URINE PRODUCTION OF THE PURPLE SHORE CRAB, *HEMIGRAPSUS NUDUS*

by

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ABSTRACT

Measurement of urine production by the crab, Hemigrapsus nudus, gives an indication of the excretion, in absolute amounts, of substances whose concentrations in the urine are presently known. In this way, it is possible to judge the degree of participation of the antennary glands in the functions of ionic and osmotic regulation.

Urine production was measured, firstly, by blockage of nephropores to prevent urination and detection of resulting weight increases. The adhesive, methyl 2-cyano-acrylate monomer (M2C-2), provided a virtually perfect block against leakage by fusing the operculum to the surrounding exoskeleton. Secondly, the rate of inulin excretion following injection was employed for determination of urine production. Blockage with M2C-2 and inulin excretion gave urine production values of 15% to 17% body weight/24 hours in 25% salinity. With M2C-2 blockage, in 75% salinity, urine production was about 6% body weight/24 hours, however, inulin excretion indicated the rate in this salinity to be 12% body weight/24 hours. The M2C-2 values are believed to be the more reliable since they were more directly obtained and were replicated. It is evident, thus, that urine production increases with decrease in salinity. In both salinities, winter and summer crabs were used. Although seasonal differences in urine production were not entirely consistent, there is

evidence to suggest that urine flow is greater in winter than in summer crabs in the low salinity. Urine production is independent of weight of crabs over the range sampled.

It appears that urine production is proportional to the osmotic gradient existing between blood and the external salinity (blood is hypertonic to the salinities considered). Thus, seasonal and salinity effects on urine production presumably are related to changes in this osmotic gradient. It is hypothesized that the antennary glands function in this species for volume regulation by excreting excess osmotic water.

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INTRODUCTION

Osmotic and ionic regulation are functionally related topics which have received considerable attention in the Crustacea. Robertson (1960) defines os motic regulation as the regulation of the total particle concentration of body fluids at levels different from those of the external environment. Pachygrapsus crassipes has been shown to regulate in both a hyposmotic salinity of 50% sea water and a hyperosmotic one of 170% (Prosser, Green & Chow, 1955). The spider crab, Maja, is a good example of a nonregulating (poikilosmotic) crustacean (Schwabe, 1933). Species may regulate in low salinities and be relatively poikilosmotic in 100% and higher salinities. Carcinus is isosmotic at high salinities, slightly hyperosmotic in 100% sea water, and hyperosmotic in dilute salinities (Duval, 1925). Most marine crustaceans are in osmotic equilibrium with their environment and the problem of osmoregulation is never met in nature.

Ionic regulation is a universal phenomenon in crustaceans. Its occurrence is recognized when the proportions of major ions in the blood are different from those that would be expected were the blood in passive, Gibbs-Donnan equilibrium with sea water. Crustaceans maintain blood concentrations of Na, K, and Ca ions higher than in the sea water, concentrations of Mg and SO_4 ions lower and Cl ion

concentration approximately the same as in the sea water (Robertson, 1960).

Knowledge of the amounts of ions excreted by the kidney and by extra-renal routes amounts of ions in blood and muscle, ion uptake from the environment, as well as fluxes due to passive diffusion, leads to a more complete understanding of the mechanisms of osmotic and ionic regulation and the relative importance of kidney function in these processes. Concentrations of ions in blood and urine in a range of seasons, salinities and temperatures have been documented by Dehnel (1962), Dehnel & Stone (1964) for Hemigrapsus nudus, the purple shore crab. Estimation of urine flow in this brackish water species provides an opportunity to determine absolute amounts of salts and water lost via the kidney and to contribute to the clarification of these mechanisms. The purpose of this study is, therefore, to estimate urine production of Hemigrapsus nudus as per cent body weight per 24 hours in two seasons and several salinities.

The excretory organs of adult decapods are paired antennary or green glands. The gland consists of three principal parts, the end-sac or coelomic sac, excretory canal and exit duct. The excretory canal has a greatly increased surface and is termed the labyrinth. The coelomic sac is much-branched and processes extend into depressions in the labyrinth wall producing a sponge-like structure (Picken, 1936). The

lower part of the excretory canal and the exit duct are enlarged to form a collecting bladder (Parry, 1960).

Urine produced by the antennary gland consists largely of water and salts. Ammonia, the principal nitrogenous waste product, is found only in traces, being lost mainly by diffusion from permeable parts of the integument (Parry, 1960). Amino acids form a major part of the excreted nitrogen and uric acid and urea are present in small amounts (Parry, 1960). However, the nature of much of the excreted nitrogen is undetermined.

The mechanics of micturition in decapods is not fully elucidated. The opercula do not function in urine retention since they receive no muscle fibres. Urine is retained in the bladder of Cambarus clarkii (Maluf, 1941b) by sphincter-like urethral fibres. The bladder of C. clarkii is supplied sparsely with unstriated fibres which are not believed to function in urination. Maluf (1941b) considers the major factor in expulsion of urine in this crayfish to be pressure exerted on the bladder by the blood and the crop-gizzard. Parry (1955) also found that the bladder in Palaemonetes has no intrinsic muscles. She hypothesized that micturition in this prawn may be accomplished by contraction of muscles running from the rostrum to the epigastric sac, an extension of the bladder.

There are three common methods by which urine production in Crustacea has been measured. The simplest is blockage of the excretory openings to prevent urination. Either weight increases are measured with the block in situ, or the volume of urine released by block removal after a time is measured. Secondly, a neutral dye or other substance is injected into the animal and its rate of excretion observed. A third method is direct cannulation which is potentially the most accurate if a cannula can be attached without leakage.

The methods chosen for the determination of urine production in Hemigrapsus nudus were blockage of nephropores, initially with dental cement, later with a physiological adhesive. Secondly, the rate of excretion of injected inulin was used as an indication of urine flow.

Inulin is considered to be an excellent substance for the determination of urine production, blood clearance rates and kidney filtration rates since it is physiologically inert (Maluf, 1941c; Smith, 1956), non-toxic (Smith, 1956), does not enter cells due to its large size (Steinitz, 1938; Riegel & Parker, 1960) and does not become bound to plasma proteins. Most importantly, it is completely filtrable through glomeruli (vertebrate) and is not secreted nor resorbed by the vertebrate kidney tubule (Smith, 1961). Its use has frequently been extended to invertebrates (Maluf, 1941c; Potts, 1954; Martin et al, 1965). Inulin is a starch-like polysaccharide, $(C_6H_{10}O_5)_n$, consisting principally of

fructose units. The molecular weight has been estimated to be 5101 (Westfall & Landis, 1936), 5600 or 7250 (Phelps, 1965).

MATERIAL AND METHODS

COLLECTION AND MAINTENANCE OF CRABS

The purple shore crab, Hemigrapsus nudus, was collected at Spanish Bank, Vancouver, B.C., during two seasons of the year, winter and summer. Winter crabs were collected from the beginning of November until the end of March. Summer animals were those collected from about the beginning of June until the first of September. Winter season was delineated by high field salinity and low temperature; summer season, by low field salinity and higher temperatures. Variations in these field parameters have been described by Dehnel (1960).

Crabs were maintained in plastic dishes in experimental salinities of 25% sea water (7.97‰ salinity, 4.41‰ chlorinity) which approximates the average summer field salinity, and 75% sea water (23.91‰ salinity, 13.24‰ chlorinity) which is close to the winter field salinity. Initially, a third salinity, 125% sea water (39.85‰ salinity, 22.06‰ chlorinity) was used but it was necessary to decrease the number of salinities to two. Crabs were held under constant temperature ($10^{\circ} \pm 1.0^{\circ}$C) and darkness from one to four days before treatment and were not fed.

All salinities are based on a 100% sea water standard of 31.88‰ salinity and 17.65‰ chlorinity. Stock sea water was prepared by adding

reagent grade inorganic salts, in the appropriate ratios given by Barnes (1954), to local sea water to bring its content to 200% of the standard. This stock water was then diluted with glass-distilled water to the required experimental salinity. Salinity was determined by titration with $AgNO_3$.

BLOCKAGE EXPERIMENTS

Blockage of Nephropores with Dental Cement

The blockage method for the determination of urine production depends upon the detection of small weight changes. It was necessary initially to develop a method of drying crabs consistently which resulted in as little error in subsequent weights as possible. Crabs were damp-dried and placed into a 6" by 2.25" Plexiglass drying cylinder. Air was dried to a consistent degree by passage through two columns of Drierite (anhydrous calcium sulfate with indicator) before entry into the cylinder. Individual crabs were weighed after a drying period of 3, 4, 5, or 7 minutes at intervals of two to four hours until five or ten weighings had been performed. The variance of the series of weights for each crab was calculated. It could not be shown (by an F test for homogeneity of variance, Steel & Torrie, 1960, p. 82) that the variance associated with one drying period was significantly lower than that for any other period. Therefore, the time of 4 minutes was chosen arbitrarily as the drying period to be used throughout all blockage and injection experiments.

Crabs were dried 4 minutes and weighed before blockage to determine the normal, pretreatment body weight. The faceplate of the crab was then roughened with a dental burr to facilitate adherence of the cement to the exoskeleton. Several coats of dental cement (S. S. White Zinc Cement made by S. S. White Dental Mfg. Co., Philadelphia, Pa.) were applied over the region between the orbits and down to the epimeral suture, the ridge above the mouth. The application of the cement and its drying took from 30 to 45 minutes. During this period of exposure, crabs were kept cool and moist. After hardening of the cement, each crab was placed into 300 ml sea water of the experimental salinity, through which air was bubbled. Constant temperature ($10^{\circ} \pm 1.0^{\circ}$C.) and darkness were maintained during the periods of immersion. The crab was allowed approximately 30 minutes before the first weighing in which to regain water lost during the period of exposure to air. After this time, the crab was damp-dried, dried in the cylinder for the 4 minute period, weighed and then reweighed at intervals which ranged from two to occasionally ten hours. Immediately after each weighing the crab was reimmersed in the sea water until the next weighing.

Controls were of two types, uncemented and cemented. Uncemented controls were dried, weighed, burred and exposed to air for the same average time as blocked crabs. They were handled as often as blocked crabs in the event that this activity caused animals to lose weight due to gastric juice release. The same regime of equilibration with the sea

water, weighing and reweighing was followed as for blocked crabs. These controls indicated whether animals continued to take up water as a result of exposure past the 30 minutes of equilibration.

Cemented controls, similarly, were dried and weighed. An amount of cement equivalent to that applied to the faceplate of blocked crabs was placed on the burred dorsal surface of the carapace. After hardening of the cement, the crab was equilibrated with sea water, weighed and reweighed. In these animals, possible absorption of water by the dental cement was not masked by weight increases due to retention of urine and a measure of the amount of water absorption could be made.

