (S.W.) of various salinities, either in the large holding tanks or in the apparatus used to measure urinary rate described in Chapter 4. The salinities of the media, calculated from hydrometer readings, were as follows: 100% S.W., 33-34 ‰; 75% S.W., 25 ‰; 50% S.W., 16-17 ‰; 30% S.W., 10 ‰. Crabs were directly exposed to 100%, 75% and 50% S.W. Acclimation of crabs to 30% S.W. was accomplished by exposing the crabs to 50% S.W. for 24 hr before transfer to 30% S.W. for a final acclimation period of 48 hr. In a few cases it was necessary to increase the salinity of the laboratory sea water to obtain 100% S.W. This was accomplished by addition of "Instant Ocean" (Aquarium Systems, Inc.) salts to a final salinity of 33-34 ‰. Temperatures in the media were 12 ^{o}C $\pm$ 2 in the large holding tanks and 12 ^{o}C $\pm$ 1 in the urinary rate apparatus described in Chapter 4.

Sampling of Serum and Urine

Hemolymph samples of 1-2 ml were obtained with a hypodermic syringe inserted into the arthrodial membrane at the base of a walking leg. Hemolymph was allowed to coagulate in small test tubes and serum was withdrawn from the clot. Urine was obtained by lifting the urinary operculum with a fine, hooked needle and removing 1-2 ml of urine with a pipette. The openings of the gill chambers were plugged with tissue paper to avoid contamination of the urine sample with sea water. Serum and urine samples were stored in small vials at -10 ^{o}C until analyses were performed.

Statistical Treatment of Data

Student's t test was used to test the hypothesis that two mean values are equivalent. The significance of the difference between two mean values was assigned according to probability values as follows: $P > .05$, not significant; $P < .05$, significant; $P < .01$, highly significant; $P < .001$, very highly significant. Regression analysis of paired data was done using the least squares method. In the tables and figures, n = the number of observations or animals, s.d. = standard deviation of the mean, s.e. = standard error of the mean and P_t = probability, by Student's t test, that the

observed difference between the indicated mean values is due to chance variation.

Miscellaneous

Crabs were routinely weighed to 0.1 g on a Mettler P 1200 balance. Prior to weighing, the crabs were wrapped in paper towels and held head-down for one minute in order to drain the gill chambers. The difference between the means of two consecutive weighings of ten crabs with an average weight of 272.5 g was 0.4 g.

Crab Ringer solution was mixed from the stock solutions given by Welsh, et al., (1968). Ionic concentrations (mEq/l) were as follows: Na^+, 499; K^+, 11; Ca^{++}, 25; Mg^{++}, 37; Cl^-, 528; $SO_4^=$, 44. The final solution had an osmotic pressure of approximately 1000 mOsm/kg H_2O and was adjusted to a pH of 7.8 with 0.5 M NaOH. Ringer solution for crabs in 75% S.W. was made by mixing 850 ml of the crab Ringer and 150 ml distilled water.

Injection of solutions was accomplished using a hypodermic syringe inserted through the arthrodial membrane at the base of a walking leg.

Eyestalk ablation was carried out by cooling the crabs in crushed ice and severing the eyestalks at the arthrodial membrane with a scalpel. The eyesockets were packed with

tissue paper to aid the formation of a clot. Bleeding was slight and mortality was less than 5%. The crabs were allowed to recover 24 hr before use in eyestalk ablation experiments.

Standards for chemical analyses were made up from reagent grade chemicals weighed to 0.001 g on a Torbal ET-1 torsion balance.

CHAPTER 3

STRUCTURE OF THE ANTENNAL GLAND

METHODS

Male, intermoult crabs weighing between 200 and 800 g were captured and maintained in 100% Coos Bay sea water as noted in Chapter 2.

The arterial supply to the antennal gland was visualized by injection of a small amount of crab Ringer solution with methylene blue into the antennal artery after removal of the dorsal carapace and hypodermis.

The approximate maximum volume of urine held by the bladder was estimated by introducing known volumes of a latex rubber solution into the previously drained bladders of two 500 g crabs. The urinary opercula were then glued closed, several holes were made in the carapace of each crab and the animals were stored in 5% formalin for one week to solidify the latex. The crabs were then dissected and checked for evidence of ruptured bladders.

The bladder area of four crabs was estimated by dissecting the entire right bladder of each crab, quickly rinsing it in distilled water and placing it in a preweighed aluminum pan. The tissues were dried to constant weight at

100 °C and weighed to the nearest 0.01 mg on a Metler H20T analytical balance. A piece of bladder tissue of known area from each crab was treated in the same way. Thus an approximation of the total area of the bladder could be made and doubled, assuming that both right and left bladders were of equal area.

Tissues for light microscopy were fixed in Bouin's solution, dehydrated in an alcohol series and infiltrated with paraffin. Serial sections of a whole antennal gland were cut at 20 μ and stained with hematoxylin-eosin. Thin sections were cut at 7 μ and stained with Mallory's trichrome stain.

Tissues for electron microscopy were fixed for 1.5 hr in 4% glutaraldehyde made isosmotic (1050 mOsm/kgH_2O) with sucrose and postfixed in 1% OsO_4, also made isosmotic with sucrose. The tissues were dehydrated in an alcohol series, infiltrated with propylene oxide and then with an epon-araldite resin mixture which was then polymerized for 12 hours at 60 °C. Thin sections were cut, transferred to grids, and stained 20 minutes in 6% uranyl acetate and 2 to 5 minutes in Reynold's aqueous lead citrate. The sections were viewed and photographed with a Phillips EM 300 or Hitachi HS-7S transmission electron microscope.

RESULTS AND DISCUSSION

Gross Anatomy

The only externally visible structures of the antennal gland are the urinary opercula which close the nephropores (Fig. 2). The bladder in C. magister is similar in structure to those of Maia squinado (Marchal, 1892) and Cancer pagurus (Pearson, 1908). The bladders are paired, unconnected, structures with several lobes, both above and below the stomach, as well as a large lateral lobe (Figs. 3 and 4). Hemolymph is supplied to the bladder by numerous, fine branches of the antennal, hepatic and sternal arteries.

When 10 ml of a latex rubber solution were injected into the empty bladders of two 500 g crabs and hardened by immersion of the crabs in a formalin solution, there was no evidence of rupture of the bladders when the crabs were dissected. In one of the crabs 12 ml of latex solution was injected into one bladder and it ruptured, thus 2 X 10 ml or 4% of the body weight (ml/g) is the approximate maximum volume contained by both bladders. Riegel et al., (1974) give figures of approximately 2% body weight for bladder volume in the crabs, Carcinus maenas and Macropipus depurator. A latex cast of the bladder of a 500 g crab is shown in Fig. 5. The mean total area of the bladders of four crabs,

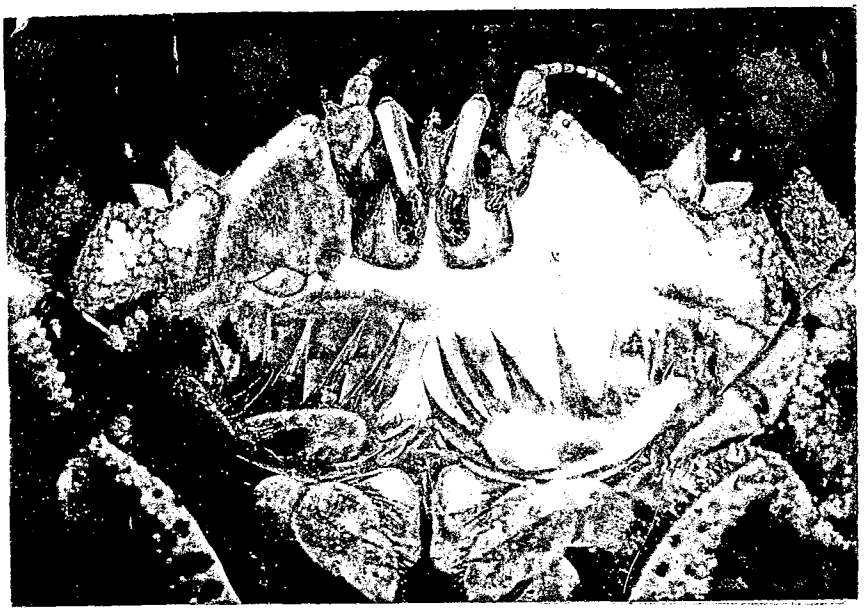


Figure 2. Frontal view of C. magister, X 2. Arrows indicate urinary opercula.

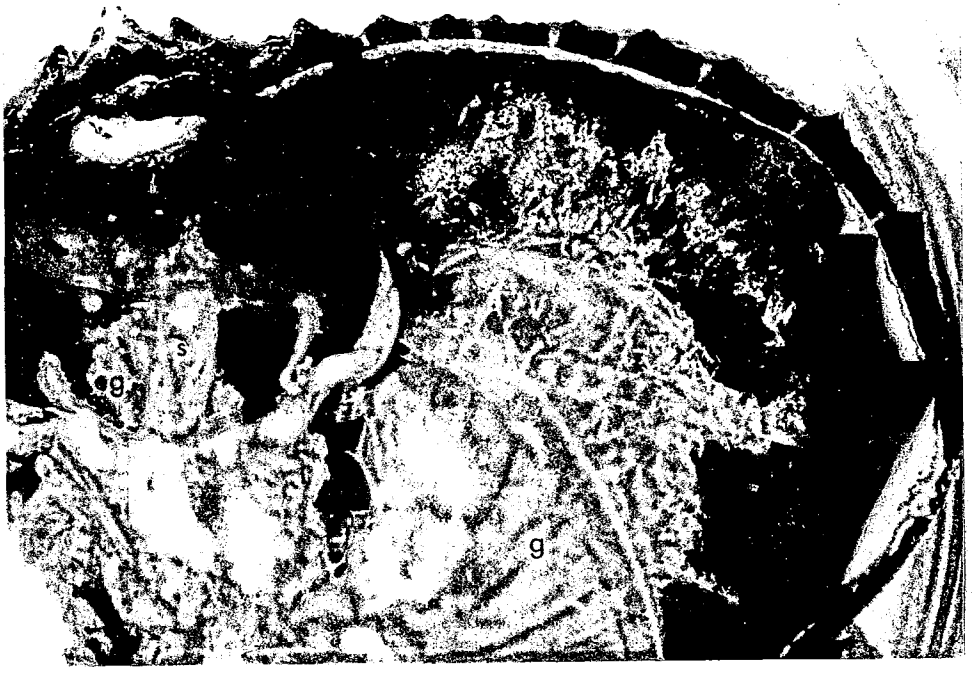


Figure 3. Dorsal dissection of C. magister, X 1. Dorsal carapace, hypodermis and midgut gland have been removed. eg, epigastric lobe of bladder; g, gills; l, lateral lobe of bladder; s, stomach.

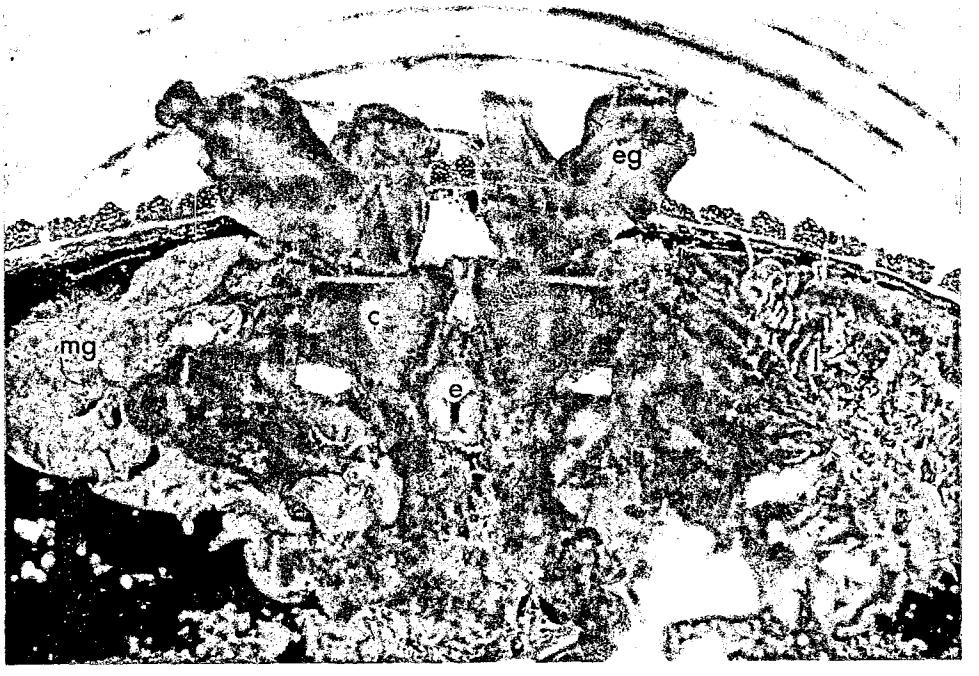


Figure 4. Dorsal dissection of the bladder of C. magister, X 0.9. Stomach has been removed. c, coelomosac/labyrinth; e, esophagus; eg, epigastric lobe of bladder; l, lateral lobe of bladder; mg, midgut gland.

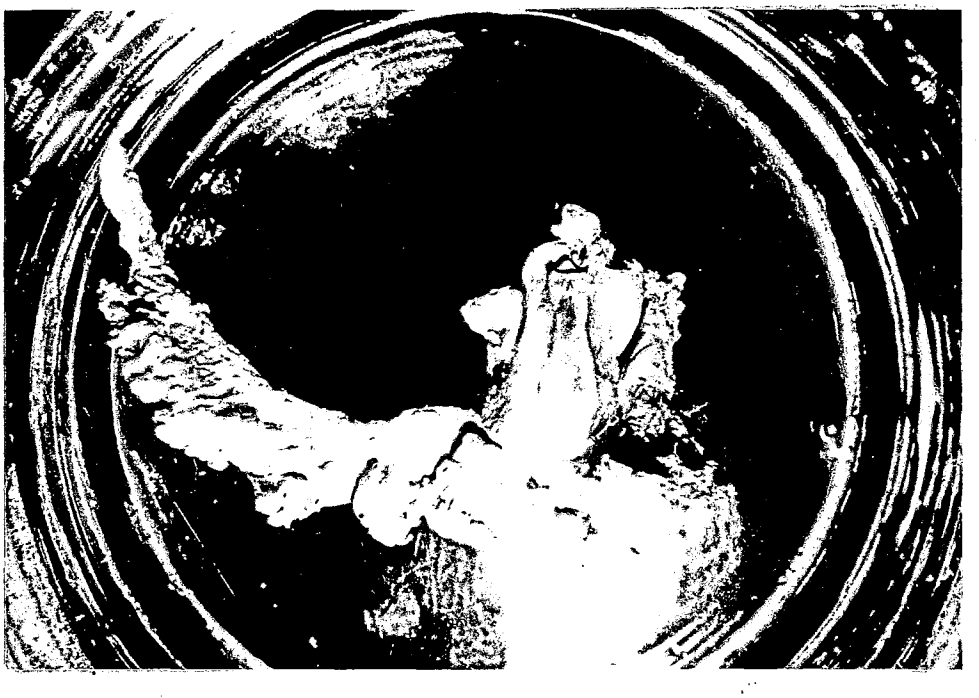


Figure 5. Latex cast of the right bladder of a 500 g crab, C. magister, X 1.2.

as estimated by dissection and weighing, was 0.867 cm^2/g body weight (Table 1).

In C. magister the coelomosac and labyrinth are closely apposed and the composite structure has a leaf-like appearance (Fig. 6). Hemolymph at arterial pressure is supplied to the coelomosac by a small branch of the antennal artery and urine is thought to be formed by a process of ultrafiltration across the coelomosac and into its lumen (Riegel, 1972). Once formed, the urine flows into the labyrinth and then into the bladder.

Light Microscopy

Serial sections of a complete antennal gland were made and examined. As is the case in other brachyurans (Marchal, 1892; Balss, 1944) the coelomosac extends ventrally into the labyrinth in a complex, branching pattern (Fig. 7). The space between the two structures is supplied with hemolymph by the coelomosac artery. In places, the coelomosac and arterial space extend nearly through the labyrinth and, presumably, the unfiltered hemolymph returns to the hemocoele via spaces between the ventral labyrinth cells at such places (Marchal, 1892). I could see no connections between the arterial space in the gland and its ventral surface in my sections. Balss (1944) has noted that Marchal's (1892)

Table 1. Estimated bladder area of four specimens of C. magister.

Body weight(g)	Weight of 0.8 cm^2 of bladder(g)	Weight of remaining bladder(g)	Area of right bladder (cm^2)	Total area of bladder in crab(cm^2/g B.W.)
650	0.00283	1.10862	313	0.964
625	0.00070	0.24202	277	0.888
627	0.00244	0.61447	201	0.643
480	0.00180	0.52527	233	0.973
Mean	0.00194	0.62259	256	0.867
S.D.	0.00093	0.36082	49	0.154

Figure 6. Dorsal view of the right antennal gland of C. magister, X 4. aa, antennal artery; ac, arterial space between coelomosac and labyrinth; ca, coelomosac artery; l, labyrinth; o, opening of labyrinth into bladder.

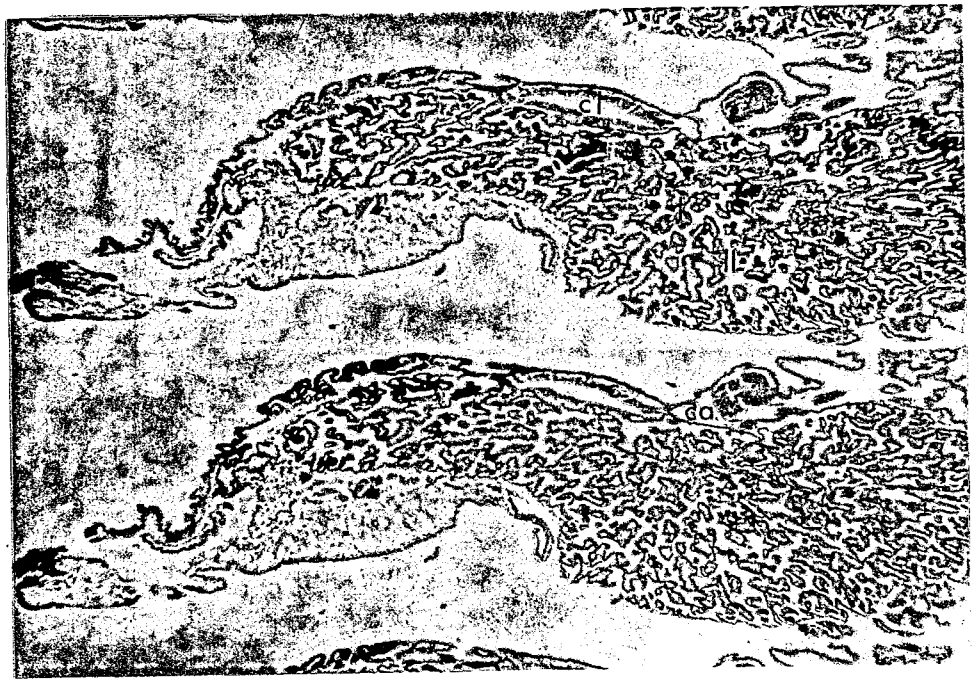


Figure 7. Light micrograph of 20 μ sections of the coelomosac and labyrinth of C. magister, X 30. c, coelomosac; ca, coelomosac artery; cl, lumen of coelomosac; l, labyrinth; ll, lumen of labyrinth.

hypothesis has not been confirmed. If Marchal is correct, then the connections in question are the functional equivalents, in terms of blood flow, of the vertebrate efferent glomerular arterioles.

The coelomosac is prevented from collapsing under arterial pressure into its own lumen by numerous connections to the labyrinth cells. The connection between the lumina of the coelomosac and labyrinth is shown in Fig. 8. At higher magnifications (Fig. 9) the coelomosac and labyrinth cells are similar in appearance to those of other brachyurans (Marchal, 1892; Balss, 1944; Flemister, 1959; Schmidt-Nielsen, et al., 1967). Riegel (1966a, 1966b) has found that the labyrinth and tubule of the crayfish antennal gland produce vessicles which are liberated into the lumen of the gland. I could find no evidence of this sort of activity in the labyrinth in my sections. Bladder tissue, however (see below), has many vacuoles in the process of being liberated into the urine and it may be that in brachyurans the bladder is responsible for the same type of apocrine excretion that is apparently accomplished by the labyrinth in macruran forms.

The bladder is a unicellular sheet of cuboidal epithelium (Fig. 10). The tissue is unusual in that it is highly vacuolated and the vacuoles, in turn, contain granules. In places the vacuoles may be seen in the process

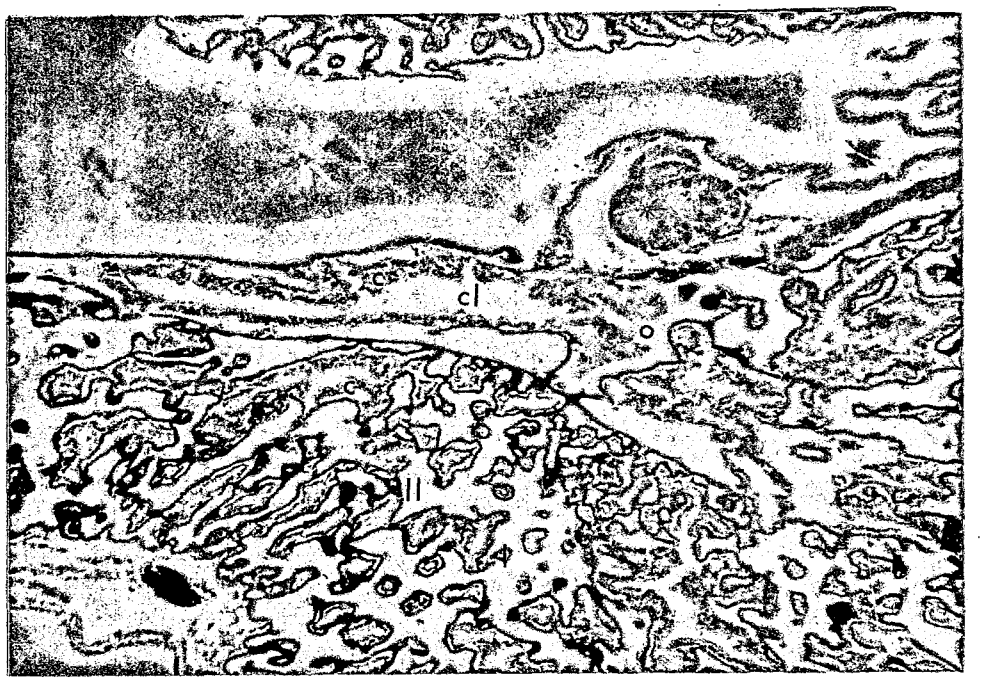


Figure 8. Light micrograph of a 20 μ section of coelomosac and labyrinth of C. magister, X 80. c, coelomosac; cl, lumen of coelomosac; l, labyrinth; ll, lumen of labyrinth; o, connection between lumina of coelomosac and labyrinth.

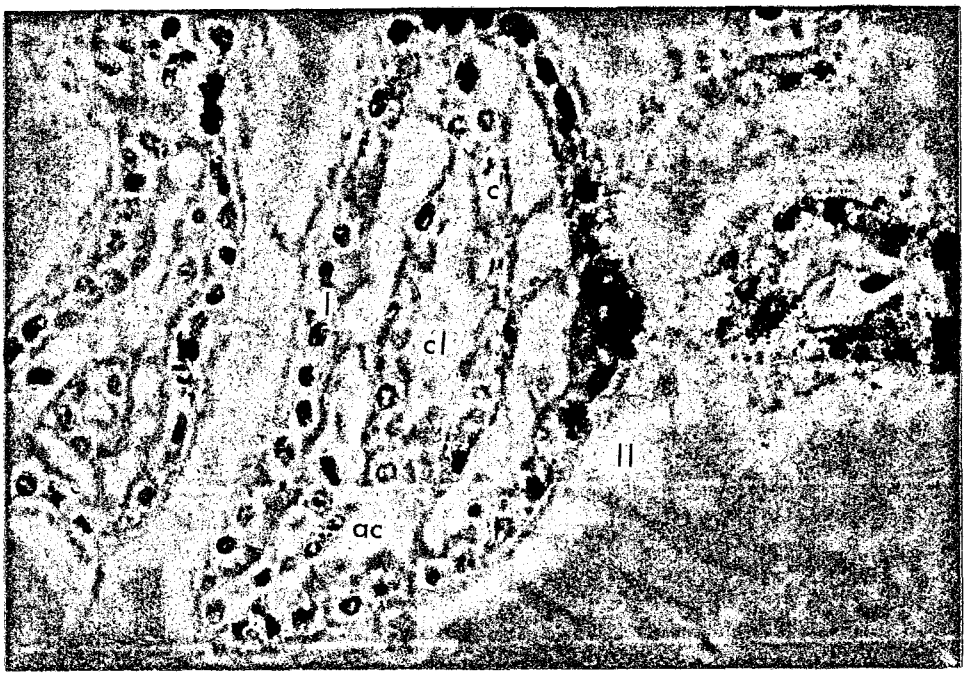


Figure 9. Light micrograph of a 7 μ section of coelomosac and labyrinth of C. magister, X 230. ac, arterial space between coelomosac and labyrinth; c, coelomosac; cl, lumen of coelomosac; l, labyrinth; ll, lumen of labyrinth.

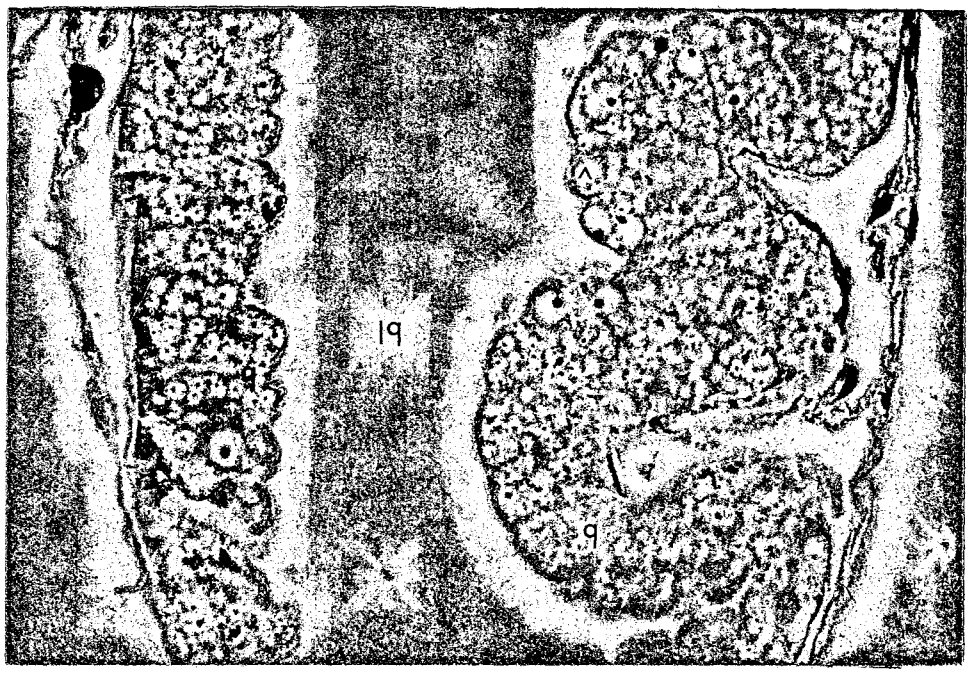


Figure 10. Light micrograph of a 7 μ section of bladder tissue of C. magister, X 570. b, bladder tissue; bl, lumen of bladder; V, vacuole.

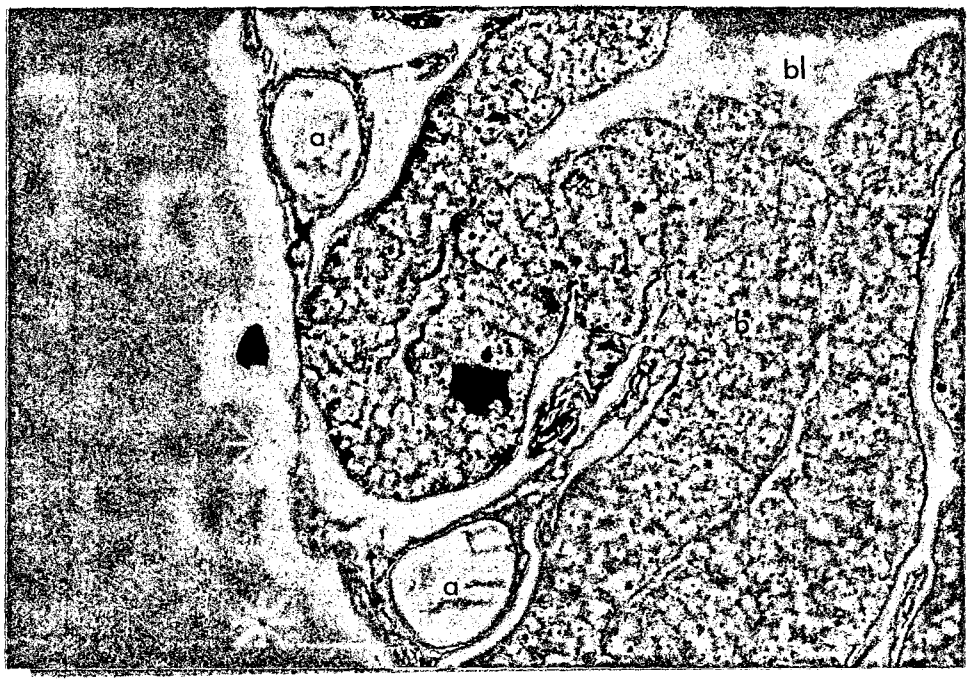


Figure 11. Light micrograph of a 7 μ section of bladder tissue of C. magister, X 570. a, artery; b, bladder tissue; bl, lumen of bladder.

of leaving the lumenal face of the bladder to enter the urine. The existence of these granule-containing vacuoles and their passage into the urine have been noted by several workers (Marchal, 1892; Fischer, 1925; Balss, 1944). The lumenal face of the cells has a brush border. As seen in Fig. 11, the bladder is well supplied with arteries which approach the size of vertebrate capillaries.

Electron Microscopy

Thin sections of coelomosac tissue (Fig. 12) show that the cells are similar in appearance to the podocytes found in the vertebrate glomerulus and in the coelomosacs of several other invertebrates (Anderson and Beams, 1956; Kummel, 1964; Koechlin, 1966; Schmidt-Nielsen, et al., 1967; Tyson, 1968; Fain-Muriel and Cassier, 1971; Peterson and Liozzi, 1973). It is thought that ultrafiltration occurs across the thin membrane between the foot processes (Fig. 13). Rodewald and Schaffner (1977) have shown that this membrane, the slit diaphragm, is the filtration barrier in the coelomosac of the crayfish, Procambarus clarkii.

Labyrinth cells have an extensive brush border on the lumenal surface (Fig. 14) and the serosal side of the tissue shows the elaborate infoldings and numerous mitochondria

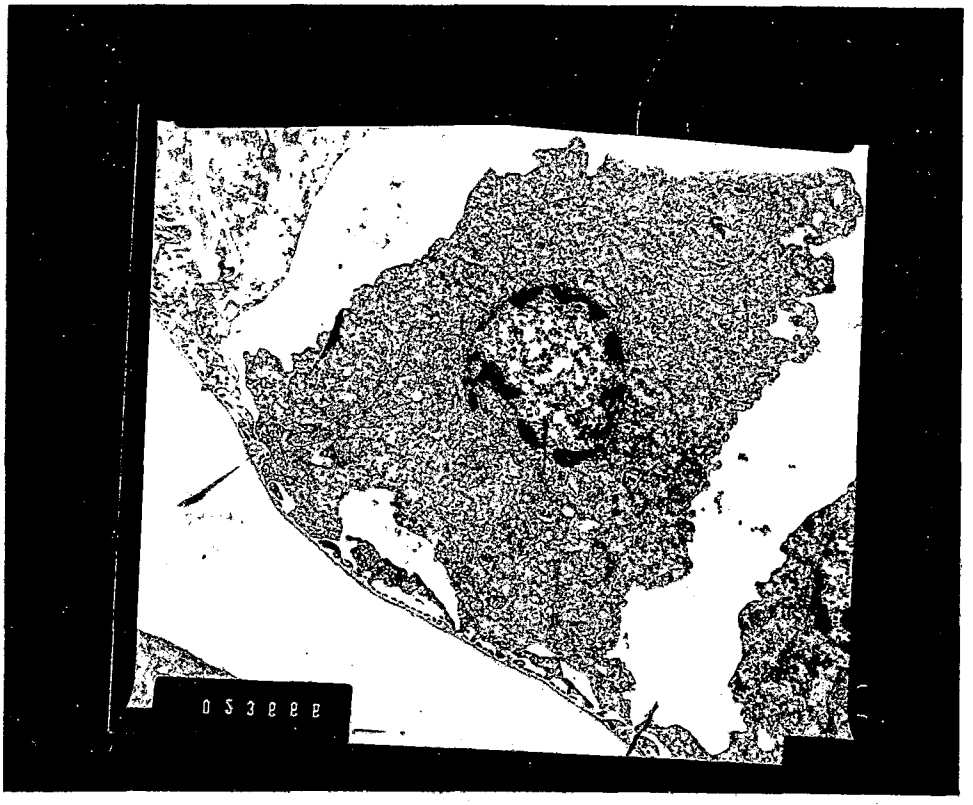


Figure 12. Electron micrograph of a podocyte in the coelomosac of C. magister, X 9200. h, hemolymph; lis, lateral intercellular space,; n, nucleus.

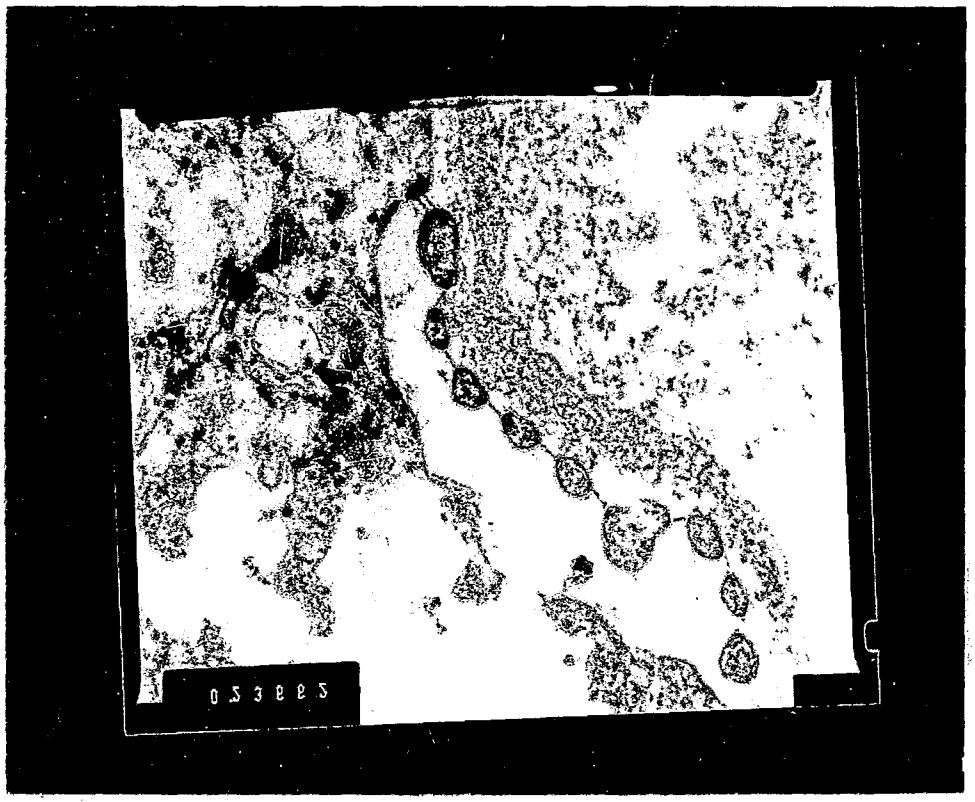


Figure 13. Electron micrograph of a podocyte in the coelomosac of C. magister, X 132,000. fm, filtration membrane; fp, foot process, h, hemolymph.

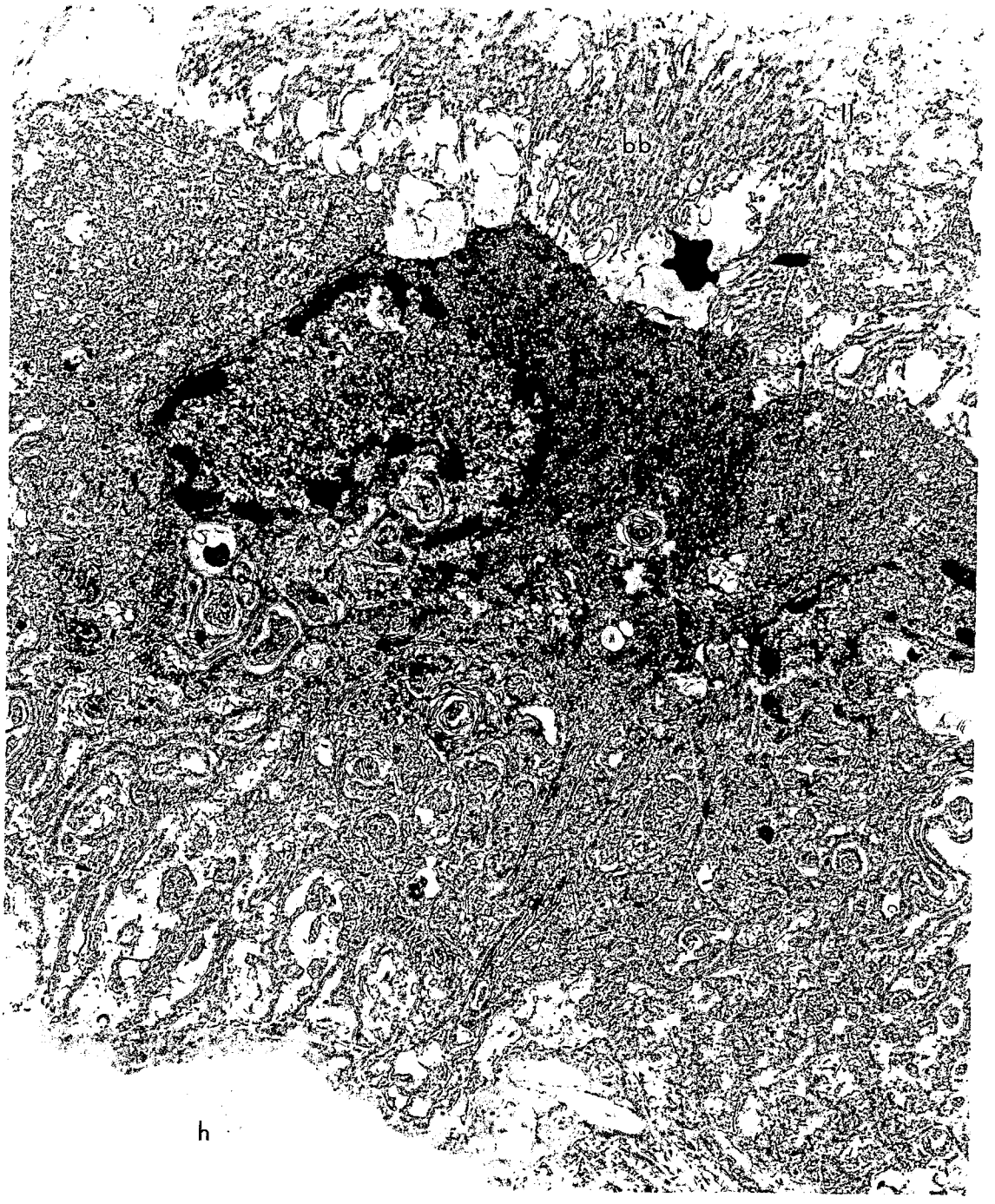


Figure 14. Electron micrograph of a labyrinth cell of C. magister, X 4800. bb, brush border; h, hemolymph; ll, lumen of labyrinth; n, nucleus.

characteristic of transporting epithelia.

Because the serosal side of the labyrinth is bathed by hemolymph which, presumably, returns to the general circulation, this tissue is in a good position to both excrete and reabsorb various substances.

Bladder cells (Fig. 15) are similar to those of the labyrinth in having a lumenal brush border and basal infoldings. Riegel (1970) has shown that, under certain conditions, the bladder of the crayfish is probably capable of reabsorbing considerable amounts of salts from the urine. Evidence that the bladder of C. magister modifies the ionic content of the urine is presented in Chapter 5 of the present study. Thus, in the crayfish and the crab there is both structural and physiological evidence for ion transport across the bladder. The numerous vacuoles containing granules seen in the light micrographs are also evident in electron micrographs. Marchal (1892) has suggested that the orange-yellow color of the bladder is due to these granules. Cuenot (1893) and Fischer (1925) have shown that various dyes are excreted by the decapod bladder and, thus, the granules may be organic wastes in the process of being excreted.