Calculations and Statistical Analysis of Blockage Data

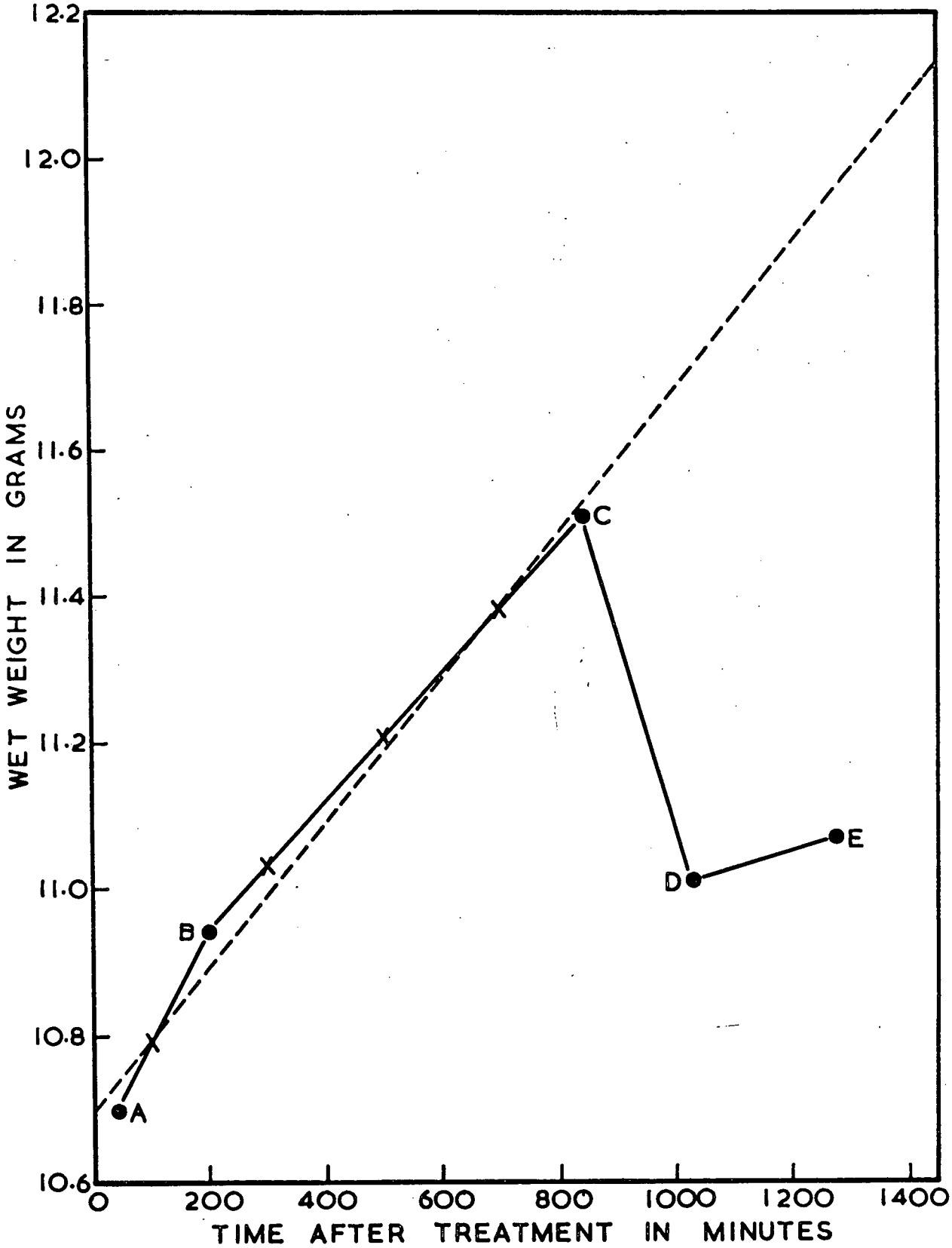
The weights of each individual uncemented or cemented control or blocked crab were plotted as a function of time after treatment. This plot is given in Figure 1 which shows the original graph for an individual blocked crab. Time zero was considered to be the point at which crabs were replaced into sea water, the cement on blocked and cemented control crabs having sufficiently hardened. The weight was not measured at time zero since the crab was desiccated following the exposure.

To illustrate the average weight changes of control and blocked crabs with time, that is, the actual "shape" of the response of each type of crab, absolute per cent weight increase was estimated for each individual crab, at arbitrary times of 100, 300, 500 and 700 minutes after

treatment. First, the weight of the crab was read from the line ABC (Fig. 1) at each of these times. Crosses on the figure indicate these weights. Subtraction of the weight at time zero (the Y-intercept of the regression equation through the points A, B and C) gave the absolute weight increase at each arbitrary time. If the first several weights, A, B or C, did not lie approximately on a straight line but on a curve, the weight at time zero was estimated as the point at which the curve fitted by eye intersected the Y-axis. Each absolute weight increase of a crab was expressed as a per cent of body weight by dividing by the preblocked weight of that crab. Per cent weight increases were averaged at each time, 100, 300, 500 and 700 minutes after treatment, for the following groups of summer crabs: uncemented controls in three salinities, cemented controls in 75% salinity, and dental cement blocked crabs in three salinities.

The calculation of urine production as per cent body weight/24 hours depends on the extrapolation of the initial rate of increase (Fig. 1, line ABC) to the time of 24 hours. Regression equations ($Y=a+bX$) were therefore calculated for the first several weights (Fig. 1, A, B and C) of each crab, control and blocked, as a function of time after treatment, by the method of least squares (Steel & Torrie, 1960, p. 163). The value of Y at X=1440 minutes (24 hours) was the theoretical weight following retention of urine for 24 hours by blocked crabs. From this was subtracted the weight at time zero (Y-intercept) to give the weight increase

Figure 1. Typical graph of the weight of a single summer dental cement blocked crab in 25% salinity as a function of time after treatment. The measured weights are indicated by closed circles and are connected by straight lines. These weights are labelled by letters for reference purposes. The dotted line indicates the regression line given by the equation Y=10.691+0.001003X, based on points A, B, and C. Crosses designate weights at the arbitrary times of 100, 300, 500 and 700 minutes after treatment. These lie on the line ABC.


12·2
12·0
11·8
11·6
11·4
11·2
11·0
10·8
10·6
WET WEIGHT IN GRAMS
0
200
400
600
800
1000
1200
1400
TIME AFTER TREATMENT IN MINUTES
A
B
C
D
E

over a 24 hour period. This weight increase in grams is numerically equivalent to the volume of urine in millilitres and the latter can be expressed as per cent body weight per 24 hours by dividing by the pre-blocked, normal weight of each crab. Per cent weight increase/24 hours was calculated for each control crab for comparison with blocked crabs. Per cent weight increase/24 hours and urine production as per cent body weight/24 hours are synonymous terms with reference to blocked crabs. Weight changes for the unblocked control crabs may be expressed only as per cent weight increase/24 hours, since they were not due to urine production.

It was necessary to make a correction in the urine production values of dental cement blocked crabs due to the fact that the cemented controls were found to take up water ($P < 0.05$). The amount of water absorption by the cement depends upon the amount of cement present. A Student's t-test showed that the weights of dental cement applied to summer cemented controls in 75% and to summer blocked crabs in the same salinity were not significantly different. Therefore, the average per cent weight increase/24 hours of the cemented controls, 2.034%, was subtracted from urine productions of individual dental cement blocked crabs in 25%, 75% and 125% salinities, assuming that similar weights of cement had been applied to crabs in all salinities. In some cases, the urine production calculated from the regression was less than 2.034% (in 125%

salinity), so the corrected U. P. was considered to be 0.000% rather than a biologically meaningless negative quantity.

To test whether the per cent weight increases/24 hours of control crabs and urine production of blocked crabs were significantly different from a zero increase, confidence intervals were calculated around the mean. These were calculated for summer uncemented controls in three salinities, cemented controls in one salinity, summer crabs blocked with dental cement in three salinities and for winter and summer crabs blocked with a physiological adhesive (M2C-2) in 25% and 75% salinities. The technique of blockage with M2C-2 is described below. Exclusion of zero from these confidence intervals indicated significance. Unless otherwise stated, statistical significance throughout all data refers to the 1% level. If significant results were obtained at the 5% level, they were indicated as such.

Per cent weight increase/24 hours of individual uncemented control crabs in each salinity, of individual cemented control crabs in 75% salinity, and urine production of individual blocked crabs in each salinity were considered as functions of weight of crab, and a regression line was calculated for each of these groups of crabs. If the F value obtained from the analysis of variance on regression (Steel & Torrie, 1960, p. 172) exceeded the tabulated F value at the 0.01 level, indication was given that per cent weight increase/24 hours or urine production depended upon weight of crab.

A one-way analysis of variance (Steel & Torrie, 1960, p. 113) was performed on per cent weight increases of uncemented control crabs and on urine productions of dental cement blocked crabs in three salinities, 25%, 75% and 125% to determine if salinity had an effect on weight increase. The urine production data were subjected further to Scheffé's test (Scheffé, 1953) to determine if the effects on urine production of the salinities, 25%, 75% and 125%, differed. Confidence intervals were calculated for comparisons of 25% with 75% and for 75% with 125%. Exclusion of zero from the 99% confidence interval indicated significance.

Urine productions determined from M2C-2 blockage data were subject to the possible effects of both season and salinity. Since a two-way analysis of variance is difficult with unequal replication, Scheffé's test was performed. Urine productions for summer crabs were compared with those of winter animals. Urine productions in 25% salinity were compared with those in 75% salinity.

Inulin Injection into Crabs Blocked with Various Materials

The adequacy of the dental cement block was questioned since the cement did not physically bind to the exoskeleton and could be easily pried off after hardening. Injection of inulin, which is excreted in the urine, and assay in the medium (100 ml aliquot of sea water) for this substance appeared to indicate substantial leakage of urine around the block. The techniques of injection and inulin assay will be given in a later section.

Natural beeswax-tree resin (50%-50%), heated and dropped onto the opercula, was then tested as a blockage medium and found to attach to the exoskeleton even less securely than the dental cement. Injection of inulin and medium assay were carried out on these blocked crabs.

There is a second possibility, other than block leakage, which could possibly account, at least in part, for the appearance of inulin in the medium of blocked crabs. As suggested by Parry (1960), substances may be excreted extrarenally when the normal route is blocked.

The possibility that inulin passes out through the gills into the medium was tested. Crabs were dried 4 minutes, burred and blocked with dental cement. They were then injected with inulin, placed in 75% sea water for 15 minutes to flood the gill chambers and removed to plastic dishes containing filter paper saturated with 75% sea water for the remainder of a two hour period. Constant temperature and darkness were maintained. Gill chamber fluid was collected by the same technique used for urine collection (described below), except that the terminal glass tubing was left blunt to minimize puncturing of the gills. Suction was gently applied to the large exhalent opening in front of the mouth. If no fluid was obtained in this way, the tubing was moved into the chamber. The fluid obtained was placed in the minimum assay volume of 2.5 ml of distilled water and assayed for inulin.

Further, the appearance of inulin in the medium may be explained as diffusion of inulin through the exoskeleton, by slight bleeding at time of injection causing contamination of the medium, or by the presence of carbohydrate-containing gastric juice.

Gross (1957) demonstrated that the permeability of the chitinous exoskeleton of Pachygrapsus was the same in both directions. Thus, diffusion of inulin into the crab would probably indicate the ability of inulin to diffuse outwardly, as well. Uninjected crabs were placed into 100 ml of 75% sea water containing 3 mg. of inulin (0. 300 mg/ml) and blood sampled at 24 hours was assayed for inulin.

A measure of the amount of inulin lost into the medium by slight bleeding at the time of injection was obtained by injecting inulin solution into dried crabs, which were each placed into 100 ml 75% sea water for 15 minutes. These "rinse media" were analyzed for inulin. The amount of bleeding at time of injection was subjectively estimated by eye and assigned a value of 0 or + to +++. A correlation of the amount of inulin in each "rinse medium" with the estimated amount of bleeding was performed as a check that the detected inulin was due to bleeding. Since the method of estimating bleeding was non-parametric, an appropriate procedure was the calculation of Spearman's coefficient of rank correlation (Steel & Torrie, 1960, p. 409). The t value associated with the calculated coefficient was compared with the tabulated t.

Undiluted gastric juice released by crabs is deep brown and was seen to color the medium to a considerable extent in some cases. The visible color contributed to the total optical density of the medium obtained with anthrone during inulin assay. Unblocked, uninjected crabs were placed into 25% salinity medium for 24 hours. The optical density

due to visible color was obtained from dilution of these medium aliquots with distilled water (1:5) equivalent to that obtained during assay with anthrone, and read against distilled water blanks at 630 mu. The correlation between the slight optical density due to visible color and the amount of inulinoid substance determined by assay with anthrone was calculated. The correlation coefficient was compared with the tabulated value.

From the results of these experiments, an indication was available of the extent to which block leakage accounted for the appearance of inulin in the medium of crabs blocked with dental cement, beeswax and a physiological adhesive.

Blockage of Nephropores with a Physiological Adhesive

The physiological adhesive, methyl 2-cyanoacrylate monomer (M2C-2), produced by Ethicon, Inc., Somerville, N.J., is a clear, colorless fluid which dried almost instantly fusing the operculum to the surrounding exoskeleton. Simultaneous injection of inulin indicated that this block allowed very little, if any, leakage. Crabs were dried 4 minutes, weighed, blocked with two or three coats of the adhesive applied in small amounts to the opercular region. After a drying period of 20 to 30 minutes crabs were returned to sea water and subjected to the same regime of equilibration, weighing and reweighing as dental cement blocked crabs. In the intervals the crabs were immersed, and maintained at constant temperature ($10^{o} \pm 1.0^{o}$C.) and darkness. Winter and summer animals

were tested in 25% and 75% salinities.

Weight as a function of time was graphed in the same manner as for dental cement blocked crabs. Similarly, per cent weight increases were averaged at the times of 100, 300, 500 and 700 minutes. Regression equations for the initial rate of weight increase were calculated for each crab in order to derive urine production.

INULIN INJECTION EXPERIMENTS

Inulin Injecting and Standard Solutions

A 5% weight/volume solution of inulin was used for injecting into crabs. Initially, this solution was prepared by dissolving powdered inulin, made by Nutritional Biochemicals Corp., Cleveland, Ohio, in distilled water. This solution was used for several sets of injections and required reheating each day of use to dissolve the precipitate.

Later, inulin was available in the form of a 10% solution containing 0.5% sodium chloride, produced by General Diagnostics Division of Warner-Chilcott Laboratories, Morris Plains, N.J. Ten ml of the dissolved, cooled inulin solution was diluted with 10 ml distilled water resulting in a 5% solution for injection. This solution was reheated on the day of use then discarded. This reduced the chance of breakdown of inulin by repeated heating.

Inulin standards were prepared initially from a 0.1% stock solution of powdered inulin. Later, a 10 ml aliquot of Warner-Chilcott

10% solution was diluted to one litre with distilled water. The stock solutions thus contained 1 mg/ml inulin and from them were prepared inulin standard solutions ranging in concentration from 0.005 mg/ml to 0.08 mg/ml inulin. New standards were prepared at least every two months. All inulin solutions were stored between 4° and 9°C.

Injection Technique

Crabs were dried for 4 minutes previous to the injection in the same manner as described for blocked crabs. The site of injection was the membrane of the coxopodite of the right fifth periopod since the sinus there is relatively large and accessible. A 0.25 cc tuberculin syringe, fitted with a limited delivery apparatus, was calibrated to deliver 0.1 ml. It was fitted with a #30 needle which was fine enough to eliminate excess bleeding yet did not become plugged in use. Immediately upon removal of the needle the wound was sprayed with Fluoro Glide, a product of Chemplast, Inc. This dried instantly, appeared to stop bleeding quickly, if it occurred, and to be non-toxic. Injected crabs were placed individually into 100 ml volume of sea water of the experimental salinity and were supplied with air. Constant temperature ($10^{\circ} \pm 1.0^{\circ}$C.) and darkness were maintained throughout the period of immersion.

The 100 ml aliquot of sea water in which crabs were maintained in the interval between injection and sampling is termed the medium. In experiments with the powdered inulin, little or no inulin was detected

in the medium, presumably due to bacterial breakdown of inulin. In experiments using Warner-Chilcott inulin, a 0.5 gm vial of penicillin G procaine, penicillin G sodium and streptomycin sulphate produced by Parke, Davis & Co., Ltd., Brockville, Ontario, was diluted to 200 ml with distilled water. One ml of this, containing about 5000 units, was added to 999 ml of sea water. The medium thus contained 50 units/ml which was sufficient to overcome bacterial breakdown.

Sampling Blood, Urine and Medium

An attempt was made to sample each of three fluids, blood, urine and medium, from the same crab, however, this was not always successful. A separate set of more than 10 crabs was sampled at each of four time periods after injection, 6, 12, 18 and 24 hours, during winter and summer seasons, in two salinities, 25% and 75%. In addition, urine collected at 24 hours from a set of crabs in each season and salinity was added to the medium of the same crabs to obtain a fluid termed medium-with-urine. Blood was sampled from each crab in the latter case, thus giving two sets of 24 hour blood samples. Analyses of these fluids indicated the concentrations of inulin present at each time. The medium-with-urine fluid gave an indication of total amount of inulin excreted after 24 hours since it included the inulin-containing urine present in the bladder at that time.

Blood was sampled by removing the left fifth periopod at the base of the coxopodite or by pricking the membrane there with a needle. The

drop of blood which flowed out was drawn up into a 100 lambda pipette and placed into 1.3 ml distilled water as quickly as possible to prevent clotting.

The urine was collected by lifting the operculum with a glass tube, drawn to a fine point, fitted into a piece of plastic tubing leading through a small cork into a 2 ml centrifuge tube. The urine was pulled into the test tube by the aspiration of running water. Samples of 30, 50 or 100 lambda volume were placed into 5 ml distilled water.

The medium required no further preparation for inulin assay after removal of the crab at the sampling time.

All the above inulin-containing fluids were stored between 4^{o} and 9^{o}C. until processing and assay one to three days later.

Precipitation of Blood Proteins

Blood proteins were precipitated to prevent cloudiness due to heating during inulin assay. The method of Young & Raisz (1952) was modified by using one-tenth of their quantities since limited quantities of blood were available from the crab. To the blood in 1.3 ml distilled water was added 0.1 ml 10% $ZnSO_4 \cdot 7H_2O$ and 0.1 ml 0.5N NaOH. The tubes were manually shaken briefly to ensure precipitation, then 3.4 ml distilled water was added to bring the total volume to 5 ml. Tubes were then placed on an automatic shaker for 30 minutes to allow maximum

recovery of inulin. Samples were centrifuged for 10 minutes at 3000 rpm and the supernat assayed for inulin.

Blood and Urine Blanks

Blood (100 lambda) and urine (30, 50 or 100 lambda) samples were obtained from normal uninjected crabs by the methods indicated above. The fluids were assayed in the same manner as inulin-containing fluids and served to indicate the amount of inulinoid substances present normally in these body fluids. The average inulinoid content of 48 blood blanks was equivalent to 0.012 mg inulin/ml blood. The blood precipitating agents contributed to this value an apparent inulin concentration of 0.003 mg/ml. The average inulinoid content of 38 urine blanks was equivalent to 0.012 mg inulin/ml urine. Blood and urine, thus, contained similar amounts of inulinoid substances. These were considered negligible compared with typical blood (0.500 to 2.500 mg/ml) and urine (1.000 to 3.500 mg/ml) inulin concentrations obtained experimentally.

Blood, Urine and Medium Inulin Standards

Since the diluted samples of urine contained very little salts, inulin standards assayed at the same time as unknowns were unmodified inulin standards in distilled water.

In order to compensate for any effect that the blood precipitation procedure might have on the inulin in this fluid, standards were processed in the same manner as the blood. To 2.5 ml of a standard of double the desired concentration, was added 0.1 ml 10% $ZnSO_4 \cdot 7H_2O$ and 0.1 ml of 0.5N NaOH. The solution was shaken briefly, then 2.3 ml distilled water was added to bring the volume to 5.0 ml. These were shaken and centrifuged as the bloods, and resulted in a blood inulin

standard curve.

The saline media contained large amounts of Cl ion, the presence of which substantially enhances the color development of carbohydrates with anthrone reagent (Bartlett, 1959; Graham & Smydzuk, 1965). In order to compensate for this effect, medium standards were prepared which contained Cl ion in the same concentrations as in the two salinities, 25% and 75%. An appropriate quantity of the streptomycin-penicillin solution was added. These standards resulted in medium standard curves.

The effect on inulin color development of the chloride ion concentrations as they occur in diluted blood and urine samples was measured. These concentrations of Cl ion increased the optical density of an inulin standard (0.02 mg/ml) by 2% or 3%, an effect which was considered negligible.

Anthrone Reagent

A 0.4% anthrone reagent in 75% sulfuric acid was prepared according to Young & Raisz (1952). To 250 ml of distilled water was added 500 ml of concentrated sulfuric acid, reagent grade, produced by Allied Chemical Canada, Ltd., sp. gr. 1.84. Four grams of anthrone, obtained from Matheson, Coleman & Bell, was dissolved in 250 ml concentrated sulfuric acid. This latter mixture was added to the cooled diluted acid. The reagent was stored in a dark bottle at room temperature and was considered to be stable for 7 to 10 days.

The spectra of day- and month-old anthrone reagent were determined spectrophotometrically and are shown in Figure 2A. The curve for the aged anthrone is shifted to the right of that for freshly prepared reagent. The cause of the displacement is a shift in the reagent from anthrone to its enol tautomer, anthranol, which gives a similar curve in sulfuric acid as does aged reagent (Zipf & Waldo, 1952). Anthranol, further, is sensitive to oxidation which accounts for darkening of the reagent with age.

Development of Color in Solutions Containing Inulin

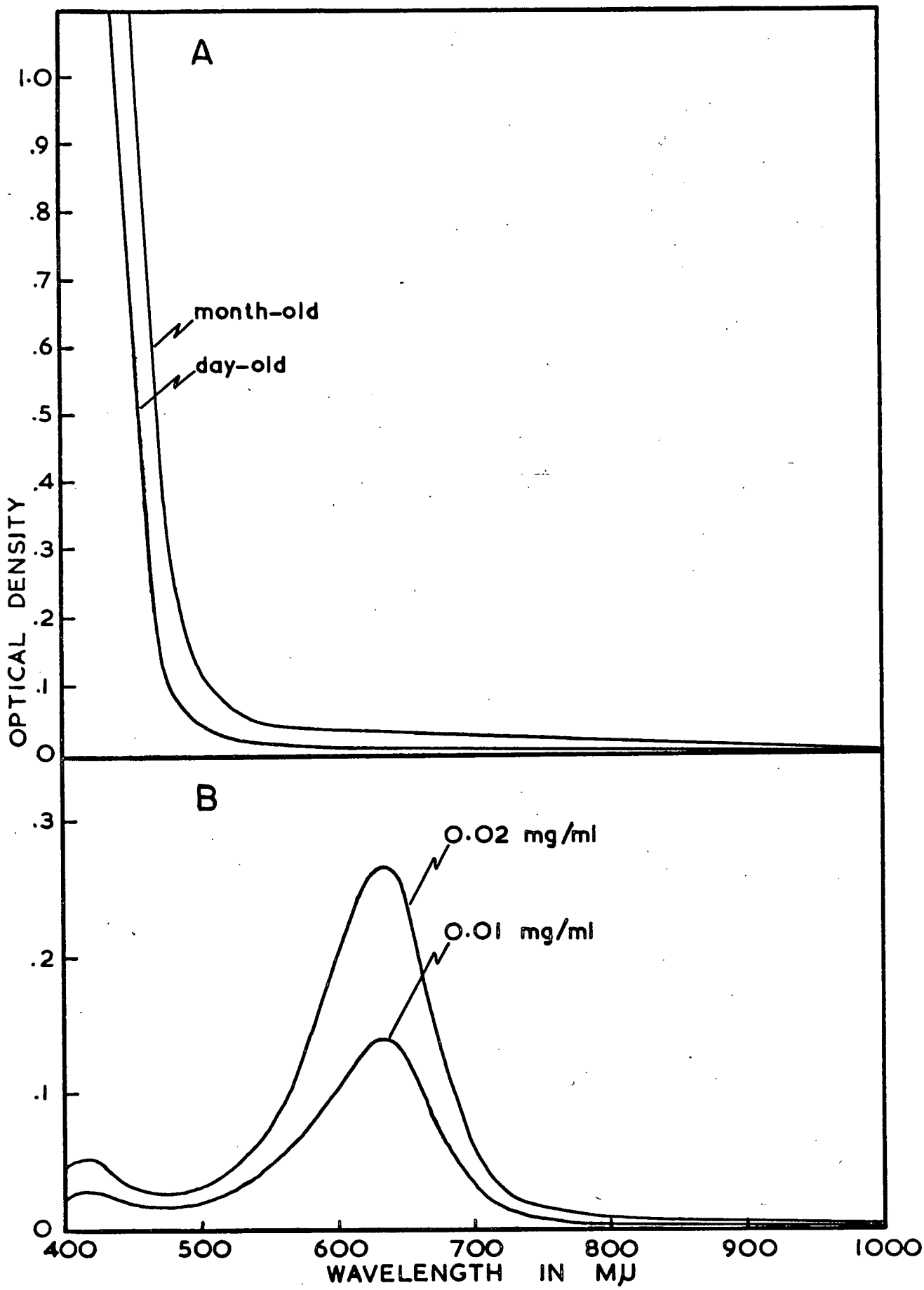
Color due to the anthrone-inulin complex was developed simultaneously in 2 ml duplicate aliquots of distilled water, three inulin standards of the appropriate type and ten unknown solutions of blood, urine or medium. Ten ml of anthrone reagent was delivered from automatic pipettes into the duplicates simultaneously. The tubes were manually shaken in a beaker of cold water during the delivery of the reagent to ensure adequate mixing and to keep the heat of mixing below that detrimental to the reagent. The tubes were then heated in a Colora water bath at 75°C. for 5 minutes and cooled in running tap water for 15 minutes. The samples were read against the distilled water-anthrone blanks (as zero) on a Beckman DU-2 spectrophotometer at 630 mu. The complete set was read, followed by the duplicate set.

Spectrum of Anthrone-Inulin Complex

The spectra of anthrone reagent complexed with inulin in two

Figure 2. A. Spectra of day- and month-old anthrone-sulfuric acid reagent.

B. Spectra of anthrone-inulin complex given for solutions containing two concentrations of inulin, 0.01 mg/ml and 0.02 mg/ml. The wavelength of maximum absorbance is 635 mu.