In summary, the antennal gland of C. magister is similar in structure to those of other brachyurans which have been studied. The bladder is by far the most extensive

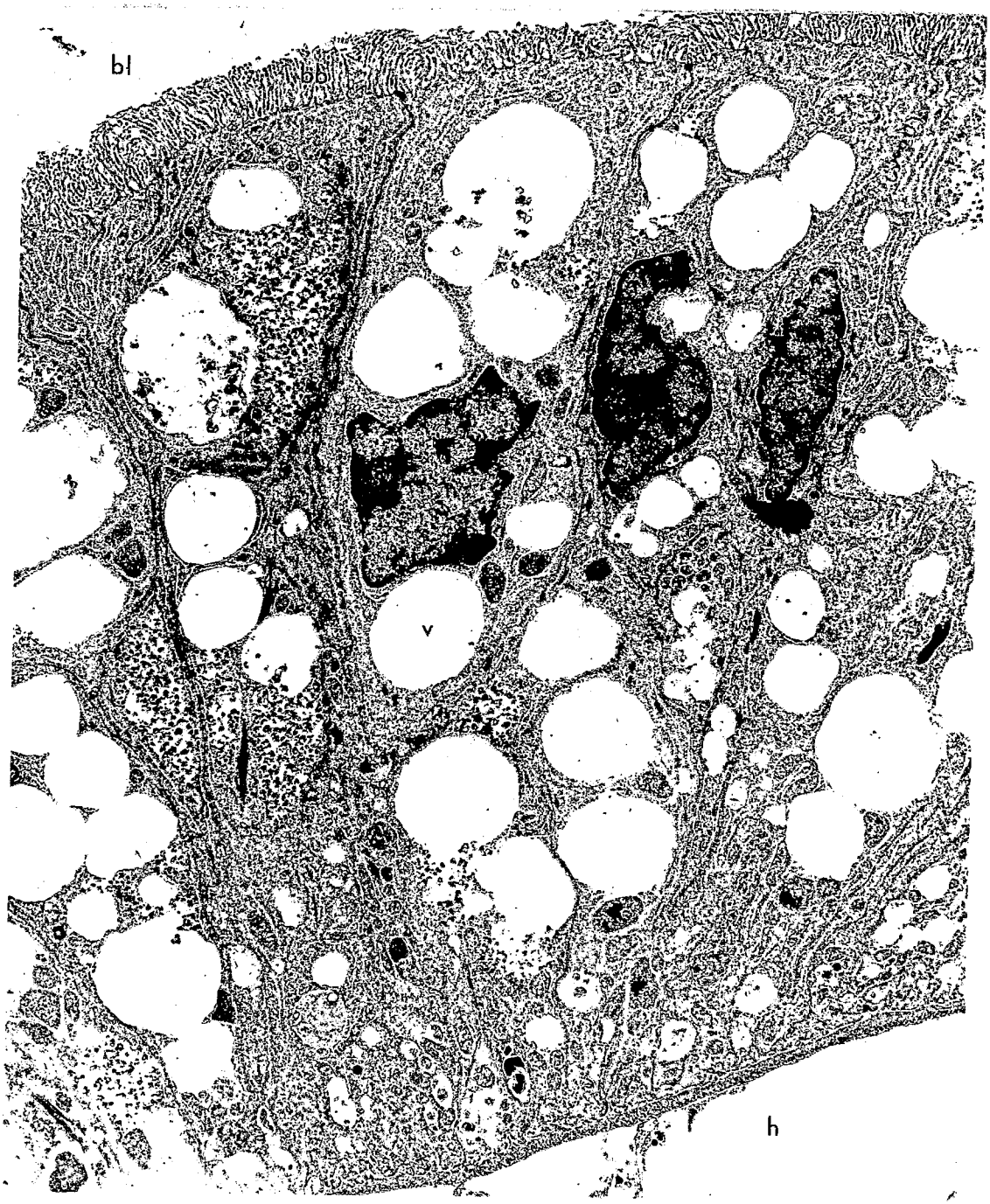


Figure 15. Electron micrograph of bladder tissue of C. magister, X 4700. bb, brush border; bl, lumen of bladder; h, hemolymph; m, mitochondrion; n, nucleus; v, vacuole.

part of the gland and it provides a large surface area across which physiological modification of the urine can occur.

CHAPTER 4

THE ROLE OF THE ANTENNAL GLAND AND GUT IN HEMOLYMPH VOLUME CONTROL

METHODS

Male intermoult crabs weighing between 200 and 800 g body weight were captured and maintained in 100% Coos Bay sea water as noted in Chapter 2. Serum osmotic pressure was measured with a Hewlett-Packard 302B vapor pressure osmometer using NaCl standards for calibration. The standard deviation of the mean of five replicate determinations of a 1025 mOsm/kg H_2O standard was $\pm$ 5 mOsm/kg H_2O.

Measurement of Urinary Rates

A method for continuously measuring the crab's urinary rate under relatively unrestrained conditions and for periods of up to one month was developed for this study (Holliday, 1977). The method is a refinement of that reported by Kamemoto and Ono (1968) and is based on external collection of voided urine. The areas around both nephropores were cleaned and 3/8" lengths of 3/8" O.D. X 3/16" I.D. latex rubber tubing were secured to the animal with α-cyanoacrylate

glue (similar to Eastman 910). This arrangement allows continuous collection of voided urine. A 12 cm length of 3/16" O.D. X 1/8" I.D. latex rubber tubing connected the tube around each nephropore to a polyethylene "Y" secured to the carapace over the heart with hot melt glue (U.S.M. Thermogrip glue). A 1/4" O.D. X 1/8" I.D. silicone rubber tube was connected to the third leg of the "Y" and conducted the voided urine out of the animal tank and into a thin-walled latex reservoir. The chelipeds were secured in the flexed position with rubber bands. The arrangement of the tubing on the crab is shown in Fig. 16. Urinary rates were measured by emptying the reservoirs at appropriate intervals. During experiments, five crabs were individually maintained in 12 l styrofoam tanks connected to a common 30 l seawater tank. The crabs were relatively unrestrained and were free to move about the 12 l tanks. The seawater was aerated and circulated through the animal tanks at the rate of 30 l/hr. Water temperature was kept at 12 $^{o}C \pm 1$ by a Lauda K-2R refrigerated constant temperature circulator connected to polyethylene cooling coils suspended in the reservoir. The 12 l animal tanks were covered with black polyethylene sheeting and each tank was supplied with a "grain of wheat" incandescent bulb which was used to maintain ambient photoperiod. Light levels in the tanks when the lights were on where not measured but were dim. When necessary the

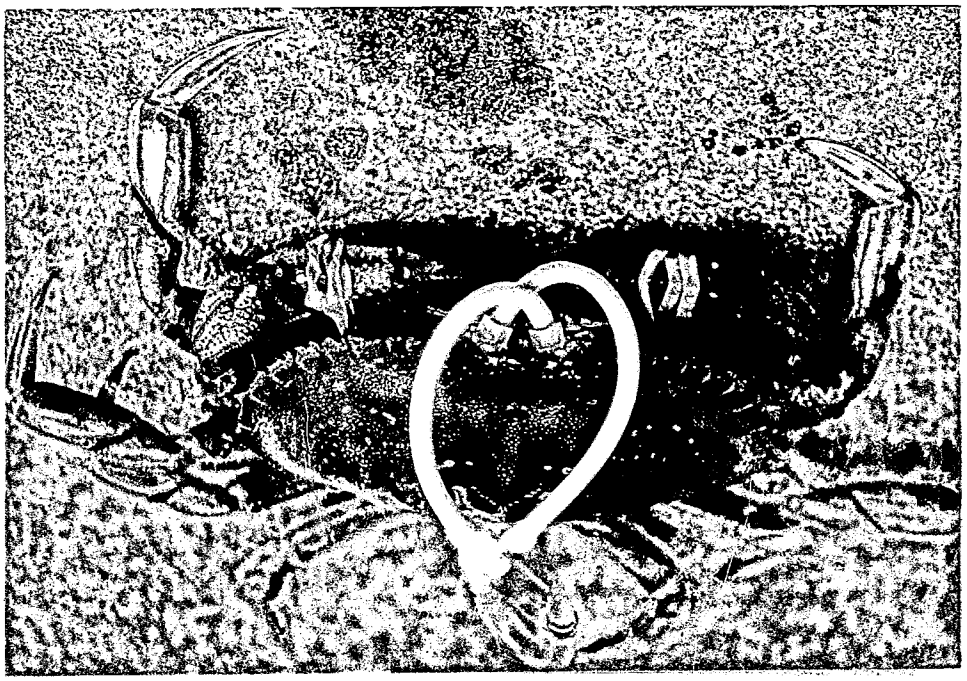


Figure 16. Placement of tubing for urinary rate measurements in C. magister, X 0.3.

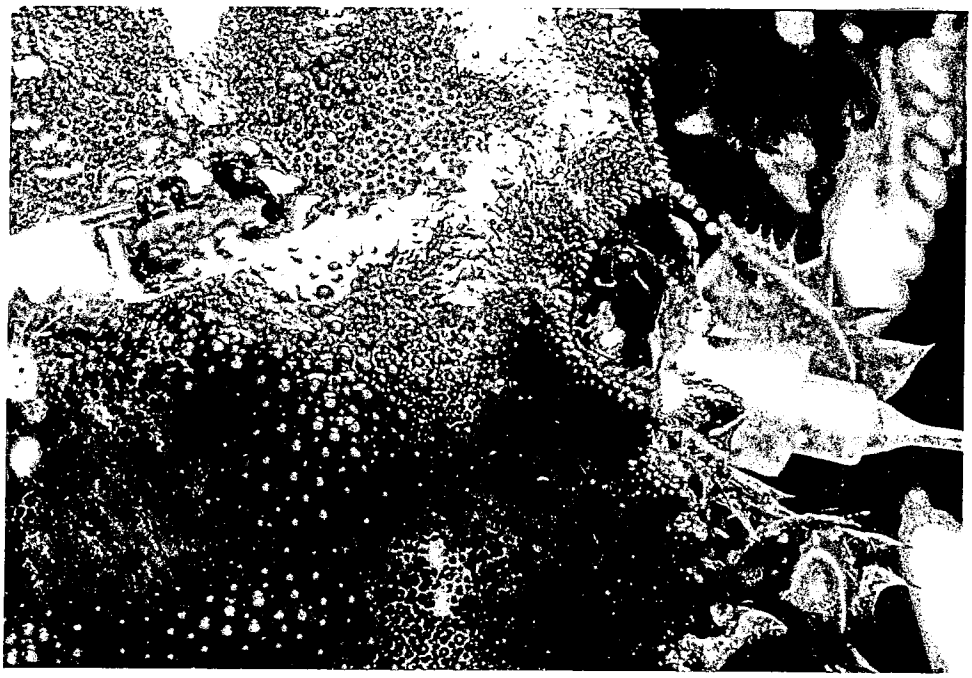


Figure 17. Placement of cannulae in nephropore and antennal artery of C. magister, X 2.

salinity of the system was lowered by removing an appropriate amount of seawater from the reservoir and replacing it with tap water of the same temperature. The salinity of the system equilibrated under these conditions in less than one hour. Unless otherwise noted, this is the method of assessing urinary rate used throughout the study.

Using the above technique it was found that the crabs voided urine infrequently in 100% S.W. Thus it was difficult to obtain meaningful data. Acclimation and maintenance of the crabs in 75% S.W., a salinity frequently encountered by the animals in nature, increases the urinary rate and frequency of micturation (Hunter, 1973) and greatly reduces the dispersion of the data. Therefore, most of the experiments were conducted in 75% S.W. It was found that acclimation to 75% S.W. was essentially complete within 48 hr. Unless otherwise noted, the crabs were affixed with the tubing and maintained in 75% S.W. for 48 hr prior to experimental manipulation. Urinary rates were monitored during acclimation and the rate during the last 12 hr period was used as a control rate to assess the effects of various experimental treatments. Urinary rates are expressed as % body weight per day.

In some experiments urinary rate was estimated by sealing the crabs' nephropores with α-cyanoacrylate glue and noting the increase in body weight over a known time period.

Preliminary experiments showed that much better data were obtained when the mouth and anus were sealed in order to prevent weight changes due to drinking or voiding gut contents. This was accomplished by glueing a 3/8" piece of latex tubing in the esophagus just behind the mandibles with ∝-cyanoacrylate glue. The tubing was blocked with hot melt glue. The anus was blocked by glueing the three fleshy lobes which surround it together with ∝-cyanoacrylate glue. When this method was used for animals in other than 100% S.W., the crabs were first acclimated to the new medium for 72 hr before blocking the nephropores and gut.

Interruption of Blood Supply to the Coelomosac

In one experiment it was necessary to interrupt the blood supply to the coelomosac in order to stop the production of urine. The crab was secured in a tray and ice was packed around it. A hole 1 cm in diameter was cut in the carapace just behind each eye and the area was slowly irrigated with crab Ringer to wash away excess hemolymph. With the aid of a dissecting microscope the bladder was retracted and the artery to the coelomosac of the antennal gland was located and broken with forceps. The wound was carefully closed with hot melt glue in such a manner that the exposed tissues were not burned. Considerable loss of

hemolymph occured during the 30 minutes required for the operation, but the crab's volume was probably unchanged, due to replacement of the hemolymph by the crab Ringer used for irrigation. Control animals were similarly operated and sealed, but the artery was not broken. Mortality over a ten day period was less than 20% in both groups. In order to maintain the animals for longer periods of time it was necessary to periodically withdraw hemolymph to return the crabs' body weights to the original values.

Hemolymph Volume Changes

Several experiments were conducted to determine the effect of a decrease or increase in hemolymph volume on urinary rates. Hemolymph volume changes were accomplished by removal of hemolymph or injection of crab Ringer with a syringe fitted with a 22 gauge hypodermic needle. The needle was inserted through the arthrodial membrane at the base of a walking leg and the required withdrawal of hemolymph or injection of crab Ringer was performed. It was found that bleeding was very slight if the needle was withdrawn slowly and a finger was pressed against the injection site for one minute. In cases where a decrease in the osmotic pressure of the hemolymph was desired without a change in hemolymph volume, hemolymph was first withdrawn

and an equivalent amount of distilled water was slowly injected. Long term infusion of crab Ringer was accomplished via a blunted 20 gauge hypodermic needle inserted through a hole in the carapace and secured with hot melt glue. This needle was connected via P.E. 90 tubing (Intramedic) leading out of the animal tank to a peristaltic pump which moved the crab Ringer from a reservoir into the crab at a mean rate of 5.0% or 11.5% of body weight per day. The needle and catheter were inserted before acclimating the crabs to 75% S.W.

Temperature Changes

Temperature changes were accomplished by adjusting the thermostat on the constant temperature circulator. Increases in temperature were complete in 4 hr. Decreases in temperature were complete in 12 hr.

Preparation of Homogenates

Brain, thoracic ganglion and muscle homogenates were prepared by grinding the tissues from five animals with a glass tissue homoginizer for five minutes at 0-2 ^{o}C. Homogenates were made up to a volume of 5 ml with crab Ringer and 1 ml was injected into each of five crabs through

the arthrodial membrane at the base of a walking leg. Thus, each crab received one brain or thoracic ganglion equivalent per injection. Tissue donors were the same size as experimental animals and had been acclimated for 72 hr in 50% or 100% S.W. prior to use. Whole brains and thoracic ganglia were used and the amount of muscle taken from each animal was of the same size as the crab's brain.

Measurements of Heart Rate and Blood Pressure

Heart rate was measured with two fine, chloridized silver wires inserted into the dorsal pericardial space through holes drilled in the carapace. One electrode was centered over the heart and another was placed 1 cm posterior to the first and both were sealed in place with hot melt glue. Signals were amplified and displayed on a Bausch and Lomb VOM 5 amplifier/recorder unit.

Blood pressure in the antennal artery was measured as follows. After acclimation for 24 hr to the appropriate medium, a piece of exoskeleton 1 cm^2 was removed from the dorsal surface of the carapace lateral to the stomach and the underlying hypodermis was removed. The crab was secured partly submerged in the medium with the dorsal part of the carapace in the air, an arrangement which allowed the animal to respire normally. A 20 gauge hypodermic needle connected

by P.E. 90 tubing to a Statham 23A pressure transducer was inserted into the antennal artery. Transducer output was amplified by a Grass 7PIF preamplifier and displayed on a Grass 79D polygraph system. The transducer was calibrated before and after each measurement with a water manometer. Zero pressure was adjusted to the level of the antennal artery. Pressure within the heart was measured by direct cardiac puncture through a hole in the carapace over the heart. In these experiments heart rate was obtained from pressure pulses.

Cannulation of the Nephropore and Antennal Artery

In some experiments designed to show the formation of urine by a process of ultrafiltration, the nephropore and antennal artery were cannulated, using a method similar to that of Norfolk (1976). Crabs were secured upside down in a tray and the tapered end of a short length of P.E. 10 tubing was inserted 3-4 mm into the nephropore while the urinary operculum was held open with a fine dissecting needle with a bent tip. When the tube had filled with urine it could be glued to a ring of hot melt glue previously placed around the nephropore in such a way as to both hold the tubing in place and seal it in the nephropore. This tubing was connected to a 20 cm length of P.E. 90 tubing

which led to a reservoir. The chelae were then secured in the flexed position with rubber bands and the animal was secured right side up in the tray. While the cannula drained the bladder the antennal artery was exposed as previously described. The artery was nicked and a 4 cm P.E. 50 cannula connected by 20 cm of P.E. 90 tubing to a reservoir of crab Ringer was inserted into the artery and secured with two ligatures around the artery and cannula. One ligature was placed distal to the insertion to seal the artery to the catheter and the second ligature was placed proximal to the insertion to prevent hemolymph loss from the artery. The crab was suspended in flowing 100% S.W. with the carapace just out of the water. The reservoir of crab Ringer was supported at various distances above the animal, resulting in an applied pressure of between 10 and 50 cm H_2O. The crabs survived 12-24 hr under these conditions. Fig. 17 illustrates a cannulated crab. Urinary rate was estimated by weighing the amount of urine produced by the crab in a given time period. Because blockage of the urinary cannula frequently occured another method was used to "cannulate" the nephropore. A length of 3/8" O.D. X 3/16" I.D. latex tubing was glued to the area around the nephropore and a 5 mm length of 1 mm diameter plastic rod was glued to the urinary operculum with α-cyanoacrylate glue. This rod was then moved laterally until it touched a drop of

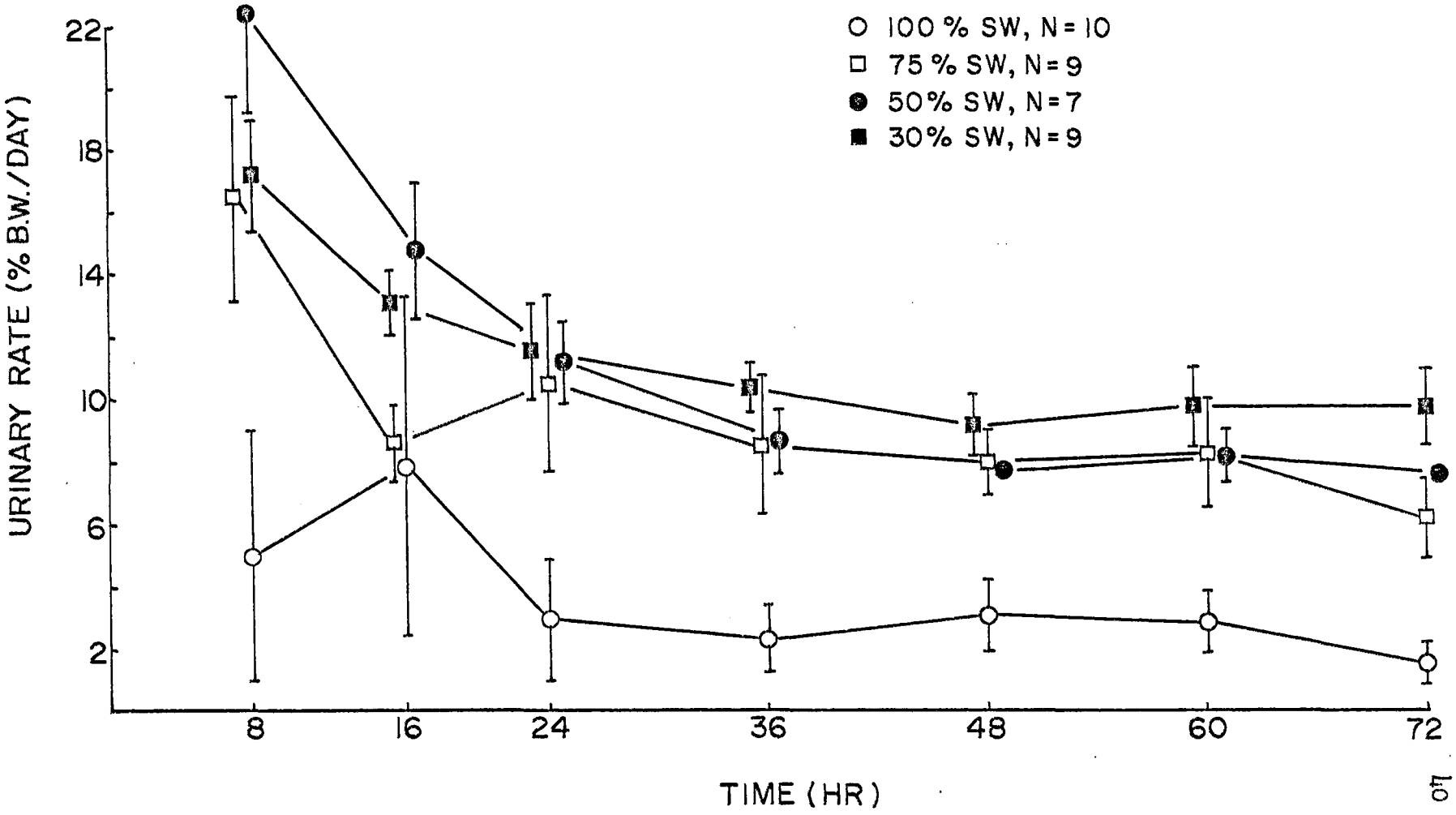
glue on the rubber tubing. A short piece of 3/16" O.D. X 1/8" I.D. latex tubing connected to 20 cm of P.E. 90 tubing was glued into the larger latex tubing and led to a reservoir which received the urine. In spite of the fact that nothing touched the nephropore, extreme problems with blockage were also encountered with this procedure.

RESULTS

Urinary Rates in Different Media

The time course of urinary rates during acclimation of four groups of crabs to four salinities is shown in Fig. 18. The animals in 30% S.W. were first exposed to 50% S.W. for 24 hr, as they will not withstand direct transfer from 100% S.W. to 30% S.W. (Hunter and Rudy, 1975). Thus, time "0" in the case of the crabs in 30% S.W. is actually the urinary rate after 24 hr in 50% S.W. It can be seen that the initial urinary rate in the dilute media was quite high, amounting to between 40 and 55 ml/12 hours for a 500 g crab. Acclimation was nearly complete after 36 hr and appeared to be completed within 72 hr. The initial high rates may be partially due to an immediate and complete urination upon placement in the apparatus. Hunter (1973) has noticed such an effect with C. magister. The urinary

Figure 18. Urinary rates of C. magister during acclimation to various media. Mean values ± 2 s.e.



rates for the final 12 hr period in each salinity are shown in Table 2. Hunter (1973) has reported similar urinary rates in C. magister using the tracer compound Glofil. For comparison, urinary rates of animals acclimated for 72 hr in the four media as measured by the blocked nephropore technique are also shown. The large difference between the two methods at 50% S.W. is difficult to explain. The significant difference between the two methods at 100% S.W. may be due to the short period (8 hr) allowed for a change in weight. It is also possible that the crabs drank the medium in 100% S.W. and, since the animals' guts were blocked, the urinary rate was lower than it would normally be. This explanation is reinforced by the data shown in the last column of Table 2. This group of crabs had had the arteries to the coelomosacs broken and weight changes were noted over a period of 72 hr. It can be seen that this rate was essentially the same as that obtained by external collection of urine (Table 2, column 1).

In Table 3 the urinary rates of C. magister and the red rock crab, C. productus acclimated to various salinities are compared. The urinary response of C. productus to acclimation in low salinity was what one would expect of an osmoconformer. C. productus could not be acclimated to 30% S.W.: the animals were very swollen and moribund after only 12 hr in this medium.

Table 2. Urinary rates (mean % B.W./day ± 2 s.e.) of C. magister acclimated in various media for 72 hr, as measured by three methods.

Medium	Method			
	Direct collection	Blocked nephropores	P_t	Broken arteries
100% S.W.	1.6 ± 1.0	0.5 ± 0.8	<.05	1.8 ± 0.8
75% S.W.	6.3 ± 1.9	6.8 ± 1.7	>.05	---
50% S.W.	7.7 ± 0.6	10.3 ± 1.8	<.01	---
30% S.W.	9.8 ± 1.7	10.6 ± 3.0	>.05	---

Table 3. Urinary rates (% B.W./day) of C. magister and C. productus after acclimation to various media. Mean values.

Medium	C. magister	C. productus
100% S.W.	1.6	4.6
75% S.W.	6.3	4.3
50% S.W.	7.7	4.3
30% S.W.	9.8	---

Because a certain amount of handling of the crabs was necessary in all experiments, the effects of handling on urinary rate were investigated. Four crabs were acclimated to 75% S.W. for 72 hr. During the next 48 hr the crabs were removed from their tanks at 12 hr intervals, inverted several times, a hemolymph sample of 0.5 ml was taken and the crabs were returned to their tanks. As shown in Fig. 19, handling and withdrawal of small amounts of blood had no significant effect on urinary rate.

The Effect of Temperature Changes on Urinary Rate

The effect of acute temperature changes on urinary rate was assessed in three experiments. Three crabs acclimated to 75% S.W. at 12 ^{o}C were cooled to 7 ^{o}C over a period of 12 hr and held at that temperature for 3 days. Then the temperature was raised to 17 ^{o}C over a period of 4 hr and held at that temperature for another 2 days. The results are displayed in Fig. 20. It can be seen that changes in temperature had an immediate effect on urinary rate. As shown in Table 4, calculated values of Q_{10} for both temperature changes are close to 2. The difference between the means at 12 and 7 ^{o}C may or may not be significant. This is probably due to the small number of animals used, as each of the three crabs showed a decrease in urinary rate of 18

Figure 19. Effect of handling on urinary rate of C. magister in 75% S.W. Crabs were inverted seceral times and a hemolymph sample was taken at each time interval, beginning at t=12 hr. Mean values ± s.d.

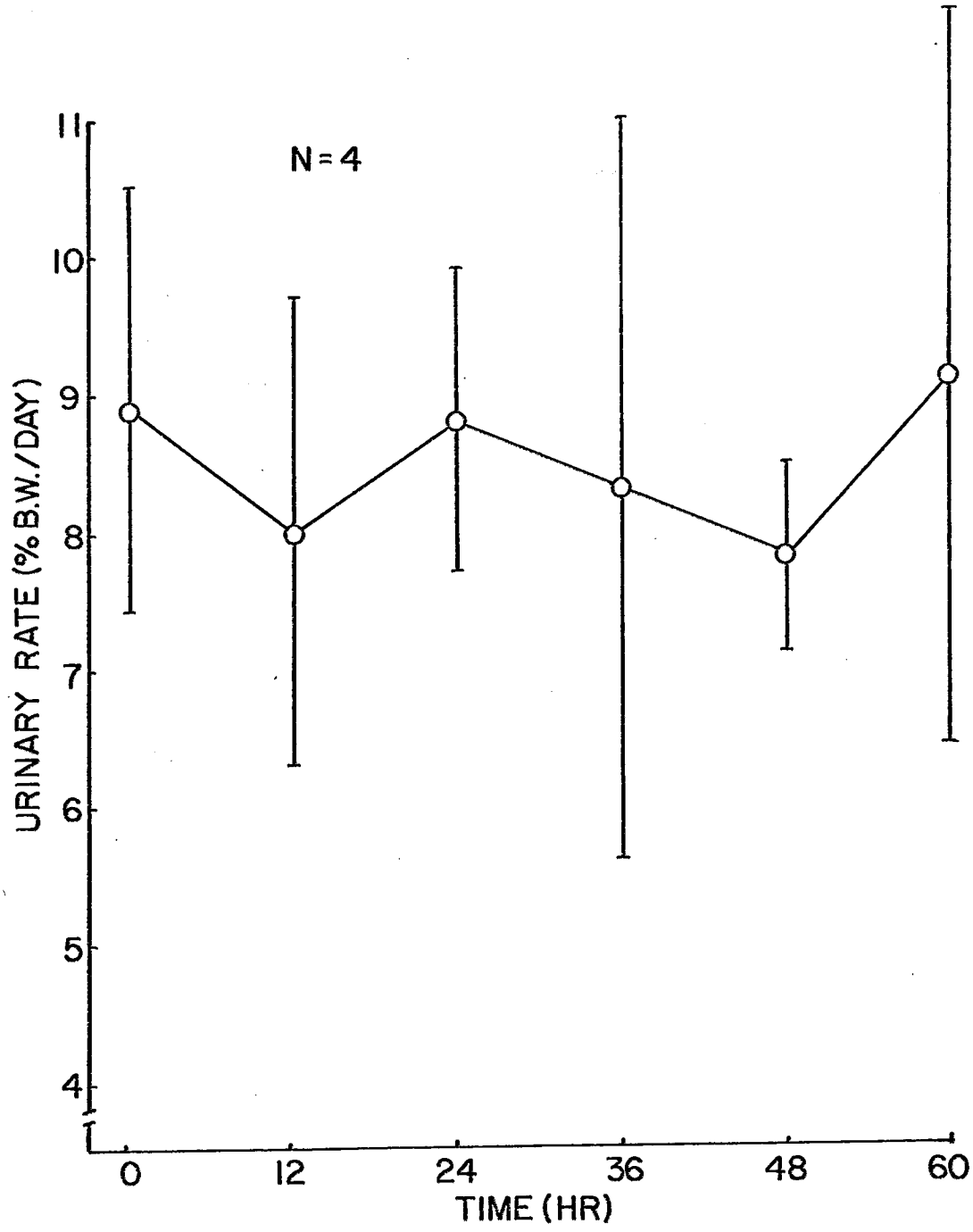


Figure 20. Effect of temperature changes on urinary rate of C. magister in 75% S.W. Mean values ± s.d.

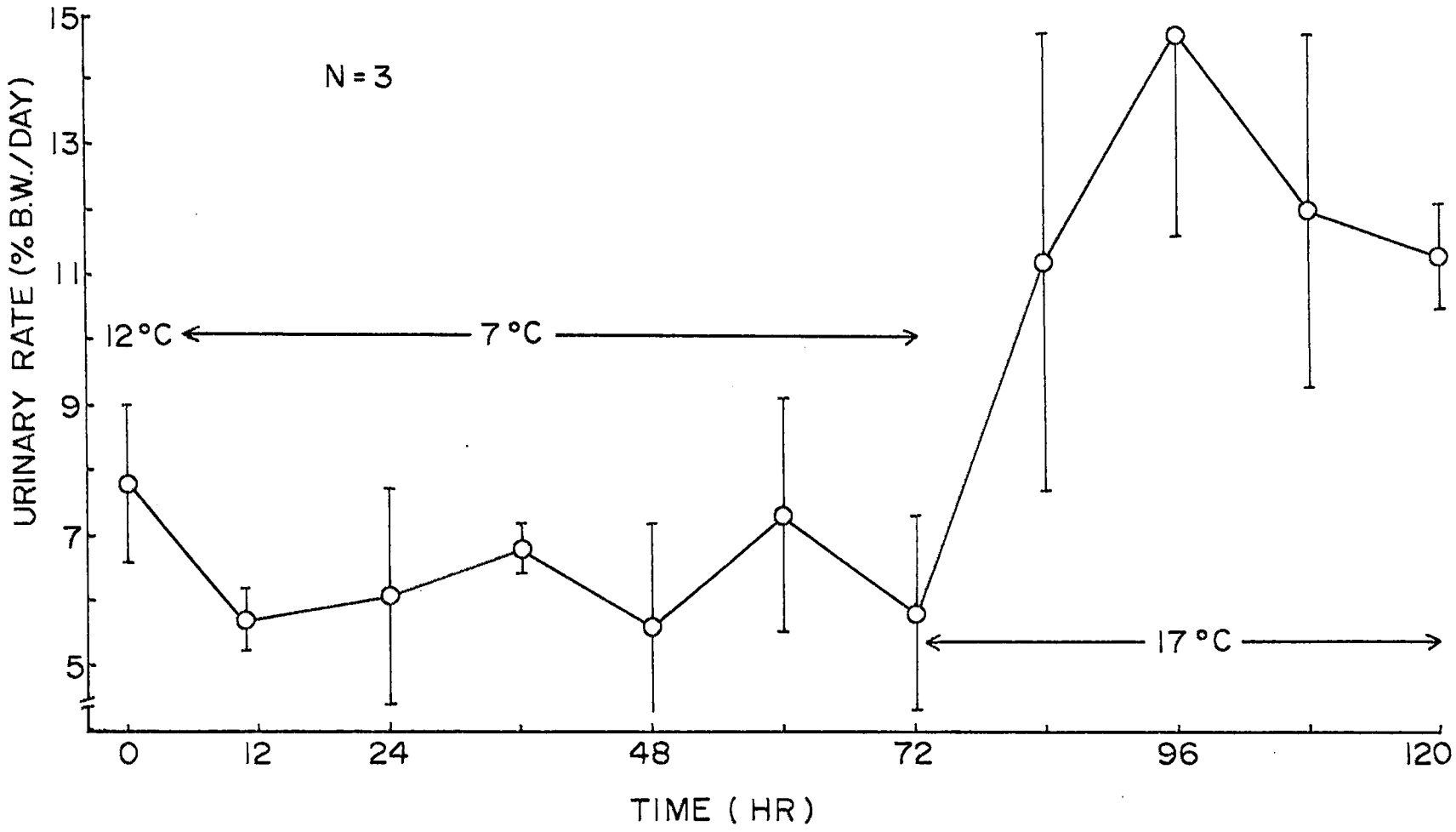


Table 4. Effect of temperature changes on the urinary rate of C. magister in 75% S.W. Mean values. Recalculated from data in Fig. 20.

	Day					
	0	1	2	3	4	5
Temperature (°C)	12	7	7	7	17	17
Urinary rate (% B.W./day)	7.8	5.9	6.2	6.6	13.0	11.6
Q_{10}		1.7			2.0	

to 32%. Acclimation to a new temperature took place slowly over a period of several days.

The effect of a 5 °C increase in temperature on the urinary rates of two crabs acclimated to 30% S.W. at 12 °C was determined and the data are displayed in Fig. 21 and Table 5. Apparently, acclimation to 30% S.W. increased the effect of an increase in temperature on urinary rate, as the Q_{10} value was 2.8.

A final experiment involved acclimation of a group of nine crabs to 75% S.W. at 6 °C. After 72 hr the crabs' nephropores and guts were blocked and urinary rates (as weight changes) were measured for an eight hr period. Data from this experiment are compared with data from crabs in 75% S.W. for 72 hr at 12 °C in Table 6. Apparently, acclimation to 6 °C was nearly complete in 72 hr, as the urinary rates of the two groups differed by only 15% and the two means were not significantly different.

The three experiments considered together show that Q_{10} values for acute changes in temperature were about 2 and that acclimation to a new temperature probably took longer than 3 days. Acclimation to 30% S.W. increased the effect of temperature and lengthened the time for the response (Fig. 21).

Figure 21. Effect of temperature changes on urinary rate of C. magister in 30% S.W. Mean values ± s.d.

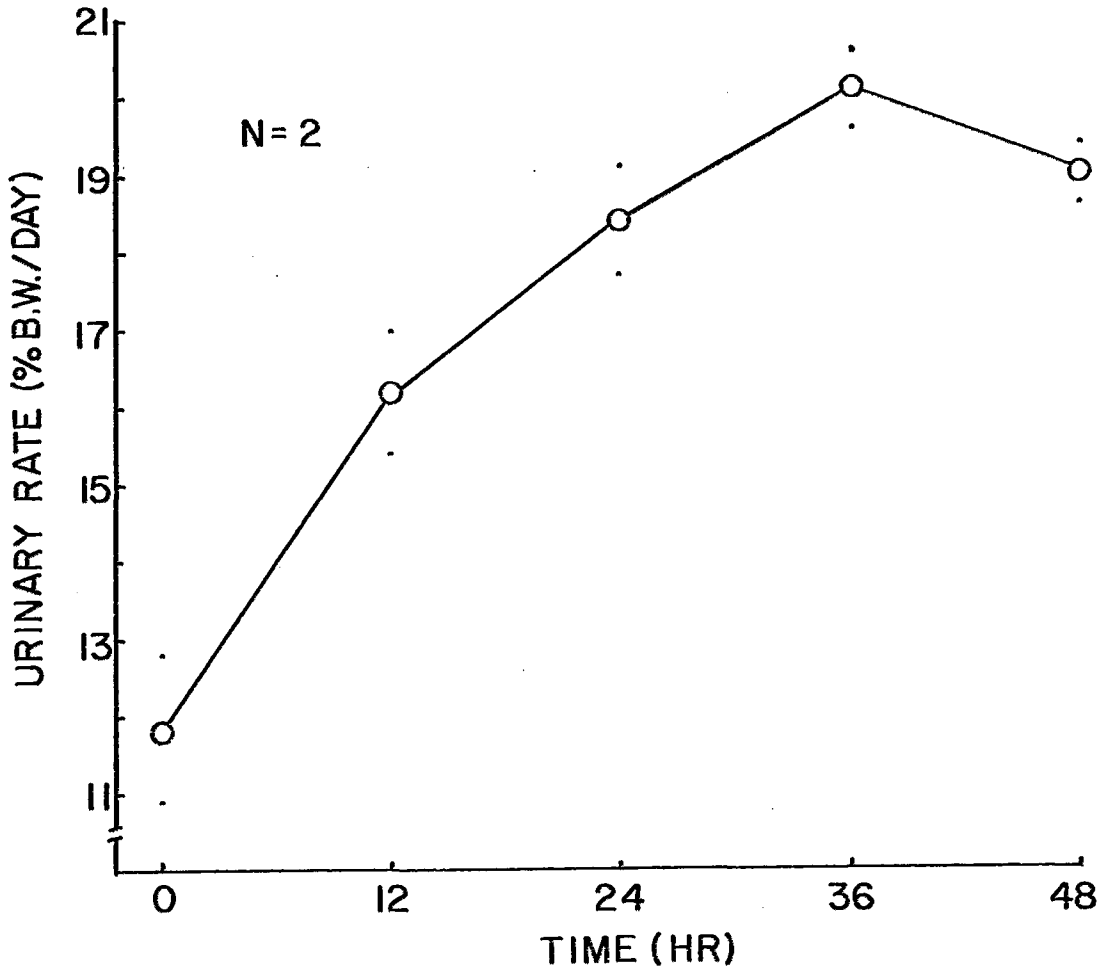


Table 5. Effect of temperature changes on urinary rates of C. magister in 30% S.W. Data recalculated from Fig 21. Mean values.

	Day		
	0	1	2
Temperature (°C)	12	17	17
Urinary rate (% B.W./day)	11.8	17.3	19.6
Q_{10}		2.1	2.8

Table 6. Urinary rates of C. magister acclimated to 75% S.W. at 12 and 6 °C. Blocked nephropore method. Mean values ± s.d.

Temperature	Urinary rate (% B.W./day)	P_t
12 °C	6.8 ± 1.7	
		>.05
6 °C	5.8 ± 0.8	

Table 7. Effect of hemolymph loss on urinary rate of C. magister in 75% S.W. Data recalculated from Fig. 24. Mean values.

	Day				
	0	1	2	3	4
Urinary rate (% B.W./day)	8.2	5.3	5.0	7.9	8.2
P_t (compared to day zero)	---	<.05	<.05	>.05	>.05

Annual Variations in Urinary Rate

Because the experimental procedure in much of this study involved preliminary acclimation of crabs to 75% S.W. for 48 hr, data on urinary rates were taken over a period of more than a year. These data are displayed in Fig. 22. There was apparently no seasonal change in urinary rate.

The Role of the Gut in Counteracting Hemolymph Loss

Fig. 23 shows the response, in terms of % initial weight of crabs in 100% S.W. subjected to a loss of hemolymph equivalent to 6-7% of the body weight. It can be seen that about 60% of the lost weight was replaced in the first hour and that occlusion of the gut essentially prevented replacement of the lost hemolymph. Presumably this unrectified decrease in hemolymph volume interfered with gill circulation, as all but one of the crabs died after 18 hr. This suggests that sudden hemolymph volume decreases such as blood loss were rectified, at least in part, by drinking the medium.

The Effect of Chronic Hemolymph Loss

Fig. 24 and Table 7 show the results of an experiment designed to examine the effect of a chronic reduction of hemolymph volume on the urinary rates of crabs in 75% S.W.

Figure 22. Annual variation of urinary rate in C. magister acclimated to 75% S.W. Mean values for groups of five crabs ± s.d.