A
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
month-old
day-old
OPTICAL DENSITY
B
.3
.2
.1
0
0.02 mg/ml
0.01 mg/ml
400
500
600
700
800
900
1000
WAVELENGTH IN MμJ

concentrations (0.01 mg/ml and 0.02 mg/ml) are shown in Figure 2B. As expected, the optical density at any wavelength is greater for the more concentrated inulin solution (0.02 mg/ml). These spectra show a larger maximum at 635 mu and a smaller one at 415 mu. These results compare reasonably with those of Viles & Silverman (1949) who obtained the larger maximum at 625 mu and a smaller one at 525 mu. Helbert & Brown (1955) also obtained a maximum for anthrone-fructose at 625 mu. The wavelength used in the present study for inulin assay (630 mu) was that given in the method of Young & Raisz (1952) and it is seen not to agree precisely with that determined here. Of greater importance than the absolute wavelength used is its constancy from one determination to the next.

The nature of the anthrone-carbohydrate complex was elucidated by Sawamura & Koyama (1964) as 10-furfurylideneanthrone. In the reaction with the anthrone reagent, the carbohydrate is first dehydrated by sulfuric acid to give furfural or a derivative (Hurd & Isenhour, 1932; Wolfrom et al, 1948). The furfural condenses (Black, 1951; Sattler & Zerban, 1948; Seifter et al, 1950) with the anthranol tautomeric form (Roe, 1955) to give the above complex.

Blood, Urine and Medium Inulin Standard Curves

The optical density obtained with anthrone assay for inulin was linearly proportional to the concentration of inulin in the standard

(Beer's law) up to an inulin concentration of 0.05 mg/ml. Average inulin standard curves are shown in Table I. Average optical density was regressed as a function of inulin concentration for each type of standard such that the curves passed through the origin and were in the form Y=bX (Steel & Torrie, 1960, p. 179). The reason for the higher optical density values of the powdered inulin standards compared with the same type using Warner-Chilcott inulin is not clear. However, since inulin concentrations in blood and urine were relative to the standards, the concentrations of inulin obtained in these two body fluids with the two types of inulin were directly comparable. The blood standards for the two types of inulin had slightly higher optical density values than the unmodified urine standards. Precipitating agents contributed a small amount to this increase. As expected, the medium standards, since they contain Cl ion, gave higher optical densities than the unmodified Warner-Chilcott inulin standards. Further, the optical density was higher for medium standards for 75% salinity, containing more Cl ion, than those for 25% salinity.

Calculation of Inulin Concentrations in Blood, Urine and Medium

Standard curves with optical density (average of the duplicates) as a function of inulin concentration were graphed from the standards included in each set of determinations. Inulin concentrations in blood, urine or medium samples were read from the respective standard curve. These values gave the quantity of inulin present in 1 ml of the sample

Table I. Average inulin standard curves for powdered and Warner-Chilcott inulin. Y is the optical density obtained with anthrone, X is the concentration of inulin in the standard assayed, in mg/ml. The standard curves were calculated to pass through the origin, thus the regression equations are in the form Y=bX. Medium standards were prepared with only Warner-Chilcott inulin.

Type of Inulin	Type of Standard Curve	Regression Representing the Standard Curve Y=bX
Powdered	Urine inulin (unmodified)	Y=14.203X
Powdered	Blood inulin	Y=14.246X
Warner-Chilcott	Urine inulin (unmodified)	Y=13.064X
Warner-Chilcott	Blood inulin	Y=13.374X
Warner-Chilcott	Medium inulin for 25% SW	Y=14.357X
Warner-Chilcott	Medium inulin for 75% SW	Y=14.667X

tested. To obtain the concentration of inulin (mg/ml) in undiluted blood, the values from the curve were multiplied by 50.00, since 1 ml of the sample assayed contained 0.02 ml blood. Urine when collected was placed into 5 ml water. Thus, a 100 lambda urine sample gave a volume of 5.1 ml from which the 2 ml aliquots were drawn. The appropriate multiplier in this case was 51.00 for 100 lambda urine, 101.00 for 50 lambda urine and 167.67 for 30 lambda urine samples. The medium was not diluted before assay, however, in order to obtain the amounts (mg) of inulin totally in the medium, the concentrations read from the standard curves were multiplied by 100.00, to give inulin content as mg/100ml.

Calculations and Statistical Analysis of Inulin Injection Data

Inulin concentrations in blood and urine (mg/ml) and inulin amounts in the medium (mg/100ml) were expressed as functions of weight of crabs. Regression equations were calculated by the method of least squares for each fluid, blood, urine and medium, at each time period after injection, 6, 12, 18 and 24 hours, in each season, winter and summer, and each salinity, 25% and 75%. The number of measurements upon which each regression was based was most often 10. The probability (P value) that the regression coefficient equals zero was determined. (Steel & Torrie, 1960, p. 172).

Nonhomogeneity of variance was frequently indicated between regression lines to be compared. (Steel & Torrie, 1960, p. 83). The

Welch test (Brownlee, 1965), a two-sample t-test for samples with unequal variances, was therefore used to compare both regression coefficients and Y-intercepts of pairs of lines. Powdered inulin winter blood and urine lines, in 75% salinity at 6, 12, 18 and 24 hours after injection, and in 25% salinity at 12, 18 and 24 hours after injection, were each compared with the corresponding Warner-Chilcott inulin regression lines. Similarly, the duplicate 24-hour blood regression lines, using Warner-Chilcott inulin, in the same season and salinity were compared with each other. Medium regression lines at 24 hours were compared with the corresponding medium-with-urine lines in the same season and salinity. Further, the regression coefficients of blood and urine lines from the same set of crabs were compared with each other. To test if these urine regression lines were above blood lines from the same sets of crabs, Y-intercepts were compared by the Welch test and, in addition, the sign test was used. This latter test indicates the significance of the frequency in a set of crabs with which the urine inulin concentration exceeded blood inulin concentration from the same crab.

To test whether Warner-Chilcott blood and urine slopes decreased with time the regression coefficients of each fluid at the four time periods, 6, 12, 18 and 24 hours after injection, in each season and salinity were considered as a function of time and a regression line was calculated for this relationship in each season-salinity combination. A significant slope of the latter regression indicated a significant effect of time

on blood and urine concentration as functions of weight of crabs.

Urine production as per cent body weight/24 hours was calculated from the Warner-Chilcott inulin injection data according to the following equation:

$$\text{Volume of urine produced during 24 hours (ml)} \approx \frac{\text{Amount of inulin excreted into medium at end of 24 hours (mg)}}{\text{Average urine inulin concentration during the 24 hour period (mg/ml)}}$$

The average urine inulin concentration over 24 hours was obtained by averaging the inulin concentrations given by the regression equations for the four time periods, for a particular weight of crab. The amount of inulin in the medium after 24 hours was obtained from the medium-with-urine regression equation for the same weight of crab. To obtain weight-specific urine production the volume of urine produced was divided by the weight of the crab. Urine production as per cent body weight/24 hours was calculated in each season and salinity for crabs weighing 6 to 16 grams since these weights represent the major part of the weight range used.

Urine productions calculated by this method do not lend themselves to an analysis of variance since they are not independent of each other and are not replicated. To determine if weight, season and salinity affected urine production, 99% confidence intervals were calculated for 6, 10 and 16 gram crabs in the two seasons and salinities. Nonoverlap of intervals indicated whether significant differences existed. Calculation of these intervals involved estimating the variance of medium-

with-urine inulin amounts (a, below) at each weight. The method is given by Snedecor (1956, p. 137). Secondly, a variance of the average urine inulin concentration (b, below) was obtained by averaging variances for each weight from the four urine lines at the four time periods. The variance of a quotient a/b is given by Beers (1953):

$$s_V^2 = \frac{s_a^2}{b^2} + \frac{a^2}{b^2} \cdot s_b^2$$

where "a" represents the medium-with-urine inulin amount and "b" represents the average urine inulin concentration; s_a^2 and s_b^2 are their variances, respectively. Using these variances of urine volume produced (s_V^2), confidence intervals were calculated on volume of urine produced for the three weights of crabs. The limits of these intervals were each divided by the respective weight of crab (not associated with a variance) to obtain the intervals on urine productions.

Inulin clearances of the blood were calculated for a 10 gram crab in the two seasons and salinities, by the formula given by Maluf, (1941c):

$$\text{Clearance} = \frac{\text{Concentration of inulin in urine X Vol. of urine}}{\text{Concentration of inulin in blood}}$$

This equation reduces to:

$$\text{Clearance (ml)} = \frac{\text{Amount of inulin in medium (mg)}}{\text{Concentration of inulin in blood (mg/ml)}}$$

since volume of urine equals inulin medium amount divided by average urine concentration.

In the experiments with powdered inulin, due to inulin breakdown in the medium, no calculation of urine production could be made. For this reason these data are not presented in the results. The relationship of blood inulin concentration to weight of crabs was found to be the same (comparing regression coefficients and Y-intercepts) using the two types of inulin at each time, season and salinity. The powdered inulin urine regression lines frequently were significantly above (comparing Y-intercepts) corresponding Warner-Chilcott regressions, however, slopes were found to be the same at each time, season and salinity.

RESULTS

BLOCKAGE OF NEPHROPORES

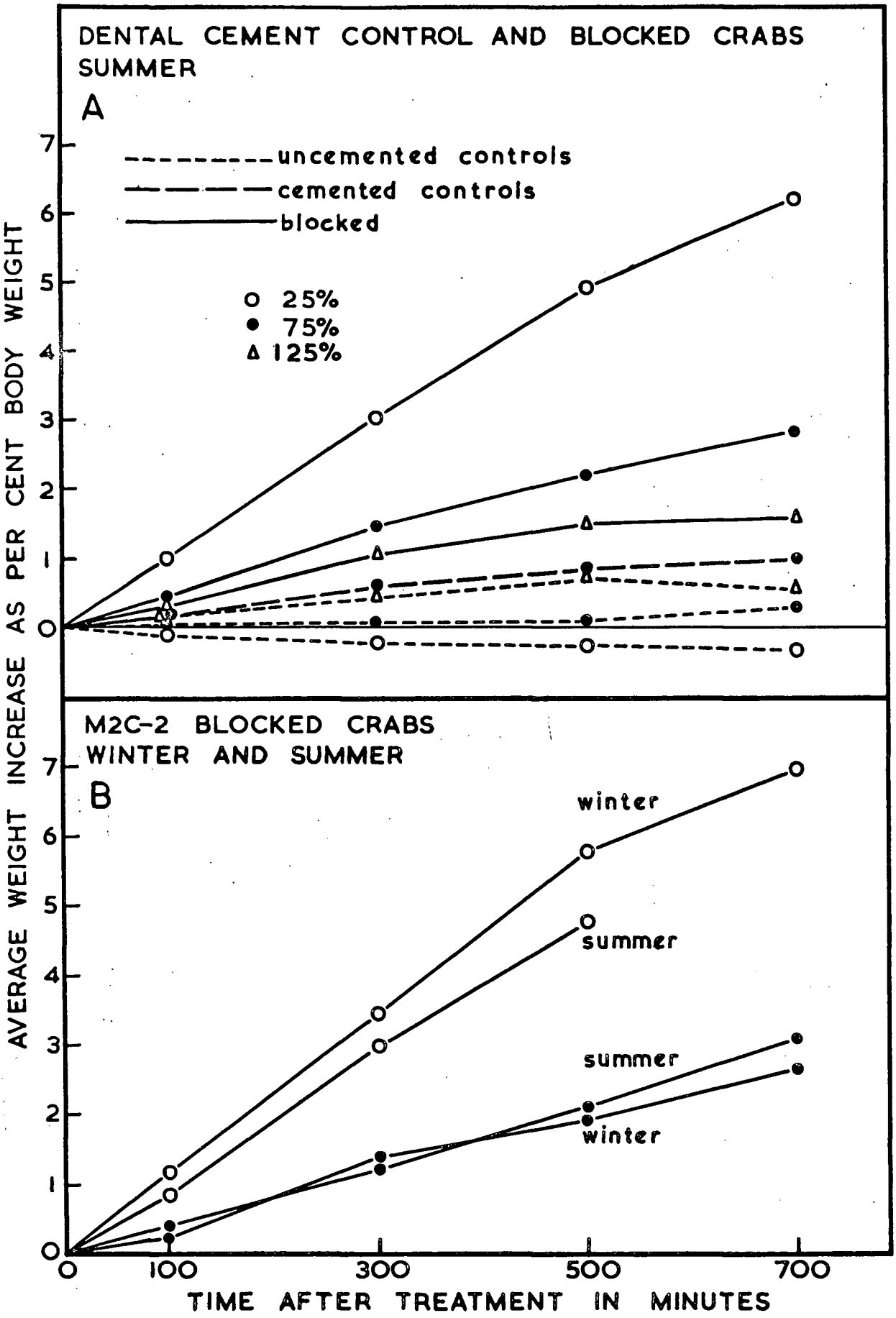
Weight Increases of Control and Blocked Crabs

Average per cent weight increase as a function of time in minutes after treatment is shown in Figure 3A for summer uncemented controls in three salinities, summer cemented controls in 75% and summer dental cement blocked crabs in three salinities. Figure 3B presents per cent weight increase for crabs blocked with M2C-2 for two seasons and salinities. Uncemented controls (Fig. 3A) showed little weight change with time and appeared to be unaffected by salinity. Cemented controls (Fig. 3A) showed a slightly greater per cent increase in weight with time than did the uncemented controls and this effect was attributed to absorption of water by the dental cement. Weight increases for dental cement blocked crabs (Fig. 3A) were greater in each salinity than those of the four groups of control crabs. Comparison of the three lines for dental cement blocked crabs shows that the increase was greatest for crabs in 25% salinity and least in 125%. For example, at 500 minutes blocked crabs in 25% salinity showed a per cent weight increase about two and one-half times greater than that of blocked crabs in 75% salinity and the increase of the latter group was about one and one-half times that of blocked crabs in 125% salinity. Reference to Figure 3B indicates that M2C-2 blocked crabs in 25% salinity exhibited greater per cent weight increase with time than those in 75%. At 500

Figure 3. Average per cent weight increases of crabs at arbitrary times, 100, 300, 500 and 700 minutes after treatment.

A. Average per cent weight increases for summer uncemented control crabs in three salinities, 25%, 75% and 125%, for summer cemented controls in 75% salinity, and for summer dental cement blocked crabs in three salinities.

B. Average per cent weight increases for winter and summer crabs blocked with M2C-2 in two salinities, 25% and 75%.


DENTAL CEMENT CONTROL AND BLOCKED CRABS
SUMMER
A
uncemented controls
cemented controls
blocked
o 25%
• 75%
Δ 125%
M2C-2 BLOCKED CRABS
WINTER AND SUMMER
B
winter
summer
summer
winter
AVERAGE WEIGHT INCREASE AS PER CENT BODY WEIGHT
0
1
2
3
4
5
6
7
0
100
300
500
700
TIME AFTER TREATMENT IN MINUTES

minutes, the increase shown by crabs in 25% salinity was two and one-half to three times greater than that of crabs in 75% salinity. Comparison of winter and summer M2C-2 blocked crabs in the same salinity (Fig. 3B) indicates that, in 25% salinity, winter animals underwent greater per cent weight increase with time than summer crabs. In 75% salinity, however, no appreciable differences were evident. Statistical significance of differences was not tested. In summary, crabs blocked with dental cement or M2C-2 demonstrated larger weight increases with time than do control crabs. Salinity has a more pronounced effect on this weight increase of blocked crabs than does season.

In each line representing blocked crabs in Figures 3A and 3B, a levelling-off of per cent weight increase with time is evident. The "shape" of these lines is a reflection of the average "shape" of responses of individual crabs. The original graphs for individual dental cement blocked crabs (see Fig. 1) indicated, in general, a linear increase in weight over the first eight or ten hours (Fig. 1, ABC) then a sharp decrease (CD) or at least a levelling-off of weight, which occurred from eight to 22 hours. This was followed by a gradual increase (DE) to still greater weights over the remainder of the period of blockage, another ten hours at most. There appeared to be no relationship between time of decrease and salinity. The drop in weight of dental cement blocked crabs is believed to be due to sufficient loosening of the dental cement block to permit leakage of urine. The drops are not observed in Figure 3A

since many occurred after the time of 700 minutes.

For crabs blocked with M2C-2, original graphs for individual crabs indicated a levelling-off with time, but no extreme drops in weight during the period of blockage of about ten hours. The levelling-off was not due to the crab becoming saturated following extensive desiccation since uncemented controls showed no pronounced initial rise in weight. Experiments with inulin injection indicated that virtually no leakage of urine occurs around this block. Therefore, to explain the levelling-off, possibly back-pressure from the full bladder increased body hydrostatic pressure. This presumably decreased the rate at which water entered the body from the medium, and consequently, the rate of weight increase.

Weight increase as per cent body weight for individual summer uncemented control crabs in three salinities, summer cemented controls in 75% salinity, and summer dental cement blocked crabs in three salinities are indicated in Tables II to IV. Table V gives the corresponding data for M2C-2 blocked crabs in two seasons and salinities. These weights increases were derived by extrapolation of the regression line for each crab (see Fig. 1) to X=24 hours. Weight increase as per cent body weight is equivalent to urine production (U.P.) of blocked crabs and is so indicated in Tables IV and V. Weight increases shown in these four tables are theoretical values which would be obtained if the initial rate of weight increase (over the first 8 or 10 hours) were to continue

Table II. Weight increase as per cent body weight/24 hours for individual uncemented control crabs during the summer season, in three salinities, 25%, 75% and 125%. Weight increase was obtained by extrapolating the regression line, based on the first several weighings of each crab, to X=24 hours. C.I. sumbolizes the confidence interval about the mean per cent weight increase/24 hours.

Summer season

25% salinity N=10	75% salinity N=19	125% salinity N=9
1.105	1.154	1.831
1.196	0.502	0.561
0.317	1.144	1.637
-0.212	-0.670	2.098
1.228	1.303	-0.387
1.128	1.667	0.639
-8.109	-0.992	2.906
-1.664	-0.031	-1.082
-1.568	3.421	8.588
-0.442	1.381	
	-1.070	95% C.I.
95% C.I.	-0.173	
-0.702±2.020%	1.841	1.866±2.163%
	-0.306	
	-0.424	
	1.438	
	-0.046	
	2.304	
	0.009	

95% C.I. 0.655±0.601%
99% C.I. 0.655±0.823%

Table III. Weight increase as per cent body weight/24 hours for individual cemented control crabs during the summer season, in 75% salinity. Weight increase was obtained by extrapolating the regression line, based on the first several weighings of each crab, to X=24 hours. C.I. sumbolizes the confidence interval about the mean per cent weight increase/24 hours.

Summer season

	75% salinity N=11
	2.306
	1.480
	0.255
	3.040
	-1.055
	6.826
	1.621
	4.158
	2.879
	0.473
	0.390
95% C.I.	2.034±1.466%
99% C.I.	2.034±2.085%

Table IV. Urine production as per cent body weight/24 hours for individual dental cement blocked crabs during the summer season, in three salinities, 25%, 75% and 125%. Urine production is equivalent to per cent weight increase obtained by extrapolating the regression line, based on the first several weighings of each crab, to X=24 hours. C.I. symbolizes the confidence interval about the mean urine production as per cent body weight/24 hours.

Summer season

25% salinity N=10	75% salinity N=18		125% salinity N=12
12.831	5.556		2.413
16.393	1.944		0.000
15.524	1.032		0.000
17.867	5.885		1.802
11.530	1.737		3.001
7.257	0.315		3.183
13.581	11.473		0.643
14.536	8.713		1.005
0.665	6.471		0.678
9.456	3.843		0.000
	3.025		10.545
99% C.I. 11.964±5.233%	7.045		1.587
	1.966		
	3.472	95% C.I.	2.071±1.840%
	1.702	99% C.I.	2.071±2.597%
	1.929		
	5.274		
	0.000		
99% C.I.	3.966±2.118%		

Table V. Urine production as per cent body weight/24 hours for individual M2C-2 blocked crabs, during two seasons, winter and summer, in two salinities, 25% and 75%. Urine production is equivalent to per cent weight increase obtained by extrapolating the regression line, based on the first several weighings of each crab, to X=24 hours. C.I. symbolizes the confidence interval about the mean urine production, as per cent body weight/24 hours.

Winter season

	25% salinity N=11		75% salinity N=11
	13.883		7.933
	16.693		5.496
	17.191		5.897
	12.236		5.720
	18.163		8.092
	20.999		5.745
	16.074		5.935
	20.037		2.782
	17.562		6.960
	13.713		6.921
	16.842		4.653
99% C.I.	16.672±2.526%	99% C.I.	6.012±1.432%

Summer season

	25% salinity N=10		75% salinity N=17
	16.425		6.424
	18.906		9.474
	16.782		1.885
	14.487		13.841
	9.999		12.788
	19.125		5.592
	12.408		10.875
	11.336		4.089
	15.886		4.687
	13.068		2.884
			8.421
99% C.I.	14.842±3.198%		4.507
			5.467
			3.298
			4.392
			7.700
			3.306
		99% C.I.	6.449±2.509%

unchanged for 24 hours. The initial rate of weight increase, however, does not continue unchanged (Fig. 3A and 3B) and reasons for this have been discussed previously. Per cent weight increases demonstrated by uncemented controls in 25% and 125% salinities (Table II) are not significant ($P > 0.05$). Weight increases of crabs in 75% salinity are, however, significant at the 0.05 level. Since this level of significance is low and occurs in only one set of uncemented controls, it is believed that these crabs showed no biologically significant weight increase with time. No effect of salinity was detected on the per cent weight increase/24 hours of uncemented controls in the three salinities (Table VI, Part 1). Cemented controls in 75% salinity (Table III) showed an average weight increase of 2.034% body weight/24 hours ($P < 0.05$) and this increase was attributed to water uptake by dental cement. Urine production values in Table IV were corrected for water uptake by the dental cement. Mean urine production of summer dental cement blocked crabs was 11.964% body weight/24 hours in 25% salinity, ($P < 0.01$); 3.966% body weight/24 hours in 75%, ($P < 0.01$); and 2.071% body weight/24 hours in 125% salinity, ($P < 0.05$). Urine production by dental cement blocked crabs was lower than that of summer M2C-2 blocked crabs (given below) in the same salinities, presumably due to leakage of urine around the dental cement block. Urine production clearly increases with decrease in salinity and this effect is significant (Table VI, Part 2). Table IV (confidence intervals) and Table VI, Part 3 show that urine production in 25% salinity is significantly higher than that in 75%, however, urine

Table VI. Results of statistical tests on per cent weight increases/24 hours of control and blocked crabs to determine the relative effects of season and salinity. ** symbolizes a P value < 0.01, * $P < 0.05$, NS $P > 0.05$.

Part 1. One-way analysis of variance of per cent weight increases/24 hours of uncemented control crabs during the summer season, in three salinities, 25‰, 75‰ and 125‰.

Source	df	Sums of Squares	Mean Square	F
Salinity	2	31.421	15.711	3.402 NS
Error	35	161.645	4.618	
Total	37	193.066		

Part 2. One-way analysis of variance on urine production of crabs blocked with dental cement during the summer season, in three salinities, 25‰, 75‰ and 125‰.

Source	df	Sums of Squares	Mean Square	F
Salinity	2	600.845	300.423	22.711 **
Error	37	489.445	13.228	
Total	39	1090.290		

Part 3. Scheffé's test on the difference in urine production of dental cement blocked crabs in three salinities. Inclusion of zero in the confidence interval indicates non-significance.

25‰ compared with 75‰
99% confidence interval: $3.290 < \mu < 12.706$

75‰ compared with 125‰
99% confidence interval: $-2.553 < \mu < 6.343$

Part 4. Scheffé's test to determine the relative effects of season and salinity on urine production values of M2C-2 blocked crabs in winter and summer seasons, in 25‰ and 75‰ salinities. Inclusion of zero in the confidence interval indicates nonsignificance.

Winter season compared with summer season pooling salinities
99% confidence interval: $0.882 < \mu < 1.904$

25‰ compared with 75‰ pooling seasons
99% confidence interval: $18.542 < \mu < 19.563$

production is not significantly different in the two salinities, 75% and 125%. This conclusion was suggested by Figure 3A in which the rates of weight increase in 125% and 75% lay closer to each other than to that in 25% salinity. Urine production values for winter M2C-2 blocked crabs given in Table V averaged 16.672% body weight/24 hours in 25% salinity ($P < 0.01$) and 6.012% body weight/24 hours in 75% salinity ($P < 0.01$). Summer M2C-2 blocked crabs produced urine at the rate of 14.842% body weight/24 hours in 25% salinity ($P < 0.01$) and 6.449% body weight/24 hours in 75% ($P < 0.01$) (Table V). It is evident that in the M2C-2 data urine production increases with decrease in salinity, and this effect is significant (Table V, confidence intervals; Table VI, Part 4). Season, also, appears to influence urine production since winter M2C-2 blocked crabs produced more urine than summer animals, in 25% salinity. In 75% salinity, urine production is virtually the same in both seasons, although, the slightly higher urine flow of summer crabs may be a real phenomenon. The effect of season on urine production is significant (Table VI, Part 4) and is presumably due to this difference in winter and summer urine production in 25% salinity. No seasonal difference is indicated by confidence intervals (Table V).