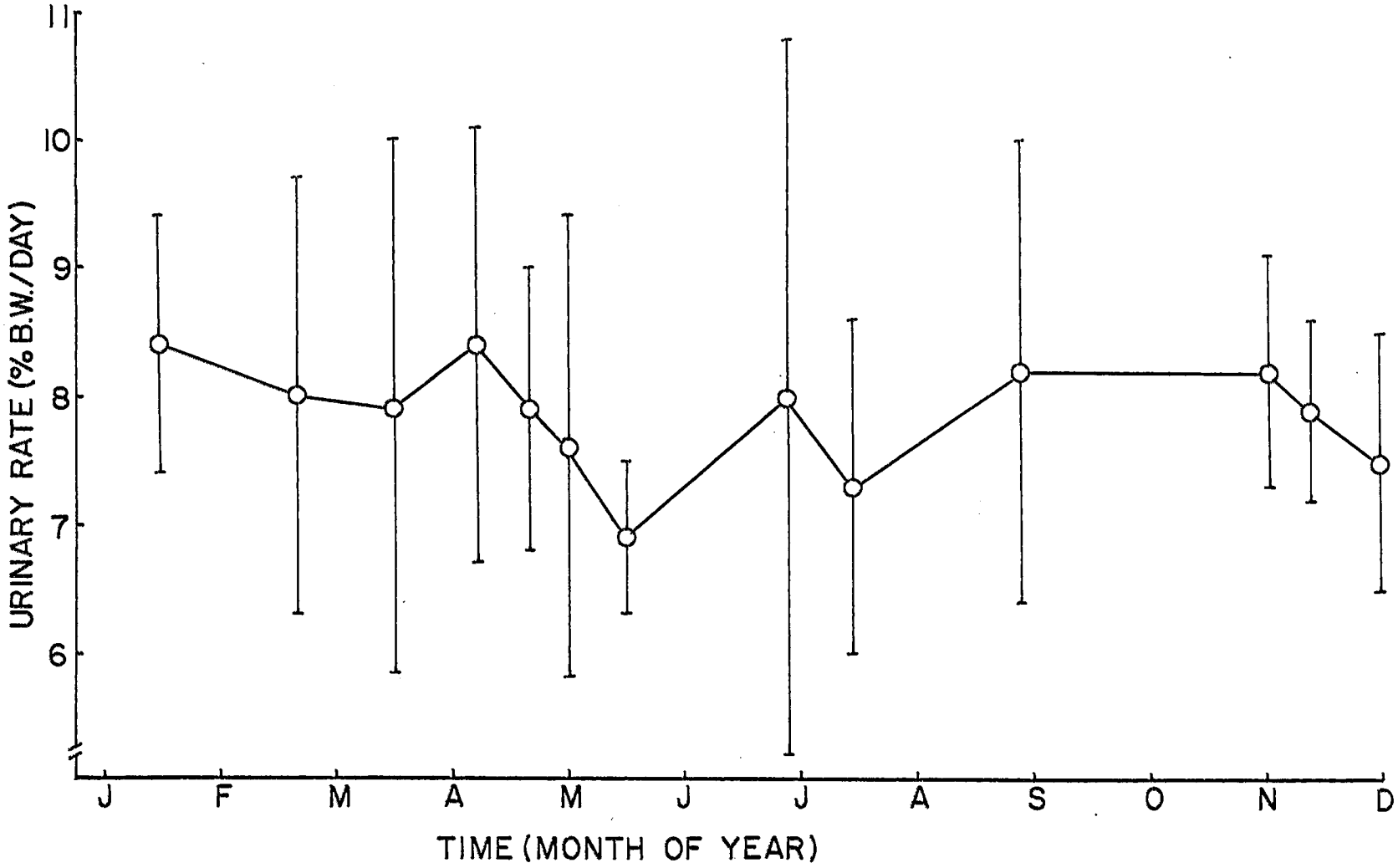


Figure 23. Time course of replacement of lost hemolymph in C. magister in 100% S.W. Mean values ± s.d.

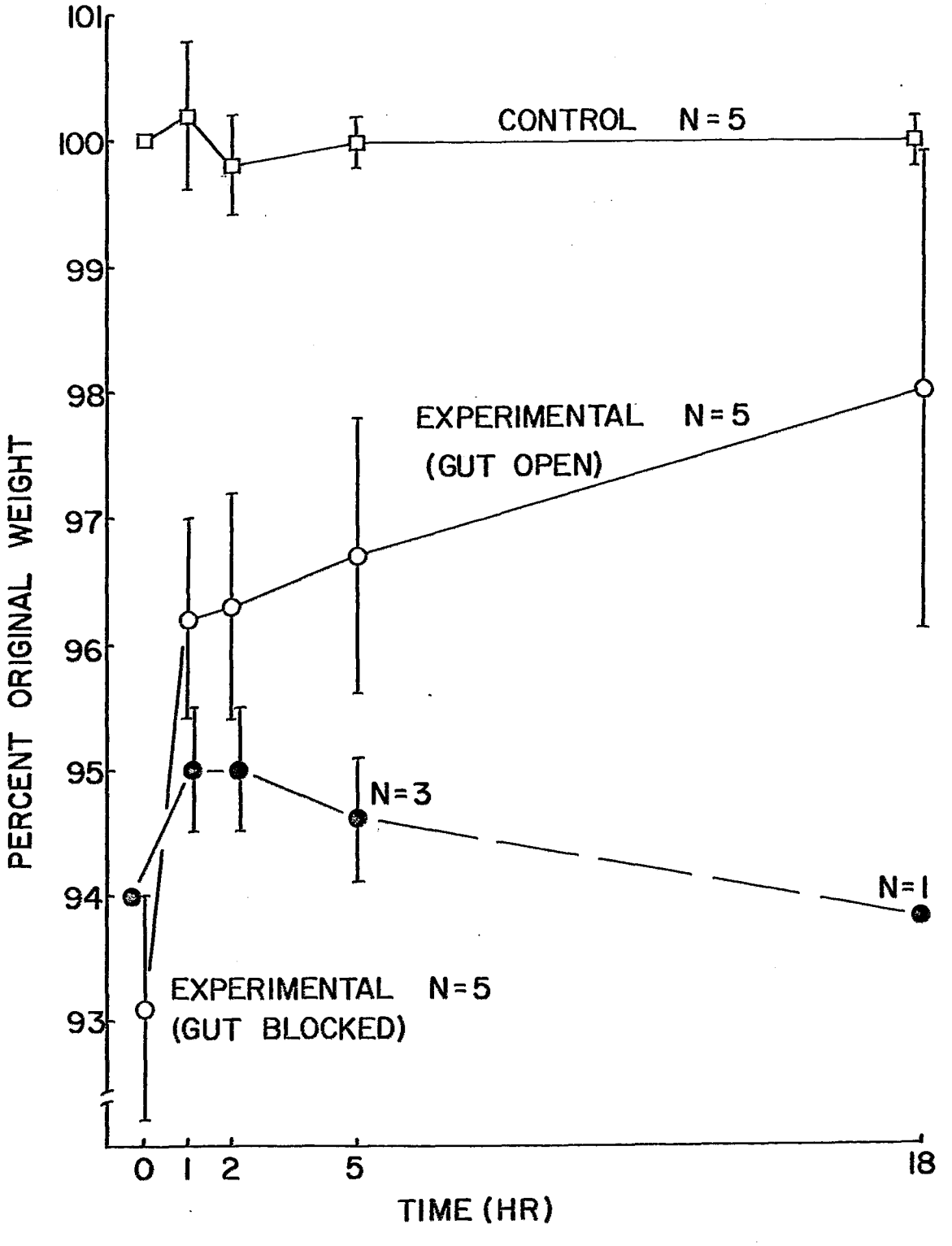
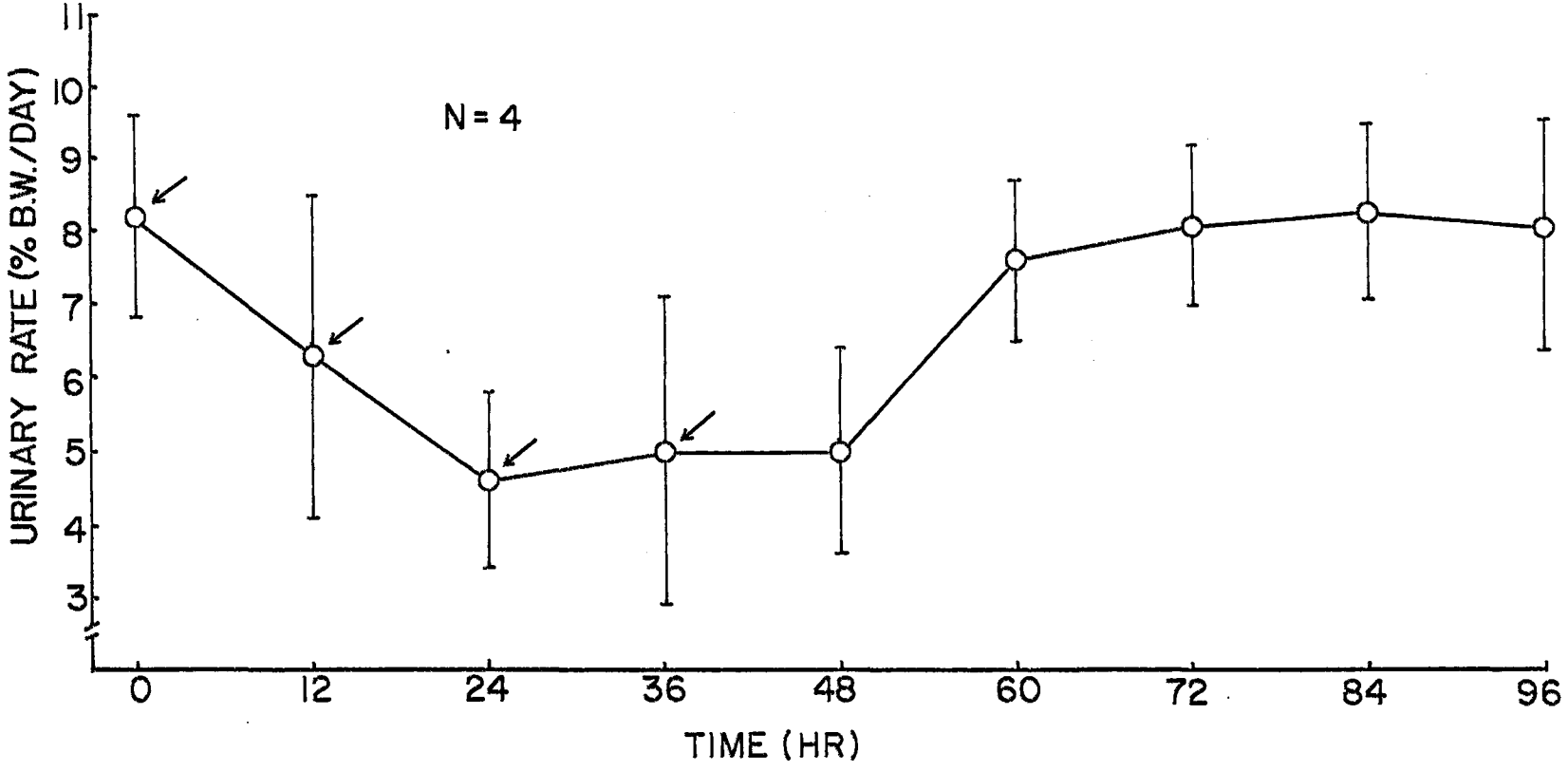


Figure 24. Effect of hemolymph loss on urinary rate of C. magister in 75% S.W. At arrows 3.6% body weight of hemolymph was withdrawn. Mean values ± s.d.



Removal of a volume of hemolymph equal to 3.6% of the body weight every 12 hr reduced the urinary rate by 36% and the reduction was significant (Table 7). It might be expected that urine production would cease under these conditions, as the normal urinary rate in 75% S.W. was 8.2% body weight per day, but this was not the case. It is probably that the volume loss was rectified by drinking between hemolymph withdrawals, a conclusion supported by the data in Fig. 23.

The Effect of Hemolymph Volume Increases

Two experiments were conducted to determine the effect of hemolymph volume increases, or volume "loads", on urinary rate. First, a volume load of 2% of the body weight of crab Ringer was administered. After 24 hr a second injection of 2% body weight of distilled water was administered after withdrawing an equivalent volume of hemolymph. This second injection constituted a lowering of the blood osmotic pressure without a concurrent volume load. If it is assumed that blood volume is 30% of body weight (Lockwood, 1967), an injection of 2% body weight of water constituted a reduction of the hemolymph osmolality by about 7%. Before the injection the mean blood osmolality was 835 mOsm/kg H_2O. Two hours after injection of the water the blood osmolality was 808 mOsm/kg H_2O. The data from this experiment are

presented in Fig. 25 and Table 8. Volume loads immediately increased urinary rate and the load was eliminated within 2 hr. Lowering of the hemolymph osmotic pressure without a change in hemolymph volume had no significant effect on urinary rate. This experiment was repeated using injection volumes of 4% body weight of crab Ringer and distilled water. The results are displayed in Fig. 26 and Table 9. Again, the urinary rate immediately increased and the volume load was eliminated within 2 hr. Dilution of the hemolymph in this experiment was apparently greater than the crabs could tolerate, as the urinary rate was halved. The crabs were very sluggish 2 hr after the injection of the distilled water and four of the five crabs were dead 24 hr after the end of the experiment.

As has been shown, C. magister has large bladders which can contain a volume of urine equivalent to 4% of the body weight (Chapter 3). It could be argued that the high urinary rates produced by volume loads were due in part to a sudden voiding of a large amount of urine held in the bladders. To determine the ability of the crab to maintain high urinary rates, two experiments were conducted in which crab Ringer was perfused into the crabs' hemocoeles at mean rates of 5.0% and 11.5% of body weight per day. The results of these two experiments are shown in Figs. 27 and 28 and Tables 10 and 11. It is clear that high urinary rates could

Figure 25. Effect of volume load and dilution of hemolymph on urinary rate of C. magister in 75% S.W. At t=0 a volume load of 2% body weight crab Ringer solution was injected. At t=24 hr 2% body weight of hemolymph was withdrawn and an equivalent amount of distilled water was injected. Mean values ± s.d.

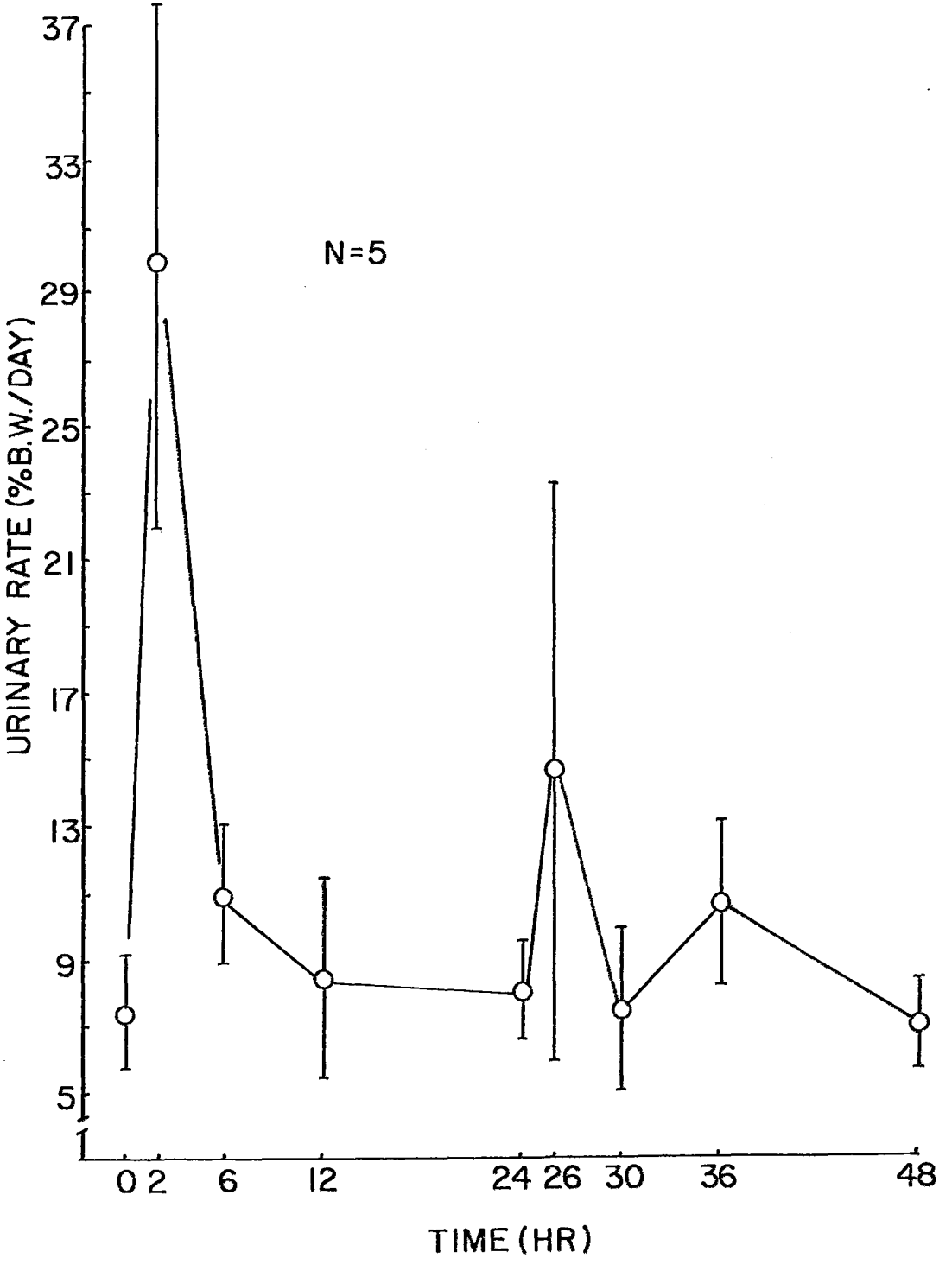


Table 8. Effect of volume load and dilution of hemolymph on urinary rate of C. magister in 75% S.W. Data recalculated from Fig 25. Mean values.

	Day		
	0	1	2
Urinary rate (% B.W./day)	7.4	10.4	8.6
P_t (compared to day zero)	---	<.05	>.05

Table 9. Effect of volume load and dilution of hemolymph on urinary rate of C. magister in 75% S.W. Data recalculated from Fig. 26. Mean values.

	Day		
	0	1	2
Urinary rate (% B.W/day)	8.2	12.2	4.6
P_t (compared to day zero)	---	<.001	<.001

Figure 26. Effect of volume load and dilution of hemolymph on urinary rate of C. magister in 75% S.W. At t=0 a volume load of 4% body weight of crab Ringer solution was injected. At t=24 hr 4% body weight of hemolymph was withdrawn and an equivalent amount of distilled water was injected. Mean values ± s.d.

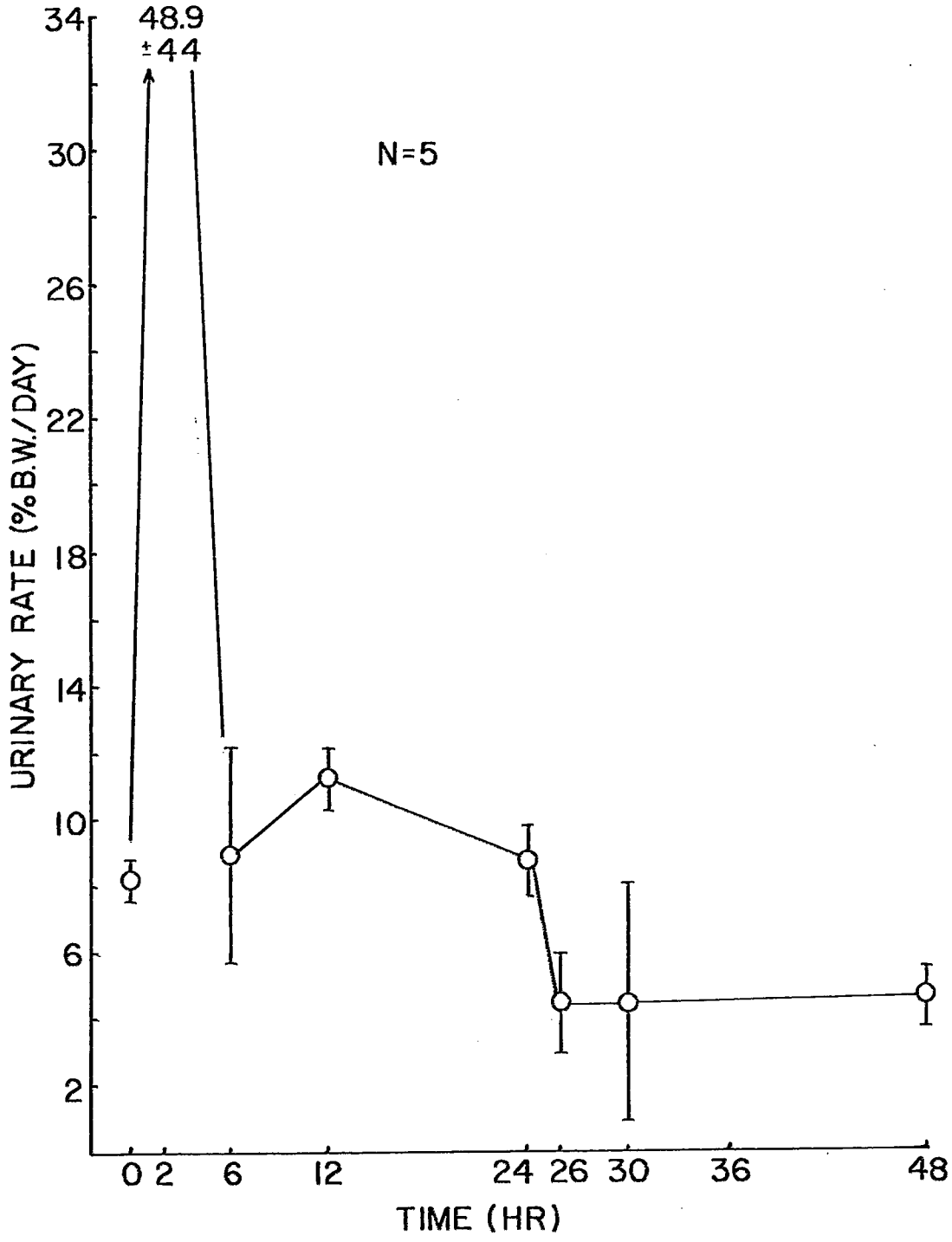


Figure 27. Effect of infusion of crab Ringer solution on urinary rate of C. magister in 75% S.W. Between t=0 and t=24 hr crab Ringer solution was infused at the rate of 5% body weight/day. Mean values ± s.d.

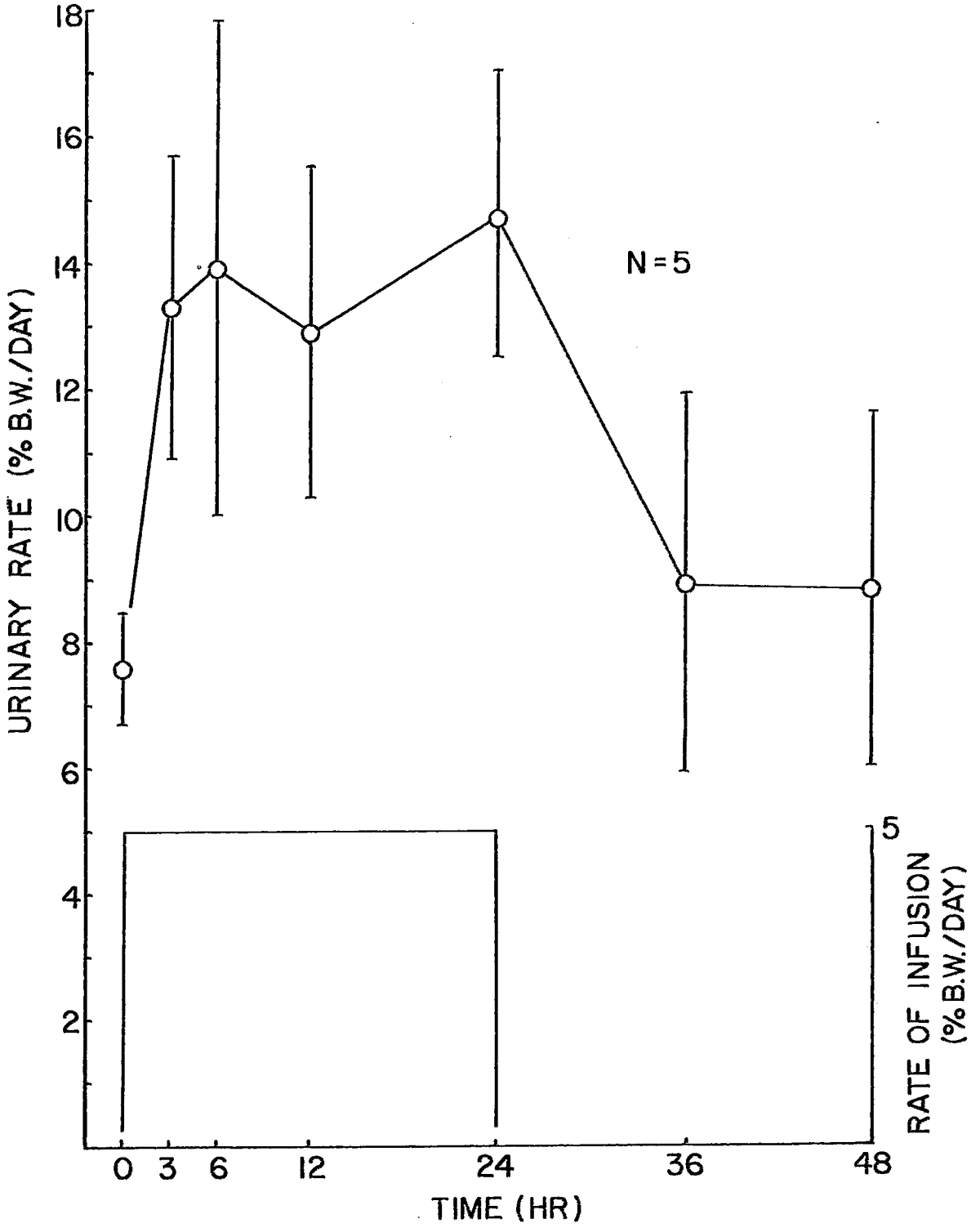


Figure 28. Effect of infusion of crab Ringer solution on urinary rate of C. magister in 75% S.W. Between t=0 and t=24 hr crab Ringer solution was infused at the rate of 11.5% body weight/day. Mean values ± s.d.

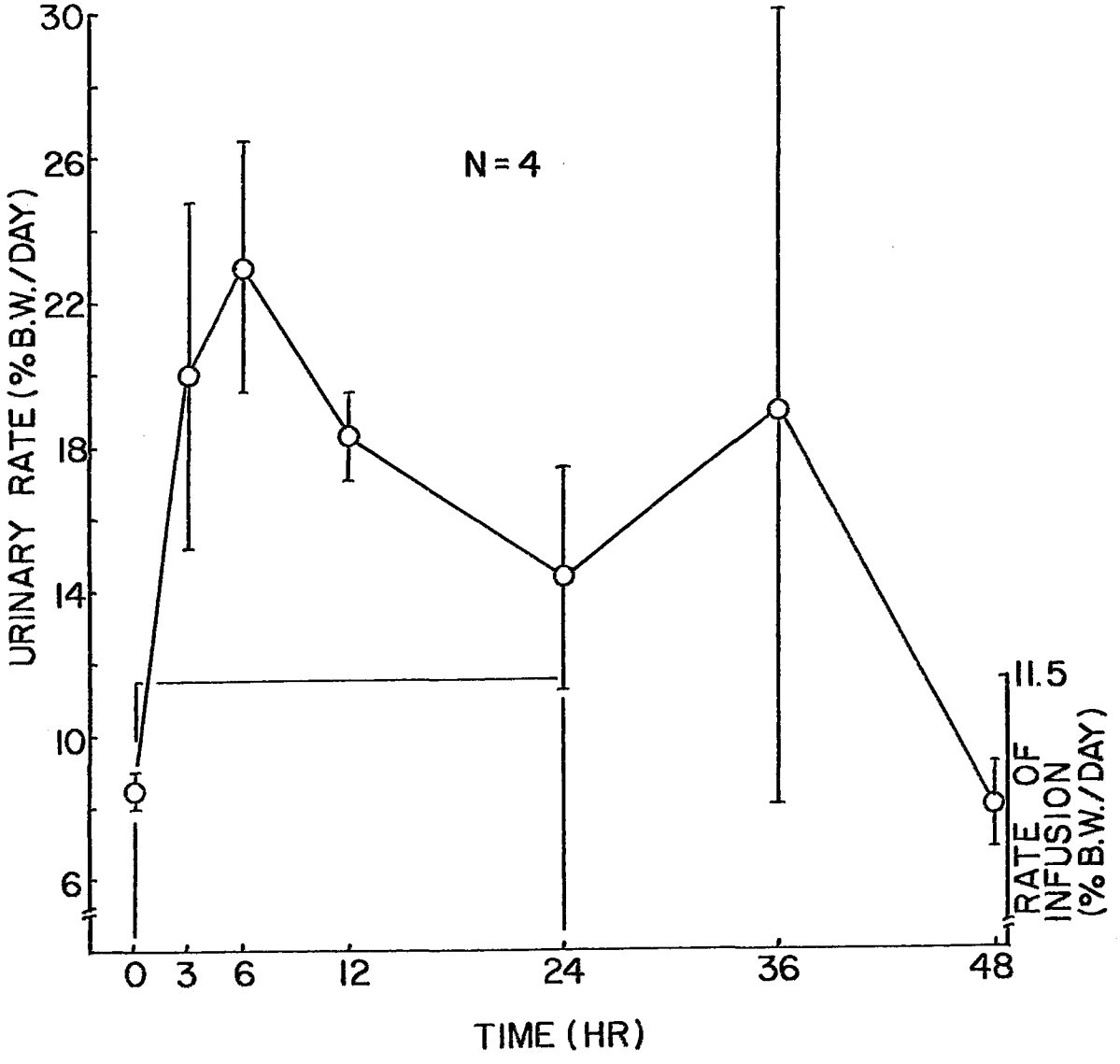


Table 10. Effect of infusion of crab Ringer solution on urinary rate of *C. magister* in 75% S.W. Data recalculated from Fig. 27. Mean values.

	Day		
	0	1	2
Urinary rate (% B.W./day)	7.6	13.9	8.8
P_t (compared to day zero)	---	<.001	>.05

Table 11. Effect of infusion of crab Ringer solution on urinary rate of *C. magister* in 75% S.W. Data recalculated from Fig. 28. Mean values.

	Day		
	0	1	2
Urinary rate (% B.W./day)	8.5	17.2	13.5
P_t (compared to day zero)	---	<.001	>.05

be maintained for long periods of time and that the urinary rate was increased to counteract the volume load applied. At the higher rate of infusion the crabs were swollen after 24 hr and one had died. This swelling was correlated with a reduction in urinary rate during the last 12 hr of the volume load and was probably due to the inability of the antennal glands to keep up with the applied volume load. Although the perfusion was stopped, the urinary rate remained high for the following 12 hr. Presumably the crabs were able to reduce their volumes to a normal level, as the urinary rates during the last 12 hr period were normal and the crabs were no longer swollen.

The preceding experiments clearly show a direct relationship between applied volume loads and increased urinary rates. They also show the absence of such a relationship between hemolymph dilution and urinary rate. C. magister apparently adjusts its urinary rate to counteract hemolymph volume changes.

Mechanism of Control of Urinary Rate

Kirschner (1967) and Riegel (1972) have reviewed the large body of evidence for the formation of urine in crustaceans by a process of ultrafiltration. If the existence in crustaceans of the functional equivalent of a

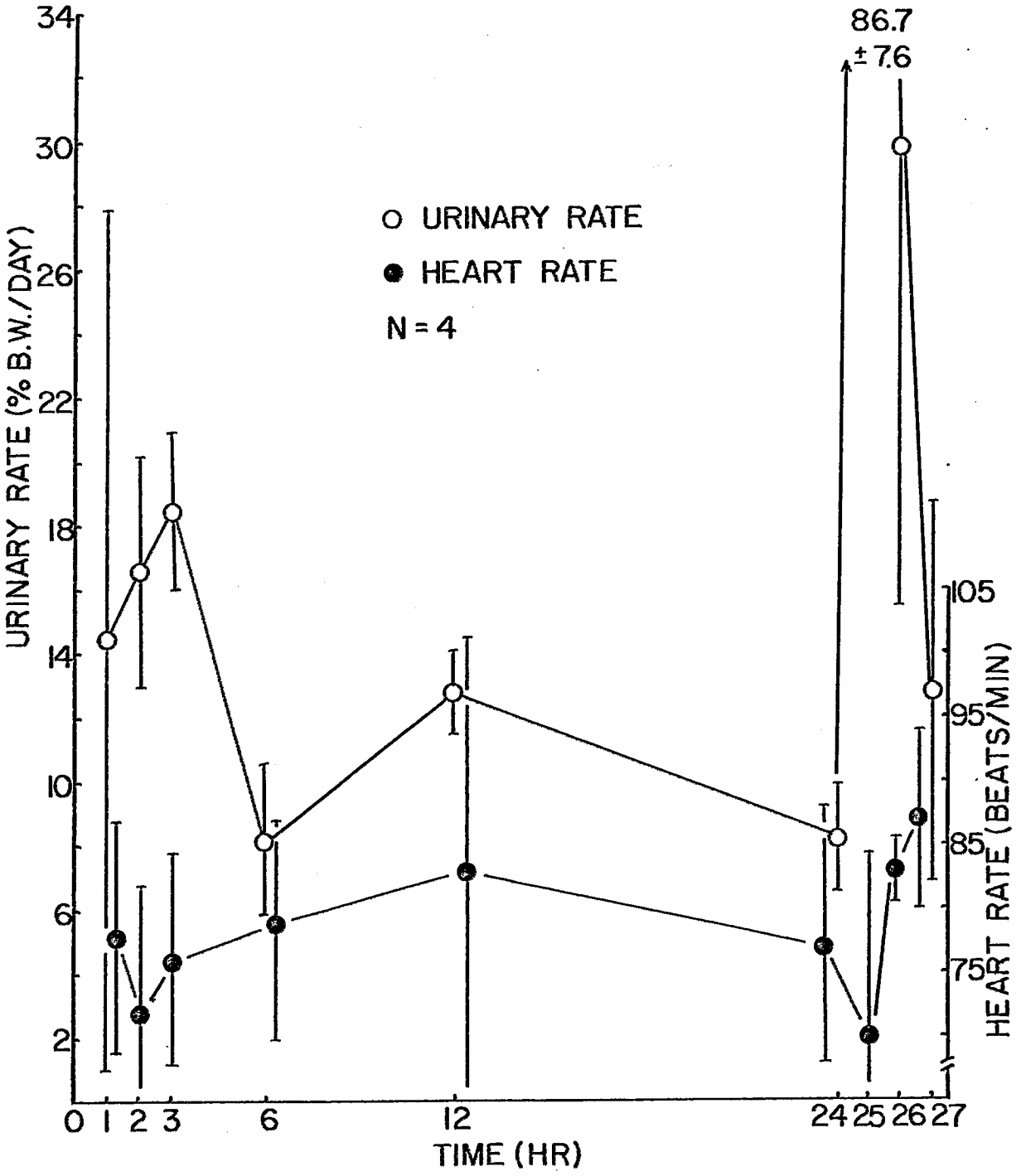
vertebrate nephron is accepted, then several parameters could possibly control the rate of urine production. Hormonally induced changes in blood pressure and/or effective pore size of the ultrafiltration membrane could cause large changes in urinary rate. Experiments were conducted to determine the effect of volume loads on heart rate and blood pressure. Additional experiments were carried out to determine the possibility of control of urinary rate by hormones produced or stored in the sinus gland, brain and thoracic ganglia.

Effect of Volume Loads on Heart Rate and Blood Pressure

Because heart rate in decapods has been shown to be under central nervous system control (Wilkens, et al., 1974) and is also influenced by the pericardial organs (Alexandrowicz and Carlisle, 1953), the effects of acclimation to lowered salinity and volume loading on heart rate were measured. The results of the experiment are shown in Fig. 29. It can be seen that no correlation exists between heart rate and urinary rate during conditions of volume loading.

Hemolymph pressure in the antennal artery, and in the heart, as well as heart rate were measured in two groups of crabs. One group was acclimated to 100% S.W. and the other

Figure 29. Effect of acclimation to 75% S.W. and volume loading on urinary rate and heart rate of C. magister. At t=0 salinity was lowered from 100% to 75% S.W. At t=24 hr a vloume load of 4% body weight of crab Ringer solution was injected. Mean values ± s.d.



group had been in 50% S.W. for 36 hr. The results are shown in Table 12. There were no significant differences in heart rate or blood pressure between the two groups, in spite of the fact that the crabs in 50% S.W. had urinary rates more than three times greater than the crabs in 100% S.W. (Fig. 18 at t=36 hr). These observations show that urinary rate was not increased under volume loading conditions by an increase in either heart rate or blood pressure.

Effect of Eyestalk Ablation on Urinary Responses to Volume Loading

The eyestalks of five crabs were ablated as described. Urinary rates were measured during acclimation to 75% S.W. and after a volume load of 4% body weight. The results of the experiments are shown with data from intact crabs in Fig. 30 and Table 13. Urinary rates of eyestalkless crabs during acclimation to 75% S.W. and during a volume load were normal. The urinary rate peak during the volume load was lower and displaced to the right because the first time period was 3 hr as opposed to 2 hr for the intact group. It is, therefore, unlikely that the sinus gland - x organ system is directly involved in maintenance of hemolymph volume.

Table 12. Effect of acclimation to 100% and 50% S.W. on heart rate and hemolymph pressure in C. magister. Mean values ± s.d. Hemolymph pressures are shown as systolic/ diastolic pressure.

Medium	N	Heart rate (beats/min)	P_t	Pressure in the heart(cm H_2O)	P_t	Antennal artery pressure(cm H_2O)	P_t
100% S.W.	5	83 ± 15		11.5 ± 2.9 / -2.1 ± 1.4		8.7 ± 2.2 / 7.5 ± 2.3	
			> .05		> .05		> .05
50% S.W.	6	73 ± 10		10.3 ± 1.5 / -3.1 ± 1.0	>.05	8.7 ± 2.2 / 7.2 ± 1.9	>.05

Figure 30. Effect of eyestalk ablation on urinary rate during acclimation to 75% S.W. and on urinary elimination of a volume load in C. magister. At t=0 salinity was lowered from 100% to 75% S.W. At t=48 hr a volume load of 4% body weight of crab Ringer solution was injected. Mean values ± s.d.

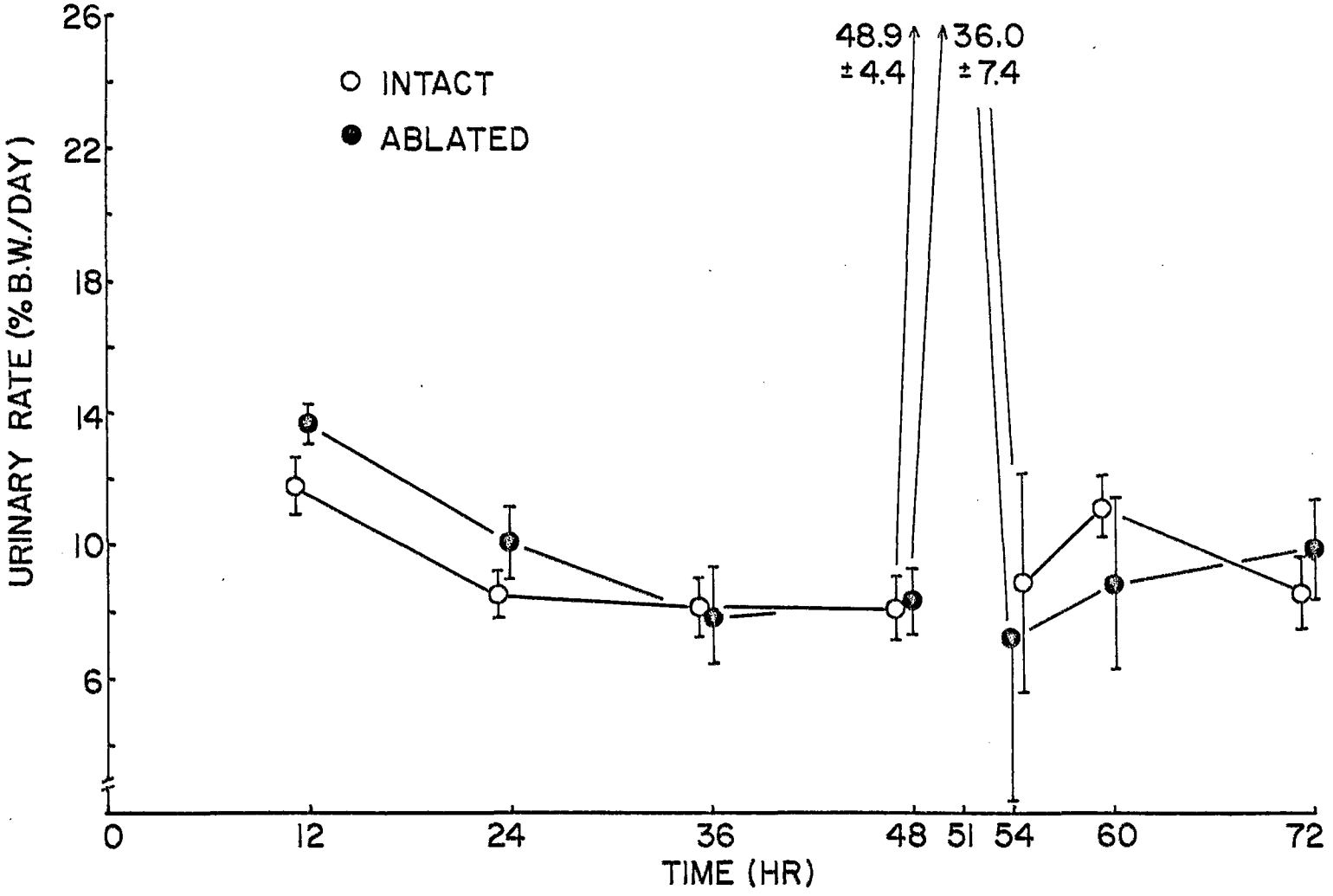


Table 13. Effect of eyestalk ablation on acclimation to 75% S.W. and urinary elimination of volume loads by C. magister. Data recalculated from Fig. 30. Mean values.