Per cent weight increase (from Tables II and III) as a function of weight of crab is shown in Figure 4 for individual uncemented control crabs in three salinities and summer cemented controls in 75%. Urine production (from Tables IV and V) as a function of weight is shown for

Figure 4. Per cent weight increase/24 hours as a function of wet weight for summer uncemented controls in three salinities, 25%, 75% and 125%, and for summer cemented controls in 75% salinity. Crosses indicate mean per cent increase for each group of crabs.

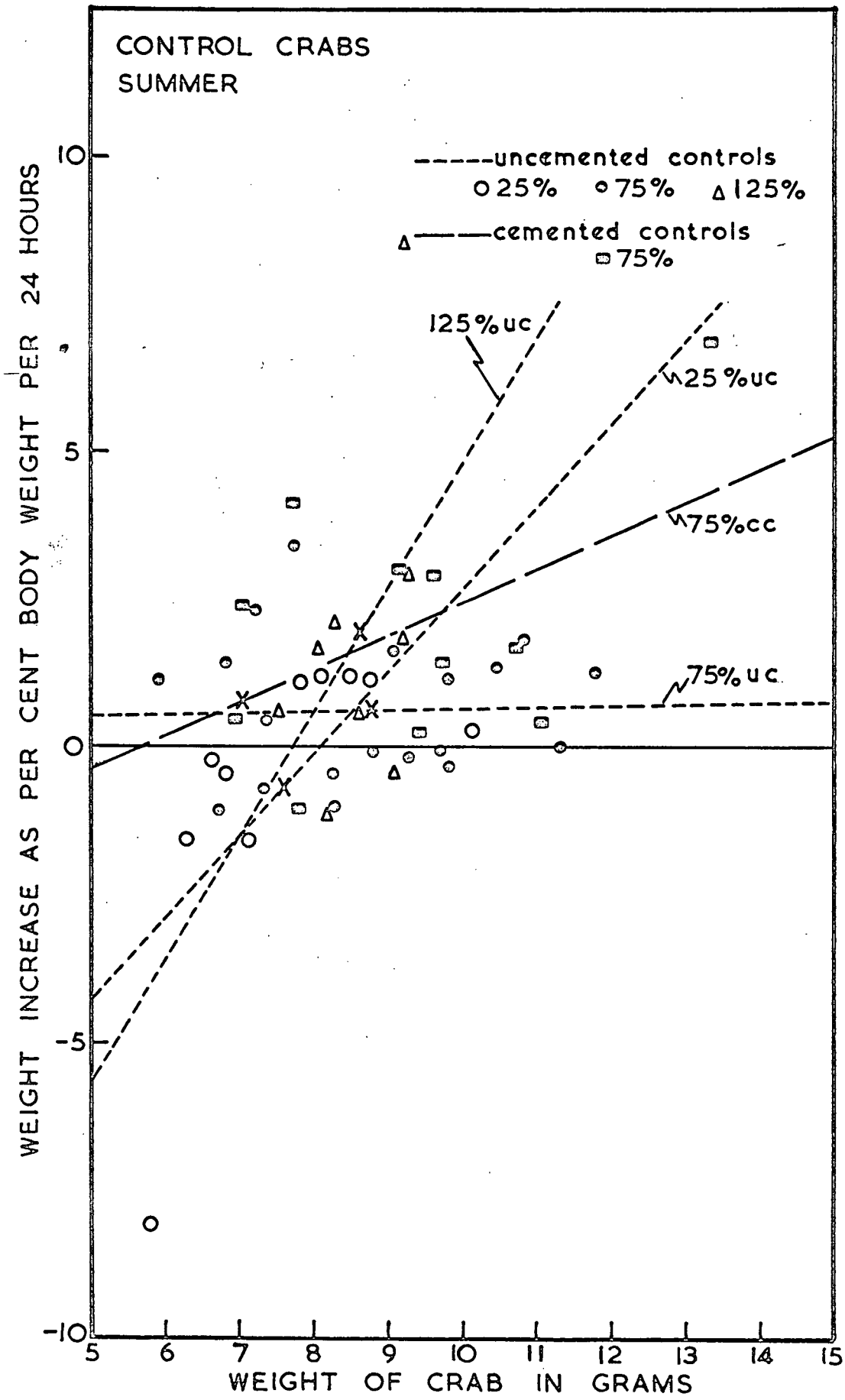

CONTROL CRABS
SUMMER
-----uncemented controls
○ 25% ○ 75% △ 125%
△——cemented controls
□ 75%
125% uc
25% uc
75% cc
75% uc
WEIGHT INCREASE AS PER CENT BODY WEIGHT PER 24 HOURS
10
5
0
-5
-10
5
6
7
8
9
10
11
12
13
14
15
WEIGHT OF CRAB IN GRAMS

Figure 5. Urine production as a function of wet weight for summer dental cement blocked crabs in three salinities, 25%, 75% and 125%. Crosses indicate mean urine production in each salinity.

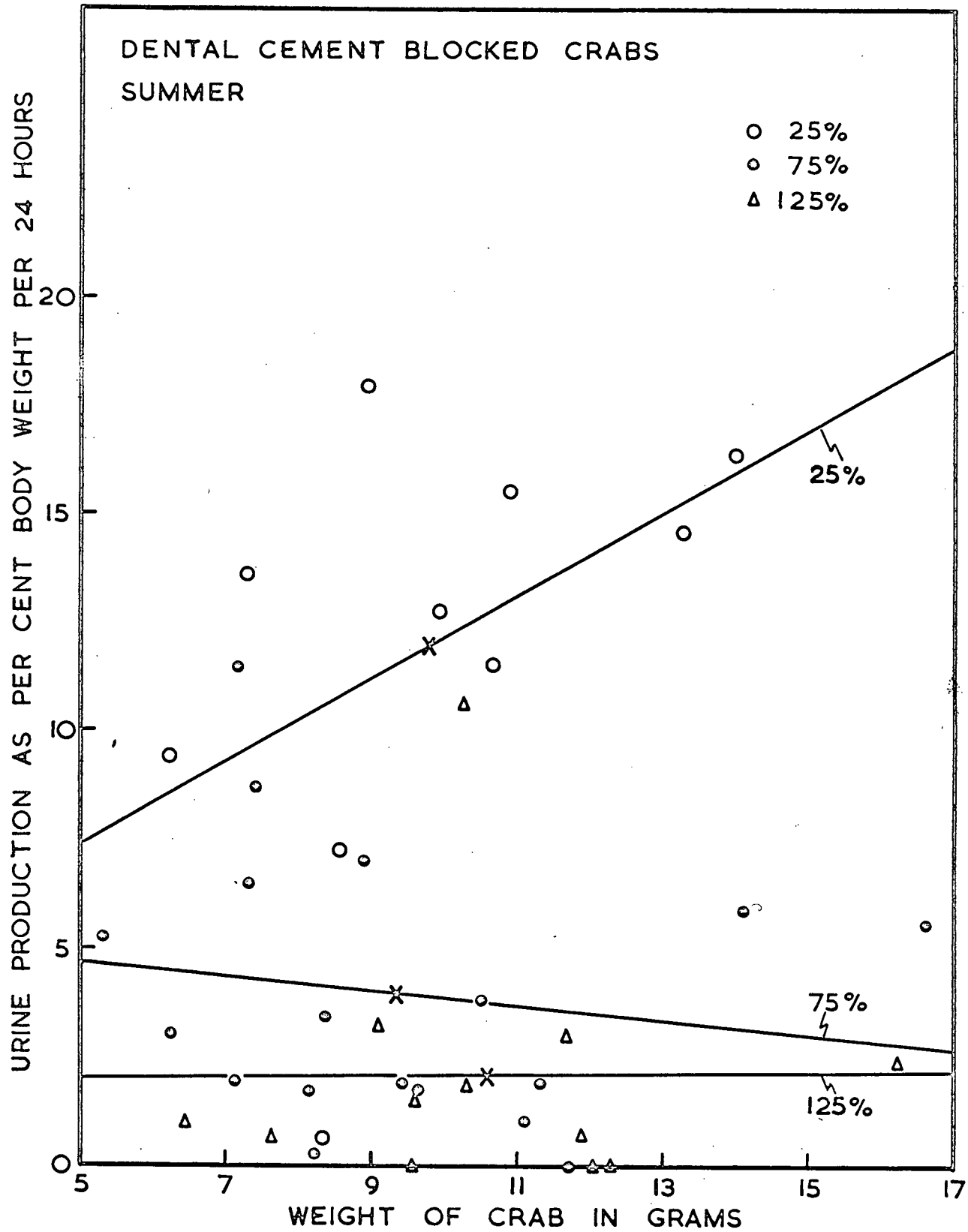

DENTAL CEMENT BLOCKED CRABS
SUMMER
25%
75%
125%
URINE PRODUCTION AS PER CENT BODY WEIGHT PER 24 HOURS
0
5
10
15
20
5
7
9
11
13
15
17
WEIGHT OF CRAB IN GRAMS
25%
75%
125%

Figure 6. Urine production as a function of wet weight for winter M2C-2 blocked crabs in two salinities, 25% and 75%. Crosses indicate mean urine production in each salinity.

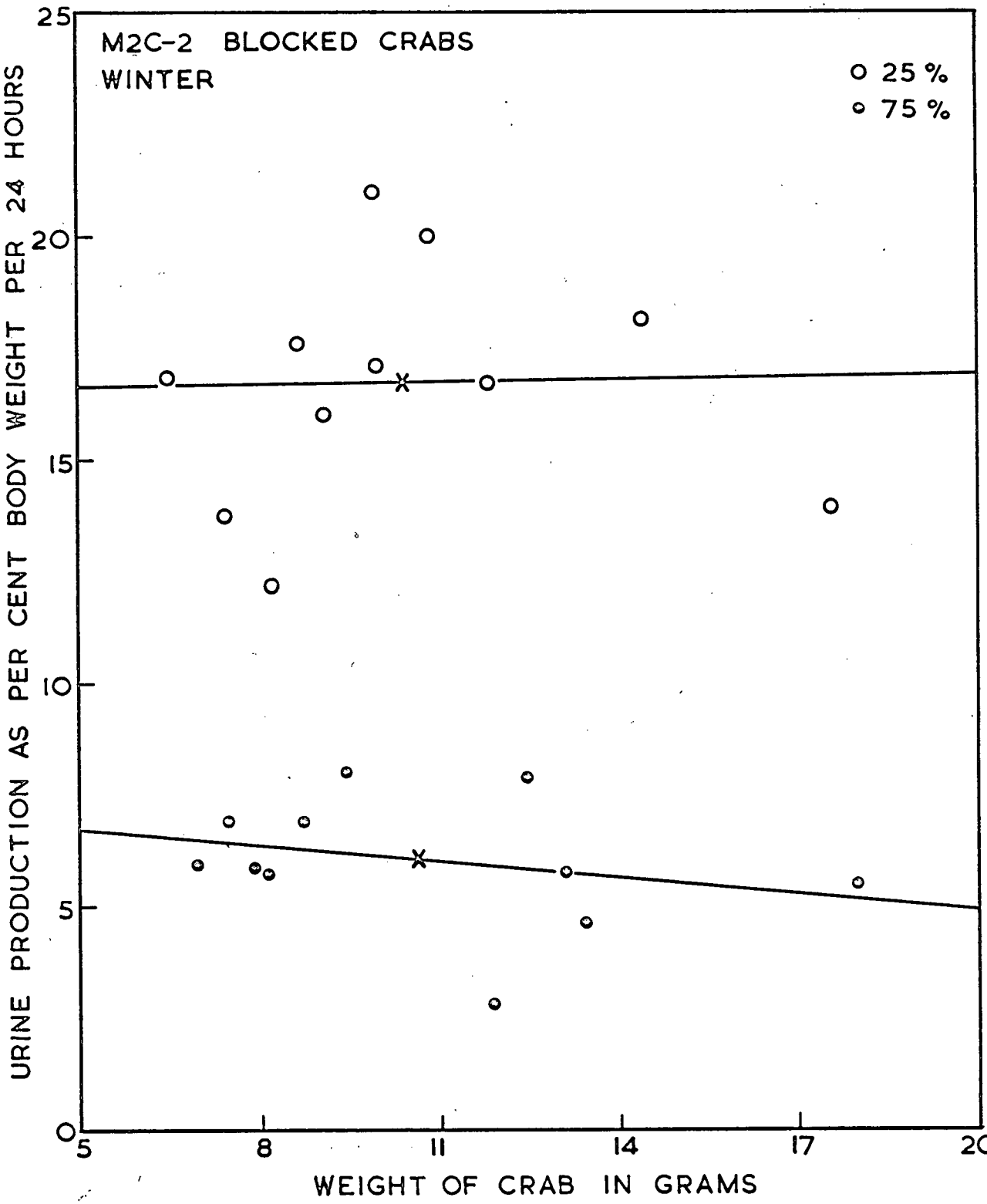
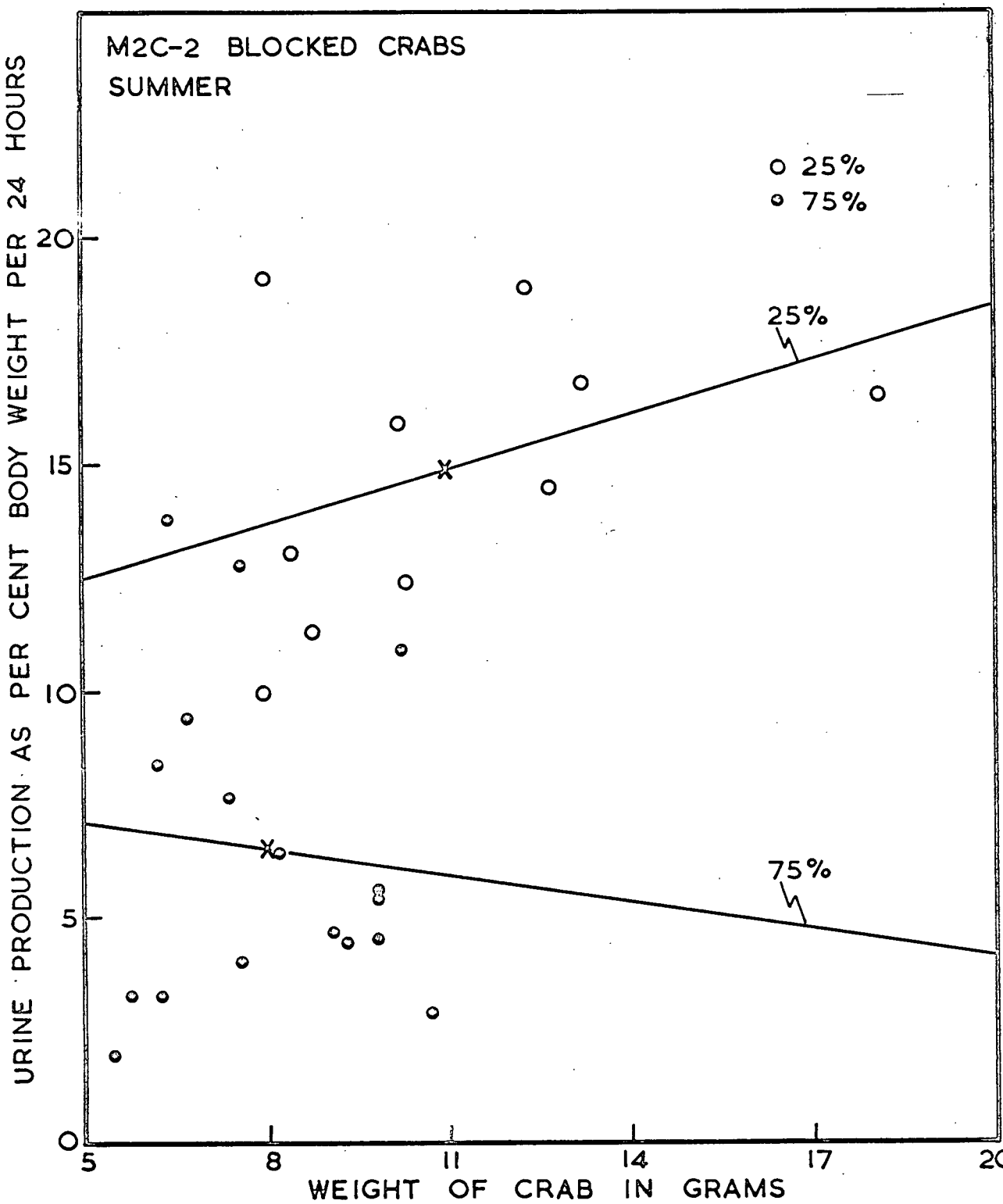

M2C-2 BLOCKED CRABS
WINTER
25 %
75 %
URINE PRODUCTION AS PER CENT BODY WEIGHT PER 24 HOURS
WEIGHT OF CRAB IN GRAMS
0
5
10
15
20
25
5
8
11
14
17
20

Figure 7. Urine production as a function of wet weight for summer M2C-2 blocked crabs in two salinities, 25% and 75%. Crosses indicate mean urine production in each salinity.


M2C-2 BLOCKED CRABS
SUMMER
25%
75%
25%
75%
URINE PRODUCTION AS PER CENT BODY WEIGHT PER 24 HOURS
0
5
10
15
20
5
8
11
14
17
20
WEIGHT OF CRAB IN GRAMS

summer dental cement blocked crabs in three salinities (Fig. 5), for winter M2C-2 blocked crabs in two salinities (Fig. 6) and for summer M2C-2 blocked crabs in two salinities (Fig. 7). The weight of each crab is that measured prior to treatment. In no case was the regression line of per cent weight increase/24 hours or urine production as a function of weight different from zero slope, except that of uncemented controls in 25% salinity ($P < 0.05$ for $b=0$). Discarding the 5.8 gm crab (shown in Fig. 4), which underwent extreme decrease during the 24 hour period, brings the regression coefficient to nonsignificance. Thus, it is concluded that urine production of blocked crabs and per cent weight increase/24 hours of control crabs is not a function of weight of crab.

It is concluded that urine production increases with decreasing salinity, appears to be lower in summer than in winter crabs in low salinities, and is not dependent upon weight of crab.

Inulin Injections into Crabs blocked with Various Materials.

The amounts of inulin assayed in the medium of crabs blocked with various materials are shown in Table VII. The dental cement did not appear to entirely prevent urine leakage into the medium as would be expected were the block effective. After 24 hours, dental cement blocked crabs on the average excreted 25% of the 5 mg inulin injected into them. Corresponding unblocked crabs had excreted 38% of injected inulin at this time. The effectiveness of the block at 6 hours was of

Table VII. Mean inulin amounts excreted into the medium by inulin-injected crabs previously blocked with various materials. Mean inulin amounts excreted by unblocked crabs in the same season and salinity are given for comparison. All values refer to Warner-Chilcott inulin, winter season and 75% salinity.

Blockage Material	Time in Hours	Mean Inulin in Medium mg/100 ml	Number of Crabs	Mean Crab wt in Gms.	Inulin in Medium as Per Cent of Inj. Inulin
Dental Cement	6	0.172	15	10.090	3.44
Dental Cement	24	1.246	11	9.394	24.92
Beeswax	24	2.517	8	10.128	50.34
M2C-2	24	0.207	6	11.000	4.14
Unblocked	6	0.843	10	9.513	16.86
Unblocked	24	1.898	10	12.060	37.96

greater importance since urine production in blocked crabs was calculated from increases during the first six or eight hours. At 6 hours dental cement blocked crabs excreted 3.5% of the inulin injected into them compared with unblocked crabs which excreted 17% of the injected inulin. The inulin excretion by blocked crabs at 6 hours compared with that of unblocked crabs (3.5%:17%) was thus, proportionately, less than that at 24 hours (25%:38%). This supports the above hypothesis that the dental cement block was most effective up to eight or ten hours of blockage.

Evidence is given (Table VII) that beeswax as applied was entirely ineffective as a blocking agent. Blocked crabs excreted over 100% as much inulin as unblocked crabs.

The M2C-2 block (Table VII) was more effective than the dental cement block. At 24 hours the amount of inulin excreted was only 4% of that injected compared with 38% by unblocked crabs. An attempt was made to account for this apparent inulin excretion in other ways than block leakage.

The results obtained by inulin assay of gill chamber fluid as a source of inulin in the medium of blocked crabs were inconclusive. In 9 out of 16 samples obtained, variable amounts of inulinoid substances were found. In 5 of these 9, however, contamination was suspected from gastric juice, inulin-containing urine, or blood from gill puncture.

The possibility remains that appreciable inulin loss occurs via the gills.

Evidence indicated that inulin did not diffuse into the crab. This same result was obtained by Flemister (1958). No inulin was found in the present study in blood taken from crabs held in an inulin-containing medium. It is assumed, then, that injected inulin does not diffuse outwardly, however, it could conceivably be secreted into the medium.

The inulin content of "rinse media" was suggested to be correlated ($P=0.05$) with the estimated amount of bleeding. The amount of inulin in "rinse media" of 11 crabs averaged 0.071 mg/100 ml. Thus, bleeding at time of injection appears to account for about one-third of the inulin (0.207 mg/100 ml) found in the media of M2C-2 blocked crabs at 24 hours (Table VII).

In those media which were visibly colored by gastric juice, the optical density due to the color was negligible compared with the total optical density determined with anthrone. However, there was a significant correlation between optical density due to the visible color and the inulinoid content of the medium determined with anthrone. The media of 17 uninjected crabs contained an average amount of inulinoid substances equivalent to 0.132 mg inulin/100 ml. This is two-thirds of the inulin found in the media of M2C-2 blocked crabs (Table VII). Thus, much of the inulin in the media of M2C-2 blocked crabs is probably due to release of carbohydrate-containing gastric juice.

It appears that the M2C-2 adhesive provides a virtually perfect block against urination by the crab. Inulin present in the medium of blocked crabs after 24 hours can be accounted for by bleeding at time of injection and by release of gastric juice. Since all the inulinoid substance in the medium can be accounted for, there is no need to hypothesize inulin loss via the gill. The dental cement block was somewhat less effective than the M2C-2 block, the excess amount of inulin present in the medium of dental cement blocked crabs presumably due to urine leakage around the block.

INULIN INJECTION EXPERIMENTS

Inulin concentrations in blood and urine (mg/ml) and amounts of inulin in the medium (mg/100 ml) as functions of weight of crabs are indicated as regression equations (Tables VIII to XI) at four time periods during winter and summer seasons, in 25% and 75% salinities. A representative set of these regression lines is indicated in Figure 8, for blood, urine and medium at four time periods for winter crabs in 25% salinity. In the tables, for each regression the P value for b=0, the number of values upon which each equation is based, and the square root of the variance about regression are indicated. No statistical differences were found between duplicate 24 hour blood regressions. The addition of urine at 24 hours to the medium (Tables VIII to XI) did not significantly change the absolute amounts of inulin in the medium, comparing corresponding medium with 24 hour medium-with-urine regressions. Thus, the amount of inulin in unmodified medium at 24

Table VIII. Regression equations of inulin concentration (Y) in blood and urine (mg/ml) and in medium (mg/100 ml) as a function of wet weight (X) for four time periods, 6, 12, 18 and 24 hours, for the winter season, in 25‰ salinity, using Warner-Chilcott inulin. Number of samples is in brackets. Probability that b equals zero and the standard error of estimate, $s_{y.x}$ (square root of the residual variance about regression) are indicated for each regression.

Winter, 25‰

	Blood	Urine	Medium
6 hrs.	$Y=3.406-0.154X$ $P<.001$ (11) $s_{y.x}$ 0.149	$Y=4.105-0.212X$ $P<.005$ (7) $s_{y.x}$ 0.247	$Y=1.084+.007X$ $P>.100$ (11) $s_{y.x}$ 0.337
12 hrs.	$Y=2.486-0.102X$ $P<.001$ (11) $s_{y.x}$ 0.088	$Y=2.920-0.101X$ $P<.050$ (9) $s_{y.x}$ 0.296	$Y=2.600=0.052X$ $P>.100$ (11) $s_{y.x}$ 0.335
18 hrs.	$Y=1.734-0.061X$ $P<.001$ (10) $s_{y.x}$ 0.069	$Y=3.609-0.182X$ $P<.001$ (10) $s_{y.x}$ 0.192	$Y=3.404-0.070X$ $P>.100$ (10) $s_{y.x}$ 0.221
24 hrs.	$Y=1.197-0.036X$ $P<.010$ (12) $s_{y.x}$ 0.095	$Y=3.293-0.152X$ $P<.005$ (9) $s_{y.x}$ 0.252	$Y=4.660-0.142X$ $P<.050$ (11) $s_{y.x}$ 0.361
			Medium-with-urine
24 hrs.	$Y=1.567-0.056X$ $P<.025$ (9) $s_{y.x}$ 0.206	added to medium	$Y=3.768-0.062X$ $P<.050$ (10) $s_{y.x}$ 0.291

Table IX. Regression equations of inulin concentration (Y) in blood and urine (mg/ml) and in medium (mg/100 ml) as a function of wet weight (X) for four time periods, 6, 12, 18 and 24 hours, for the winter season, in 75% salinity, using Warner-Chilcott inulin. Number of samples is in brackets. Probability that b equals zero and the standard error of estimate, $s_{y.x}$ (square root of the residual variance about regression) are indicated for each regression.