	Day	
	2	3
Urinary rate (% B.W./day), intact	8.2	12.7
Urinary rate (% B.W./day), ablated	8.2	12.6
P_t	>.05	>.05

The Effect of C.N.S. Homogenates on Urinary Rate

The effect of injections of brain and thoracic ganglion homogenates from crabs acclimated to 100% S.W. and 50% S.W. on the urinary rates of crabs in 75% S.W. was measured. One homogenized brain or thoracic ganglion was injected at 24 hr intervals. The results are shown in Fig. 31. Brain homogenates from crabs in 50% S.W. produced a pronounced increase in urinary rate, whereas brain homogenates from crabs in 100% S.W. produced a smaller effect. Thoracic ganglion homogenates from both groups showed a large effect on urinary rate. The effect of injection of homogenates was variable and some animals showed no effect at all. Because the effects of the homogenates were evidently short-lived, the experiment was repeated. One brain or thoracic ganglion equivalent was injected every 2 hr for a total of 3 injections and the urinary rate was monitored for 24 hr. The results of this experiment are shown in Fig. 32. Again, the effect was variable. Injection of homogenates of brains and thoracic ganglia from animals in 100% S.W. and of brains from animals in 50% S.W. greatly increased urinary rates while homogenates of the thoracic ganglia from animals in 50% S.W. had a smaller effect. In a third, similar experiment, single brain equivalents from animals in 50% or 100% S.W. or muscle homogenates were injected at 2 hr intervals

Figure 31. Effect of injected homogenates of brain and thoracic ganglion on urinary rate of C. magister in 75% S.W. At arrows one homogenized brain(B.) or thoracic ganglion(T.G.) from a crab in the indicated salinity was injected. Mean values ± s.d.

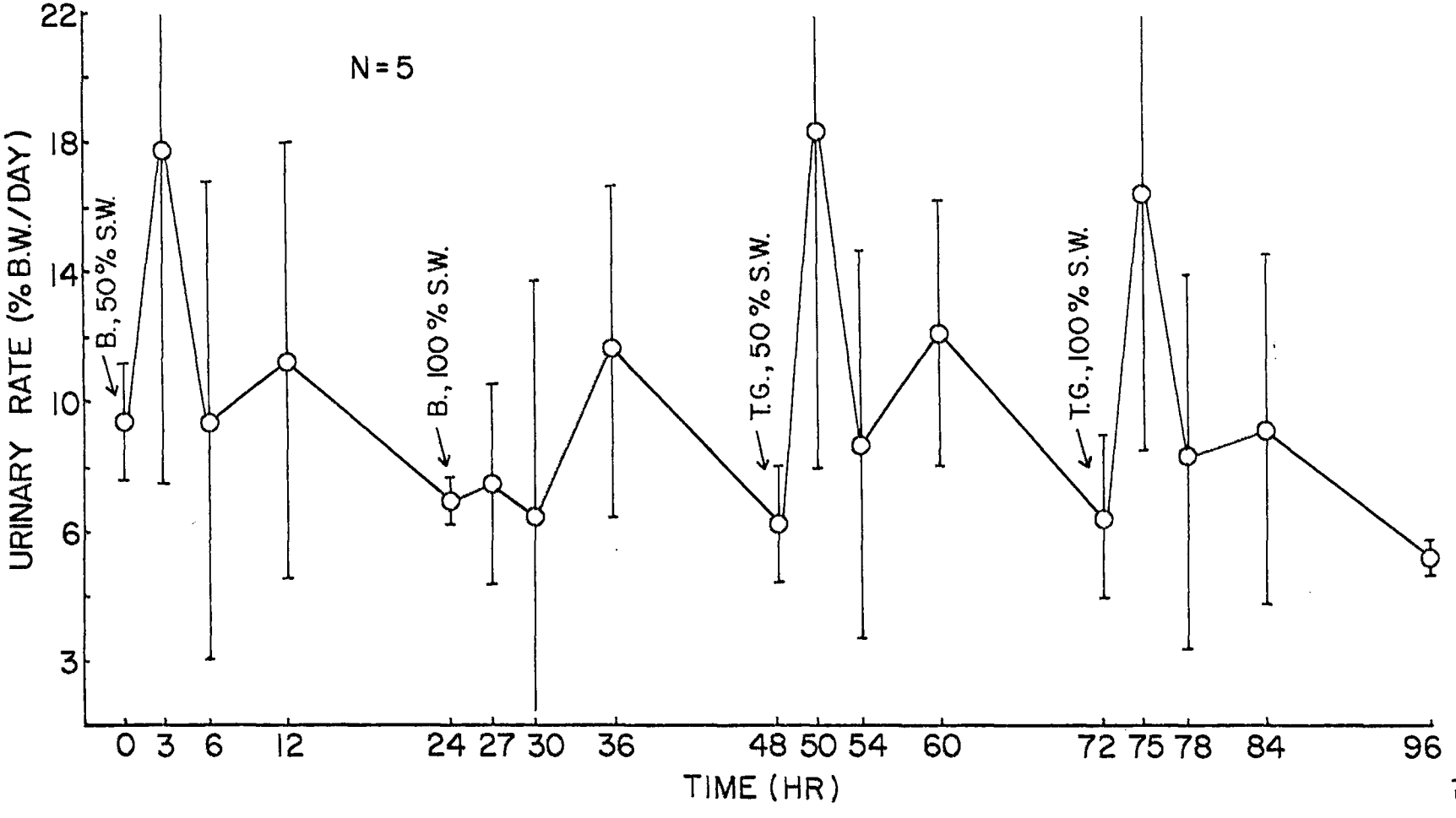
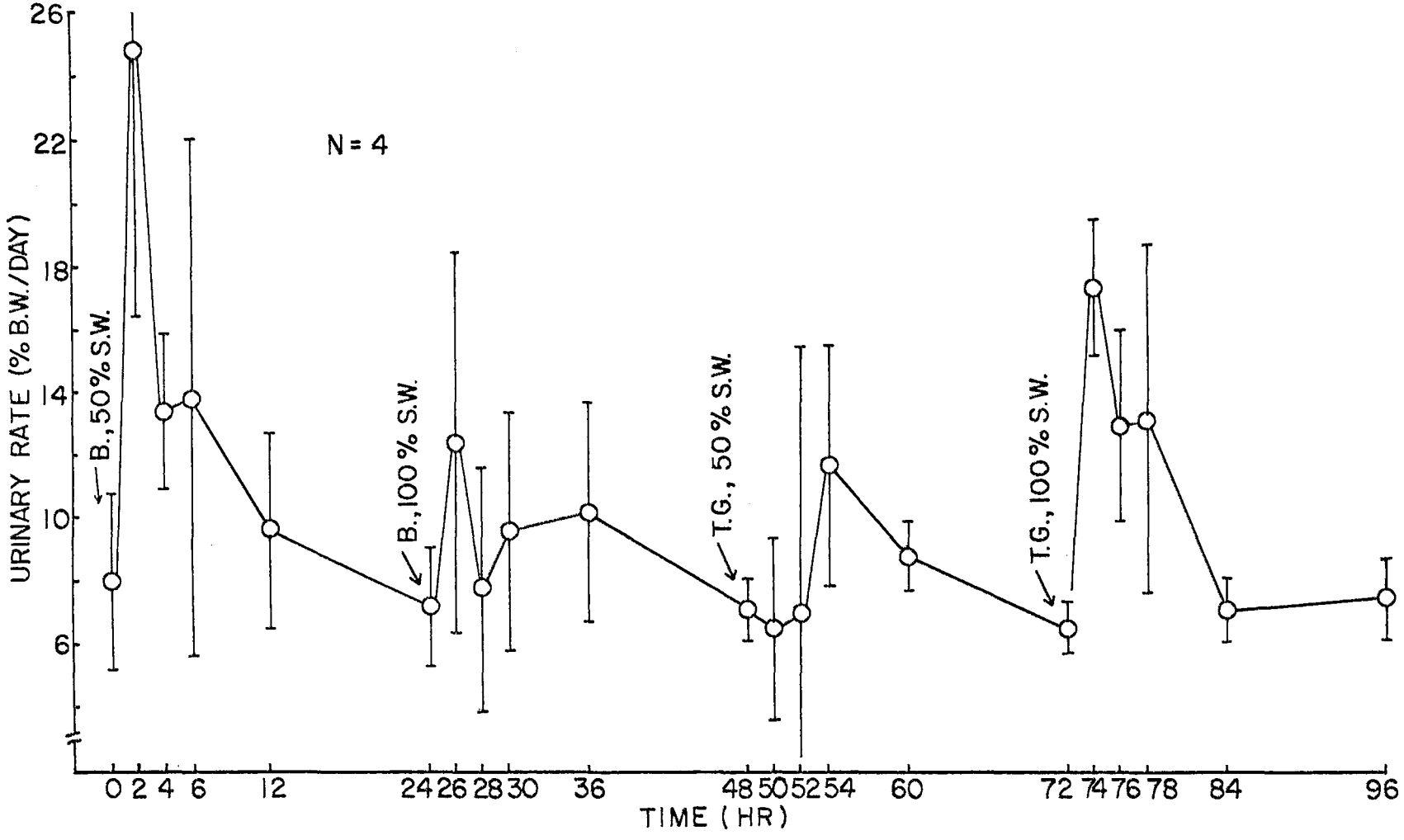


Figure 32. Effect of injected homogenates of brain and thoracic ganglion on urinary rate of C. magister in 75% S.W. At arrows a series of three injections at two hour intervals of one brain(B.) or thoracic ganglion(T.G.) homogenate from crabs in the indicated salinity was begun. Mean values ± s.d.



for a total of 3 injections and the urinary rate was monitored for 24 hr. The results of this experiment are shown in Fig. 33. Again, brain homogenates from animals in 50% and 100% S.W. caused a large increase in urinary rate. Injection of muscle homogenates also caused a large increase in urinary rate, and the data from all treatments were quite dispersed. The mean urinary rates during the first 6 hr of each treatment in the three experiments are shown in Table 14. The data from these experiments are difficult to interpret. The results were variable and there was no apparent dose-response relationship. Most significant was the finding that muscle homogenates were often quite as effective as brain or thoracic ganglion homogenates in causing increased urinary rates. In light of the effect of muscle homogenates, it is possible that all of the observed increases in urinary rates were at least partially due to a reaction to a cellular injury substance released from the tissues by homogenization. The effect of 50% S.W. brain homogenates (Figs. 31-33, Table 14) was large and it is possible that a "diuretic" hormone elaborated by the brain in response to dilute media is responsible for this effect.

Because a change in the pore size of the ultrafiltration membrane could cause a change in the rate of production of urine, an attempt was made to develop a perfused antennal

Figure 33. Effect of injected homogenates of brain and muscle on urinary rate of C. magister in 75% S.W. At arrows a series of three injections at two hour intervals of one brain(B.) or muscle tissue (M.) homogenate from crabs in the indicated salinity was begun. Mean values ± s.d.

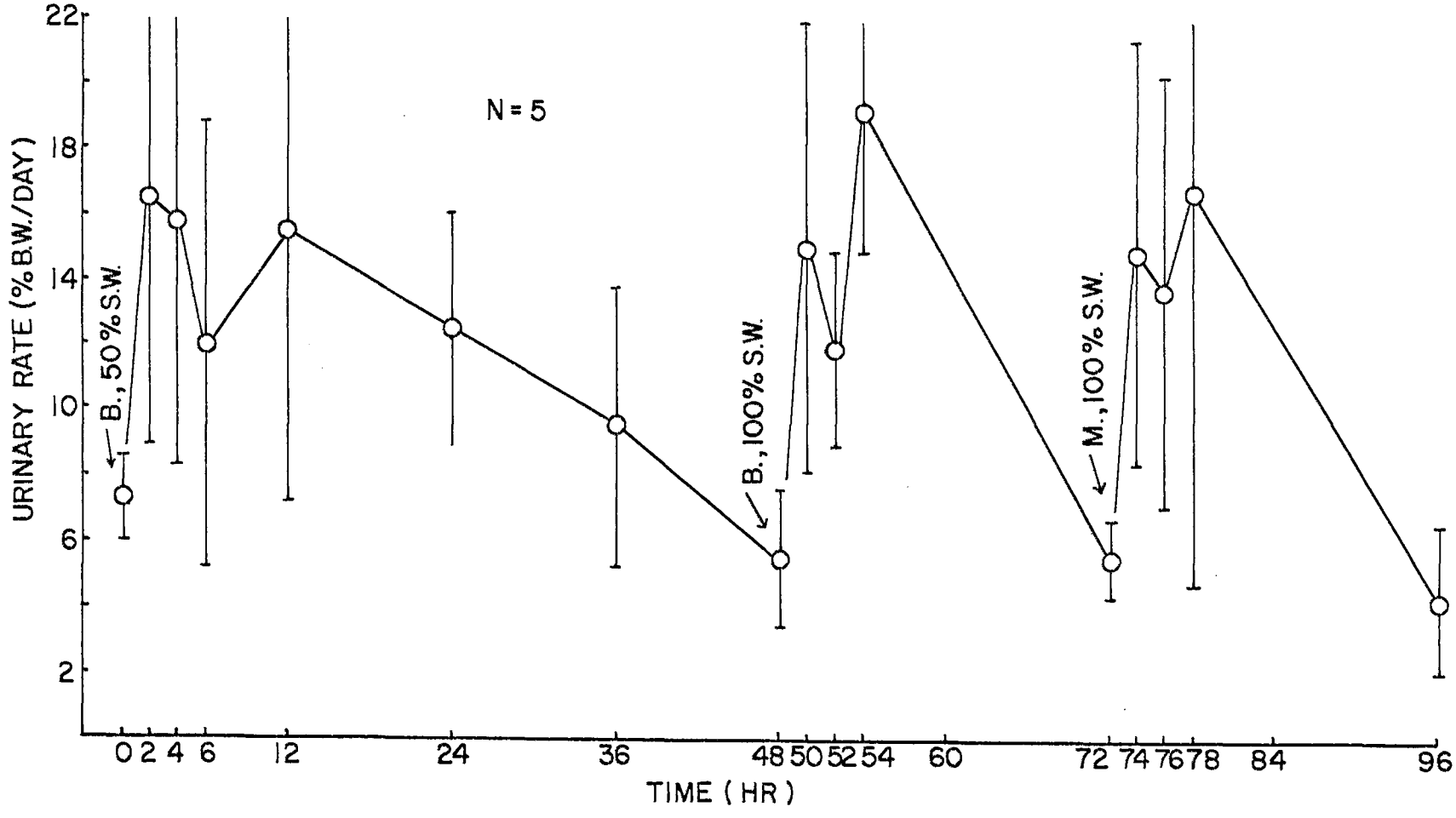


Table 14. Effects of injection of homogenates of brain, thoracic ganglion and muscle from crabs in 100% and 50% S.W. on urinary rate in C. magister in 75% S.W. Mean values ± s.d. for the first six hours after injection are shown.

	Tissue homogenate				
Reference	Brain- 50%SW	Brain- 100%SW	Thoracic ganglion-50%SW	Thoracic ganglion- 100%SW	Muscle
Fig. 31	13.6 ± 5.7	7.0 ± 4.0	14.1 ± 6.6	12.4 ± 5.6	---
Fig. 32	17.3 ± 1.4	10.0 ± 2.9	8.4 ± 1.9	14.4 ± 0.5	---
Fig. 33	14.8 ± 5.4	15.4 ± 3.4	---	---	15.1 ± 5.7

gland preparation similar to that devised by Norfolk (1976) with Carcinus maenas. The bladder and antennal artery were cannulated as described and an attempt was made to measure the rate of urine production in response to different perfusion pressures applied to the antennal artery. It was hoped that, using this preparation, the effects of brain homogenates on urine production at a constant perfusion pressure could be measured. Unfortunately, extreme difficulties with urinary cannula blockage were encountered. In only 3 of 20 experiments was the amount of "urine" produced greater than the amount that might have been held by a full bladder before cannulation, and, even in these experiments, the cannulae required frequent back-flushing to prevent occlusion. Further, there was no evident relationship between perfusion pressure and "urine" production rate. The results of one of the three successful experiments are shown in Table 15. The successful experiments do, however, provide evidence for urine production in C. magister by a process of ultrafiltration.

DISCUSSION

Urinary Rates

The urinary rates reported in this study for C. magister

Table 15. Urine production as a function of applied pressure in the perfused antennal artery of C. magister.

Perfusion pressure (cm H_2O)	Time period (min)	Urine produced (ml)	Urinary rate (% B.W./day)	Comments
50	30	0.25	1.6	
30	30	0	0	urinary cannula blocked, flushed
50	15	9.65	128	
50	15	2.16	28.6	
50	15	0.39	5.2	
20	60	0.06	0.2	urinary cannula blocked, flushed
50	15	2.37	31.4	urinary cannula blocked, flushed
50	15	3.40	45.0	urinary cannula blocked, flushed
50	15	3.28	43.4	urinary cannula blocked, flushed

in varying salinities agree well with published values for this species and other marine and brackish water brachyurans (Table 16). Hunter (1973) has obtained similar urinary rates for C. magister, and has shown that the urinary rate is directly related to the mole-fraction difference between the hemolymph and the external medium. A correlation between urinary rate and the sustained osmotic differential across the body surface has also been shown by Werntz (1963) in two species of the amphipod, Gammarus. The same author has shown, by recalculation of data from Parry (1955) and Panikkar (1941) that a similar relationship between urinary rate and osmotic differential exists in the shrimp, Palaemonetes varians. This constant relationship between mole fraction difference and urinary rate is what one would expect to find if an animal does not change its permeability to water in dilute media, and Hunter (1973) has, in fact, found that the hourly water exchange fraction (K_i) was unchanged in C. magister exposed to 30% S.W. Rudy (1967) found that Palaemonetes varians also does not reduce its K_i on acclimation to dilute media. In contrast to Rudy's results, Roesijadi, et al., (1976) found that Palaemonetes pugio does lower K_i in dilute media. Rudy (1967) also found that Carcinus maenas did not significantly reduce its permeability in dilute media, while Smith (1970) found that C. maenas did lower its water permeability in similar

Table 16. Urinary rates of brachyurans acclimated to various media.

Species	Medium (%SW)	Urinary rate (%BW/day)	Method	Reference
Cancer magister	100	1.6	Direct collection	Present study
	75	6.3		
	50	7.7		
	30	9.8		
C. magister	100	1.4	Glofil clearance	Hunter, 1973
	75	5.3		
	50	11.8		
	30	12.2		
Carcinus maenas	100	4.4	Inulin clearance	Binns, 1969a
	75	10.8		
	50	16.9		
	40	21.1		
C. maenas	100	3.6	$^{35}SO_4$ efflux	Shaw, 1961
	75	11.1		
	50	16.5		
	40	31.3		
Pachygrapsus crassipes	150	1.5	Mg efflux to Mg-free medium	Gross & Marshall, 1960
	100	3.9		
	50	58		

Table 16. (continued)

Soecies	Medium (%SW)	Urinary rate (%BW/day)	Method	Reference
Rithropanopeus harisi	50	25.2	Cl efflux to distilled water	Smith, 1967
	10	23.3		
Maia squinado	100	2.9	Collection of available urine	Bialaszewicz, 1931
Cancer pagurus	100	3.1-10.1	Collection of available urine	Robertson, 1939
Cancer productus	100	4.6	Direct collection	Present study
	75	4.3		
	50	4.3		

dilute media. Cantelmo (1978) has shown that the K_i of excised gill and gut from Cancer irroratus and Callinectes sapidus is reduced in tissues from crabs acclimated to dilute media. Although Smith (1976) proposes that the phenomenon of adaptive reduction of water permeability observed in many crustaceans may be a general occurence, the response may instead be species-specific. Hannan and Evans (1973) have found that 3 species of the more terrestrial and extremely euryhaline fiddler crab, Uca, have similar K_i values in 100%, 50% and 3% S.W. Smith (1976), in discussing unpublished experiments by J.C. Cornell, raises the possibility that adaptive changes in apparent water permeability may actually be due to changes in the rate of hemolymph flow through the gills. Berlind and Kamemoto (1978) also mention the possibility that changes in hemolymph flow through the gills or in ventilation of the gill chamber may be responsible for changes in K_i.

The crabs cited in Table 16 are all isosmotic or hypoosmotic to 100% S.W., yet all produce urine at rates of between 1.4 and 4.6% body weight per day. Shaw (1961) has suggested that in C. maenas the necessary water is taken up from the medium by an active process in response to blood sulfate levels. Rudy (1967) has measured water permeabilities in several crustaceans, and has calculated net water influxes from these data. For C. maenas and Astacus

fluviatilis the calculated net water influx was significantly less than the published values for urinary rate. Rudy suggested that the difference could be accounted for if the animals were drinking. Smith (1967) has also found that in the crab, Rithropanopeus harrisi, the urinary rate exceeded the calculated net diffusional influx of water in 10% S.W., and Capen (1972) has presented evidence that R. harrisi does not drink to any significant extent in 70% and 10% S.W. Smith (1976) notes: "That urinary output of water occurs even in the absence of an 'osmotic' inflow resulting from a water-concentration difference has been so well known that it is usually not commented upon." Drinking is a well established phenomenon in crustaceans. Hannan and Evans (1973) found that Uca sp. drinks about 14% of the body weight per day in 100% S.W. and that Penaeus drinks 41% of the body weight per day. Lockwood (1970) has found that Gammarus duebeni drinks 48-96% body weight per day when subjected to a loss of hemolymph volume. Dall (1967) has shown that the shrimp, Metapenaeus bennettae, and the crabs, Metapograpsus gracillipes and Macropthalmus crassipes, all drink the medium at a rate of about 16% body weight per day. Dall also showed that some regurgitation occurs, indicating that the drinking rate may be greater than actual water uptake via the gut. It is possible that the production of urine at a greater rate than required to

balance osmotic influx may facilitate the secretion of Mg^{++} and $SO_4^{=}$ ions by the bladder in the more marine crabs. Gross and Capen (1966) have provided evidence that Mg^{++} is secreted into the urine by the bladder of the crab, Pachygrapsus crassipes, and evidence for Mg^{++} secretion by the bladder of C. magister is shown in the present study (Chapter 5). Given that the bladder secretes Mg^{++} ions into the urine, a reduction of the urine/hemolymph gradient by the flushing of the bladder with newly formed urine which has the same low Mg^{++} concentration as the hemolymph would seem advantageous. In Chapter 5 of the present study, evidence is presented which shows that such a flushing of the bladder is necessary for the maintenance of normal blood Mg^{++} levels. The continuous production of urine would also remove toxic and useless metabolites from the hemolymph in the absence of specific secretory pathways for these substances (Burger, 1957; Lockwood, 1967, p. 43). Drinking of the medium may supply the "extra" water needed to produce urine in the absence of a net osmotic influx of water (Potts and Parry, 1964, p. 110), although this is apparently not the case in two species of the lobster, Panulirus (Dall, 1974; Malley, 1977a, 1977b). Alternatively, the "extra" water may be due to water uptake accompanying active inward ion transport, even in isosmotic media (Potts and Parry, 1964, p. 110; Lockwood and Inman, 1975; Smith,

1970, 1976; Malley, 1977). Ono and Kamemoto (1969) have suggested that seasonal variations in urine production in the crayfish, Procambarus clarkii, may be due to differences in feeding and drinking habits. The regression line derived by Hunter (1973) which relates urinary rate to mole-fraction difference in C. magister shows urinary rates of 1% body weight per day in the absence of an osmotic gradient, possibly indicating that in 100% S.W. the crabs drink 1% of the body weight per day. Evidence that C. magister drinks in 100% S.W. has been provided in the present study (Table 2). Crabs with blocked guts and nephropores showed a weight gain in 100% S.W. that is about half the urinary rate of intact crabs. Further, crabs made anuric by breaking the arteries to the antennal glands gained weight at a rate equal to the urinary rate of the intact crabs. Therefore, it seems likely that C. magister drinks at a rate equal to about 1% body weight per day when in 100% S.W. Drinking evidently did not occur to any significant extent in dilute media, as the weight gains of crabs with blocked guts and nephropores were equal to or exceeded the urinary rates of intact crabs in these media (Table 2).

Because urinary rates were measured by direct collection in this study, it was possible to determine the time course of urinary rate changes during acclimation of crabs to dilute media (Fig. 18). The initial urinary rates were

quite high, but fell rapidly during the first 24 hours. Huf (1936) and Norfolk (1976) have observed similar high initial urinary rates in C. maenas on transfer to 50% S.W. Spaargaren (1974) and Hunter and Rudy (1975) have shown that urine and hemolymph are isosmotic in all media tolerated by C. maenas and C. magister, respectively, a common condition in brachyurans (Potts and Parry, 1964). Further, Hunter (1973) has shown that C. magister did not decrease its apparent water permeability when acclimated to 30% S.W., and that sodium uptake was maximally activated in 50% and 30% S.W. It seems evident from these data that acclimation to dilute media in C. magister consists of a rapid increase in urinary rate to control swelling caused by osmotic entry of water. Because the urine is isosmotic with the hemolymph, the high urinary rate causes a loss of salts which in turn reduces the osmotic gradient. The urinary rate tapers off as the osmotic gradient across the epithelia falls and a new equilibrium is reached in 48-72 hr, in which the urinary and diffusional efflux of salts is balanced by active uptake from the medium, and net water influx is balanced by urine production.

The urinary response of a related crab, C. productus, to acclimation in low salinity media showed it to be less of an osmoregulator than C. magister (Table 3). C. productus could not be acclimated to 30% S.W.: animals exposed to

this medium for 12 hr were very swollen. Gross (1957) has shown that two closely related crabs, Cancer antennarius and Cancer gracilis, are osmoconformers. C. productus is apparently an osmoconformer also, as the urinary rate returned to the normal (100% S.W.) value after acclimation to 75% and 50% S.W. (Table 3).

Handling has been shown to cause anuria in the crayfish, Orconectes virilis (Riegel and Kirschner, 1960), and Burger (1957) has found that the lobster, Homarus americanus, may be anuric in captivity for periods of one month. Hannan and Evans (1973) found that stress and leg autonomy had no effect on the water influx constant in 3 species of the crab, Uca,and, presumably, the urinary rate was unaffected as well. As shown in Fig. 19 of the present study, handling and blood withdrawal at 12 hr intervals had no effect on the urinary rate of C. magister. Apparently macruran decapods are, for some reason, more sensitive to handling than are brachyurans.

Only a few workers have investigated the effect of temperature changes on urinary rates in crustaceans. Preliminary experiments by Werntz (1963) showed a Q_{10} of 2 for urinary rates of two species of the amphipod, Gammarus. Riegel (1960) has shown that the urinary rate of the crayfish Orconectes virilis, was depressed by lowered temperatures. In the present study acute temperature changes

showed Q_{10} values close to 2, and acclimation to a change in temperature from 12 °C to 7 °C had not been completed in three days (Figs. 20 and 21). Because the temperature changes lasted for only 2 or 3 days, it is not possible to say whether complete or partial acclimation occurs in this species. Using the blocked nephropore technique, the urinary rates of crabs acclimated to 12 °C and 6 °C for 72 hr were compared (Table 6). In this case, acclimation had been nearly completed, as the means for the two groups differed by only 15%, a non-significant value. Acclimation to a new temperature is a variable process and apparently takes at least three days and may take much longer.

Several investigators have found Q_{10} values of approximately 2 for heart rate in crustaceans (Maynard, 1960; Florey and Kriebel, 1974). It is possible that temperature-induced changes in urinary rate are actually due to changes in arterial blood pressure. Burger and Smythe (1953) found that arterial and hemocoelar pressures and heart rate in the lobster, H. americanus, were correlated. However, Burger (1957) found no correlation between urinary rate and hemocoele pressure in H. americanus. Norfolk (1976) has found a direct relationship between applied pressure and urinary rate in the perfused antennal gland of C. maenas, and has suggested that arterial pressure partially controls

the crab's urinary rate. The effect of temperature changes on urinary rate may also be due to temperature-induced changes in the permeability of the crab to water. Smith and Rudy (1972) found that the hourly water exchange fraction (K_i) in the crab, Hemigrapsus nudus, had an acute Q_{10} value of 1.6. Hannan and Evans (1973) have found the Q_{10} of K_i in the horseshoe crab, Limulus polypnemis, and the crab, Uca sp., after two weeks of acclimation at 10 °C or 20 °C to be approximately 2. It is interesting that Uca sp. and L. polypnemis show no acclimation of K_i after 2 weeks, whereas the urinary rate of C. magister and, presumably the K_i as well, shows at least partial acclimation (Table 6, Figs. 20 and 21).

Ono and Kamemoto (1969) have reported that the crayfish P. clarkii shows annual variations in urinary rate in fresh water, and Wong and Freeman (1976) have shown seasonal changes in the hemolymph osmotic concentration of the crayfish, Paranephrops zealandicus. Kamemoto and Tullis (1972) have also suggested that seasonal cycles in hemolymph chloride levels occur in the crabs, Thalamita crenata and Potamon dehaani. Englehardt and Dehnel (1973) have shown slight seasonal variations in ionic regulation in C. magister. From the data in Fig. 7 of the present study, it is apparent that there were no seasonal effects on the urinary rate of C. magister acclimated to 75% S.W. It may

be that the seasonal effects in this species noted by Englehardt and Dehnel (1973) were due to temperature changes, as their summer and winter crabs were acclimated to temperatures of 14-15 ^{o}C and 8 ^{o}C respectively. Wong and Freeman (1976) also attributed seasonal changes in hemolymph osmotic concentration in the crayfish, P. zealandicus, to changes in environmental temperature.

Urinary and Drinking Rate Changes in Response to Hemolymph Volume Loss

In C. magister the gut plays a significant role in rectifying losses of hemolymph (Fig. 23). Within one hour of withdrawal of 6-7% body weight of hemolymph, half of the lost fluid had been replaced. Blockage of the gut prevented the crab from drinking and caused death under these conditions. Chronic withdrawal of blood at 12 hr intervals from crabs in 75% S.W. at a rate nearly equal to the normal urinary rate in that medium reduced the urinary rate by only 36% (Fig. 24). Under these circumstances one would expect urine production to cease, as was the case with two species of the crab, Ocypode (Flemister, 1958; Gifford, 1962; Harris, 1977) and the lobster, H. americanus (Burger, 1957), when held out of water. It is probable that the crabs rapidly replaced a large part of the lost

hemolymph by drinking and that, after a short period of time, the osmotic influx of water from the hypoosmotic medium necessitated production of urine to maintain normal volume. T. H. Matthews (Lockwood, 1967, p. 48) has found that the crab, C. maenas, regained most of the weight loss caused by withdrawal of hemolymph within minutes of transfer back to sea water and that the increase in weight was due to drinking. Burger (1957) found that urine production in H. americanus was unaffected by withdrawal of 10% of the hemolymph and that the lobster normally drank sea water at a rate equal to about one fifth of the urinary rate. It is possible that Burger's animals also rapidly replaced the lost hemolymph by drinking. Burger and Smythe (1953) found that urine production in the lobster, H. americanus, was suppressed when the lobsters were held out of water, a treatment which must have caused desiccation and a reduction of hemolymph volume. Lockwood (1970) has found that the amphipod, G. duebeni, increased its drinking rate when subjected to an osmotic stress which caused a reduction in blood volume. The crab, P. crassipes, continued to produce urine in 150% S.W. and the blood volume was shown to increase as the tissues lost water (Gross and Marshall, 1960). Because P. crassipes is a good hypoosmotic regulator in 150% S.W. (Gross, 1957) and tends, therefore, to lose water to the medium, it seems likely that the hemolymph volume

loss caused by urine production and osmosis was rectified by drinking. Dall (1967) has shown that this is, indeed, the case in the shrimp, M. benettae, and that the gut is also a site of excretion of excess salts. The crabs, Cancer pagurus and Maia squinado, have been shown to drink sea water at moult (Drach, 1939), and Robertson (1960b) has found that C. maenas drinks sea water during moulting and that this drinking is responsible for the crabs' increase in size at moult. Mykles (1977, and personal communication) has made similar observations on the lobster, H. americanus. It is clear from the foregoing discussion that urine production is lowered or stopped under conditions causing a decrease in hemolymph volume and that the crustacean gut is a major organ of volume control which is used to counteract hemolymph volume loss.

Urinary and Drinking Rate Changes in Response to Hemolymph Volume Expansion

C. magister has been shown to increase its urinary rate in response to hemolymph volume expansion and to rapidly eliminate volume loads (Figs. 25-28). Dilution of the hemolymph caused no significant increase in urinary rate (Figs. 25 and 26). It seems likely that the crab monitors its hemolymph volume, possibly via stretch receptors, and

adjusts its urinary rate accordingly to maintain normal volume. Hemolymph osmotic pressure is apparently not monitored. Capen (1972) has shown that hemolymph chloride concentration and, presumably, osmotic pressure are not control parameters for adjusting K_i in the crab, R. harrisi. Burger (1957) found that injection of salt solutions and transfusion of 10-15 ml of blood from another animal restored urine production in anuric specimens of the lobster, H. americanus. In light of the response of C. magister to to volume loads it seems likely that Burger's animals were responding to a volume load, a possibility noted by Riegel (1961). In the crayfish, O. virilis, inulin clearance and, presumably, urinary rate were greatly increased by injection of distilled water (Riegel, 1961). Kamemoto and Ono (1969) have reported that water loading caused diuresis in P. clarkii. Using direct cannulation of the nephropore, Norfolk (1976) has shown that both injection of water and volume loads applied via a stomach tube in the crab, C. maenas caused an increase in urinary rate. Conversely, Hannan and Evans (1973) have shown that 3 species of the crab, Uca, drink less when transferred from 100% S.W. to 3% S.W., indicating a suppression of drinking under conditions of volume increase. Dall (1967) has also shown a reduction of drinking in dilute media in the shrimp, M. bennettae, and the crabs, M. gracilipes and M. crassipes.

It is clear that rapid urinary elimination of applied volume loads is a widespread phenomenon in the crustacea, and that drinking is reduced under conditions of hemolymph volume expansion.

Control of Urinary Rate

Several experiments were conducted in an effort to determine the mechanism by which C. magister increases its urinary rate in response to a volume load. Heart rate (Fig. 29) and arterial pressure (Table 12) were not significantly affected by volume loads. Thus, pericardial organ or cardial pacemaker control of arterial pressure and, hence, urinary rate are unlikely as control mechanisms for urinary rate. Spaargaren (1973) found a positive correlation between heart rate and the osmotic differential across the body surface in the shrimp, Palaemon serratus, and suggested that hemolymph pressure also increased. This effect evidently does not exist in C. magister. Florey and Kriebel (1974) found that stroke volume decreased as heart rate increased in C. magister and their finding makes a simple relationship between heart rate and arterial pressure unlikely. Further, the heart rate of the crab, Libinia emarginata, decreases during acclimation to 80% S.W., while the urinary rate shows a six-fold increase (Cornell, 1973).

Spaargaren (1974) found that heart rate and cardiac output increased in C. maenas acclimated to 50% S.W. Heart rate in C. maenas has also been shown to undergo an increase during exposure to 15% S.W. In experiments of longer duration, Taylor, et al., (1977) found that changes in salinity had no effect on heart rate or ventilation volume in C. maenas. Norfolk (1976) found that the net pressure (systole-diastole) in the heart of C. maenas is unchanged in dilute media, but suggested that urinary rate is partially controlled by arterial pressure. Heart rate is probably a poor indicator of antennal gland function, as it can be changed by increased locomotory activity and the relationships between heart rate, arterial pressure and urinary rate are uncertain. It is quite possible that the increased heart rates seen in many crustaceans in dilute media are due to increased activity in an attempt to avoid exposure to osmotic stress. Taylor, et al., (1977) have, in fact, found this to be the case in C. maenas. Cantelmo, et al., (1975) have found the heart rate of the crab, C. irroratus, to be quite variable and conclude that heart rate is ". . . not a reliable indicator of the physiological state of these crabs under osmotic stress." Because of the variability of the effects of dilute media on heart rate and the finding in the present study that pressure in the antennal artery (within 3 cm of the antennal gland) is unchanged by acclimation to 50% S.W.,

(Table 12) it seems unlikely that arterial pressure is a normal control parameter for urinary rate.

Eyestalk ablation did not interfere with acclimation to 75% S.W., nor with the elimination of an injected volume load of 4% body weight in C. magister (Fig. 30). Presumably the permeability of the crab to water and salts was also unaffected. Eyestalk ablation has been shown by Kamemoto and Ono (1969) to result in nearly doubled urinary rates in the crayfish, P. clarkii, when tested in the summer. However, Ono and Kamemoto (1969) have reported that there is a yearly cycle in urinary rate with a winter peak of 13% body weight per day and a summer low of 7% body weight/day. Further, eyestalk ablation had no significant effect on urinary rate in the winter crayfish. As the authors note, it is possible that eyestalk ablation may affect seasonal eating and drinking habits instead of the permeability of the crayfish to water. Ehrenfeld and Isaia (1974) also found that ligation of the eyestalks of the crayfish, Astacus leptodactylus, caused increased urinary rates. DeLeersnyder (1967) has found that eyestalk ablation in the crab, Eriochier sinensis, increases the urinary rate of animals in both sea water and fresh water, although the osmotic pressure of the hemolymph was not lowered by eyestalk ablation (DeLeersnyder, 1970). Eyestalk-ablated specimens of the crab, M. messor, have been shown to gain

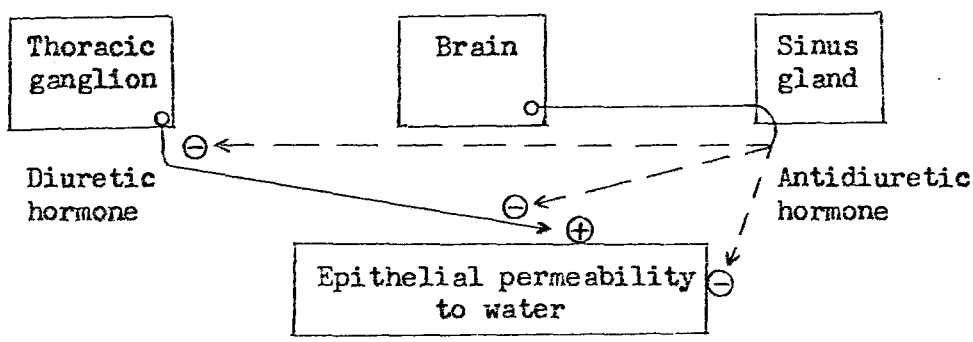
weight faster than normal specimens when the nephropores were blocked (Kato and Kamemoto, 1969). It is curious that C. magister did not show a similar urinary response to eyestalk ablation. Perhaps the freshwater P. clarkii, A. leptodactylus and E. sinensis and the more terrestrial M. messor have evolved more complex hormonal control mechanisms involving the sinus gland-x organ complex.

Although injections of brain and thoracic ganglion homogenates caused large increases in urinary rate in C. magister (Figs. 31 and 32), the injection of equivalent amounts of muscle homogenate also caused a large increase in urinary rate (Fig. 33). Presumably, the net water influx was also increased by these treatments. The possibility that increased urinary rate is a consequence of injection of any homogenized foreign tissue cannot be excluded. It is quite possible that crustacean tissues release an injury substance upon homogenation and that the crab's urinary rate is increased in order to clear this substance from the hemolymph. As has been noted, the data from these experiments were quite variable: some animals showed a great increase in urinary rate, others showed none at all. Handling has been shown to have no significant effect on urinary rate (Fig. 19), thus the increased urinary rates are not a consequence of experimental manipulation. In one of the experiments (Fig. 33) brain homogenates from

crabs in 50% S.W. had a much greater effect on urinary rate than 100% S.W. brain or muscle homogenates, possibly indicating the existence of a "diuretic" hormone in the brain. Burger (1957) found that injection of 10-15 ml of hemolymph from another lobster into an anuric lobster resulted in resumption of urine flow in H. americanus. Although, as previously discussed, this treatment constituted a volume load, it is also possible to interpret the effect as a reaction to foreign tissue. It has been shown that C. magister can replace lost hemolymph by drinking and, thus, the hemolymph volume loss caused by the increased urinary rates in crabs injected with tissue homogenates was probably made good via the gut. It is also possible that the action of the tissue homogenates was to increase the drinking rate, which in turn caused an increase in the urinary rate.

The effects of ablation, reimplantation and injection of homogenates of various neural tissues on salt and water balance in crustaceans have been studied by several workers. Early experiments by a number of workers (reviewed by Knowles and Carlisle, 1956) have shown that eyestalk ablation causes a greater than normal increase in size at moult. From these experiments the existence of a permeability-decreasing or "antidiuretic" hormone which is produced in the x-organ and released at the sinus gland was deduced. Bliss, et al., (1966) proposed the existence of a second,

"diuretic" hormone which causes the release of previously retained water after moult in the terrestrial crab, Gecarcinus lateralis. Mantel (1968) found that thoracic ganglion homogenates increased the permeability of the foregut of G. lateralis to tritiated water and she postulated that the hormone involved in the response is the "diuretic" hormone proposed by Bliss, et al., (1966), and that it is axonally transported to the sinus gland for release. The results of a series of experiments done by Kamemoto and his associates with the crayfish, P. clarkii, and the crab, M. messor (Kamemoto, et al., 1966; Kamemoto and Ono, 1969; Kato and Kamemoto, 1969), have led them to propose a tentative scheme for the hormonal control of water balance in crustaceans which may be graphically summarized as follows:



The evidence for involvement of the thoracic ganglia is partly based on the effect of injections of thoracic ganglion extracts on blood osmotic pressure in M. messor (Kamemoto,

et al., 1966). It is possible that their deduced increase in permeability to water could be instead due to an increased urinary rate and compensatory drinking caused by a reaction to cell-injury substances present in the extracts. Kamemoto and Tullis (1972) and Tullis and Kamemoto (1974) have found 2 fractions in extracts from the brain and thoracic ganglia of the crab, T. crennata, which have opposite effects on tritiated water fluxes across the gills. Their findings necessitate the addition to the above diagram of a second "antidiuretic" hormone produced by the thoracic ganglia. Mantel, et al., (1975) have found that eyestalk ablation lowers hemolymph osmolality in the crab, G. lateralis, a finding which is in accord with the graphic model presented above. Kamemoto (1976) has reviewed the literature on neuroendocrine control of osmoregulation in crustaceans. As he notes, actual hormones and release sites have not yet been identified and caution must be used in interpreting the existing data. Recently Berlind and Kamemoto (1978) have shown that the sinus gland-x organ complex is not involved in the rapid decrease in apparent water permeability shown by C. maenas when placed in dilute media. In the same study, extracts of thoracic ganglion were shown to reduce the K_i of isolated, perfused gills, while extracts of sinus glands, pericardial glands and hemolymph from crabs in dilute media were without effect.

In all of the works cited above, except that of Tullis and Kamemoto (1974), in which injections of brain or thoracic ganglion homogenates were used, the control injection was distilled water or Ringer solution. The existence of a urinary response to injection of homogenates of non-endocrine tissue homogenates similar to that found in C. magister in the present study cannot be excluded. Apparently, T. crenata (Tullis and Kamemoto, 1974) does not show the same response to injected muscle homogenates as C. magister. Further, it is possible that eyestalk ablation in some crustaceans may cause increased drinking of the medium (Ono and Kamemoto, 1969), which would raise the animals' urinary rates. No studies have been done to determine the possibility of control of drinking by the eyestalk, brain, or thoracic ganglia in crustaceans. Bliss, et al., (1966) have noted that it is possible that crustecdysone itself causes uptake of water at moult. This increased water uptake could be due to increased drinking caused by crustecdysone. As has been noted, many marine crustaceans are isosmotic in sea water, thus an increase in epithelial permeability to water would cause little if any increase in size at moult.

It is interesting to note that Stobbart (1977) has shown that in the mosquito, Aedes aegypti, diuresis after a blood meal is controlled by the nervous system. Retrograde movements of the midgut, which lead to reabsorption

of the urine produced by the Malphigian tubules and, therefore, to antidiuresis, are inhibited after a blood meal and diuresis results. The retrograde gut movements are apparently controlled by the output of stretch receptors in the mosquito's abdominal wall. Maddrell (1964 a, b, c) has shown that abdominal stretch receptors control the release of a diuretic hormone in the insect, Rhodnius prolixus. Pilgrim (1974) has found several paired thoracic stretch receptors in the anomuran, Pagurus bernhardus, and has suggested a volume-sensing function for the most anterior pair. Neurons extend from these stretch receptors into the thoracic ganglion. It is possible that there are two hormonal control systems for water balance in crustaceans: one system may control hemolymph volume by modulating drinking and urinary rates (Burger and Smythe, 1953), possibly in response to stretch receptor output, and the other may control epithelial permeability to water and/or salts. If this is the case, then the eyestalk is not involved in the first system, as eyestalk ablation does not impair the ability of C. magister to eliminate volume loads (Fig. 30).

It has been previously stated that adaptive reduction of water permeability may be a species-specific phenomenon and not a general characteristic of the crustacea. The ability to hormonally control water permeability would

obviously be advantageous, as water influx and, hence, urinary salt losses could be reduced in a hypoosmotic environment. It may be that this second, indirect mechanism of hemolymph volume control has evolved in some crustaceans and is integrated with a more primitive, universal mechanism of volume control based on adjustment of drinking and urinary rates.

Norfolk (1976) has shown that when the antennal artery of C. maenas was perfused with crab Ringer at a constant pressure, the urinary rate was increased by the addition of brain homogenates from crabs in 50% S.W. to the perfusate. His findings indicate the possible existence of a brain hormone which acts directly at the filtration site. The hormone is apparently not liberated into the hemolymph at the brain, as perfusion of hemolymph from crabs in 50% S.W. did not increase the urinary rate. Norfolk suggests that the diuretic hormone is released into the hemolymph at or near the arterial supply to the antennal glands. Further, his experiments showed that high molecular weight tracer compounds were cleared more rapidly when the brain homogenate was added, indicating that the hormone acts by increasing the pore size of the filtration site. It is interesting that Flemister (1958) found that the crab, O. albicans, does not clear inulin when held out of water, indicating that the crab stops urine formation. This finding is

consistent with Norfolk's (1976) postulated control of urinary rate by adjustment of pore size at the filtration site. Flemister (1958) also found that the terrestrial crab, G. lateralis, apparently reabsorbs water and salts from its urine when denied access to water, as it continues to clear inulin but no urine is obtainable, under these conditions. Harris (1977) has shown that the inulin urine/serum ratio in G. lateralis does not exceed one, even when the crab is severely desiccated. Her findings do not support Flemister's (1958) suggestion of water and salt reabsorption from the urine of this crab.

It is unfortunate that Norfolk (1976) did not use homogenates of muscle or other non-endocrine tissue as a control. Similar perfusions with C. magister were attempted in this study in an effort to confirm Norfolk's findings with C. maenas. Extreme difficulty was encountered with urinary cannula blockage, despite the fact that C. magister is much larger than C. maenas. Kato and Kamemoto (1969) and Smith (1967) were also unsuccessful in attempts to cannulate the nephropores of the crabs, M. messor and R. harrisi, respectively. Because reliable data were impossible to obtain, further experiments with brain and muscle homogenates were not attempted. Norfolk (1976) also conducted experiments to determine what triggered increases in urinary rate. Although his data are few and quite

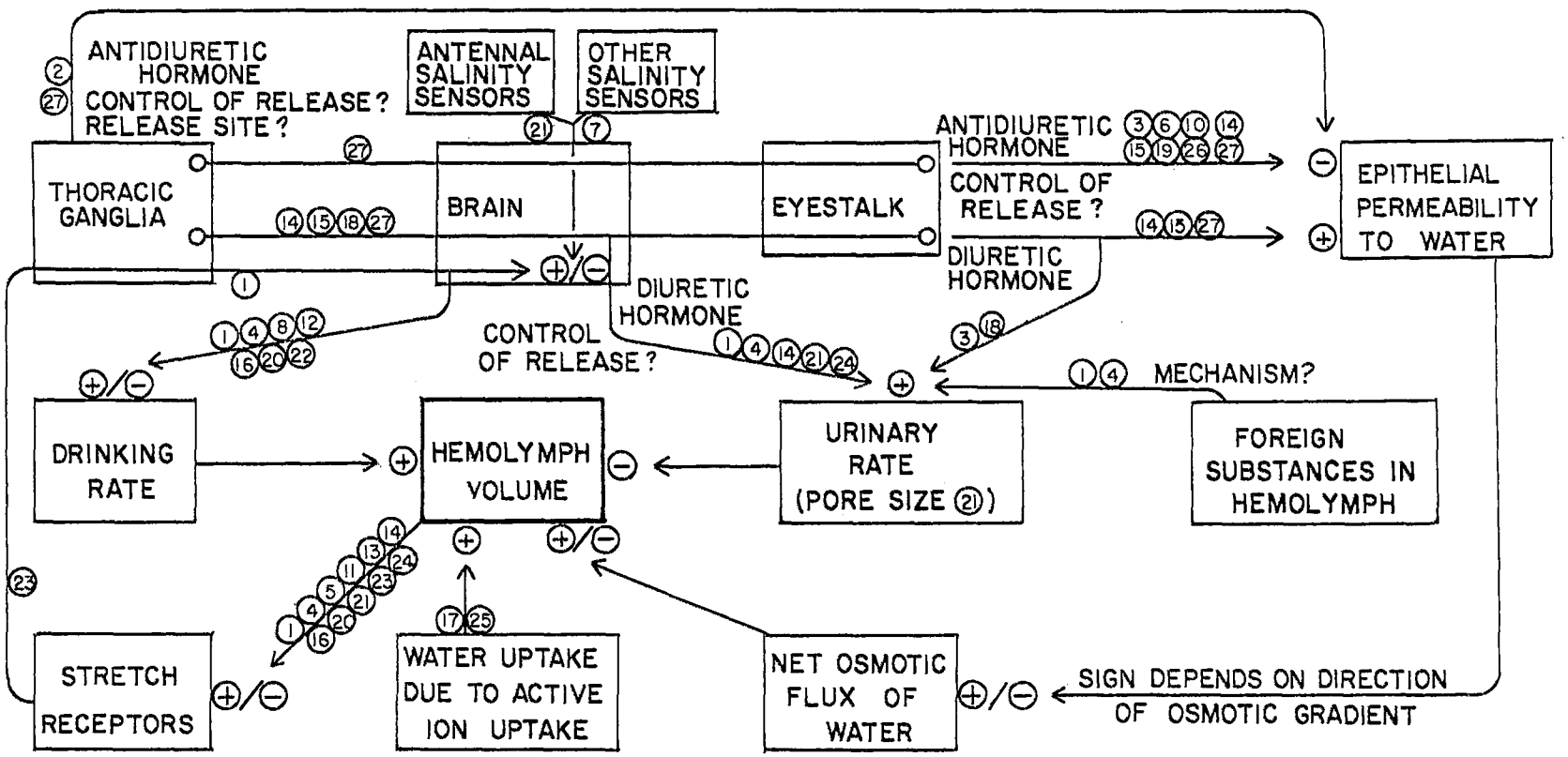
variable, it appears that a certain threshold level of internal pressure, or more properly, volume must be exceeded to trigger an increase in urine production. The crab also rapidly changed its rate of urine production in response to changes in the salinity of the medium and the antennae were apparently involved in this second mechanism. Lockwood (1968) has presented evidence that the amphipod, G. duebeni, may monitor the salinity of the medium and adjust the degree of dilution of its urine accordingly. Davenport (1972) has shown that when the anomuran, Eupagurus bernhardus, was placed in dilute media, urine production did not increase until substantial swelling had occurred, a finding which supports Norfolk's postulated volume threshold for increased urinary rate. Evidently, external salinity is not a control parameter for urinary rate in E. bernhardus. The results of the volume loading experiments in the present study (Figs. 25-30) are consistent with Norfolk's dual control mechanism. Volume loads were rapidly eliminated by C. magister, but the urinary rate was not changed significantly by dilution of the hemolymph without an accompanying volume load, which shows that hemolymph osmotic concentration is not monitored to control urinary rate.

Using data from all available studies, a tentative model of the possible mechanisms of control of hemolymph volume in malacostracan crustaceans has been constructed

(Fig. 34). The circled numbers in this figure refer to the studies listed in the legend which provide evidence for or are consistent with the indicated hormone or effect. In constructing the model, the minimum number of hormones which could account for all of the observed effects has been used. Thus, the diuretic brain hormone postulated by Norfolk (1976) is represented as the same diuretic hormone postulated by Mantel (1968) to be produced in the thoracic ganglion and transported axonally through the brain for release at the sinus gland. An additional, non-sinus gland release site for Norfolk's diuretic brain hormone is shown because of the finding in the present study that crabs without eyestalks can eliminate volume loads and acclimate to 75% S.W. normally. Further, I have not found any mention of swelling in any of the cited studies in which eyestalk ablation was performed. Therefore, urinary rate cannot be directly controlled by a hormone released at the sinus gland. Available data on hormonal regulation of hemolymph levels of individual ions (reviewed by Kamemoto, 1976) have been omitted in order to keep the model reasonably uncomplicated. The effect of such hormones would be to increase or decrease the osmotic gradient across the body surface.

It is to be stressed that the model is a composite one and that all crustaceans may not possess all of the

Figure 34. Diagram illustrating possible mechanisms of control of hemolymph volume in malacostracan crustaceans. Numbers indicate references listed below in which the indicated control is demonstrated or postulated, or which could be interpreted as consistent with the existence of the indicated control.



1, present study; 2, Berlind & Kamemoto('78); 3, Bliss et al.('66); 4, Burger('57); 5, Burger & Smythe('53); 6, Cantelmo('78); 7, Capen('72); 8, Dall('67); 9, Davenport('72); 10, DeLeersnyder('67); 11, Flemister('58); 12, Hannan & Evans('73); 13, Harris('77); 14, Kamemoto & Ono('69); 15, Kato & Kamemoto('68); 16, Lockwood ('70); 17, Lockwood & Inman('73); 18, Mantel('68); 19, Mantel et al.('75); 20, Matthews(unpub.); 21, Norfolk ('76); 22, Ono & Kamemoto('69); 23, Pilgrim('74); 24, Riegel('61); 25, Smith('76); 26, Thompson('67); 27, Tullis & Kamemoto('74).

hormones or effects shown. The knowledge of endocrine control of water balance in crustaceans is still limited and much more work is necessary for clarification of the mechanisms involved. As Kamemoto (1976) has noted, the diversity of findings to date may be due to the existence of different mechanisms of control in crustaceans inhabiting fresh water, brackish water, marine and semi-terrestrial environments, and to the existence of seasonal cycles in responses to certain experimental treatments.

The most important findings of the present study of C. magister are two. First, the existence of a non-eyestalk mediated system of volume control which operates by varying both urinary and drinking rates has been demonstrated. Second, it has been shown that injected homogenates of non-endocrine tissues cause increased urinary rates. It is hoped that both findings will influence the design of future experiments in endocrine control of water balance in crustaceans.

CHAPTER 5

ANTENNAL GLAND CONTROL OF HEMOLYMPH MAGNESIUM LEVELS

METHODS

Male, intermoult crabs weighing between 200 and 800 g were captured and maintained in 100% Coos Bay sea water as noted in Chapter 2. Unless otherwise noted, details of capture and maintenance of crabs, sampling of blood and urine, eyestalk ablation, injection of solutions and statistical treatment of data were as stated in Chapter 2. Urinary rates were measured by the external collection method given in Chapter 3. Because of the low urinary rate and irregularity of micturation of crabs in 100% S.W. (Chapter 4), many of the experiments were conducted with crabs acclimated to 75% S.W. All experiments were performed at 12 $^{o}C \pm 1$.

In one experiment the urinary opercula were temporarily blocked by covering them with a small drop of hot melt glue (U.S.M., Thermogrip). The glue was removed, a 2 ml sample of urine taken and the glue replaced at each subsequent sampling of the urine.

The antennal gland arteries were broken, when necessary, by the procedure described in Chapter 4.

Ion Analyses

Magnesium levels in serum, urine and stomach fluid ($[Mg^{++}]_s$, $[Mg^{++}]_u$ and $[Mg^{++}]_{sf}$, respectively) were measured using the spectrophotometric thiazole yellow method of Sky-Peck (1964), as modified by Hunter and Rudy (1975) for use with small (20 µl) aliquots. All samples were deproteinized with 5% trichloroacetic acid prior to analysis. Measurements were made with a Zeiss PMQ-II spectrophotometer. Mg^{++} standards were made up in Mg^{++}-free crab Ringer solution in which NaCl was substituted for $MgCl_2$ to allow for interference by other ions. The standard deviation of the mean of five determinations of a 100 mEq/l standard was $\pm$ 1.6 mEq/l. Tissue for measurements of Mg^{++} levels in muscle ($[Mg^{++}]_m$) was obtained by crushing the carpus of a walking leg with a hemostat to induce autonomy of the leg. Approximately 0.2 g of muscle tissue was dissected from the merus, blotted, weighed to 0.001 g, homogenized in a glass tissue grinder and made up to 10.0 ml with distilled water. The analysis for $[Mg^{++}]_m$ was conducted as previously described for $[Mg^{++}]_s$ and $[Mg^{++}]_u$, with the exception that a 500 µl aliquot of the hemogenate was used. The hemolymph space of the muscle tissue was assumed to be equal to the extracellular space, which was estimated by injection of 5 µCi of the tracer compound Glofil (^{125}I-labeled sodium

iothalamate, Abbott Laboratories) into each of ten crabs. After a two hour equilibration period, samples of hemolymph and muscle tissue were taken and counted to at least 1000 counts in a Picker Nuclear 2840E NaI crystal solid scintillation well counter connected to a Picker Nuclear 628-057 dual pulse height analyzer and a Picker Nuclear 628-145 dual rate computer. From these figures the mean hemolymph volume of muscle tissue was calculated to be 12% $\pm$ 4 (ml hemolymph/g tissue). This figure and the $[Mg^{++}]_s$ at the time of tissue sampling were used to correct the apparent $[Mg^{++}]_m$. Levels of Mg^{++} in midgut gland (hepatopancreas) were also measured. Approximately 0.2 g of tissue was rinsed quickly in distilled water and treated in the same manner as the muscle tissue. No correction for $[Mg^{++}]_s$ was made. Mg^{++} levels in feces were measured in a manner similar to that for $[Mg^{++}]_m$. Approximately 0.2 g of feces were homogenized, made up to 10.0 ml and a 500 µl aliquot was assayed for Mg^{++}.

Sodium levels in serum and urine ($[Na^+]_s$ and $[Na^+]_u$, respectively) were measured with a Coleman Model 21 flame photometer connected to a Coleman 6/20 Junior II spectrophotometer wired for use as a galvanometer. A 100 µl aliquot was diluted in 10.0 ml distilled water and used in the analysis. The standard deviation of five replicate determinations of a 400 mEq/l standard was $\pm$ 2 mEq/l.

Magnesium Depletion

Crabs were depleted of magnesium by maintenance in Mg^{++}-free 75% S.W. for six days, with two changes of the medium. Full-strength sea water was made from reagent grade salts according to the formula given by Welsh, et al., (1968), except that NaCl was substituted for $MgCl_2$ and the trace elements were omitted. The resulting Mg^{++}-free 100% S.W. was then diluted to 75% S.W. The ionic composition, in mEq/l of the final medium was: Na^+, 421; K^+, 7.5; Ca^{++}, 15; Cl^-, 401; HCO_3^-, 1.5; $SO_4^=$, 41. Crabs held in this medium for six days showed no signs of distress, although they did appear to be aggressive and easily excited.

Magnesium Loading

In several experiments crabs were given a Mg^{++} load. This was accomplished in all cases by injection of 0.12% body weight (ml/g) of a 3.26 M solution of $MgCl_2$. Preliminary experiments in which blood volume was measured by dilution of injected $MgCl_2$ and Na_2SO_4 indicated that the blood volume of C. magister is approximately 30% body weight (ml/g). Given this figure, the amount of 3.26 M $MgCl_2$ injected in the loading experiments was calculated to be sufficient to double the $[Mg^{++}]_s$ in crabs acclimated to

75% S.W.

Magnesium Loss to Magnesium-Free S.W.

The rate of Mg^{++} loss to Mg^{++}-free 75% S.W. by crabs with blocked nephropores was measured to check the possibility of extrarenal elimination of Mg^{++} loads. The urinary opercula of crabs previously acclimated to 75% S.W. were blocked with hot melt glue as described above. The experimental crabs were given a Mg^{++} load and all crabs were rinsed three times in Mg^{++}-free S.W. and placed in individual aquaria with 1 l. of aerated, Mg^{++}-free 75% S.W. The Mg^{++} levels in the media were measured at 1, 2, 4, 6, 12, and 24 hr after injection of the Mg^{++} load and Mg^{++} efflux (μEq/g/hr) was calculated.

Oxygen Consumption by Excised Bladder Tissue

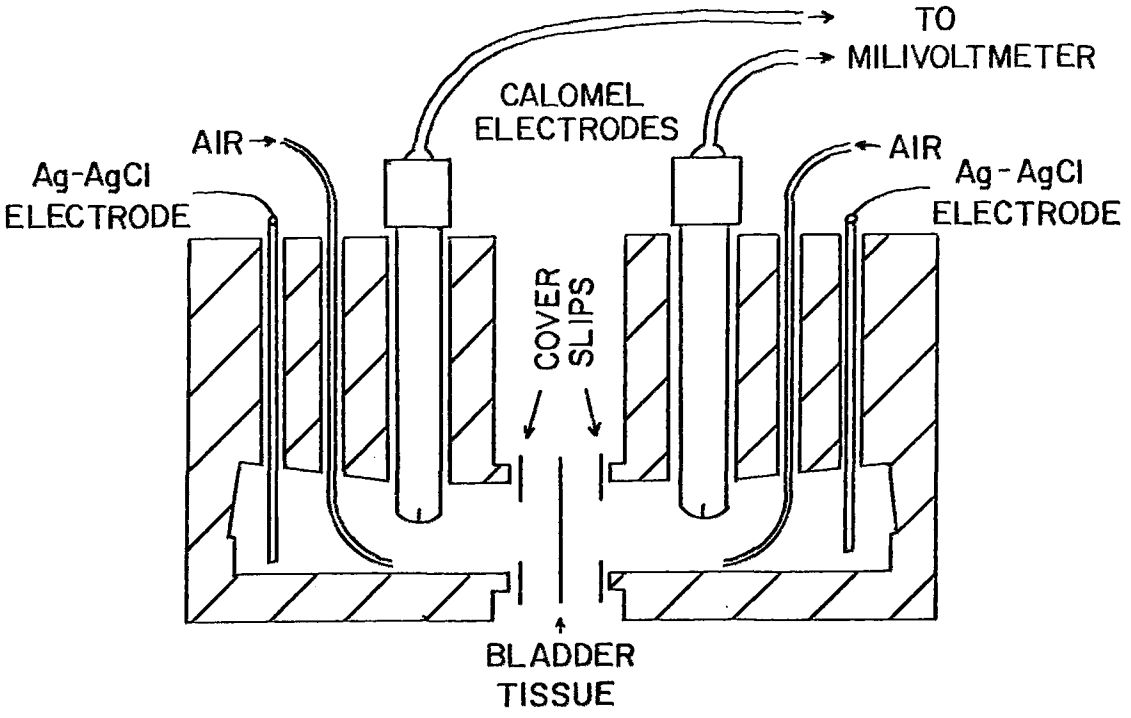
Oxygen consumption by excised bladder tissue was measured using a Gilson Model DRP-14 differential respirometer. Pieces of bladder tissue approximately 3 cm^2 in area were dissected from the epigastric lobe of the bladder and placed in 5 ml of crab Ringer solution with 5 mg% glucose in a 25 ml Gilson reaction vessel. During the measurements the reaction vessels were shaken at the rate

of 70 cycles/minute. Oxygen consumption was measured in consecutive one and two hour periods. A control reaction vessel with no bladder tissue showed no oxygen consumption during the period of the experiment. At the end of the experiment the tissues were dried to constant weight at 100 ^{o}C in preweighed aluminum pans and weighed to the nearest 0.01 mg on a Mettler H2OT analytical balance.

Electrical Parameters of Excised Bladder Tissue

Measurements of transepithelial potential difference (T.E.P.), resistance (R) and short circuit current (S.C.C.) across excised bladder tissue were made in a modified Ussing cell (Fig. 35). Pieces of bladder tissue approximately 2 cm^2 in area were dissected from the epigastric lobe of the bladder, mounted between plastic cover slips which had centered holes 0.80 cm^2 in area, and clamped between the two halves of the cell. Miniature calomel electrodes (Sargeant-Welch S-30080-17) were positioned within 1 cm of the tissue. The electrodes were connected to a high impedance millivolt meter/voltage clamp device (Menninger, 1972). The chambers on either side of the tissue held 25 ml of crab Ringer solution which was vigorously stirred by a stream of air. The tissue was set up in the apparatus and left for ten minutes before readings were taken.

Figure 35. Ussing cell used for measurement of T.E.P., R. and S.C.C. in excised bladder tissue from C. magister.



Immediately prior to measurements, the millivolt meter was zeroed by shorting the two chambers together with a 3 M KCl-agar bridge contained in a 4 mm diameter polyethylene tube.

Resistance of the tissue was calculated using Ohm's law. A current of 25 μA was applied across the tissue with the Ag-AgCl electrodes and the resulting change in T.E.P. across the tissue was measured. After each experiment the apparatus was set up without the tissue and the resistance was again measured and an appropriate correction made to the apparent tissue resistance.

In all cases the S.C.C. of the tissue was less than 9 μA, which, due to the design of the circuit used, was the smallest amount of current that the voltage clamp device could deliver.

In Vitro Ion Fluxes

The fluxes of $^{28}Mg^{++}$, $^{22}Na^{+}$ and $^{36}Cl^{-}$ across pairs of isolated bladder tissue were measured in a dual lucite chamber. A cross section through two of the four chambers is shown in Fig. 36A.

The second pair of chambers was machined into the lucite blocks in the same configuration (Fig. 36B). Thus,

Figure 36 A. Dual lucite cell used to measure *in vitro* ion fluxes across matched pairs of excised bladder tissue from *C. magister*. End view.

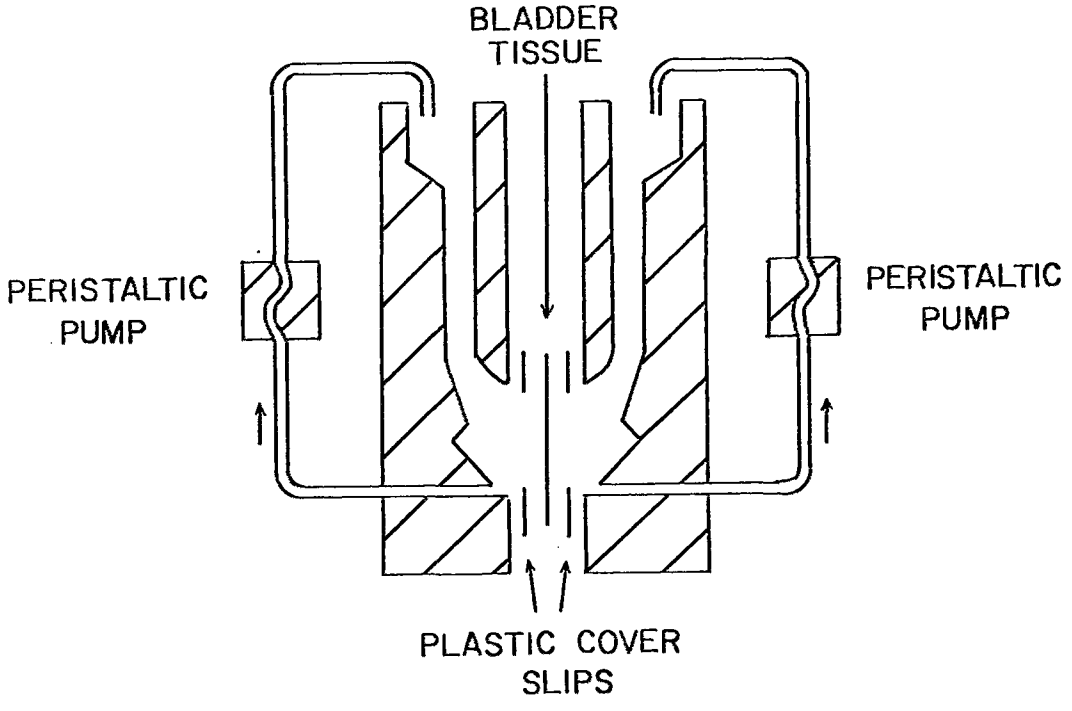
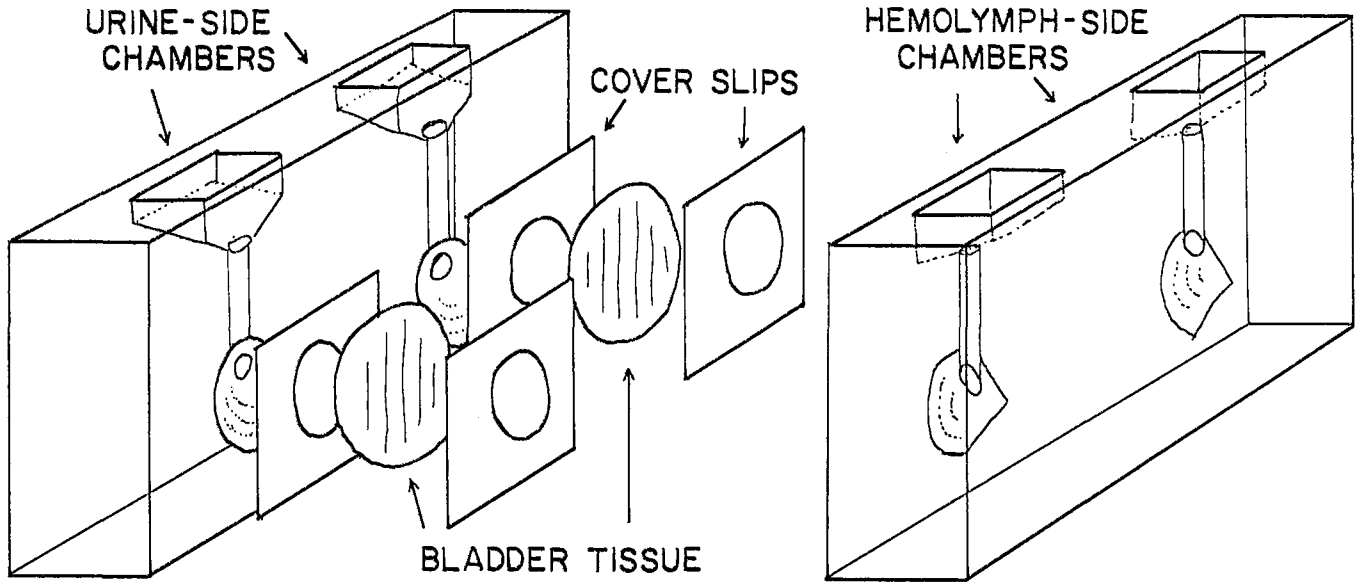


Figure 36 B. Dual lucite cell used to measure *in vitro* ion fluxes across matched pairs of excised bladder tissue from *C. magister*. Exploded view.



two pieces of bladder tissue from the same crab, mounted between pairs of plastic cover clips with centered holes 0.80 cm^2 in area, were each mounted between pairs of separate chambers when the two lucite blocks were clamped together. Both sides of the two pieces of bladder tissue were bathed by separate 1.00 ml aliquots of crab Ringer solution which were aerated and circulated by a peristaltic pump at the rate of 2 ml/min. At the beginning of the experiment a small amount (20-50 µl) of isotope solution was added to the hemolymph-side chamber of one tissue and to the urine-side chamber of the other tissue. The unidirectional flux of the isotope across the tissue in either direction was defined as the rate of appearance of the isotope in the crab Ringer solution which was originally without it. All fluxes were calculated as µEq/cm^2/hr using the equation below:

$$\frac{\Delta \text{cpm/ml}}{\text{S.A.} \times 0.80 \times t} = \text{unidirectional flux } (\mu\text{Eq/cm}^2\text{/hr})$$

where: Δcpm/ml = the change in counts/min/ml in the originally unlabeled crab Ringer solution, adjusted for changes in volume due to removal of aliquots at time "0".

S.A. = the specific activity of the labeled crab Ringer solution in cpm/µEq.

0.80 = area of the bladder tissue in cm^2.

t = time in hr.

Because the T.E.P. and S.C.C. were so small, the tissues were not voltage clamped at zero T.E.P. during ion flux measurements.

$^{28}Mg^{++}$ was obtained as $^{28}MgCl_2$ from Brookhaven National Laboratories, Upton, N.Y. Samples containing $^{28}Mg^{++}$ were counted to at least 1000 counts with a Bicron NaI crystal solid scintillation detector connected to a Canberra Industries 802-9 preamplifier and a Canberra Omega-I multichannel analyzer, or with the above-mentioned Picker Nuclear equipment. In both cases 100 µl aliquots were counted in a narrow "window" centered on the 1.33 MeV gamma emission of $^{28}Mg^{++}$. Decay corrections were made by counting a $^{28}Mg^{++}$ standard of known cpm at the start of each experiment and with each set of aliquots and adjusting the apparent cpm accordingly. The specific activity of the labeled crab Ringer solution was 1000-2000 cpm/µEq Mg^{++}. Unidirectional fluxes were measured over a period of 3 hr. In some experiments, ouabain (Sigma Chemical Co.) was added to the crab Ringer solution at a concentration of 5×10^{-3} M.

$^{22}Na^{+}$ was obtained as $^{22}NaCl$ from ICN Pharmaceuticals, Irvine, CA. Samples containing $^{22}Na^{+}$ were counted in the

abovementioned Picker Nuclear equipment between 0.4 and 1.4 MeV using 50 µl aliquots. The specific activity of the labeled crab Ringer solution was 400-2000 cpm/µEq Na^+. Unidirectional fluxes were measured over periods of 2 or 3 hr. In some experiments, ouabain (5×10^{-5} M) crab Ringer was used. Simulated "normal" Mg^{++} and Na^+ gradients were imposed across excised bladder tissue in some experiments by filling the hemolymph-side chambers of the dual lucite cell with a simulated crab urine of the following composition (mEq/l): Na^+, 449; K^+, 11; Ca^{++}, 25; Mg^{++}, 103; Cl^-, 544; $SO_4^=$, 44. Under these conditions there was a 50 mEq/l (499/449) hemolymph-to-urine (H→U) Na^+ gradient and a 66 mEq/l (103/37) urine-to-hemolymph (U→H) Mg^{++} gradient across the bladder tissue. When necessary, the bladder tissue was deprived of oxygen by placing the dual lucite cell in an atmosphere of nitrogen for one hour before addition of $^{22}Na^+$ and during the flux measurements.

$^{36}Cl^-$ was obtained as $Na^{36}Cl$, also from ICN Pharmaceuticals. Samples containing $^{36}Cl^-$ were dissolved in a liquid scintillation counting fluid of the following composition: 900 ml toluene, 7.16 g Beckman Fluoralloy, Formula TLA, and 51 ml Beckman Bio-Solv Soluibilizer, Formula BBS-3. Aliquots of 20 µl were dissolved in 10.0 ml of the counting fluid and counted to at least 1600 counts in a Beckman LS-150 liquid scintillation system.

Quench corrections were made using the external channels ratio feature of the counter. Counting was done in a wide "window" approximately between 0.02 and 1.7 MeV (^{14}C and ^{32}P above ^{3}H "Isoset"). The specific activity of the labeled crab Ringer solution was approximately 3500 cpm/μEq Cl^-. Unidirectional fluxes were measured over a 1 or 2 hr period. In some experiments a modified crab Ringer solution was used in which choline chloride replaced 90% of the NaCl. The composition of the modified crab Ringer solution (mEq/l) was as follows: Na^+, 50; K^+, 11; Ca^{++}, 25; Mg^{++}, 37; $choline^-$, 449; Cl^-, 528; $SO_4^=$, 44.

RESULTS

In Vivo Mg^{++} Excretion by the Bladder

The normal values of $[Mg^{++}]_s$ and $[Mg^{++}]_u$ in crabs acclimated to 100% and 75% S.W. are shown with data from two other studies of C. magister in Table 17. It is clear that the crab maintains its $[Mg^{++}]_s$ at a level between 1/2 and 1/3 that of the medium. $[Mg^{++}]_u$ is much greater than $[Mg^{++}]_s$, and urine:serum ratios (U:S) are between 1.8 and 5.3, the highest U/S values occurring in crabs acclimated to 100% S.W.

If the antennal gland is responsible for hyporegulation

Table 17. Magnesium levels in sea water and urine and serum of *C. magister*. Mean values ± s.d.

Salinity (% S.W.)	Mg concentration (mEq/l or mEq/kg H_2O)				Reference
	Medium	Serum	Urine	U/S	
100	100	39 ± 7	138 ± 20	3.7	Present study
75	75	21 ± 5	54 ± 16	2.7	
100	103	42 ± 12	156 ± 29	3.9	Hunter & Rudy, 1975
75	76	29 ± 12	94 ± 44	3.6	
100	98	≅ 30	≅ 120	4.3	Englehardt & Dehnel, 1973 (winter)
75	73	≅ 20	≅ 45	2.4	
100	98	≅ 45	≅ 220	5.3	(summer)
75	73	≅ 25	≅ 60	1.8	

of Mg^{++}, then cessation of urine production should cause $[Mg^{++}]_s$ to increase to the level of the medium. When the arteries which supply the coelomosacs of the antennal glands were broken, urine production stopped (Chapter 4) and $[Mg^{++}]_s$ rose steadily over a period of 26 days, but remained 20-25 mEq/l below the Mg^{++} level of the medium (Fig. 37). The slowness of the rise in $[Mg^{++}]_s$ is probably due to a low permeability of the crab to Mg^{++} and possibly, to the operation of a low-capacity, extrarenal mechanism for Mg^{++} excretion. Sham-operated crabs showed a transitory rise in $[Mg^{++}]_s$ five days after surgery, due, perhaps, to the trauma of the operation (Fig. 37). Normal hyporegulatory ability was recovered within ten days.

Evidence that the bladder is the site of Mg^{++} excretion is provided by the data shown in Fig. 38. The $[Mg^{++}]_u$ in crabs with initially empty bladders and blocked nephropores was a function of residence time in the baldder. If Mg^{++} is transported into the urine by a more proximal part of the antennal gland, then urine entering the bladder would already have a high Mg^{++} content and $[Mg^{++}]_u$ would not increase with residence time in the bladder. As $[Mg^{++}]_u$ increased, $[Na^{+}]_u$ fell and there is a highly significant negative correlation between these two variables (Fig. 39). An estimate of the ratio of exchange of Mg^{++} for Na^{+} across the bladder may be obtained by recalculation of

Figure 37. Effect of cutting coelomosac artery on serum Mg levels in C. magister in 100% S.W. Mean values ± s.d. Controls were sham-operated.

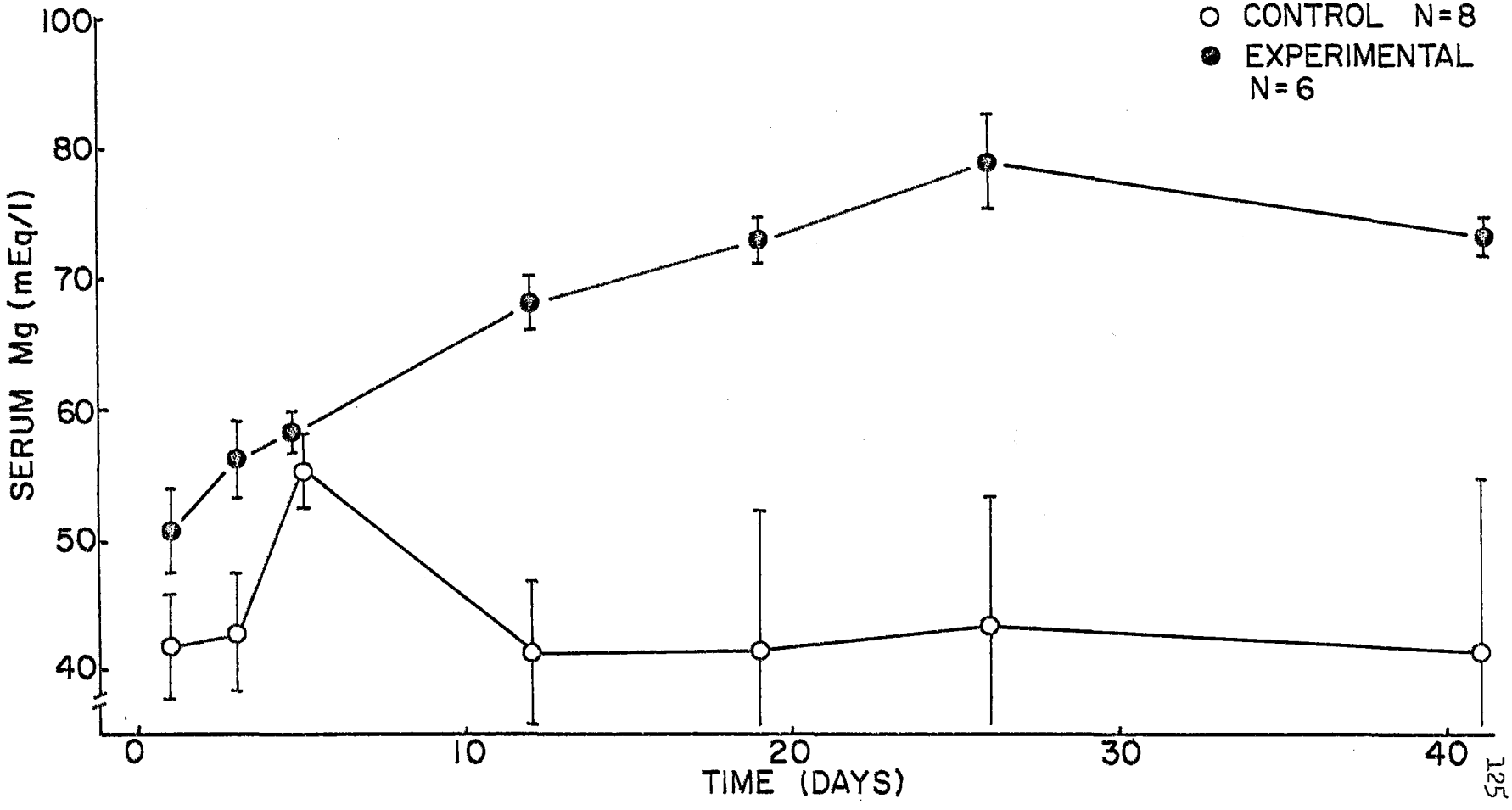


Figure 38. Effect of residence time in bladder on Mg and Na levels in the urine of C. magister with blocked nephropores in 100% S.W. Mean values ± s.d.

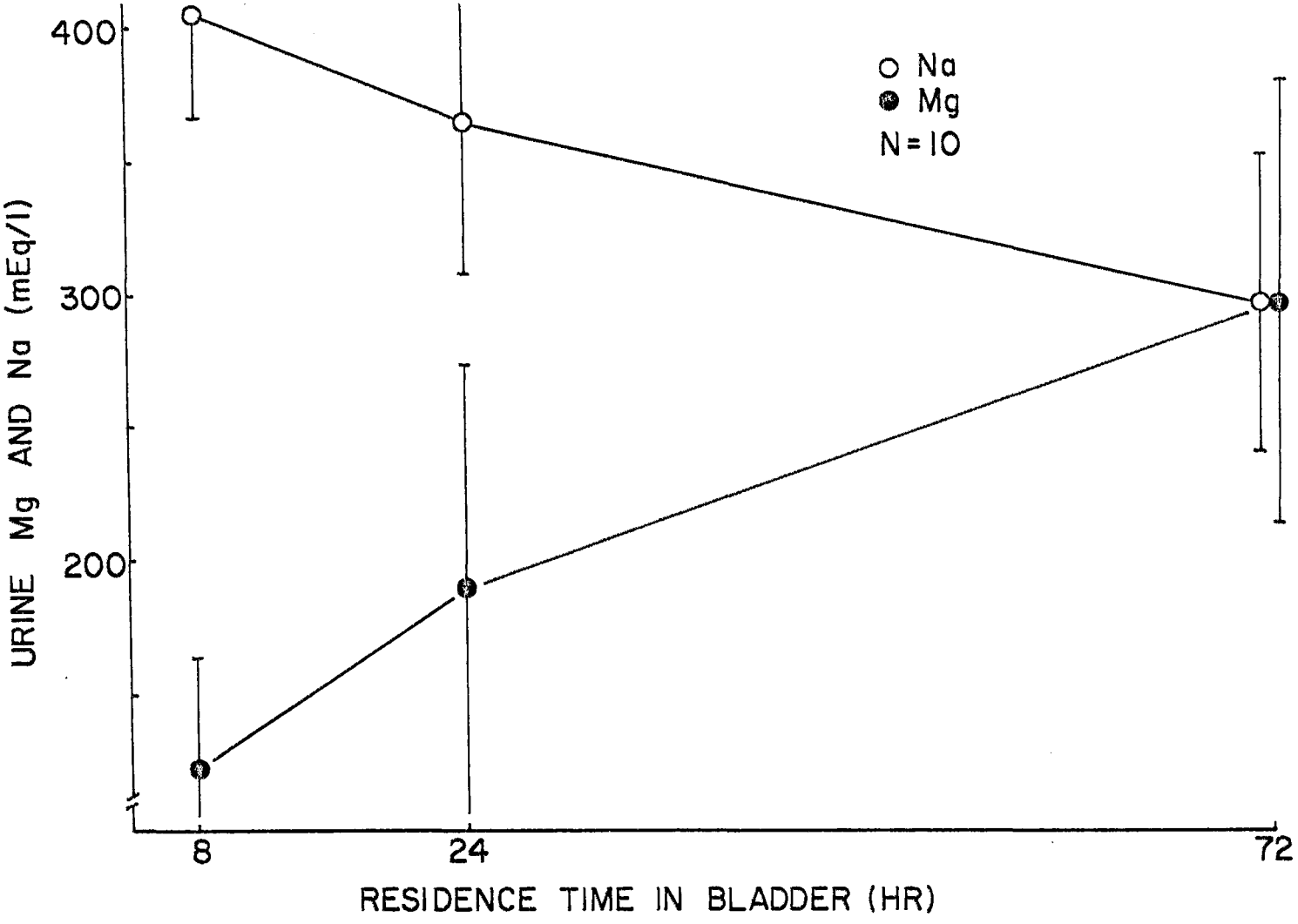
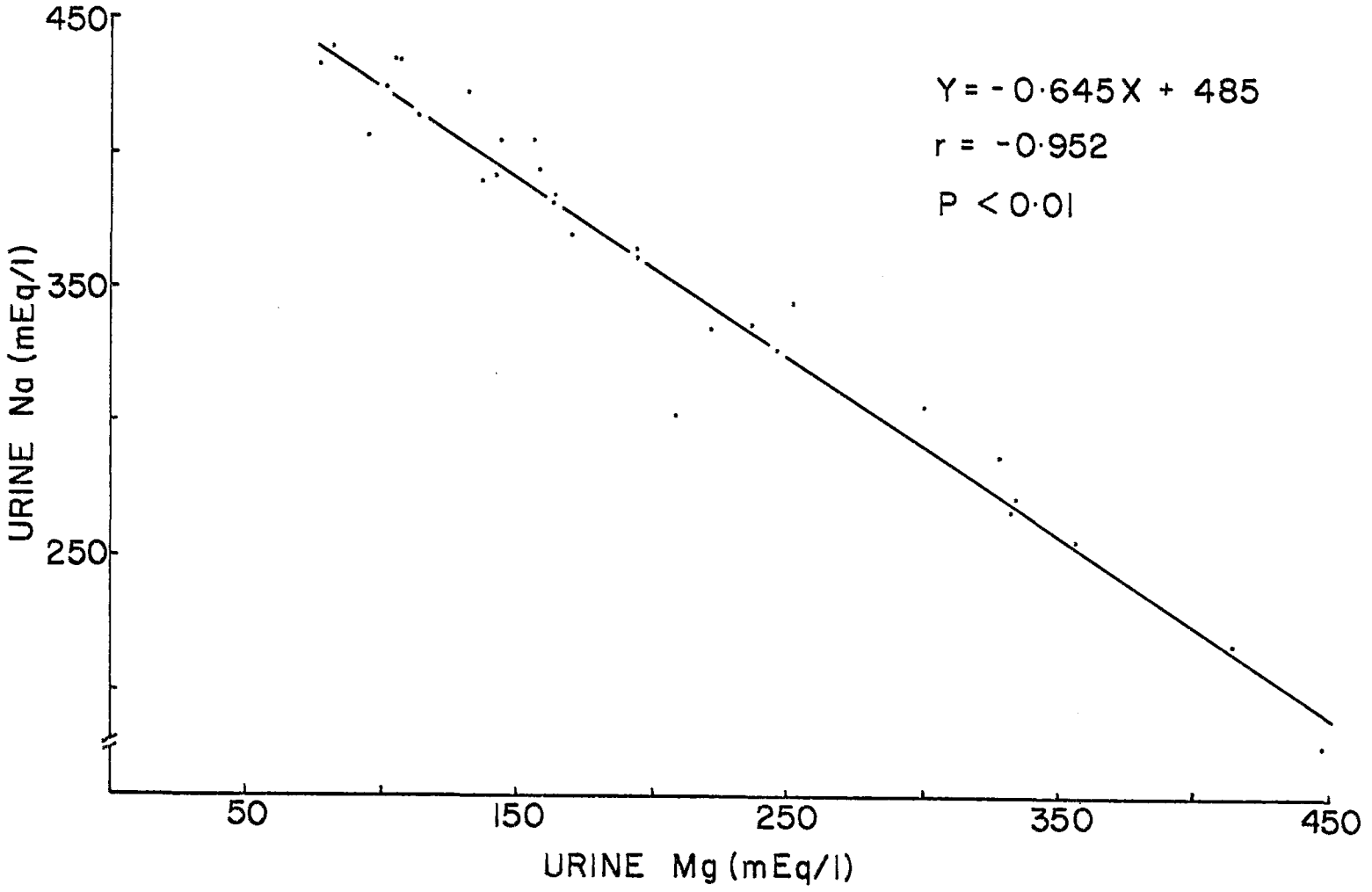


Figure 39. Correlation between Mg and Na levels in the urine of C. magister with blocked nephropores in 100% S.W. Data replotted from Fig. 38. Each point represents one urine sample. See text.



the data shown in Fig. 38. As shown in Table 18, the change in $[Mg^{++}]_u$ divided by the change in $[Na^+]_u$ for the two time periods gives in both cases an exchange ratio of approximately 1.6 Eq Mg^{++}:Eq Na^+.

Because the urinary rate in 100% S.W. (0.067% B.W./hr) and the area of the bladder (0.867 cm^2/gm B.W.) are known (Chapters 4 and 3), the rates of Mg^{++} and Na^+ transport across the bladder may be calculated from the data shown in Fig. 38. The equation used to calculate ion transport in $\mu Eq/cm^2$ bladder/hr is:

$$\frac{\Delta[ion]_u \left(\left[\frac{B.W. \times 0.0067}{1000} \times t \right] - S \right)}{(B.W. \times 0.867)t}$$

where: $\Delta[ion]_u$ = the change in $[Mg^{++}]_u$ or $[Na^+]_u$ ($\mu Eq/l$).

B.W. = body weight (g).

S = the cumulative volume of urine samples removed in liters.

t = time period (hr).

$\left[\frac{B.W. \times 0.00067}{1000} \times t \right] - S$ = the volume of urine in liters produced between t = 0 and the time of sampling, corrected for urine samples removed.

Table 18. Calculated ratios of exchange of Mg for Na across the bladder of C. magister with blocked nephropores. Data recalculated from Fig. 38. Mean values.

8 to 24 hr			24 to 72 hr		
$\Delta[Mg]_u$ (mEq/l)	$\Delta[Na]_u$ (mEq/l)	Ratio	$\Delta[Mg]_u$ (mEq/l)	$\Delta[Na]_u$ (mEq/l)	Ratio
67	41	1.6:1	107	68	1.6:1

(B.W. X 0.867) = area of the bladder in cm^2.

This equation makes no allowance for reabsorption of water and salts from the urine. The results for two time periods (8 to 24 hr and 24 to 72 hr) are shown in Table 19. The calculations based on the first time period may be more accurate, for reasons considered in the discussion. It is interesting that in both time periods the apparent ratio of Mg^{++} transported into the urine to Na^{+} transported out of the urine is, again, approximately 1.6:1 on a μEq basis. When the individual calculated rates of Mg^{++} and Na^{+} transport from both time periods are plotted together, there is a highly significant correlation between them (Fig. 40). Again, the apparent ratio of Mg^{++} to Na^{+} transport on a μEq basis is 1/0.642 or about 1.6:1. The high degree of correlation observed over a decade change in rate is good evidence that *in vivo* Mg^{++} and Na^{+} transport across the bladder in opposite directions are linked in some manner.

In Vitro Ion Movements Across Bladder Tissue

When measured *in vitro*, the values for T.E.P., R. and S.C.C. were low (Table 20). Values for T.E.P. usually declined to zero within 1 hr. Preliminary experiments in

Table 19. Calculated *in vivo* Mg and Na transport by the bladder of *C. magister* with blocked nephropores. Data recalculated from Fig. 38 (see text). Mean values ± s.d.

Period	Mg transport H→U(μEq/cm²/hr)	Na transport U→H(μEq/cm²/hr)	Mg:Na ratio
8 to 24 hr	0.039 ± 0.036 n= 10	0.024 ± 0.025 n= 10	1.6:1
24 to 72 hr	0.080 ± 0.024 n= 10	0.052 ± 0.018 n= 10	1.5:1

Figure 40. Correlation between calculated Mg and Na transport by the bladder of C. magister with blocked nephropores in 100% S.W. Recalculated from data shown in Fig. 38. Each point represents calculations based on one urine sample. See text.

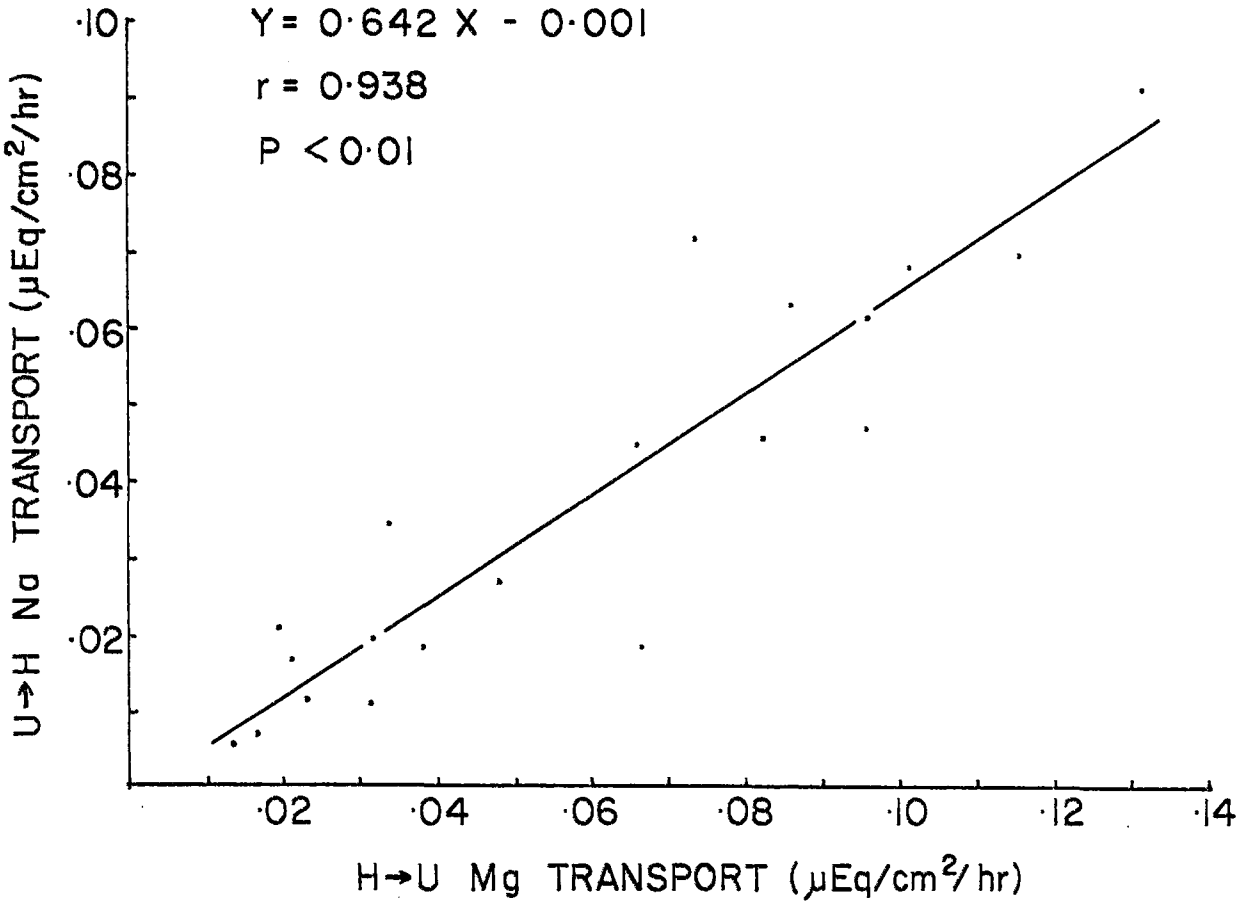


Table 20. In vitro electrical and metabolic parameters of excised bladder tissue of C. magister. Mean values ± s.d. Area of tissue = 0.80 cm^2.

T.E.P. (mV) inside negative	Resistance (Ohm)	Short circuit current (μA)*	O_2 consumption** (ml/g dry wt/hr)
-1.8 ± 0.6 n=22	147 ± 38 n=5	< 9 n=5	1.00 ± 0.26 n=8

* Apparatus could not deliver less than 9 μA.

** Mean of two measurements each on 4 pieces of bladder tissue.

which the movements of $^{28}Mg^{++}$, $^{22}Na^{+}$, and $^{36}Cl^{-}$ were measured at half-hour intervals over periods of 6 hours showed relatively constant values. Thus, the T.E.P. is apparently not associated with the movements of Mg^{++}, Na^{+} or Cl^{-}. Bladder tissue is approximately 30 μ thick and, thus, it might be expected that the values for these parameters would be small. Excised bladder tissue is metabolically active, as oxygen consumption by the tissue was relatively high (Table 20). Oxygen consumption was stable over the 3 hour period of measurement.

Unidirectional fluxes of $^{28}Mg^{++}$ across excised bladder tissue in normal crab Ringer solution were relatively low and there was a highly significant net hemolymph-to-urine (H→U) flux of the isotope (Row A, Table 21). Thus, the bladder is apparently the site of Mg^{++} excretion into the urine. Further evidence that the bladder is the site of Mg^{++} excretion is provided by the data in Row B of Table 21. Crabs maintained in Mg^{++}-free S.W. stopped excreting Mg^{++} into the urine, i.e., $[Mg^{++}]_u / [Mg^{++}]_s = 1$ (see below). Unidirectional $^{28}Mg^{++}$ fluxes across bladder tissues from such crabs were not significantly different. The observed decrease in the net flux was caused by a nearly significant decrease in the H→U flux. Thus, Mg^{++} excretion by the bladder is under physiological control and is curtailed under conditions of Mg^{++}-depletion. Mg^{++} transport across

Table 21. *In vitro* fluxes of ^{28}Mg across excised bladder tissue of *C. magister*. Mean values ± s.d.

Treatment	N	U→H flux (μEq/cm²/hr)	P_t*	H→U flux (μEq/cm²/hr)	P_t**	Net flux (μEq/cm²/hr)	P_t***
A. Normal crab Ringer	9	0.132 ± 0.051	---	0.412 ± 0.205	---	H→U 0.280 ± 0.176	<.01
B. Mg-depleted crabs	5	0.171 ± 0.099	A vs B >.05	0.224 ± 0.131	A vs B nearly .05	H→U 0.052 ± 0.113	>.05
C. Ouabain crab Ringer	6	0.144 ± 0.021	A vs C >.05	0.240 ± 0.123	A vs C nearly .05	0.096 ± 0.106	>.05

* P_t for comparison of U→H fluxes between treatments.

** P_t for comparison of H→U fluxes between treatments.

*** P_t for comparison of unidirectional fluxes within treatments.

the bladder may be coupled to Na^+ transport in the opposite direction, as 5 X 10^{-3} M ouabain eliminated the normal, significant difference between the unidirectional $^{28}Mg^{++}$ fluxes (Row C, Table 21), although it is possible that ouabain acted indirectly by disturbing intracellular levels of Na^+. Alternatively, Mg^{++} transport may be directly inhibited by ouabain. Again, the decrease was due to a nearly significant decrease in the H→U flux.

Unidirectional fluxes of $^{22}Na^+$ across excised bladder tissue in normal crab Ringer solution were much greater than the corresponding fluxes of $^{28}Mg^{++}$ (Row A, Table 22), and there was a large, highly significant net U→H flux of $^{22}Na^+$. Ouabain (Row B, Table 22) had no significant effect on the unidirectional or net fluxes. When flux measurements were made under a nitrogen atmosphere (Row C, Table 22) the unidirectional U→H $^{22}Na^+$ flux was reduced by a highly significant amount and the net $^{22}Na^+$ flux was not significant. Net U→H $^{22}Na^+$ transport is apparently dependent on aerobic metabolism. In a final experiment $^{22}Na^+$ fluxes were measured under simulated "normal" conditions, that is, with crab Ringer solution in the hemolymph-side chambers and simulated "urine" in the urine-side chamber of the dual lucite cell (Row D., Table 22). Under these conditions the unidirectional U→H $^{22}Na^+$ flux was reduced, but not significantly so, and the net U→H $^{22}Na^+$ flux was reduced

Table 22. *In vitro* fluxes of ^{22}Na across excised bladder tissue of *C. magister*. Mean values ± s.d.

Treatment	N	U→H flux ($\mu Eq/cm^2/hr$)	P_t*	H→U flux ($\mu Eq/cm^2/hr$)	P_t**	Net flux ($\mu Eq/cm^2/hr$)	P_t***
A. Normal crab Ringer	9	3.77 ± 0.98	---	2.44 ± 0.66	---	U→H 1.33 ± 0.56	<.01
B. Ouabain crab Ringer	6	3.42 ± 1.21	A vs B >.05	2.16 ± 0.42	A vs B >.05	U→H 1.26 ± 0.83	<.05
C. N_2 gas	5	2.29 ± 0.54	A vs C <.01	2.55 ± 0.76	A vs C >.05	H→U 0.26 ± 0.51	>.05
D. Normal Mg and Na gradients	6	2.76 ± 0.99	A vs D >.05	2.26 ± 0.92	A vs D >.05	U→H 0.50 ± 0.57	>.05
E. 0.1 normal Na	6	0.29 ± 0.05	A vs E <.001	0.20 ± 0.02	A vs E <.001	U→H 0.10 ± 0.04	<.01

* P_t for comparison of U→H fluxes between treatments.

** P_t for comparison of H→U fluxes between treatments.

*** P_t for comparison of unidirectional fluxes within treatments.

to an insignificant value. Although it is not of a significant magnitude, the net U→H $^{22}Na^+$ flux took place against a 50 mEq/l Na^+ gradient. It would be expected that, under these conditions, the net flux would have been in the opposite direction if only passive processes were involved. Because of the large standard deviation of the data, all that can be definitely concluded is that imposition of a chemical Na^+ gradient across the tissue did not cause a net movement of $^{22}Na^+$ down the gradient. When compared with the data in Row A of Table 22, the data from the last two experiments (Rows C and D, Table 22) provide good evidence that the net U→H $^{22}Na^+$ flux is an active process. When $^{22}Na^+$ fluxes were measured in 0.1 normal (50 mEq Na^+/l) Na^+ crab Ringer solution (Row E, Table 22) the unidirectional fluxes were decreased ten-fold and there was a highly significant net U→H Na^+ flux.

Unidirectional fluxes of $^{36}Cl^-$ across excised bladder tissue in normal crab Ringer solution were much greater than the corresponding $^{22}Na^+$ fluxes (Row A, Table 23). There was a net U→H flux of $^{36}Cl^-$ which was of the same size and direction as the net $^{22}Na^+$ flux under similar conditions, but which was slightly less than significant. Thus, the net U→H $^{36}Cl^-$ flux may be linked with the net U→H $^{22}Na^+$ flux. Alternatively, it is also possible that the net U→H $^{22}Na^+$ flux is the passive result of net U→H

Table 23. *In vitro* fluxes of ^{36}Cl across excised bladder tissue of *C. magister*. Mean values ± s.d.

Treatment	N	U→H flux ($\mu Eq/cm^2/hr$)	H→U flux ($\mu Eq/cm^2/hr$)	Net flux ($\mu Eq/cm^2/hr$)	P_t
				U→H	
Normal crab Ringer	7	7.39 ± 1.05	6.23 ± 1.17	1.16 ± 1.12	approaches .05

transport of $^{36}Cl^-$.

No attempt was made to determine what portion of the unidirectional isotope fluxes was due to exchange diffusion.

Control of Mg^{++} Excretion by the Bladder

Maintenance of crabs in Mg^{++}-free 75% S.W. for 6 days halved $[Mg^{++}]_s$ and resulted in cessation of Mg^{++} excretion into the urine (Table 24). These data, in combination with those shown in Row B of Table 21, provide good evidence for the existence of physiological control of Mg^{++} transport by the bladder.

Eyestalk ablation had no significant effect on $[Mg^{++}]_s$ or $[Mg^{++}]_u$ in crabs acclimated to 100% S.W. (Table 25). It is thus unlikely that the eyestalk is directly involved in the control of Mg^{++} excretion by the bladder.

Mg^{++} Loading Experiments

If it is accepted that the bladder is the major site of Mg^{++} excretion, then it would be expected that elevation of $[Mg^{++}]_s$ would result in an increase of Mg^{++} excretion in the urine. Experiments were conducted to determine the time course of elimination of Mg^{++} loads injected into the hemolymph and the role of the antennal gland in elimination

Table 24. Effect of Mg depletion on Mg levels (mEq/l) of serum and urine of C. magister in 75% S.W. Mean values ± s.d.

Normal crabs*			Mg-depleted		
Serum	Urine	P_t	Serum	Urine	P_t
21 ± 5 n=34	54 ± 16 n=34	<.01	11 ± 3 n=10	12 ± 2 n=10	>.05

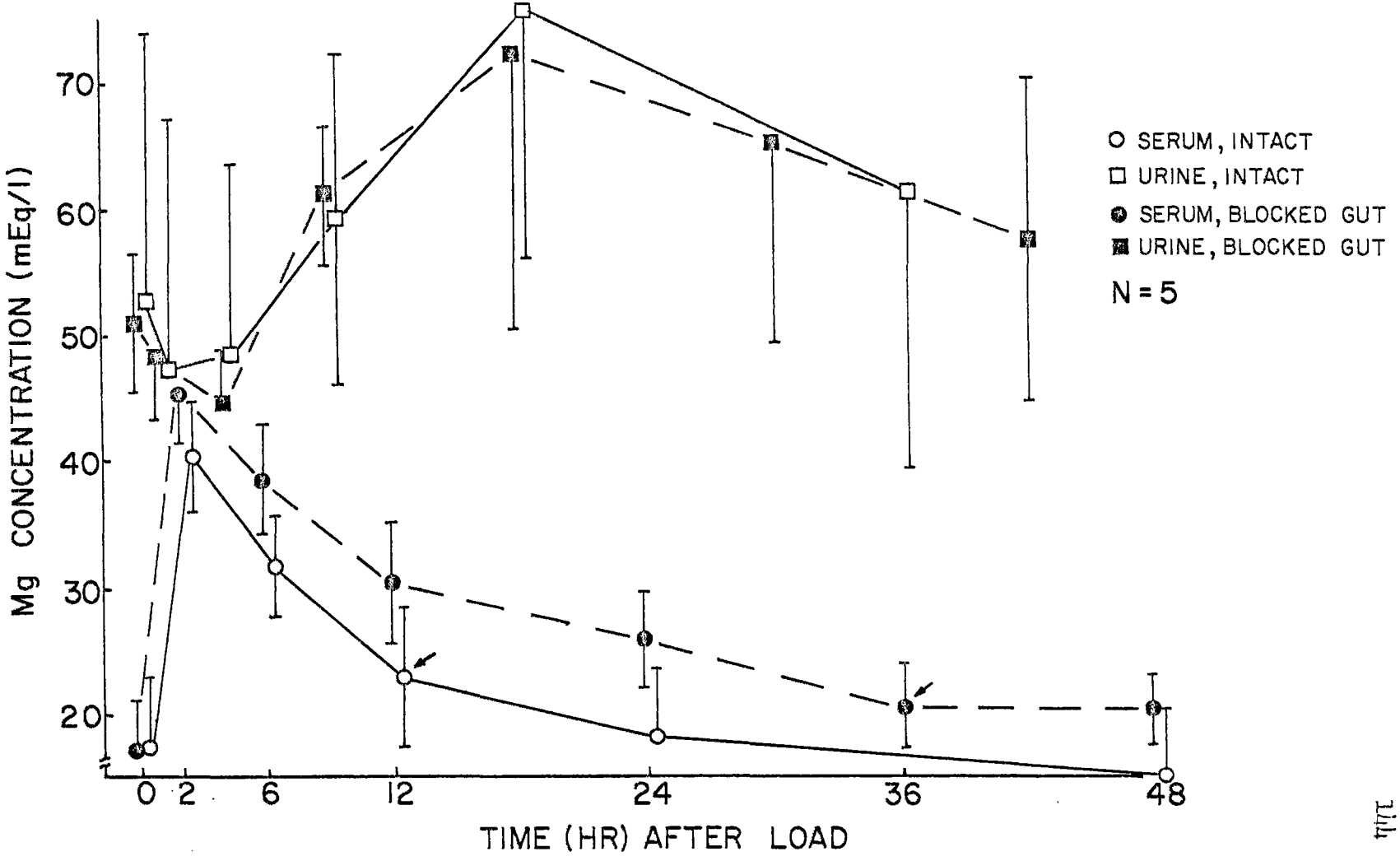
* Data from Table 17 (75% S.W.)

Table 25. Effect of eyestalk ablation on serum and urine Mg levels (mEq/l) in C. magister in 100% S.W. Mean values ± s.d.

	Intact	Ablated	P_t
Serum	39 ± 7 n=15	38 ± 2 n=10	>.05
Urine	138 ± 20 n=15	128 ± 14 n=10	>.05

of the load. Crabs were fitted with rubber tubes for external collection of voided urine (Methods, Chapter 4) and acclimated in 75% S.W. for 48 hr. After acclimation the crabs received injections of $MgCl_2$ solution and urinary rate, $[Mg^{++}]_s$ and $[Mg^{++}]_u$ were monitored for 48 hr. A second experiment to determine the role of the gut in Mg^{++} excretion was conducted in the same way, with the exception that both ends of the crabs' guts were blocked (Methods, Chapter 4). The results are shown in Fig. 41. Because urine was collected from a reservoir at the end of each collection period, $[Mg^{++}]_u$ is actually an average value during the time period. Accordingly, values of $[Mg^{++}]_u$ are plotted at the middle of each collection period. Intact crabs eliminated the Mg^{++} load in 12 hr, while crabs with blocked guts required 36 hr to eliminate the load. Thus, the gut may also be involved in Mg^{++} excretion. In both experiments $[Mg^{++}]_u$ rose steadily after an initial drop, reached a peak after approximately 16 hr and then fell toward the original value. Urinary rates in both experiments (data not shown) were unchanged. Allowing for the fact that urine is retained for several hours before voiding (Hunter, 1973), one would expect to find a lag between a decrease in $[Mg^{++}]_s$ and an increase in $[Mg^{++}]_u$ due to the method of urine collection and this is, in fact, the case. Thus, at first glance, it would

Figure 41. Effect of Mg load on serum and urine Mg levels in C. magister in 75% S.W. See text. Arrows indicate time at which serum Mg level does not differ significantly from value at t=0. Mean values ± s.d.



seem that most of the Mg^{++} load was eliminated via the urine. If the normal rate of urinary Mg^{++} efflux (urinary rate X $[Mg^{++}]_u$) is taken to be the rate during the last 12 hr of acclimation to 75% S.W. in these experiments and if the Mg^{++} load is assumed to be eliminated in the urine, then the rate of urinary Mg^{++} excretion after the load should increase greatly. As shown in Table 26, this is not the case for intact crabs or for those with blocked guts. Apparently, the Mg^{++} load was removed from the hemolymph at an extrarenal site at a rate nearly equal to the normal rate of Mg^{++} excretion via the urine. Alternatively, the Mg^{++} load could have been taken up by the tissues or deposited in the exoskeleton. If the Mg^{++} load had been excreted via the gut, then the stomach fluid and feces from crabs with blocked guts would be expected to have very high levels of Mg^{++}. As shown in Table 27, Mg^{++} levels in stomach fluid and feces from these crabs were greater than $[Mg^{++}]_s$. However, the amounts of stomach fluid (about 2 ml) and feces (about 1 g) present were far too small to account for elimination of the Mg^{++} load.

It could be argued that the calculated rates of urinary Mg^{++} efflux shown in Table 26 are an inaccurate measure of Mg^{++} excretion by the bladder because some of the Mg^{++} in the urine was already present in the primary urine formed by ultrafiltration at the coelomosac. Thus,

Table 26. Effect of Mg load on urinary Mg excretion in *C. magister* in 75% S.W. Mean values ± s.d. See text.

Treatment	Rate of normal Mg efflux (mEq/48 hr)	Mg load (mEq)	Rate of Mg efflux during 48 hr after load (mEq/48 hr)	Calculated extrarenal removal of Mg during 48 hr after load* (mEq/48 hr)
Gut open n=5	3.60 ± 1.12	3.50 ± 0.47	4.53 ± 1.16	2.57 ± 0.68
Gut blocked n=5	3.48 ± 0.94	3.55 ± 0.37	4.40 ± 0.62	2.62 ± 1.45

* Extrarenal removal of Mg during 48 hr after load is:

$$\left[\begin{array}{l}\text{Normal rate of urinary}\\ \text{Mg efflux in mEq/48 hr}\end{array}\right] + \left[\begin{array}{l}\text{Mg load}\\ \text{in mEq}\end{array}\right] - \left[\begin{array}{l}\text{Rate of urinary efflux during}\\ \text{48 hr after load in mEq/48 hr}\end{array}\right]$$

Table 27. Mg levels in stomach fluid and feces of C. magister with blocked gut 60 hr after injection of Mg load. Mean values ± s.d.

Stomach fluid (mEq/l)	Feces (mEq/l)
35 ± 4 n=5	63 ± 4 n=5

apparent rates of Mg^{++} excretion would be spuriously high when $[Mg^{++}]_s$ was increased by injection of the Mg^{++} load. A more accurate estimate of the rate of Mg^{++} excretion into the urine by the bladder may be calculated using the formula:

$$\frac{\left(\bar{x}\,[Mg^{++}]_u - \bar{x}\,[Mg^{++}]_s\right) \times U.V.}{B.W. \times t}$$

where:

$\bar{x}\,[Mg^{++}]_u$ and $\bar{x}\,[Mg^{++}]_s$ are, respectively, the MEAN urine and serum magnesium concentrations (μEq/l) during the time period.

U.V. = urine voided in liters during the time period.

B.W. = body weight (g).

t = time period (hr).

The data shown in Fig. 41 were recalculated using this equation and the results are shown in Table 28. The rate of Mg^{++} transport by the bladder during the 48 hr after injection of the load is not significantly different in either group of crabs from the normal rate, which is, again, based on the last 12 hr of acclimation to 75% S.W. The rate of extrarenal excretion of Mg^{++}, calculated by

Table 28. The effect of Mg load on Mg excretion into the urine by C. magister in 75% S.W. Mean values ± s.d. See text.

Treatment	Normal rate of urinary Mg efflux (µEq/g/hr)	Rate of urinary Mg efflux during 48 hr after load (µEq/g/hr)	P_t	Mg load (µEq/g)	Extrarenal removal of Mg during 48 hr after load (µEq/g/hr)*
Gut open (n=5)	0.115 ± 0.081	0.136 ± 0.072	>.05	7.73 ± 0.03	0.154 ± 0.045
Gut blocked (n=5)	0.107 ± 0.041	0.119 ± 0.038	>.05	7.84 ± 0.04	0.152 ± 0.071

* Extrarenal removal of Mg during 48 hr after load is:

$$\left[\begin{array}{l}\text{Normal urinary Mg}\\ \text{efflux in µEq/g/hr}\end{array} + \frac{\text{Mg load in µEq/g}}{\text{48 hr}}\right] - \left[\begin{array}{l}\text{Urinary Mg efflux during 48 hr}\\ \text{after load in µEq/g/hr}\end{array}\right]$$

the equation shown in the footnote in Table 28, is in both cases greater than the normal rate of Mg^{++} excretion by the bladder.

A final experiment was conducted to prove that Mg^{++} loads are eliminated by extrarenal means. The coelomosac arteries of seven crabs were surgically broken (Methods, Chapter 4), rendering the crabs anuric. One day after surgery a Mg^{++} load was administered and $[Mg^{++}]_s$ was monitored for several days. As shown in Fig. 42, the Mg^{++} load was eliminated in approximately 24 hr and $[Mg^{++}]_s$ continued to rise slowly, as is normal in surgically anuric crabs (compare with Fig. 37). The Mg^{++} load could not have been eliminated via the antennal glands, as the crabs were anuric.

Mg^{++} levels in muscle tissue $[Mg^{++}]_m$ from autonomized walking legs were measured before and 24 hr after a Mg^{++} load. As shown in Table 29, $[Mg^{++}]_m$, corrected for tissue hemolymph volume and $[Mg^{++}]_s$ (Methods), did not change. Uncorrected Mg^{++} levels in midgut gland 24 hr after administration of the Mg^{++} load were not high. It is, thus, unlikely that either tissue serves as a "sink" for injected Mg^{++} loads.

If short-term Mg^{++} loads are removed from the crabs by an extrarenal mechanism, then it would be expected that Mg^{++} efflux from Mg^{++}-loaded crabs would be greater than

Figure 42. Effect of Mg load on serum Mg in C. magister with coelomosac arteries broken. Mean values ± s.d. Arrow indicates injection of Mg load.

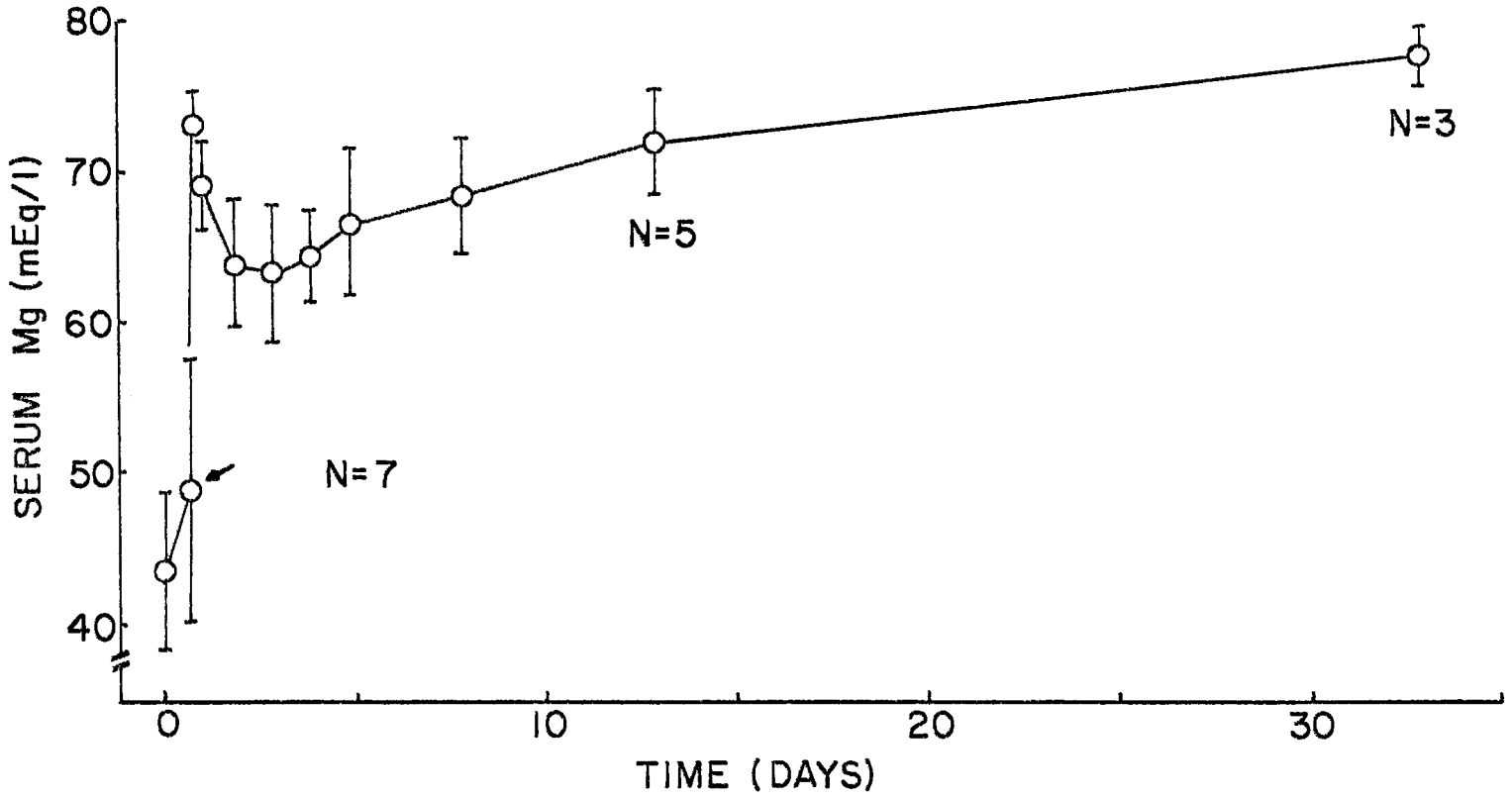


Table 29. Effect of Mg load on muscle and midgut gland Mg levels in *C. magister* in 75% S.W. Mean values ± s.d.

Control (t=0)		Mg load (t=24 hr)		
Serum Mg (mEq/l)	Muscle Mg* (mEq/l)	Serum Mg (mEq/l)	Muscle Mg* (mEq/l)	Midgut gland Mg (mEq/l)
27 ± 2 n=5	24 ± 2 n=5	37 ± 2 n=5	24 ± 2 n=5	29 ± 9 n=5

* Corrected for E.C.S. and $[Mg]_s$

normal. Mg^{++} efflux to Mg^{++}-free 75% S.W. was measured in normal and Mg^{++}-loaded crabs over a 24 hr period. In order to eliminate changes in the Mg^{++} level in the medium due to voiding of urine, the crabs' urinary opercula were sealed. As shown in Table 30, Mg^{++} efflux was not significantly changed by a Mg^{++} load. Thus, in the short term, injected Mg^{++} loads must be sequestered within the crab. It may be that the sequestered Mg^{++} is slowly released into the hemolymph over a period of days and then eliminated by the bladder. An increased rate of Mg^{++} excretion by the bladder is apparently the result of a longer period of elevated $[Mg^{++}]_s$ than is provided by single, injected Mg^{++} loads.

DISCUSSION

The normal values of $[Mg^{++}]_s$ reported in the present study is well within the range of reported values of other marine crustaceans (Burton, 1967). Robertson (1949, 1953, 1960a) has noted that there is an apparent inverse correlation between normal $[Mg^{++}]_s$ and activity in decapods. As noted by Englehardt and Dehnel (1973), C. magister is well described by Robertson's rule, as it is an active crab with low $[Mg^{++}]_s$. Mg^{++} levels in the urine of marine crustacea are usually greater than $[Mg^{++}]_s$ (Potts and Parry,

Table 30. Effect of Mg load on Mg efflux from C. magister to Mg-free 75% S.W.(nephropores blocked). Mean values ± s.d.

Mg efflux from normal crabs (μEq/g/hr)	Mg efflux from Mg-loaded crabs (μEq/g/hr)	P_t
0.208 ± 0.053 n=10	0.181 ± 0.040 n=10	>.05

1964; Riegel, 1972) and C. magister is typical in this respect (Table 17).

Marine crustaceans usually hyporegulate $[Mg^{++}]_s$ and, because urine:serum (U/S) ratios of Mg^{++} are usually much greater than U/S inulin ratios, the antennal gland is commonly regarded as the organ responsible for this phenomenon (Lockwood, 1967; Riegel, 1972). Additional evidence for this hypothesis is provided by the data shown in Fig. 37. Crabs made anuric by surgical interruption of the blood supply to the coelomosac showed a slow rise in $[Mg^{++}]_s$. It is interesting that $[Mg^{++}]_s$ did not eventually equilibrate with the external medium, but remained 20-25 mEq/l below it. Hunter (1973) has shown that C. magister has a small, inside-negative potential difference across its epithelia in 100% S.W., and it would seem that, in the absence of an active process of Mg^{++} extrusion, $[Mg^{++}]_s$ would at least equal the Mg^{++} concentration of the external medium. Further, Webb (1940) has shown that when the blood of the crab, Carcinus maenas, is dialysed against 100% S.W., the Mg^{++} concentration in the dialysate slightly exceeded that of the medium. Roesijadi (personal communication) has recently shown that low concentrations of chlorine gas in 100% S.W. completely eliminate the ability of C. magister to hyporegulate Mg^{++}. Thus, it seems likely that in C. magister the antennal gland is responsible for the

greater part of Mg^{++} hyporegulation, but that there also exists a low-capacity, extrarenal site of Mg^{++} excretion. Burger (1957) found that naturally anuric lobsters, Homarus americanus, maintained normal $[Mg^{++}]_s$. Although Burger interpreted this finding to show that the lobster was relatively impermeable to Mg^{++}, it could also be taken as evidence for the existence of an extrarenal site of Mg^{++} excretion.

In Vivo Mg^{++} Excretion by the Bladder

Although Schmidt-Nielsen, et al., (1968) favor the labyrinth as the site of Mg^{++} transport into the urine, the present study and that of Gross and Capen (1966) provide good evidence that Mg^{++} is transported into the urine by the bladder. In both C. magister (Fig. 38) and Pachygrapsus crassipes (Gross and Capen, 1966) $[Mg^{++}]_u$ is a function of the residence time of the urine in the bladder, increasing with increased residence time in the bladder. Riegel and Lockwood (1961) obtained similar results with C. maenas with blocked nephropores but did not comment on this phenomenon as evidence that the bladder is responsible for Mg^{++} excretion into the urine. As Gross and Capen (1966) have noted, if Mg^{++} were concentrated in the urine by a more proximal part of the antennal gland, then $[Mg^{++}]_u$

would not be a function of residence time in the bladder. These authors have also noted that Mg^{++} transport by the bladder would also account for the extreme variability of $[Mg^{++}]_u$ in decapods: urine sampled a short time after micturation would have been in the bladder only a short time and would have low levels of Mg^{++}, while urine that had been in the bladder for several hours would have high levels of Mg^{++}. Hunter (1973) has found that C. magister micturates only once every 10 hours in 100% S.W. and that a relatively large amount of urine (0.6% B.W.) is voided at each micturation. Given that Mg^{++} is transported into the urine by the bladder, then it would be advantageous for the crab to retain newly formed urine in the bladder for a period of time to reduce the U/S Mg^{++} gradient opposing transport by diluting the Mg^{++} in as large a volume of urine as possible.

In the blocked-nephropore experiment shown in Fig. 38, $[Na^{+}]_u$ fell as $[Mg^{++}]_u$ rose. These data are similar to those reported by Riegel and Lockwood (1961, Table 2) and Gross and Capen (1966, Fig. 4), although the experiments are not strictly comparable to the present study as, in both cases, the crabs were kept out of water for several days during the experiments and were, therefore, not subject to normal Mg^{++} influx and were experiencing desiccation as well. As shown in Fig. 39 of the present study, there is a

highly significant negative correlation between $[Mg^{++}]_u$ and $[Na^+]_u$. Several authors have noted the reciprocal relationship between $[Mg^{++}]_u$ and $[Na^+]_u$ in decapods (Webb, 1940; Prosser, et al., 1955; Gross, 1957, 1959; Green, et al., 1959; Gross and Marshall, 1960; Riegel and Lockwood, 1961; Gifford, 1962, Gross, 1964; Gross and Capen, 1966; Gross, et al., 1966; Lockwood and Riegel, 1969; Riegel, et al., 1974). Hunter and Rudy (1975) have found a significant negative correlation between $[Mg^{++}]_u$ and $[Na^+]_u$ in C. magister. DeLeersnyder (1967) has shown that there is a significant negative correlation between the urine-serum differences of Mg^{++} and Na^+ in Eriocheir sinensis. Given that the urine is produced by ultrafiltration of the hemolymph and the Mg^{++} and Na^+ movements occur across the bladder, DeLeersnyder's expression is probably the most descriptive of actual Mg^{++} and Na^+ movements into and out of the urine.

When the data shown in Fig. 38 are recalculated and the change in $[Mg^{++}]_u$ is compared with the change in $[Na^+]_u$ for each time period, the apparent exchange ratio is approximately 1.6 Eq Mg^{++} : Eq Na^+ (Table 18). It would seem, on the basis of this exchange ratio, that electrical neutrality across the bladder would not be preserved and that such an exchange of Mg^{++} for Na^+ would generate an inside-positive potential difference across the bladder.

However, as Hunter and Rudy (1975) note, neither Mg^{++} nor Na^{+} salts are fully ionized at normal urine concentrations, and it is the degree to which a salt is ionized, the activity coefficient, that determines its effective ionic concentration in solution (Morris, 1968). The activity coefficient of the major Na^{+} salt in crab urine, NaCl, at 0.5 M is approximately 0.7 and that for the major Mg^{++} salt, $MgCl_2$, at 0.1 M is approximately 0.5 (Harned and Owen, 1958). When these activity coefficients are applied to the calculated exchange ratio, the effective raio is 1.6 X 0.5 : 1 X 0.7 or 1.1 Eq Mg^{++} : Eq Na^{+}. Thus, the observed exchange ratio of 1.6 Eq Mg^{++} : Eq Na^{+} would produce little, if any, potential difference across the bladder. It is to be stressed that activity coefficients for pure, single-salt solutions were used in the above calculations. Actual Na^{+} and Mg^{++} activity coefficients in the urine may be greatly different from these values due to interactions with other ions.

Several authors have considered the stoichiometry of the apparent exchange of Mg^{++} for Na^{+} in the urine of decapods. Prosser, et al., (1955) and Gifford (1962) have noted that $[Mg^{++}]_u$ and $[Na^{+}]_u$ are reciprocally related, but interpreted their findings as indicating competition between Mg^{++} and Na^{+} for transport into the urine, which they took to be formed by a process of secretion instead of

ultrafiltration. Green, et al., (1959) have rejected an an equivalent-for-equivalent (0.5 M Mg^{++} : M Na^{+}) exchange in Uca pugnax and U. pugilator in 75% S.W., but their data may have been reported as mEq Mg^{++}/l instead of the indicated mM Mg^{++}/l (e.g., 100% S.W. is reported to have 88 mM Mg^{++}/l, an unreasonably high value). Using the assumption that all of the values for Mg^{++} reported by these authors were in mEq Mg^{++}/l, a recalculated value of 1.3 Eq Mg^{++} : Eq Na^{+} is obtained. Green, et al., (1959) did not take into account the difference between the activity coefficients of Mg^{++} and Na^{+}. If these are applied as before, the final ratio is 0.9 Eq Mg^{++} : Eq Na^{+}, which is close to an electrically neutral exchange. Recalculation of the data presented by these authors for crabs in 100% S.W. yields similar results. Riegel and Lockwood (1961) found that in C. maenas held four days in water-saturated air the fall in $[Na^{+}]_u$ was too small to account for the observed rise in $[Mg^{++}]_u$ on an equivalence basis. These authors did note that Mg^{++} salts are less fully ionized than Na^{+} salts, but did not apply the activity coefficients in their calculations. In this case, when the proper activity coefficients are applied the Mg^{++} : Na^{+} ratios are 1.3 - 2 Eq Mg^{++} : Eq Na^{+}. Recalculation of these authors' data to compare urine-serum differences yields similar, high Mg^{++} : Na^{+} ratios. In discussing their findings Riegel and Lockwood (1961) mention

the possibility that some of the Mg^{++} excreted into the urine may have been exchanged with ions other than Na^{+} or metabolites not measured in their study. As the authors note, the crabs had undergone considerable desiccation and inulin U/S ratios were 2.5:1, indicating considerable reabsorption of water and salts from the urine, which was isosmotic with the hemolymph. It is interesting that under these conditions $[Cl^{-}]_u$ was considerably greater than $[Cl^{-}]_s$. Gross and Capen (1966) concluded that a 1 Eq Mg^{++} : Eq Na^{+} exchange does, indeed, occur across the bladder of P. crassipes and that Cl^{-} moves into the urine to partially compensate for the osmotic imbalance created by removal of 2 Na^{+} for each Mg^{++} transported into the urine. These authors did not consider activity coefficients in their calculations. The experiments of Riegel and Lockwood (1961) and Gross and Capen (1966) were both conducted with crabs held out of water for 2-4 days, and under these conditions both C. maenas and P. crassipes reabsorb greater than normal amounts of water and salts from the urine. Further, crabs held out of water are not subject to the normal influx of Mg^{++} from the medium. Thus, Mg^{++} and Na^{+} movements may have been disturbed under these extreme conditions. Because $[Cl^{-}]_u$ under normal conditions is not greatly different from $[Cl^{-}]_s$ in C. maenas (Webb, 1940) or in most decapods (Riegel, 1972) it seems unlikely that the hypothesis

of Gross and Capen (1966) applies in decapods under normal conditions. Their hypothesis may, however, apply to Uca, which has been shown to have U/S ratios for Cl^- that are significantly greater than unity (Green, et al., 1959; Schmidt-Nielsen, et al., 1968). Hunter and Rudy (1975) found that the levels of $[Mg^{++}]_u$ and $[Na^+]_u$ in the urine of C. magister are consistent with a 1 Eq Mg^{++} : Eq Na^+ exchange. The results of the present study indicate a slightly higher exchange ratio of 1.6 Eq Mg^{++} : Eq Na^+, and the difference between the calculated exchange ratios is due to the use of different activity coefficients for Mg^{++} and Na^+ in the present study.

Reabsorption of water and salts from the urine is a common phenomenon in decapods (Riegel, 1972) and has been shown to occur in C. magister, even when the urinary rate is high (Hunter, 1973). Riegel and Lockwood (1961) have suggested that in C. maenas water withdrawn from the urine is accompanied by Na^+, K^+, Ca^{++} and Cl^-, but not by Mg^{++}. Thus, any calculated values for Mg^{++} and Na^+ transport or the exchange ratio of these ions will reflect the net results of at least four processes: Mg^{++} transport into the urine, Na^+ transport out of the urine, urinary rate and the rate of water and salt reabsorption from the urine. As Riegel and Lockwood (1961) note, if water reabsorption from the urine occurs, and if, as seems probable, Mg^{++} is left

behind, the apparent increase in $[Mg^{++}]_u$ will be greater than that due to Mg^{++} transport into the urine. For this reason Mg^{++} : Na^{+} exchange ratios and rates of Mg^{++} and Na^{+} transport calculated on the basis of changes in $[Mg^{++}]_u$ and $[Na^{+}]_u$ in crabs with blocked nephropores or on the basis of urine-serum differences in normally-micturating crabs represent upper limits instead of actual values. As Hunter (1973, p. 95) notes, "Movements of water and salts directed at providing electrochemical neutrality and urine-serum isosmoticity in the production of the final excretory product may disguise the true character of an active exchange pathway for magnesium ions."

In order to facilitate a comparison between in vivo and in vitro rates of Mg^{++} and Na^{+} transport across the bladder, the data shown in Fig. 38 were recalculated to provide rates of ion transport in $\mu Eq/cm^2/hr$ (Table 19). For the reasons stated above, these values are maximum ones, as it is assumed in the calculation that no reabsorption of water from the urine occurred. Because less water reabsorption would have occurred during the first time period, the calculated rates of Mg^{++} and Na^{+} transport for that time period are probably more accurate. If water reabsorption did, in fact, take place to a greater extent during the second time period in this experiment, the relative movements of Mg^{++} and Na^{+} across the bladder were

unchanged, as Mg^{++} : Na^{+} ratios were approximately 1.6:1 in both cases. There is a highly significant correlation between the calculated rates of Mg^{++} and Na^{+} transport over a decade change in rate (Fig. 40), indicating that Mg^{++} and Na^{+} movements across the bladder are linked.

In Vitro Mg^{++} Excretion by the Bladder

As might be expected in such a thin (30 μ) epithelium, *in vitro* values for T.E.P., R. and S.C.C. were all low (Table 20). The T.E.P. may have been an artifact, as it usually declined to zero within one hr. In these respects crab bladder is similar to vertebrate gallbladder (Hénin, *et al.*, 1977), a tissue thought to be involved in isosmotic fluid transport.

Excised bladder tissue is metabolically active (Table 20) and shows values for oxygen consumption similar to those for excised crustacean gills (Dehnel and McCaughran, 1964; Hulbert, *et al.*, 1976).

Unidirectional fluxes of $^{28}Mg^{++}$ across excised bladder tissue in normal crab Ringer solution were relatively small, but there was a highly significant net H→U flux (Table 21). As shown in Table 31, the observed *in vitro* net flux is 2-7 times greater than the calculated *in vivo* rates. This seems a reasonable finding, as the *in vitro* rates were

Table 31. Comparison of *in vivo* and *in vitro* Mg and Na transport by the bladder of *C. magister*. Mean values ± s.d.

Ion	Blocked nephropores* (8 to 24 hr)		Blocked nephropores* (24 to 72 hr)		External collection ** of urine		*In vitro***	
	Transport (μEq/cm^2/hr)	Opposing gradient	Transport (μEq/cm^2/hr)	Opposing gradient	Transport (μEq/cm^2/hr)	Opposing gradient	Transport (μEq/cm^2/hr)	Opposing gradient
Mg	H→U 0.039 ± 0.036 (n=10)	4.9:1	H→U 0.080 ± 0.024 (n=10)	7.6:1	H→U 0.132 ± 0.093 (n=5)	3.5:1	H→U 0.280 ± 0.176 (n=9)	1:1
Na	U→H 0.024 ± 0.025 (n=10)	1.1:1	U→H 0.052 ± 0.018 (n=10)	1.3:1	----	----	U→H 1.33 ± 0.56 (n=9)	1:1
Mg:Na ratio	1.6:1		1.5:1				0.2:1	

* From Table 19.
** Recalculated from data in Table 28 on the basis of 0.867 cm^2 bladder/g body weight.
*** From Tables 21 and 22.

measured with no Mg^{++} gradient across the tissue, whereas in vivo Mg^{++} transport occurred against a considerable U→H Mg^{++} gradient. Further, the rate of H→U Mg^{++} transport is under physiological control (Row B, Table 21) and it might be expected that in different types of experiments the bladder would show different rate of H→U Mg^{++} transport. Given the great differences in the methods used, the estimates of Mg^{++} transport agree surprisingly well.

The data in Rows A and B of Table 21 confirm the hypotheses of Gross and Capen (1966) that the bladder is the site of Mg^{++} transport into the urine and that Mg^{++} transport in under physiological control. Significant H→U Mg^{++} transport was eliminated in the present study by ouabain, (Row C, Table 21), a finding contrary to that of Gross and Capen (1966). These authors found that ouabain introduced in perfusion fluid into the bladder of P. crassipes did not eliminate the ability of the crab to concentrate Mg^{++} in the urine. Unfortunately, data from a similar experiment which could have served as a control for assessing the effect of ouabain were presented in a form that prevents comparison (Gross and Capen, 1966, p. 282). Thus, it may well be that ouabain did, in fact, partially inhibit Mg^{++} transport into the urine in P. crassipes. Because of the high degree of variability in the results in the present study (Row C, Table 21), the statistical treatment of the

data is misleading, as five of the six preparations used showed net H→U Mg^{++} fluxes and in three of these preparations H→U Mg^{++} fluxes were twice as great as U→H fluxes. Thus, ouabain inhibits but probably does not eliminate transport of Mg^{++} into the urine. Transport of Mg^{++} into the urine may, therefore, be driven by Na-K activated ATPase-mediated Na^{+} transport in the opposite direction. Alternatively, Mg^{++} transport may be via a separate, ouabain-sensitive mechanism not functionally linked to Na^{+} transport in the opposite direction. It is, of course, possible that the relatively high levels of ouabain used (5×10^{-3} M) had an indirect effect on Mg^{++} transport by disturbing intracellular Na^{+} levels, although the lack of effect of ouabain on Na^{+} fluxes (see below) makes this unlikely.

In contrast to Mg^{++} fluxes, the unidirectional fluxes of $^{22}Na^{+}$ across excised bladder tissue in normal crab Ringer solution were large and there was a highly significant net U→H Na^{+} flux (Row A, Table 22). Thus, as Hunter (1973) has noted, the relative movements of Mg^{++} and Na^{+} across the bladder may be such that Na^{+} efflux from the urine is too large, rather than too small (Green, et al., 1959; Riegel and Lockwood, 1961; Lockwood and Riegel, 1969) to account for a 1 Eq Mg^{++} : Eq Na^{+} exchange. Riegel (1970) has presented evidence that the bladder of the

crayfish is the site of salt reabsorption from the urine. Riegel, et al., (1974) have shown that ethacrynic acid, an inhibitor of water reabsorption in the mammalian kidney, causes lowered U/S ratios for the tracer sodium diatrizoate in C. maenas. Because tracer U/S ratios have been shown to increase with residence time in the bladder (Riegel and Lockwood, 1961; Gross and Capen, 1966), it is tempting to speculate that most of the net U→H Na^+ flux observed in the present study is the driving force for reabsorption of water and salts by the bladder. Thus, crab bladder may be similar to vertebrate gallbladder and reabsorption of water and salts, deduced from inulin U/S ratios of 1.2-2 and the isosmoticity of urine and serum (Riegel, 1972), may be via a standing-gradient mechanism similar to that proposed by Diamond and Bossert (1967).

If it is assumed that the bladder of C. magister is engaged in reabsorption of water and salts, that the reabsorbate has the same Na^+ concentration as the hemolymph (0.5 μEq/l) and if allowance is made for a 1.6 Eq Mg^{++} : Eq Na^+ exchange across the bladder, then (1.33-0.18) μEq $Na^+/cm^2/hr$ divided by 0.5 μEq $Na^+/\mu l$ or approximately 2 $\mu l/cm^2$ bladder/hr of fluid would be reabsorbed from the urine. Thus, the volume of urine held in one bladder of a 500 g crab would be reduced by:

$$\frac{500 \text{ gm} \times 0.867 \text{ cm}^2 \text{ bladder/gm B.W.}}{2} \times 2 \ \mu l/cm^2/hr$$

or approximately 0.4 ml/hr. If it is assumed that the mean amount of urine held in each bladder of a 500 g crab is approximately 5 ml (maximum volume = 10 ml, Chapter 3; volume/micturation = 1.5 ml, Hunter, 1973), then the calculated rate of water reabsorption of 0.4 ml/hr is quite sufficient to account for the inulin U/S ratios of 1.2-2 commonly found in decapods (Riegel, 1972). Although these calculations suggest a possible mechanism for reabsorption of water and salts from the urine of C. magister, the present study provides no information on the reason for the phenomenon. As Riegel, et al., (1974, p. 179) have noted, ". . . there is at present no apparent reason why marine crabs should withdraw water from their urine."

An unexpected finding in the present study was that ouabain, which inhibits Mg^{++} transport by the bladder (Row C, Table 21), had no significant effect on Na^{+} transport (Row B, Table 22). Thus, although Na^{+} transport is dependent on aerobic metabolism (Row C, Table 22), it apparently does not occur via the ouabain-sensitive, Na^{+}-K^{+} activated ATPase-dependent sodium pump which is widely distributed in the animal kingdom (Glynn and Karlish, 1975). However, on the basis of the observed, electrically neutral in vivo exchange ratio of 1.6 Eq Mg^{++} : Eq Na^{+} and the observed in vitro rate of Mg^{++} transport, it would be expected that the rate of Na^{+} transport out of the bladder would be

reduced by 0.28 μEq Mg^{++}/cm^2hr divided by 1.6 or 0.18 μEq $Na^+/cm^2/hr$ if inhibition of Mg^{++} transport was due to inhibition of a ouabain-sensitive Na^+ pump. As has been noted, the *in vitro* value for U→H Na^+ transport and its standard deviation are large in comparison to the *in vitro* H→U Mg^{++} transport (i.e., 1.33 ± 0.88 μEq $Na^+/cm^2/hr$ as opposed to 0.28 μEq $Mg^{++}/cm^2/hr$). Therefore, a change in net U→H Na^+ transport of only 0.18 μEq $Na^+/cm^2/hr$ would not be statistically significant and might easily be obscured by chance variation in the data. For this reason the lack of a significant effect of ouabain on Na^+ transport does not provide evidence for or against the hypothesis that Mg^{++} transport into the urine is driven by or coupled with ouabain-sensitive Na^+ transport in the opposite direction.

Kamemoto, *et al.*, (1962) have shown that eserine, a cholinesterase and Na^+ transport inhibitor (Florey, 1968) slows *in vivo* U→H $^{22}Na^+$ movement in the crayfish, *Orconectes virilis*, and that levels of cholinesterase activity in the crayfish kidney are highest in the bladder. Riegel, *et al.*, (1974) have shown that ethacrynic acid lowers U/S ratios for the tracer sodium diatrizoate in *C. maenas*, leading to the conclusion that it is an inhibitor of isosmotic fluid reabsorption. In light of these findings it is curious that ouabain had no significant effect on Na^+ transport by excised bladder in the present study. Na^+ transport by

the bladder of C. magister is definitely the result of an active process, as it is dependent on the presence of oxygen (Row C, Table 22).

$^{22}Na^{+}$ fluxes were also measured under the relatively normal conditions of opposed Mg^{++} (U→H, 66 mEq/l) and Na^{+} (H→U, 50 mEq/l) gradients across the tissue (Row D, Table 22). Under these conditions U→H Na^{+} transport was reduced to a statistically insignificant value, but still proceeded against the Na^{+} gradient instead of moving with it. In five of the six preparations used, the net Na^{+} flux was U→H and two of these preparations showed normal values. Thus, it is felt that imposition of the dual Mg^{++} and Na^{+} gradients slows but does not eliminate U→H Na^{+} transport across the bladder. Because of the relatively large standard deviation of the data, all that can be definitely concluded is that Na^{+} shows no significant net movement down the Na^{+} gradient under these conditions. If the observed U→H Na^{+} transport under conditions of normal Mg^{++} and Na^{+} gradients (Row D, Table 22) is assumed to be the driving force for reabsorption of water and salts as before, the calculated rate of reabsorption from the urine held in one bladder of a 500 g crab is reduced from 0.4 to 0.2 ml/hr. This value is still of sufficient magnitude to account for inulin U/S ratios of 1.2-2.

In vitro fluxes of $^{22}Na^{+}$ across bladder tissue were

also measured in crab Ringer solution with choline substituted for 90% of the Na^+ (Row E, Table 22). This ten-fold reduction in Na^+ in the medium caused a uniform decrease in the unidirectional and net Na^+ fluxes to slightly less than 1/10 of their normal values. Thus, Na^+ fluxes across the bladder are proportional to the Na^+ concentration in the medium, and it seems likely that Na^+ movement is due to the activity of a sodium "pump".

Further evidence for reabsorption of water and salts by the bladder is provided by the data shown in Table 23. The unidirectional fluxes of $^{36}Cl^-$ across excised bladder were large and there was a nearly significant net U→H flux of Cl^- of the same magnitude as the observed U→H Na^+ flux. Thus, it appears that most of the Na^+ which is transported out of the urine is accompanied by Cl^-, resulting in electrical and osmotic neutrality across the tissue. It is also possible that the observed net U→H movement of Na^+ is the passive result of active U→H transport of Cl^-, perhaps by a mechanism similar to that in frog cornea (Zadunaisky, 1966). The fact that the U→H Na^+ flux across the bladder tissue is directly related to the availability of Na^+ in the medium, however, makes this possibility seem unlikely.

The large fluxes of Cl^- across the bladder found in the present study do not lend support to the speculation of

Gross and Capen (1966) that the bladder of P. crassipes is relatively impermeable to Cl^-.

Control of Mg^{++} Excretion by the Bladder

Transport of Mg^{++} by the bladder of C. magister is under physiological control, as Mg^{++} depletion results in Mg^{++} U/S ratios approaching unity in crabs held in Mg^{++}-free 75% S.W. (Table 24). Hunter (1973) has found that significant reabsorption of water from the urine occurs in C. magister in 75% S.W. (i.e., Glofil U/S ratio = 1.3). Thus, the crabs in the experiment shown in Table 8 may have been reabsorbing Mg^{++} from the urine. Further, excised bladder tissue from Mg^{++}-depleted crabs showed no significant H→U Mg^{++} transport (Row B, Table 5). Magnesium is an activator for a large number of enzymes (MacIntyre, 1963) and the ability to conserve this important ion under conditions favoring its loss to the environment would obviously be advantageous.

Several decapods have been shown to stop excreting Mg^{++} in the urine in dilute or Mg^{++}-free media (Gifford, 1962; DeLeersnyder and Hoestelandt, 1963; Dehnel and Carefoot, 1965; Lockwood and Riegel, 1969; Hunter and Rudy, 1975). Further, a few decapods have been shown to reabsorb Mg^{++} from the urine under these conditions (Scholles, 1933;

Burger, 1957; Gifford, 1962; DeLeersnyder and Hoestelandt, 1963; DeLeersnyder, 1967; an unnamed crustacean mentioned by Riegel, 1972, p. 121), and reabsorption of Mg^{++} and other salts from the urine is common in crayfish (Parry, 1960). On the other hand, several decapods have been shown to continue Mg^{++} excretion in the urine at high rates in dilute media (Prosser, et al., 1955; Gross and Marshall, 1960; Gross, 1964; Gross and Capen, 1966; Gross, et al., 1966). However, in all of these studies, with the exception of Prosser, et al., (1955), only short periods were allowed for acclimation and, thus, the animals may not have had time to become depleted of Mg^{++}. P. crassipes (Prosser, et al., 1955) continues to excrete Mg^{++} at high rates after 5 days acclimation in 50% S.W.

Acclimation to high-salinity or high-Mg^{++} media has been shown to result in increased U/S ratios of Mg^{++} in several decapods (Prosser, et al., 1955; Green, et al., 1959; Gross and Marshall, 1960; Gross, 1964; Dehnel and Carefoot, 1965; Gross and Capen, 1966; Gross, et al., 1966) and a few studies have shown that the increased $[Mg^{++}]_u$ under these conditions was too great to have been caused by increased fluid reabsorption from the urine (Webb, 1940; Gross, 1964, experiments on Cancer antennarius; Lockwood and Riegel, 1969). Thus, control of Mg^{++} excretion in decapods is well documented and the findings of Gross and

Capen, 1966, and the present study show that changes in the rate of Mg^{++} transport into the urine by the bladder are responsible for this phenomenon.

Although it seems likely that Mg^{++} excretion by the bladder is controlled by a mechanism which is sensitive to changes in $[Mg^{++}]_s$, the control mechanism has not been studied. Eyestalk ablation in crabs maintained in 100% S.W. had no effect on $[Mg^{++}]_s$ or $[Mg^{++}]_u$ in C. magister (Table 25). Thus, it seems unlikely that neurosecretory sites in the eyestalk control the rate of Mg^{++} excretion by the bladder. This, however, may be a premature conclusion, as there is incontrovertable evidence that there is x-organ-sinus gland control of blood sugar levels in decapods via a hyperglycemic hormone (reviewed by Kleinhelz, 1976), yet eyestalk ablation often has no effect on blood sugar levels (Kleinholz, 1976; present study, Chaper 7).

Mg^{++} Loading Experiments

Although, at first glance (Fig. 41), Mg^{++} loads administered to C. magister appeared to be eliminated by increased Mg^{++} excretion into the urine, this was not the case. Total urinary Mg^{++} efflux (Table 10) and calculated Mg^{++} excretion into the urine (Table 28) showed no significant increases and, by both calculations, there

must have been a large, extrarenal component of Mg^{++} excretion when Mg^{++} loads were injected. Several workers have shown that Mg^{++} loads are not eliminated via the antennal gland (Bielaszewicz, 1931; Burger, 1957; Gross, 1964; Dall, 1974), thus rapid, non-urinary elimination of injected Mg^{++} loads is a common phenomenon in decapods. Lockwood and Riegel (1969) have shown that in C. maenas at least part of an injected Mg^{++} load was excreted by an increase in Mg^{++} excretion into the urine. These authors did not calculate actual levels of Mg^{++} excretion before and after administration of the Mg^{++} load, and, thus, the portion of the Mg^{++} load excreted via the antennal gland is unknown.

Crabs made anuric by surgically breaking the coelomosac arteries were able to eliminate injected Mg^{++} loads (compare Figures 37 and 42), within the limits imposed by steadily increasing $[Mg^{++}]_s$. Thus, the antennal gland is definitely not responsible for elimination of the Mg^{++} load.

Mg^{++} levels in muscle tissue, $[Mg^{++}]_m$, which were similar to those reported by Gunderley (1977) in C. magister, were unchanged by Mg^{++}-loading and Mg^{++} levels in midgut gland after Mg^{++}-loading were not much greater than $[Mg^{++}]_m$ (Table 29). Thus, Mg^{++} loads are not sequestered in the two major tissues of the crab. Dall (1974) also found that $[Mg^{++}]_m$ in Panulirus longipes was unchanged by injection

of a mixed salt load which contained Mg^{++}, and Martin (1977) has found that $[Mg^{++}]_m$ in Cancer irroratus is unchanged during the moult cycle, while $[Mg^{++}]_s$ shows large variations.

Although the decapod gut has been implicated in excretion of salts (Webb, 1940; Green, et al., 1959; Gifford, 1962; Dall, 1967, 1974), the gut of C. magister does not participate in elimination of Mg^{++} loads. Mg^{++} levels in stomach fluid and feces in crabs with blocked guts are greater than $[Mg^{++}]_s$ after a Mg^{++} load, but the quantities of gut fluid and feces in the gut are far too small to account for elimination of the load (Table 27 and Results). Dall (1974) has also found that injected Mg^{++} is not eliminated via the gut in P. longipes.

Mg^{++} efflux to Mg^{++}-free 75% S.W. by crabs with blocked nephropores was not significantly changed by Mg^{++} loads (Table 30). Thus, the Mg^{++} must have been sequestered within the crabs, but not in muscle or midgut gland. In light of the finding by Martin (1977) that Mg^{++} levels in the exoskeleton of C. irroratus steadily increase over the moult cycle it may be that the hypodermis deposits injected Mg^{++} loads in the exoskeleton. In any case, the results of the present study indicate that short-term Mg^{++} loads are an inappropriate tool for study of Mg^{++} excretion by the antennal gland, as $[Mg^{++}]_s$ is rapidly returned to normal levels by extrarenal processes. Apparently a longer period

of elevated $[Mg^{++}]_s$ is required to increase rates of Mg^{++} excretion.

An interesting finding in the present study is that non-renal Mg^{++} efflux to 75% Mg^{++}-free S.W. exceeds normal renal Mg^{++} efflux in 75% S.W. (compare Tables 28 and 30). This finding may explain the very large urinary rates found by Gross and Marshall (1960) in P. crassipes in 50% S.W., as these authors used Mg^{++} efflux to Mg^{++}-free media as a measure of urinary rate.

In summary, it has been shown in both in vivo and in vitro experiments that the bladder of C. magister is responsible for excretion of Mg^{++} into the urine. C. magister is similar to other crustaceans in the Mg^{++} is concentrated in the urine at the expense of Na^{+} and it has been calculated that the observed in vivo exchange ratio of 1.6 Eq Mg^{++} : Eq Na^{+} is close to that which would preserve electrical neutrality. Mg^{++} transport by the bladder in vitro is inhibited by ouabain, but because of the large fluxes of Na^{+} across the tissue under these conditions it is not possible to determine if the inhibitor acts directly on Mg^{++} transport or on ouabain-sensitive Na^{+} transport. There are large in vitro U→H movements of Na^{+} and Cl^{-} across the bladder and these ion fluxes may be responsible for the commonly observed reabsorption of water and salts from the urine in decapods.

CHAPTER 6

ANTENNAL GLAND CONTROL OF HEMOLYMPH SULFATE LEVELS

METHODS

Male, intermoult crabs weighing between 200 and 800 g were captured and maintained in 100% Coos Bay sea water as noted in Chapter 2. Urinary rates were measured by the external collection method given in Chapter 4. Because of the low urinary rate and irregularity of micturation of crabs in 100% S.W. (Chapter 4), some of the experiments were conducted with crabs acclimated to 75% S.W. All experiments were conducted at 12 $^{o}C \pm 1$.

In one experiment the urinary opercula were temporarily blocked by covering them with a small drop of hot melt glue (U.S.M., Thermogrip). The glue was removed, a 2 ml sample of urine taken and the glue replaced at each subsequent sampling of the urine.

Crabs were rendered anuric, when necessary, by surgically breaking the coelomosac arteries (Methods, Chapter 4).

Sulfate Ion Analyses

Levels of inorganic sulfate ion in serum and urine ($[SO_4^=]_s$ and $[SO_4^=]_u$, respectively) and in the medium were measured using the turbidometric method of Berglund and Sorbo (1960), as modified by Hunter and Rudy (1975) for use with small (50 µl) aliquots. All samples were deproteinized with 5% trichloroacetic acid. Measurements were made with a Zeiss PMQ-II spectrophotometer. Sulfate standards were made up in sulfate-free crab Ringer solution in which NaCl was substituted for Na_2SO_4 to allow for interference by other ions. The standard deviation of the mean of five determinations of a 100 mEq/l standard was $\pm$ 1.3 mEq/l.

In Vitro Sulfate Fluxes Across Excised Bladder Tissue

The unidirectional fluxes of $^{35}SO_4^=$ across pairs of isolated bladder tissue were measured in the dual lucite cell described in Chapter 5. At the beginning of the experiment 20 µl of $Na_2{}^{35}SO_4$ solution (ICN Pharmaceuticals) waa added to the hemolymph-side chamber of one tissue and to the urine-side chamber of the other tissue. The specific activity in the crab Ringer solution was 30,000-40,000 cpm/µEq$SO_4^=$. The unidirectional flux of the isotope across the tissue in either direction was defined as the rate of

appearance of the isotope in the crab Ringer solutions which were originally without it. Unidirectional fluxes were measured over a two hour period and were calculated as $\mu Eq/cm^2/hr$. The equation used is given in the Methods section of Chapter 4. Aliquots of 200 µl were dissolved in the same liquid scintillation counting fluid used to count $^{36}Cl^-$ (Methods, Chapter 5) and counted to at least 1600 counts in a Beckman LS-150 liquid scintillation system. Counting was done in a wide "window" approximately between 0.2 and 1.7 MeV (^{14}C and ^{32}P above ^{3}H "Isoset").

Sulfate Loading

Crabs were given sulfate loads by injection of 0.2% body weight (ml/g) of a 280 mM/l solution of Na_2SO_4. Urinary rate, $[SO_4^=]_s$ and $[SO_4^=]_u$ were monitored after administration of the $SO_4^=$ load. The amount of Na_2SO_4 injected was calculated to be sufficient to raise $[SO_4^=]_s$ of crabs acclimated to 75% S.W. to equal the sulfate level in the medium.

RESULTS

The normal values of $[SO_4^=]_s$ and $[SO_4^=]_u$ in crabs acclimated to 100% and 75% S.W. are shown in Table 32.

Table 32. Sulfate levels in the medium, serum and urine of C. magister. Mean values ± s.d.

Salinity (%S.W.)	SO_4 concentration (mEq/l or mEq/kg H_2O)			
	Medium	Serum	Urine	U/S
100%	54	48 ± 8	68 ± 10	1.4:1
75%	38	26 ± 7	37 ± 9	1.4:1

C. magister hyporegulates $[SO_4^=]_s$ to a small extent in 100% and 75% S.W. Because $[SO_4^=]_u$ is greater than $[SO_4^=]_s$, it would seem that the antennal gland is responsible for hyporegulation of $[SO_4^=]_s$.

If, as is the case of Mg^{++} (Chapter 5), the antennal gland is responsible for hyporegulation of $[SO_4^=]_s$, then anuria caused by breaking the coelomosac arteries should eliminate the ability of the crab to hyporegulate $[SO_4^=]_s$. As shown in Fig. 43, this was not the case. Anuric crabs showed no significant differences from controls in their ability to hyporegulate $[SO_4^=]_s$. Thus, the antennal gland is probably not the site of significant $SO_4^=$ excretion in C. magister.

When urine is allowed to accumulate in the previously-drained bladders of crabs with blocked nephropores, $[SO_4^=]_u$ increases much more slowly with residence time in the bladder than does $[Mg^{++}]_u$ (Fig. 44). Thus, it could be argued that $SO_4^=$ is slowly excreted into the urine by the bladder. For reasons discussed below it seems most likely that the observed increases in $[SO_4^=]_u$ and $[Mg^{++}]_u$ were due to absorption of water and salts other than $SO_4^=$ or Mg^{++} from the urine by the bladder.

In vitro measurements of unidirectional $^{35}SO_4^=$ fluxes across excised bladder lend support to the hypothesis that $SO_4^=$ is not concentrated in the urine by the bladder

Figure 43. Effect of cutting coelomosac artery on serum SO_4 levels in C. magister in 100% S.W. Mean values ± s.d. Controls were sham-operated.

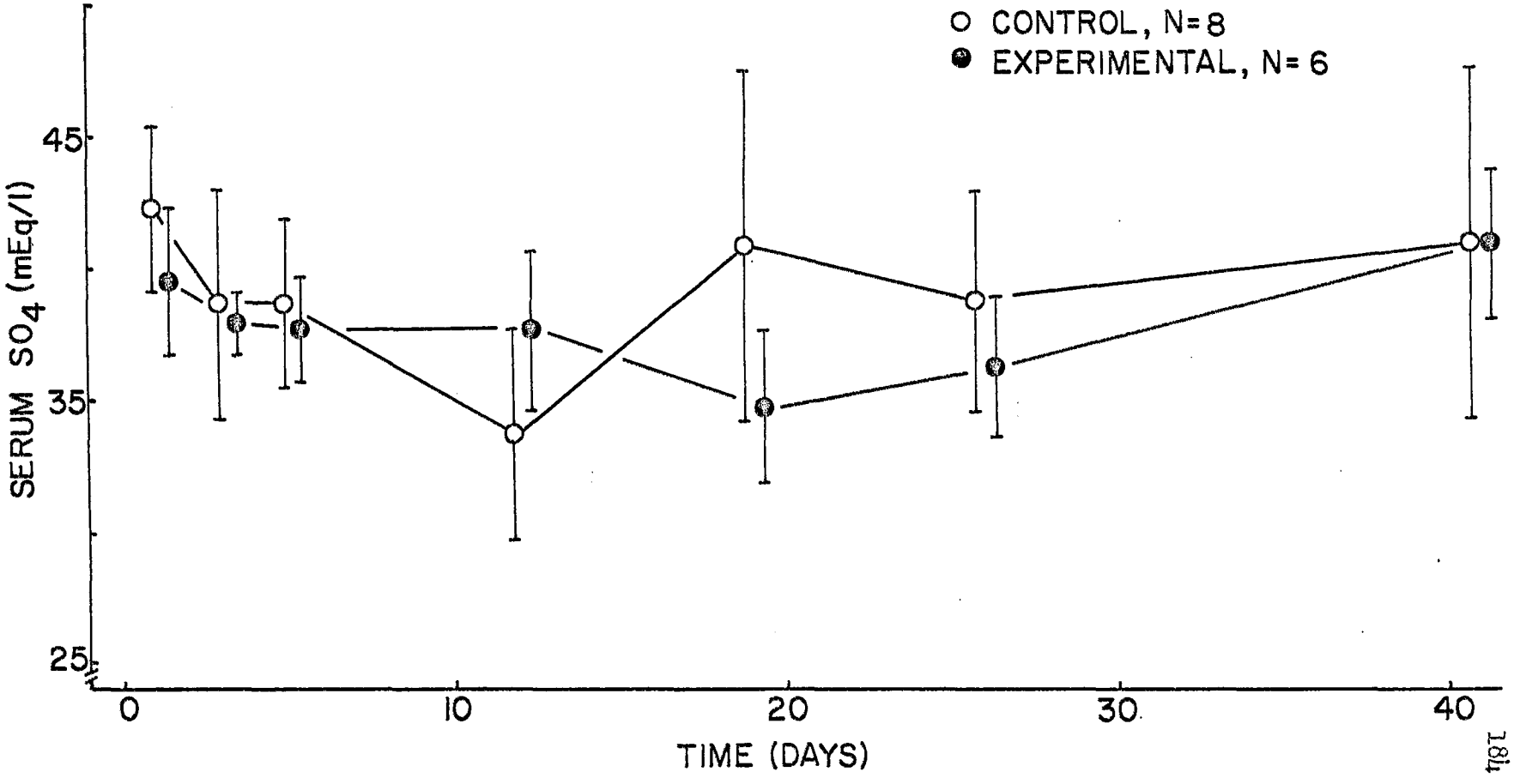
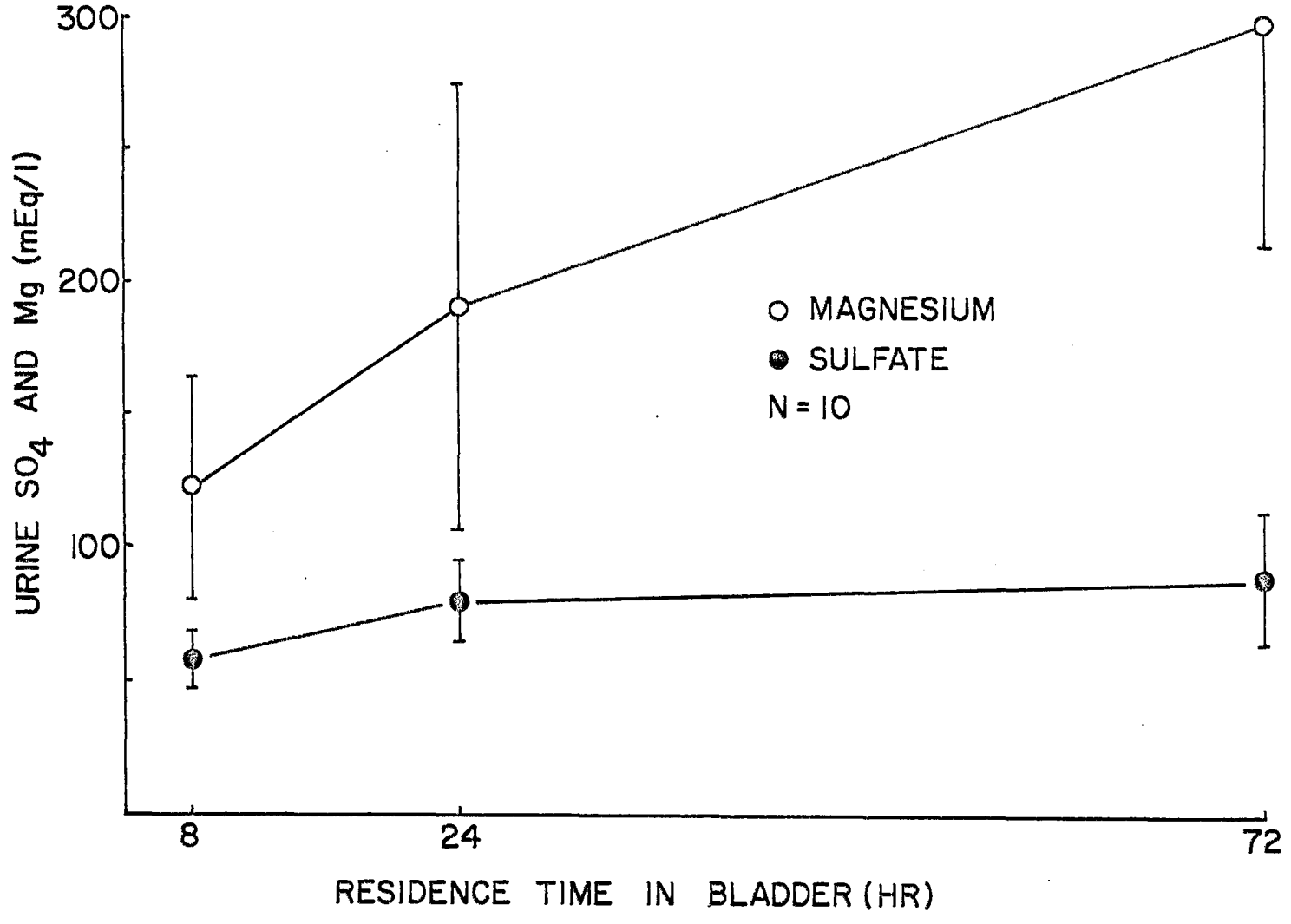


Figure 44. Effect of residence time in bladder on SO_4 and Mg levels in the urine of C. magister with blocked nephropores in 100% S.W. Mean values ± s.d.



(Table 33). The unidirectional fluxes were small and the difference between them was not significant. Thus, at least under in vitro conditions, $SO_4^=$ is not transported into the urine by the bladder.

As shown in Fig. 45, injected $SO_4^=$ loads did not result in elevated levels of $SO_4^=$ in the urine. Because urine was collected from a reservoir at the end of each period, $[SO_4^=]_u$ is actually an average value for the collection period. Accordingly, values of $[SO_4^=]_u$ are plotted at the middle of each collection period. None of the values for $[SO_4^=]_u$ are significantly different from the value before injection of the load. Serum sulfate levels fell slowly and had not returned to the pre-load value after 36 hr, although the difference between the values at 0 and 12 hr was not significant. Urinary rates (not shown) were not affected. Injected $SO_4^=$ loads are apparently eliminated at an extrarenal site or taken into the tissues of the crab.

DISCUSSION

The values of $[SO_4^=]_s$ and $[SO_4^=]_u$ found in the present study are well within the range of values reported in the literature (Table 34). Robertson (1939, 1949, 1953) has shown that hyporegulation of $[SO_4^=]_s$ in many decapods is the result of an active process, as dialysis of serum

Table 33. *In vitro* fluxes of $^{35}SO_4$ across excised bladder tissue of *C. magister*. Mean values ± s.d.

N	U→H (μEq/cm²/hr)	H→U (μEq/cm²/hr)	Net flux (μEq/cm²/hr)	P_t
8	0.140 ± 0.056	0.150 ± 0.053	0.011 ± 0.039	>.05

Figure 45. Effect of SO_4 load on serum and urine SO_4 levels. Mean values ± s.d. Load injected at t=0.

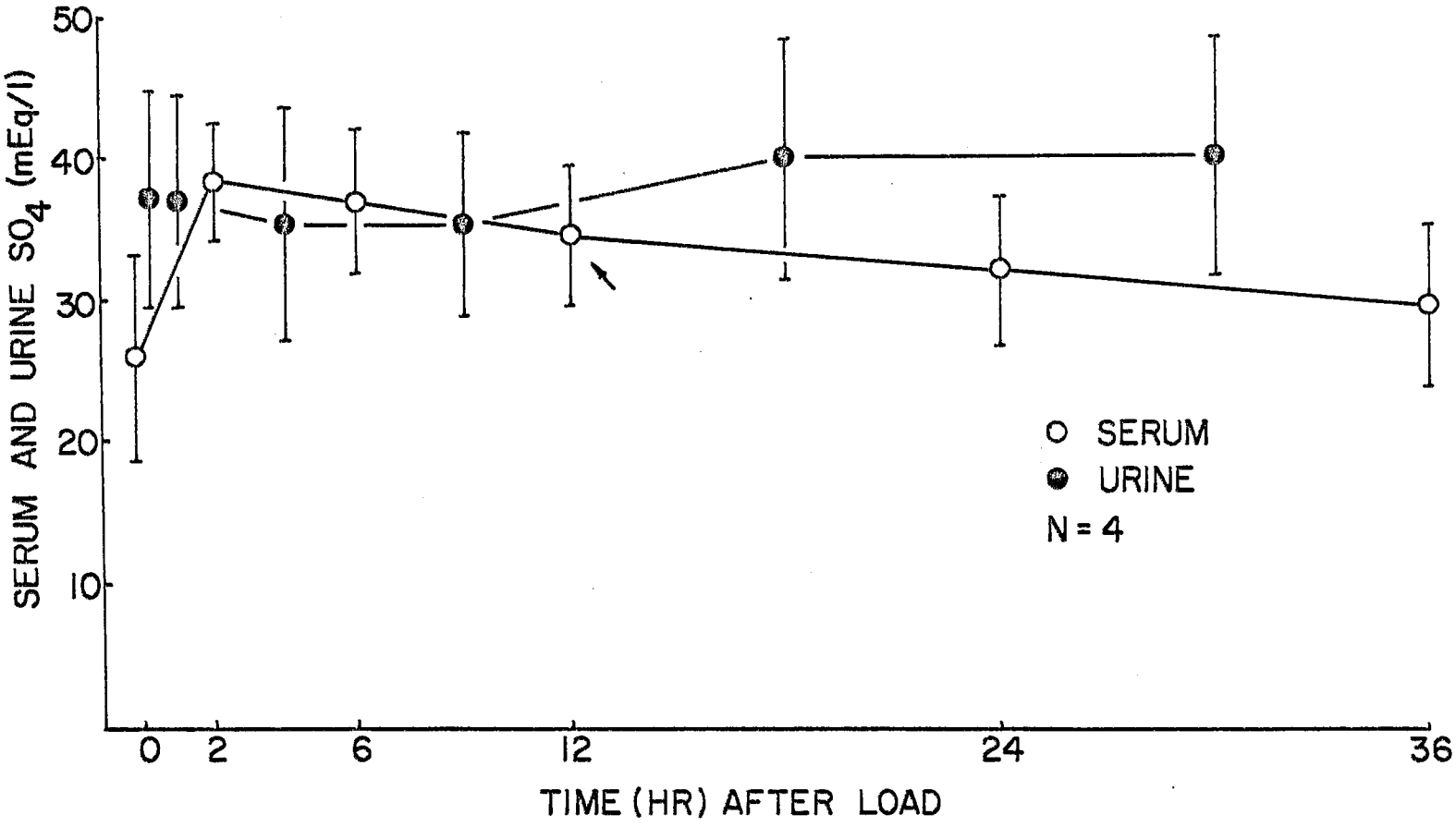


Table 34. Levels of SO_4 in the medium, serum and urine of decapods. Mean values.

Species	(%S.W.)	SO_4 concentration (mEq/l or mEq/kg H_2O) Medium	Serum	Urine	U/S	Reference
Cancer magister	100%	55	47	75	1.6	Hunter & Rudy, 1975
	75%	41	27	48	2.0	
	50%	25	19	40	1.8	
	30%	16	21	25	1.3	
Carcinus maenas	100%	--	--	--	4.1	Shaw. 1961
C. maenas	100%	56	31.8	71.2	2.2	Webb, 1940
Cancer pagurus	100%	--	--	--	1.3	Robertson, 1939
Maja squinado	100%	59	29.9	63.8	2.1	Robertson, 1939
Ocypode albicans	100%	56	48.2	38.2	0.8	Gifford, 1962

Table 34. (continued)

Species	Medium (%S.W.)	SO_4 concentration (mEq/l or mEq/kg H_2O) Medium	Serum	Urine	U/S	Reference
Uca pugnax and U. pugilator	100%	44	84	94	1.1	Green, et al., 1959
	175	58	98	240	2.4	
Homarus gammarus	100%	--	--	--	1.6	Robertson, 1939
H. americanus	100	--	--	--	1-2	Burger, 1957
Nephrops norvegicus	100%	--	--	--	1.1	Robertson, 1949
Palinurus elephas	100%	--	--	--	1.0	Robertson, 1949
Palaemon serratus	120%	--	12.8	64	5.0	Parry, 1954
	100%	--	10.4	39.6	3.8	
	50%	--	4.6	7.2	1.6	

against sea water results in many cases in increased levels of $SO_4^=$ in the dialysate. Because $[SO_4^=]_u$ is usually greater than $[SO_4^=]_s$ the antennal gland is thought to be responsible for hyporegulation of $SO_4^=$ (Potts and Parry, 1964; Riegel, 1972). As shown in Table 3, U/S ratios of $SO_4^=$ reported in the literature range from 0.8 to 5.0 in decapods subjected to various conditions, with most values between 1.1 and 2 for animals in 100% S.W. Experiments in which decapods were maintained in concentrated sea water (Parry, 1954; Green, et al., 1959; Gifford, 1962) or in sea water with elevated levels of $SO_4^=$ (Webb, 1940) have shown that under conditions of increased $SO_4^=$ influx, U/S ratios for $SO_4^=$ increase. These findings lend support to the hypothesis that the antennal gland is responsible for hyporegulation of $SO_4^=$.

Injection of Na_2SO_4 (Bialaszewicz, 1931) and administration of $MgSO_4$ via the stomach (Burger, 1957) have produced conflicting results. Bialaszewicz (1931) found that injected $SO_4^=$ loads were not eliminated via the antennal gland of the crab, Maja squinado, a finding in accord with the results of the present study (Fig. 45). Burger (1957) found that $SO_4^=$ loads administered via the stomach caused U/S ratios of $SO_4^=$ to increase in Homarus gammarus. This would seem to indicate that in the lobster the antennal gland is responsible for hyporegulation of $[SO_4^=]_u$.

The findings in the present study that surgically-induced anuria has no effect on the ability of C. magister to hyporegulate $[SO_4^=]_s$ (Fig. 1), and that excised bladder tissue does not demonstrate net transport of $SO_4^=$ into the urine (Table 33) indicate that, at least C. magister, the antennal gland is not responsible for hyporegulation of $[SO_4^=]_s$. Sulfate levels in the urine did, however, increase with residence time of the urine in the bladder (Fig. 44). Withdrawal of water and salts from crustacean urine is a well-documented phenomenon, and reported values of U/S ratios for inulin and other markers of water reabsorption range between 1.2-2 in most decapods (Riegel, 1972). Further, inulin U/S ratios have been shown to increase with residence time of the urine in the bladders of crabs (Riegel and Lockwood, 1961; Gross and Capen, 1966), and evidence is produced in the present study (Chapter 5) that salts and water are reabsorbed from the urine by the bladder. As shown in Tables 1 and 3, U/S ratios for $SO_4^=$ in most of the decapods studied fall within the range of 1.1-2. Thus it may be that under normal conditions in most decapods the concentration of $SO_4^=$ in the urine is the result of absorption of water and salts other than $SO_4^=$ from the urine by the bladder. Hunter (1973) has found that U/S ratios for Glofil, a marker for water movements, are close to the U/S ratios for $SO_4^=$. Recalculation of

his data using Students' t test shows a significant difference between the U/S ratios of Glofil and $SO_4^=$ in only one of the four acclimation salinities (50% S.W.). Thus, in C. magister and several other decapods in dilute and normal media the observed U/S ratios of $SO_4^=$ may be due to reabsorption of water and salts from the urine. Although $SO_4^=$ is concentrated in the urine of C. magister (Table 32), the antennal gland is not responsible, under normal conditions, for hyporegulation of $SO_4^=$, as anuric crabs continue to hyporegulate $SO_4^=$ for long periods (Fig. 43). Active excretion of $SO_4^=$ into the urine may occur only in response to greater than normal levels of $SO_4^=$ in the external medium.

CHAPTER 7

REABSORPTION OF GLUCOSE FROM THE URINE

METHODS

Male, intermoult crabs weighing between 200 and 800 g body weight were captured and maintained in 100% Coos Bay sea water as noted in Chapter 2. All experiments were done with crabs in 100% Coos Bay sea water at a temperature of 12 $^{o}C \pm 2$.

Glucose concentrations in serum, urine and crab Ringer solutions were measured using a glucose oxidase and peroxidase enzymatic-colorimetric method (Sigma Chemical Co., Bulletin #510). A Zeiss PMQ2 spectrophotometer was used in the determinations and appropriate blanks were used for all samples. Seven determinations of a 10 mg% glucose standard had a standard deviation of $\pm$ 0.3 mg%.

When necessary, serum glucose levels were increased to calculated values of 100 and 300 mg% by injection of 0.07% and 0.5% body weight (ml/gm) respectively of a 0.75 M glucose solution.

Crab Ringer solution containing glucose (20 mg%) and/or phlorizin (100 mg%) was introduced into the bladder via the nephropore in the following manner. The left urinary

operculum was lifted with a fine, hooked needle and the bladder was drained as completely as possible. The crab was then secured ventral side up in a dissecting tray. Crab Ringer solution (3 or 5 ml) containing the test compound(s) was introduced into the bladder using a hypodermic syringe connected to a fine polyethylene cannula (P.E. 10) which was inserted 2-3 mm into the nephropore. A few crabs were rendered anuric by this technique and were discarded. In order to keep the solution in the bladder for the required time, the urinary operculum was temporarily blocked by a drop of hot melt glue (U.S.M., Thermogrip). At the end of the experimental period the glue was removed and a sample taken. In some of these experiments 0.4 ml of a 175 mg% solution of phlorizin in crab Ringer solution was injected into each crab.

In vitro glucose uptake by and fluxes across pairs of excised bladder tissues from the same crab were measured in the previously described dual chamber lucite cell (Chapter 5). Both sides of the two pieces of bladder tissue were bathed by separate 1 ml aliquots of crab Ringer solution which were aerated and circulated by a peristaltic pump at the rate of 2 ml/min. One ml of crab Ringer solution with 20 mg% glucose was added to the hemolymph-side chamber of one bladder tissue and to the urine-side chamber of the other bladder tissue. The two remaining

chambers were filled with 1 ml each of crab Ringer without glucose. After 1 or, in some cases, 2 hr the contents of the four chambers were tested for glucose. Glucose uptake was taken to be the rate of disappearance of glucose from the glucose crab Ringer solutions. Glucose flux across the tissue was taken to be the rate of appearance of glucose in the crab Ringer solutions originally without glucose. The area of the tissue exposed to the bathing media was 0.80 cm^2. Uptake and flux rates are expressed as $\mu M/cm^2/hr$. In experiments to measure the effect of low (0.01 normal) Na^+ media on glucose uptake and fluxes *in vitro*, a modified crab Ringer solution in which choline chloride was substituted for all of the NaCl and most of the Na_2SO_4 was used. Ionic concentrations (in mEq/l) were: Na^+, 5; K^+, 11; Ca^{++}, 25; Mg^{++}, 37; $choline^-$, 494; Cl^-, 567; $SO_4^=$,5.

RESULTS

Glucose Loading and Glucosuria

The mean serum glucose level of 46 crabs was 5.3 mg% (Table 36, Row G). Glucose is rarely present in the urine of *C. magister*: only 2 of 35 crabs tested had measurable amounts of glucose in their urine (Table 36, Row A). When serum glucose levels were raised by injection to calculated

Table 35. Glucose concentrations (mg % ± s.d.) in serum and urine after glucose injection in C. magister.

Calculated initial serum glucose level	N	Time							
		0 hr		1 hr		4 hr		13.5 hr	
		Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine
100	10	7.5 (2.9)	0 (0)	71.6 (12.7)	1.1 (0.3)	26.0 (16.5)	0.2 (0.8)	7.1 (3.3)	0.1 (0.3)
300	10	5.6 (2.3)	0 (0)	176.0 (24.3)	12.6 (4.9)	70.1 (43.5)	5.2 (4.5)	5.3 (1.4)	1.0 (2.1)

Table 36. Glucose concentrations (mg %) in fluids of C. magister.

Initial bladder fluid	Time	N	Mean	S.D.
Bladder fluid				
A. Normal urine	--	35	0.1	0.4
B. Glucose (20 mg %) in 5 ml crab Ringer	4 hr	8	1.6	1.6
C. Glucose (20 mg %) + phlorizin (100 mg %) in 5 ml crab Ringer	4 hr	8	10.4	1.4
D. Phlorizin (100 mg %) in 5 ml crab Ringer + injection of phlorizin	4 hr	8	0.6	0.2
E. Normal urine (crabs injected with phlorizin at 12, 6 and 1.5 hr previous to sampling)	12 hr	6	1.4	1.4
F. Normal urine mixed with equal volume of crab Ringer with glucose (20 mg %), incubated in vitro at 12° C.	2 hr	7	10.0	0.3
Serum				
G. Normal crabs in 100% S.W.	--	46	5.3	2.8

values of 100 or 300 mg%, relatively small amounts of glucose appeared in the urine (Table 35). Glucose is obviously rapidly metabolized, as serum glucose levels returned to normal values within 13.5 hr in both cases. Assuming a urinary rate of 1.6% body weight per day in 100% S.W. (Holliday, 1977), then a volume of urine equivalent to only 0.9% of the body weight was produced during the 13.5 hr. Given a hemolymph volume equivalent to 35% of the body weight (Hunter, 1973), this amount of urine is equivalent to less than 5% of the blood volume, so urinary glucose loss was a negligible factor in the lowering of serum glucose levels. One hour after elevation of serum glucose levels to a calculated value of 100 mg%, small amounts of glucose appeared in the urine, but by 4 hours after the injection, all but one of the ten crabs had no glucose in their urine, even though the mean serum glucose level at that time was 26 mg% (Table 35). As expected, higher concentrations of glucose occurred in the urine when serum glucose levels were elevated to a calculated 300 mg% (Table 35). In this case, after 13.5 hr, all but 3 of the ten crabs had no glucose in their urine.

In Vivo Glucose Uptake by the Bladder

When crab Ringer solution containing 20 mg% glucose was introduced into the bladder, nearly all of the glucose was absorbed in 4 hours (Table 36, Row B). Addition of phlorizin (100 mg%) to the crab Ringer solution caused a large decrease in the amount of glucose absorbed (Table 36, Row C). When crab Ringer solution with phlorizin (100 mg%) alone was introduced into the bladders of the crabs which were previously injected with phlorizin, only small amounts of glucose appeared in the urine (Table 36, Row D). Greater amounts of glucose appeared in the urine of crabs injected with phlorizin 3 times over a period of 12 hr (Table 36, Row E). As has been previously noted, it is probable that only small amounts of urine were formed during the 4 or 12 hr periods in rows D and E of Table 36, and, given the normal serum glucose level of about 5 mg% it would thus be expected that only small amounts of glucose would appear in the urine when phlorizin was present. Mixtures of equal parts of crab urine and crab Ringer solution with 20 mg% glucose showed no decrease in glucose concentration after 2 hr incubation at 12 ^{o}C (Table 36, Row F), thus it is unlikely that the glucose levels in experiments A and B of Table 36 fell because of bacterial activity in the bladder. These data clearly show that the bladder has the

capacity to absorb large amounts of glucose from the urine. The fact that phlorizin inhibits glucose uptake is evidence that the process is one of active transport and not due simply to diffusion.

In Vitro Glucose Uptake and Transport by the Bladder

In vitro glucose uptake by the urine-side of the bladder was nearly twice that of the hemolymph-side (Table 37). The flux across the tissue in either direction was so small as to be negligible when compared to the values for glucose uptake, and the difference between the fluxes was not significant. In 4 of the 7 pairs of bladder tissues tested, no glucose at all appeared in the crab Ringer solution in the chambers opposite those which had glucose, despite the large (20 mg%) gradient favoring diffusion. Thus it is unlikely that diffusion of glucose across the bladder into the urine normally occurs in intact crabs. Further, if glucose is actually transported from the urine to the hemolymph it is not transported as such, but is metabolically altered.

Glucose uptake by the bladder apparently has at least a partial requirement for Na^+, as such uptake is halved by a hundred-fold reduction of Na^+ in the crab Ringer solution (Table 37).

Table 37. *In vitro* uptake and fluxes of glucose ($\mu M/cm^2/hr \pm$ s.d.) in matched pairs of bladder tissue of *C. magister*.

Glucose uptake	N	Normal crab Ringer	N	0.01 Normal crab Ringer	P_t
Hemolymph side	7	0.63 ± 0.18	8	0.35 ± 0.15	<.01
Urine side	7	1.10 ± 0.29	8	0.67 ± 0.08	<.01
Difference		0.47 ± 0.18		0.32 ± 0.09	>.05
P_t		<.01		<.001	
Glucose fluxes					
Hemolymph to urine side	7	0.010 ± 0.013	8	0.008 ± 0.009	>.05
Urine to hemolymph side	7	0.002 ± 0.005	8	0.018 ± 0.017	<.05
Difference		0.008 ± 0.011		0.010 ± 0.021	>.05
P_t		>.05		>.05	

Control of Serum and Urine Glucose Levels

Because the crustacean eyestalk has been implicated in the control of hemolymph glucose concentration, experiments were conducted to determine the effect of eyestalk abalation on serum and urine glucose levels. Eyestalk ablation had no significant effect on serum glucose levels at any time within 4 days of ablation (Fig. 46; Table 38, Rows E and F). There was no glucose present in the urine of ablated crabs, and ablation did not significantly change glucose uptake from crab Ringer solution introduced into the bladder (Table 38, Rows C and D).

DISCUSSION

The values for serum and urine glucose found in the present study of C. magister (Table 36, Rows A and G) are well within the range of published values for other crustaceans (reviewed by Florkin, 1960; Hohnke and Scheer, 1970; Jeunieux, 1971). Serum glucose levels found in the present study were nearly identical to those found by Meenakshi and Scheer (1961) in the same crab. Injected glucose is rapidly cleared from the hemolymph by C. magister (Table 35). Rapid clearance of injected glucose has been observed in several crustaceans (Burger, 1957; Riegel and

Figure 46. Serum glucose levels after eyestalk ablation in C. magister. Mean values ± s.d. Eyestalks ablated at t=0.

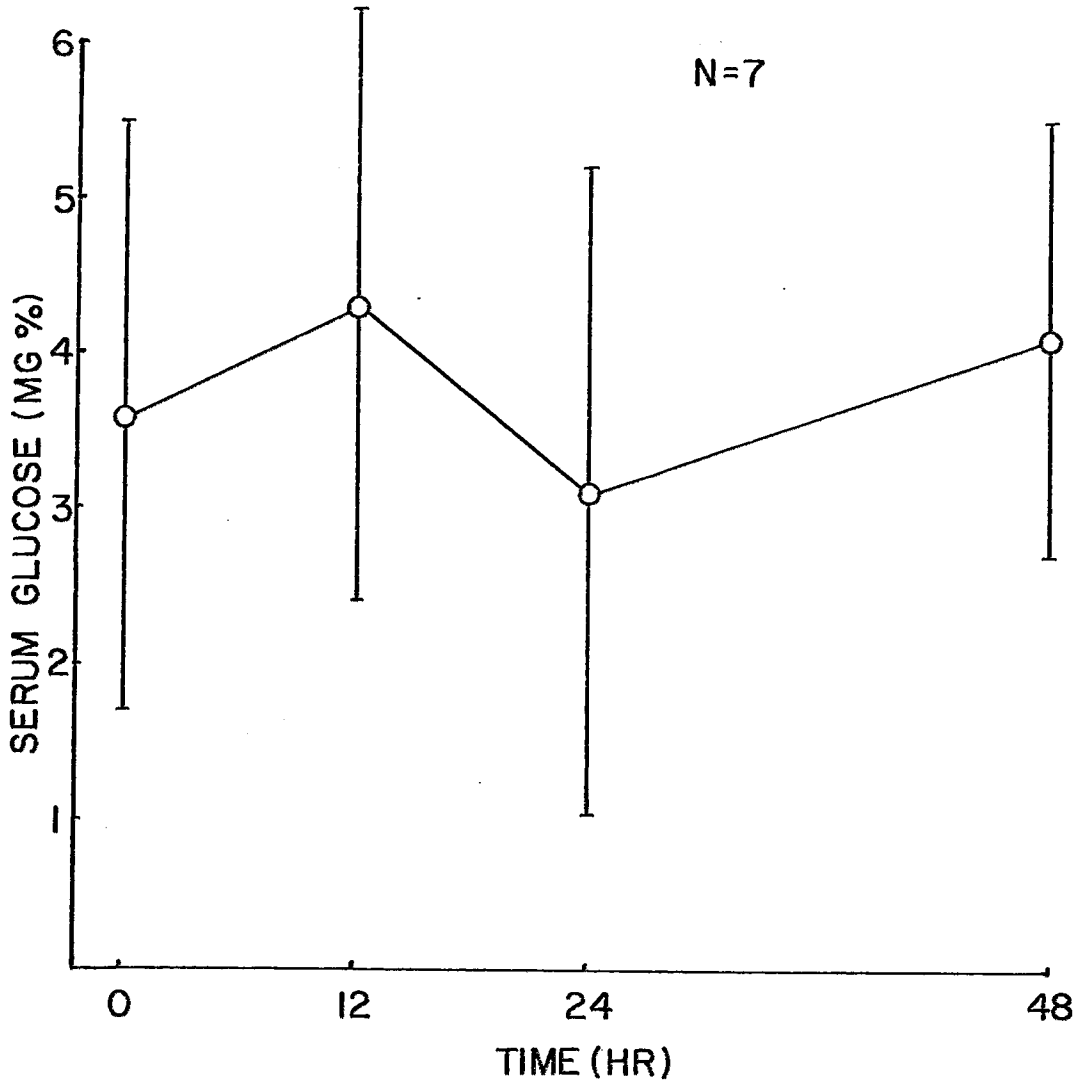


Table 38. Effect of eyestalk ablation on glucose concentrations (mg %) in fluids of C. magister.

Initial bladder fluid	Time	N	Mean	S.D.	Pt
Bladder fluid					
A. Urine from intact crabs 4 days after capture	--	10	0	0	> .05
B. Urine from eyestalkless crabs 4 days after capture and ablation	--	10	0	0	
C. Glucose (20 mg %) in 3 ml crab Ringer, intact crabs	2 hr	10	6.5	1.8	>.05
D. Glucose (20 mg %) in 3 ml crab Ringer, eyestalk ablated crabs	2 hr	10	4.9	3.3	
Serum					
E. Intact crabs 4 days after capture	--	10	5.7	2.4	>.05
F. Eyestalkless crabs 4 days after capture and ablation	--	10	6.0	1.7	

Kirschner, 1960; Meenakshi and Scheer, 1961; Binns, 1969b; Weber, 1971; Madhyastha and Rangneeker, 1976).

Riegel (1972) has reviewed the extensive evidence for the formation or urine in crustaceans by a process of ultrafiltration followed by secretory and reabsorptive modification of the primary urine. Glucose and phlorizin injections have been shown to cause glucosuria in several crustaceans (Burger, 1957; Riegel and Kirschner, 1960; Gross, 1967; Binns, 1969b; Weber, 1971) and apparent threshold hemolymph glucose levels for glucosuria are 100-200 mg% (Riegel, 1972). The hemolymph glucose threshold in C. magister is approximately 100 mg%, as significant amounts of glucose appeared in the urine when hemolymph glucose was raised to an initial concentration of 100 mg% (Table 35). The existence of a threshold for glucosuria is consistent with the filtration/reabsorption-secretion theory of urine formation. Binns (1969b) has found that the rate of glucose reabsorption from the urine of Carcinus maenas is influenced by the urinary rate and the nutritional state of the crab, findings which indicate feedback control of glucose reabsorption. Thus, as Binns (1969b) notes, the concept of a fixed threshold value which determines the presence or absence of glucose in the urine is an over-simplification.

Since the urinary rate in 100% S.W. and glucose threshold are known, the rate of glucose uptake at the threshold

may be calculated. The data are shown in Table 39 with data from Binns' (1969b) study of C. maenas. Because the urinary rate and glucose threshold value are lower in C. magister than in C. maenas, the calculated rate of glucose uptake is also lower. The normal hemolymph glucose level in C. magister is about 5 mg% and thus the antennal gland has ample reabsorptive capacity to prevent glucose loss via the urine.

The brachyuran antennal gland consists of three major parts: coelomosac, labyrinth and bladder (Marchal, 1892; Balss, 1944). Due, no doubt, to technical difficulties, glucose reabsorption has been investigated only in the bladder (Gross, 1967). Glucose is reabsorbed from perfusion fluid introduced into the bladder via the nephropore in the crab Pachygrapsus crassipes, and this reabsorption is inhibited by phlorizin. In similar experiments with C. magister phlorizin-inhibited glucose reabsorption by the bladder was also observed (Table 36). C. magister is apparently not as sensitive as P. crassipes to phlorizin. Only small amounts of glucose (0.6 mg%, Table 36, Row D) were present in the urine of crabs which had received phlorizin crab Ringer and an injection of phlorizin, while 4.6 mg% (Gross, 1967, Table 1, Row C) were present in the urine of P. crassipes under similar conditions. Gross attributed the appearance of such large amounts of glucose

Table 39. Rates of glucose reabsorption at the hemolymph threshold level in C. magister and Carcinus maenas. Mean values.

Species	Urinary rate (% B.W./day)	Approximate glucose threshold (mg %)	Rate of glucose reabsorption (mg/hr/100 g)
C. magister	1.6	100	0.07
Carcinus maenas *	4.35	150	0.27

* From Binns, 1969b.

in the urine of P. crassipes to inward diffusion from the hemolymph when reabsorption was blocked by phlorizin. Because the urinary rate, mean body weight and normal hemolymph glucose level of C. magister are known, it is possible to calculate the amount of glucose filtered into the 5 ml of phlorizin crab Ringer in the bladder during the 4 hr experimental period (Table 36, Row D). The amount of glucose calculated to have been filtered is 0.022 mg, and this amount diluted in the 5 ml of originally glucose-free phlorizin crab Ringer solution in the bladder would give a concentration of 0.44 mg%, a value close to the 0.6 mg% observed. Thus it seems unlikely that diffusion of glucose into the urine was significant. Further evidence against the diffusion of glucose into the urine is provided by the in vitro experiments with excised bladder tissues (Table 37). Glucose fluxes across the excised bladder in either direction were so small as to be negligible, despite the large gradient (initially 20 mg%) across the tissues. Hyperglycemia has been shown to result when crabs are held out of water (Roche and Dumazert, 1935; Kleinholz and Little, 1949; Lynch and Webb, 1973). Gross' (1967) animals were held out of water for three hours during his experiments and may have been hyperglycemic, despite the fact that P. crassipes is an intertidal crab. If this was the case, then much greater amounts of glucose would have been

filtered into the bladder during the experiment and may have contributed to the higher levels of glucose in the originally glucose-free bladder fluid of crabs receiving phlorizin.

Glucose was absorbed by both sides of the bladder tissue *in vitro* and the urine-side rate of uptake was nearly twice that of the hemolymph-side (Table 37). It is uncertain whether the urine-side rate of uptake represents a normal *in vivo* value, as the hemolymph-side chamber of the tissue used to measure urine-side uptake had no glucose in these experiments; the urine-side chamber was the tissue's only source of glucose. Under these circumstances glucose uptake may have been greater than normal, as the hemolymph-side of the tissue is exposed *in vivo* to a 5 mg% solution of glucose. The value for glucose uptake by the urine-side of the bladder is 1.10 $\mu M/cm^2/hr$, (Table 37), which is equivalent to 0.2 $mg/cm^2/hr$. A 500 g crab has approximately 435 cm^2 of bladder (Chapter 3). At the hemolymph glucose threshold level of 100 mg% such a crab would filter 0.33 mg of glucose per hour and, if it is assumed that all of this glucose is reabsorbed by the bladder, the rate of reabsorption is only 0.0008 $mg/cm^2/hr$ or 0.0042 $\mu M/cm^2/hr$. This "maximum" *in vivo* rate is more than 250 times smaller than the observed *in vitro* rate (1.10 $\mu M/cm^2/hr$). It is unfortunate that further experiments

were not done to determine the effect of hemolymph-side glucose levels on urine-side glucose uptake. It could be argued that bacterial breakdown of glucose in the in vitro apparatus caused a spuriously high apparent rate of glucose uptake by the bladder tissue, but this is unlikely for two reasons. First, mixtures of a glucose crab Ringer solution and crab urine showed no decrease in glucose levels after 2 hr at 12 ^{o}C (Table 36, Row F). Second, the in vitro rate of the two sides of the bladder differed by a factor of 2, a situation which would be unlikely if most of the apparent glucose uptake was due instead to bacterial breakdown. It has previously been mentioned that crustaceans rapidly metabolize injected glucose and the bladder apparently shares this capacity with other crustacean tissues.

Although glucose is rapidly taken up by the bladder, it is not transported across the tissue to any significant extent (Table 37). Thus, if glucose is transported from the urine to the hemolymph it is not moved as free glucose, but as a metabolite. Meenakshi and Scheer (1961) found greater concentrations of glucose-6-P than glucose (7.2 and 5.7 mg%, respectively) in the hemolymph of C. magister. Further, they found that radioactive label from injected glucose appeared rapidly in hemolymph glucose-6-P and more slowly in maltose and maltose oligosaccharides. Maginniss (1976) and Ahearn and Maginniss (1977) have reported that

tritiated glucose is taken up by the midgut of the prawn, Macrobrachium rosenbergii, and that the label was accumulated by the tissue in the form of hexose phosphates, while only a small fraction of the accumulated label was in free glucose. These authors also found that transmural glucose flux was much smaller than uptake by the mucosal side of the tissue, that transport had a Na^+ requirement, and that only 50% of the label transported from the mucosal to the serosal side of the tissue was in free glucose. Because glucose uptake in the present study was not measured at a constant glucose concentration, a comparison of the uptake rates of C. magister bladder and M. rosenbergii midgut is not possible, but it appears that the processes in the two tissues may have several similarities.

Eyestalk ablation had no significant effect on serum or urine glucose levels (Fig. 46) or on glucose uptake by the bladder (Table 38). Glucose uptake by the bladder is thus probably not influenced by the hyperglycemic hormone (HGH) known to be released at the sinus gland, although, for reasons explained below, the insignificant effects of ablation may not mean that HGH does not control glucose uptake. In light of the impressive body of evidence for the existence of a crustacean HGH (reviewed by Kleinholz, 1976), the normal maintenance of serum glucose levels by eyestalkless crabs is perplexing, but not without precedent.

McWhinnie and Scheer (1958) found that eyestalk ablation had no significant effect on serum glucose levels in the crab, Hemigrapsus nudus. Surprisingly, several workers have shown that eyestalk ablation in some crabs leads to an increase in "blood sugar" (total reducing substances or T.R.S.) (Abramowitz, et al., 1944; Keinholz and Little, 1949; Rangneeker, et al., 1961). The significance of a change in T.R.S. is, however, doubtful, as Kleinholz and Little (1949) found that only 20-25% of the T.R.S. was actually fermentable sugar and that this fraction showed an insignificant change after eyestalk ablation in the crab Libinia. Binns (1969b) has also commented upon the necessity of using caution in the interpretation of changes in blood sugar levels when only T.R.S. is measured. Menon and Savidas (1967) with Scylla and Madhyastha and Rangneeker (1976) with Varuna have found that eyestalk ablation lowered T.R.S., which is what one would expect if the HGH is involved in the maintenance of normal serum sugar levels. Again, because the T.R.S. method was used in these two studies, it is uncertain that actual sugar levels were changed. Johnson and Fisher (1968) found that eyestalk ablation causes the hemolymph levels of several sugars to decrease in the crab Libinia emarginata, a finding contrary to that of Kleinholz and Little (1949). It is interesting that Parvathy (1972) found that the effect of eyestalk

ablation in the crab, Ocypoda platytarsis, varied during the moult cycle. Intermoult and postmoult crabs were rendered hypoglycemic by eyestalk ablation, premoult crabs were unaffected, and early postmoult crabs were rendered hyperglycemic. The situation in macrurans seems to be more well defined as eyestalk ablation causes a decrease both in levels of serum T.R.S. (Scheer and Scheer, 1951; Rangneeker and Madhyastha, 1971) and glucose (McWhinnie and Saller, 1960; Quilter, 1977). Keller (1969) and Kleinholz and Keller (1973) have shown that eyestalk extracts of macrurans do not induce significant hyperglycemia in brachyurans and that the same is true of brachyuran eyestalk extracts when injected into macrurans. Thus, there is evidence that the HGH is different in the two groups and there may be a difference in the mechanisms of action between the two groups as well. Kleinholz (1976) has noted that in vivo deficiency studies with brachyurans may be difficult or impossible to conduct.

From the data presented in this study it is clear that the bladder of C. magister has the capacity to reabsorb large amounts of glucose from the urine. At any normal level of hemolymph gulucose all filtered glucose is reabsorbed. As Gross (1967) has noted, it is possible that reabsorption of filtered glucose normally occurs in the labyrinth and that glucose uptake by the bladder is merely

fortuitous. A resolution of the relative roles of the two structures awaits the development and application of suitable micropuncture techniques for *in vivo* studies of labyrinth function.

CHAPTER 8

CONCLUSION

The structure of the antennal gland of C. magister and its role in the maintenance of hemolymph volume, hemolymph levels of magnesium and sulfate ions, and in reabsorption of glucose from the urine has been examined.

The morphology of the antennal gland is similar to that of other crabs. The coelomosac (filtration site) contains polocytes with foot-processes which support a filtration membrane. Both the labyrinth and the bladder have the well developed brush borders, extensive basal infoldings and numerous mitochondria characteristic of transporting epithelia. The bladder in C. magister is very extensive and has an area of 0.87 cm^2/g body weight.

Hemolymph volume expansion and contraction are rapidly rectified by changes in urinary and drinking rates. Urinary rate is apparently not controlled by changes in heart rate or arterial pressure, nor is it directly controlled by neurosecretory centers in the eyestalk. Although injected homogenates of brains and thoracic ganglia from crabs in normal and dilute media cause variable changes in urinary rate, there is some indication that brain homogenates from crabs in dilute media have a greater effect than other

tissues. A speculative model for control of hemolymph volume is presented. Two integrated control mechanisms are incorporated into the model: a universal mechanism based on non-eyestalk mediated control of urinary and drinking rates, and a mechanism based on adaptive hormonal control of permeability to water by neurosecretory centers in the eyestalk and/or other tissues.

As is the case in many marine decapods, C. magister hyporegulates serum magnesium levels. Urine levels of magnesium are much greater than serum levels and production of urine is necessary for hyporegulation of magnesium. Magnesium levels increase and sodium levels decrease with residence time of the urine in the bladder, indicating that the bladder is responsible for excretion of magnesium into the urine. The observed in vivo exchange ratio of magnesium and sodium across the bladder epithelium is calculated to be non-electrogenic. In vitro measurements of $^{28}Mg^{++}$ fluxes in paired bladder tissues show a highly significant, ouabain-sensitive net flux of magnesium into the bladder, indicating linkage of magnesium excretion with ouabain-sensitive sodium reabsorption or a direct effect of ouabain on the magnesium transport mechanism. Crabs depleted of magnesium do not excrete it and excised bladder tissue from such crabs shows no significant net flux of $^{28}Mg^{++}$, indicating physiological control of magnesium excretion by

the bladder. *In vitro* fluxes of $^{22}Na^{+}$ across excised bladder tissue are large and show a highly significant, ouabain-insensitive net flux of sodium out of the urine. The net sodium flux is dependent on oxidative metabolism. The net *in vitro* flux of $^{36}Cl^{-}$ is of the same size and direction as the net $^{22}Na^{+}$ flux. It is concluded that only a small fraction of the net $^{22}Na^{+}$ flux out of the urine may be associated with magnesium excretion into the urine. The large net fluxes of sodium and chloride out of the urine may be the driving force for the commonly observed phenomenon of reabsorption of water and salts from the urine in decapods.

Although *C. magister* hyporegulates serum sulfate levels below those of the medium, anuric crabs show no changes in serum sulfate levels. *In vitro* fluxes of $^{35}SO_4^{=}$ across excised bladder tissue in both directions are small and equal. It is concluded that under normal conditions sulfate is concentrated in the urine by withdrawal of water.

Injection of glucose in *C. magister* causes glucosuria with an apparent threshold of approximately 100 mg%. Glucose is absorbed from crab Ringer solution introduced into the bladder. Phlorizin blocks glucose absorption by the bladder and causes glucosuria when injected. Glucose is absorbed *in vitro* by both sides of the bladder, but the

flux of free glucose across the tissue is negligible. Glucose absorption from the urine is apparently not controlled by neurosecretory centers in the eyestalk.

In conclusion, the antennal gland of C. magister is responsible for maintenance of hemolymph volume, hyporegulation of serum magnesium concentration below that of the medium and reabsorption of filtered glucose from the urine.

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