Winter, 75%

	Blood	Urine	Medium
6 hrs.	$Y=3.142-0.140X$ $P \leq .001$ (10) $s_{y.x.}$ 0.156	$Y=3.722-0.199X$ $P < .005$ (10) $s_{y.x}$ 0.319	$Y=1.896-0.111X$ $P < .005$ (10) $s_{y.x}$ 0.187
12 hrs.	$Y=2.737-0.111X$ $P < .005$ (10) $s_{y.x.}$ 0.234	$Y=3.508-0.167X$ $P < .001$ (10) $s_{y.x}$ 0.255	$Y=1.421-0.032X$ $P > .100$ (10) $s_{y.x}$ 0.490
18 hrs.	$Y=2.877-0.141X$ $P < .001$ (10) $s_{y.x}$ 0.141	$Y=4.007-0.218X$ $P < .001$ (10) $s_{y.x}$ 0.307	$Y=1.640-0.041X$ $P < .050$ (10) $s_{y.x}$ 0.142
24 hrs.	$Y=1.999-0.072X$ $P < .010$ (10) $s_{y.x}$ 0.172	$Y=2.421-0.071X$ $P < .050$ (10) $s_{y.x}$ 0.249	$Y=2.186-0.024X$ $P > .100$ (10) $s_{y.x}$ 0.548
24 hrs.	$Y=2.159-0.097X$ $P < .005$ (10) $s_{y.x}$ 0.115	added to medium	medium-with-urine $Y=3.414-0.130X$ $P < .025$ (10) $s_{y.x}$ 0.223

Table X. Regression equations of inulin concentration (Y) in blood and urine (mg/ml) and in medium (mg/100 ml) as a function of wet weight (X) for four time periods, 6, 12, 18 and 24 hours, for the summer season, in 25‰ salinity, using Warner-Chilcott inulin. Number of samples is in brackets. Probability that b equals zero and the standard error of estimate, $s_{y.x}$ (square root of the residual variance about regression)are indicated for each regression.

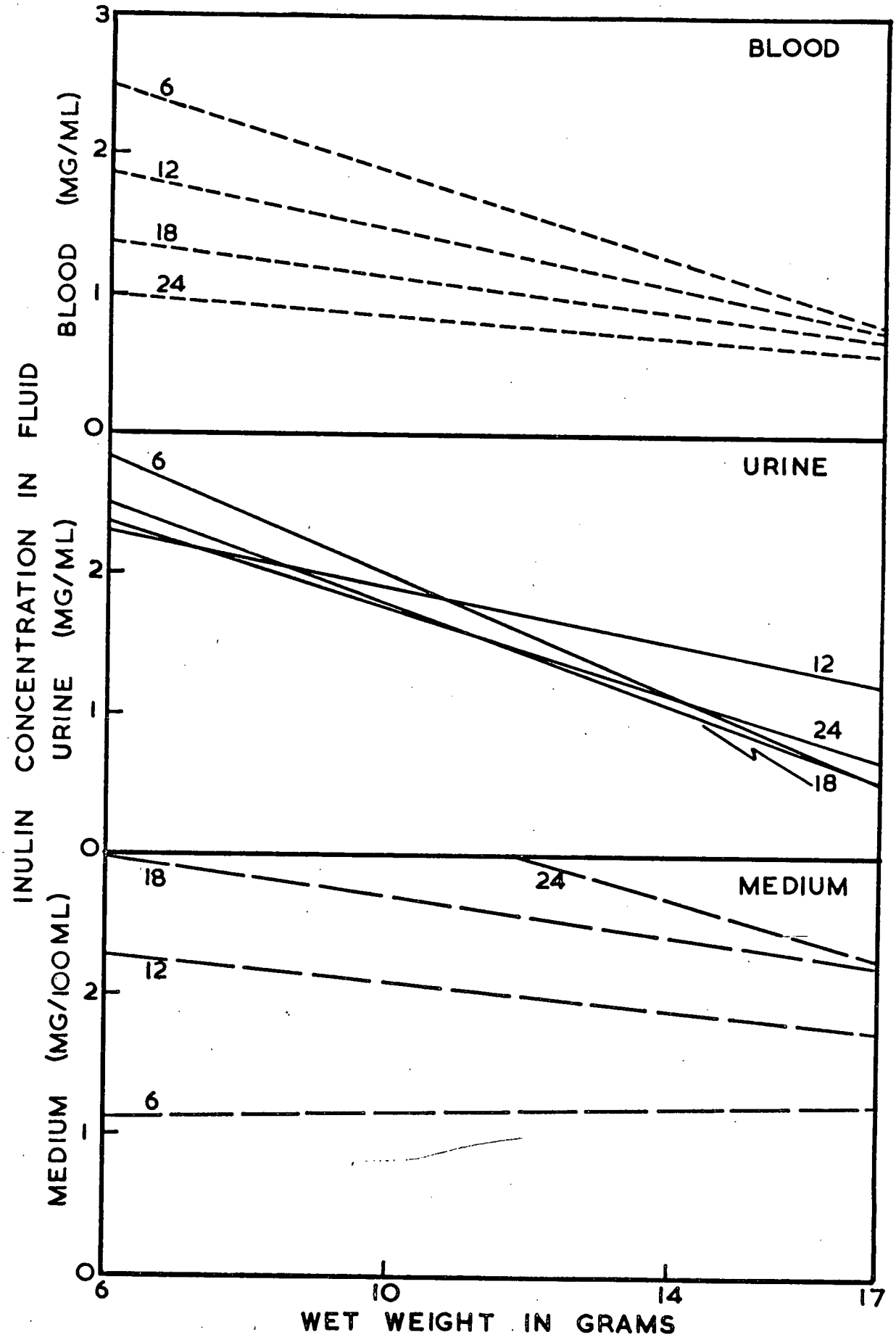
Summer, 25‰

	Blood	Urine	Medium
6 hrs.	$Y=3.396-0.153X$ $P<.001$ (12) $s_{y.x}$ 0.277	$Y=3.215-0.136X$ $P>.100$ (7) $s_{y.x}$ 0.609	$Y=1.680-0.052X$ $P<.050$ (12) $s_{y.x}$ 0.240
12 hrs.	$Y=3.442-0.202X$ $P<.001$ (10) $s_{y.x}$ 0.225	$Y=3.225-0.177X$ $P<.005$ (10) $s_{y.x}$ 0.348	$Y=1.836-0.031X$ $P>.100$ (10) $s_{y.x}$ 0.570
18 hrs.	$Y=1.902-0.064X$ $P<.025$ (9) $s_{y.x}$ 0.120	$Y=3.837-0.211X$ $P<.025$ (9) $s_{y.x}$ 0.386	$Y=3.719-0.185X$ $P>.100$ (9) $s_{y.x}$ 0.609
24 hrs.	$Y=1.220-0.040X$ $P<.025$ (10) $s_{y.x}$ 0.142	$Y=1.886-0.072X$ $P<.025$ (10) $s_{y.x}$ 0.234	$Y=3.856-0.106X$ $P>.100$ (10) $s_{y.x}$ 0.707
			medium-with-urine
24 hrs.	$Y=1.466-0.066X$ $P<.050$ (9) $s_{y.x}$ 0.124	added to medium	$Y=5.156-0.253X$ $P<.100$ (9) $s_{y.x}$ 0.582

Table XI. Regression equations of inulin concentration (Y) in blood and urine (mg/ml) and in medium (mg/100 ml) as a function of wet weight (X) for four time periods, 6, 12, 18 and 24 hours, for the summer season, in 75% salinity, using Warner-Chilcott inulin. Number of samples is in brackets. Probability that b equals zero and the standard error of estimate, $s_{y.x}$ (square root of the residual variance about regression) are indicated for each regression.

Summer, 75%	Blood	Urine	Medium
6 hrs.	$Y=4.235-0.249X$ $P<.005$ (10) $s_{y.x}$ 0.370	$Y=5.088-0.299X$ $P<.050$ (10) $s_{y.x}$ 0.782	$Y=1.023-0.028X$ $P>.100$ (10) $s_{y.x}$ 0.118
12 hrs.	$Y=1.832-0.056X$ $P>.100$ (10) $s_{y.x}$ 0.313	$Y=2.503-0.089X$ $P<.100$ (10) $s_{y.x}$ 0.418	$Y=3.183-0.118X$ $P<.050$ (10) $s_{y.x}$ 0.472
18 hrs.	$Y=2.191-0.111X$ $P<.025$ (15) $s_{y.x}$ 0.350	$Y=3.128-0.153X$ $P<.025$ (15) $s_{y.x}$ 0.524	$Y=2.245-0.038X$ $P>.100$ (15) $s_{y.x}$ 0.973
24 hrs.	$Y=1.661-0.063X$ $P<.001$ (12) $s_{y.x}$ 0.114	$Y=2.872-0.110X$ $P<.025$ (12) $s_{y.x}$ 0.474	$Y=2.058-0.0.013X$ $P>.100$ (12) $s_{y.x}$ 0.458
24 hrs.	$Y=1.672-0.058X$ $P<.050$ (12) $s_{y.x}$ 0.224	added to medium	medium-with-urine $Y=2.874-0.085X$ $P>.100$ (12) $s_{y.x}$ 0.500

Figure 8. Regression lines for inulin concentrations in blood and urine (mg/ml) and inulin amounts in the medium (mg/100 ml) as a function of wet weight. Lines, given for four time periods after injection, 6, 12, 18 and 24 hours, for each of three fluids, blood, urine and medium, during winter, in 25% salinity, are taken from Table VIII.


BLOOD
URINE
MEDIUM
6
12
18
24
INULIN CONCENTRATION IN FLUID
BLOOD (MG/ML)
URINE (MG/ML)
MEDIUM (MG/100 ML)
0
1
2
3
6
10
14
17
WET WEIGHT IN GRAMS

hours was equally as good a measure as medium-with-urine inulin amounts of the total amount of inulin excreted at 24 hours. It can be seen ($s_{y.x}$ values, Tables VIII to XI) that inulin concentration in the blood tended to be less variable than in the other body fluids and amount of inulin in the medium is the most variable quantity. Tables VIII to XI show that in most cases blood and urine inulin concentrations decreased significantly with increasing weight (P values). Further, inulin concentrations in both blood and urine, pooled over all times, seasons, and salinities, decreased significantly with increase in weight (P values, Table XII). This relationship between weight and inulin concentration in the blood, illustrated in Figure 8 for winter crabs in 25% salinity, was a direct consequence of the fact that small animals have a smaller volume for distribution of a given amount of inulin than large crabs. Similarly, greater urine inulin concentration in a small crab at any time after injection is expected since the blood which is filtered by the kidney of the small crab contains a higher concentration of inulin than that filtered by the kidney of the large crab. Medium inulin amounts (Tables VIII to XI) in most cases did not statistically decrease with increase in weight of crab (P values). Nor did pooled medium and pooled medium-with-urine inulin amounts decrease significantly with increase in weight (P values, Table XII). However, a decrease in the amount of inulin in the medium is associated consistently with an increase in weight of crab, except in winter, 25% at 6 hours (Table XIII). Further, Figure 8 shows that medium inulin amounts decrease approximately at

Table XII. Pooled regression equations of inulin concentration in blood and urine (mg/ml) and in medium (mg/100 ml), combined over time, season and salinity, as a function of wet weight. Number of samples is in brackets. Probability that b equals zero and the standard error of estimate, $s_{y.x}$ (square root of the residual variance about regression) are indicated for each regression.

Corresponding blood, urine concentrations and medium amounts

Blood	Urine	Medium
$Y=2.511-0.112X$	$Y=3.188-0.143X$	$Y=2.046-0.029X$
$P<.001$ (172)	$P<.001$ (158)	$P>.100$ (171)
$s_{y.x}$ 0.399	$s_{y.x}$ 0.445	$s_{y.x}$ 0.823

Corresponding 24 hr. blood concentrations and medium-with-urine amounts

Blood	Medium-with-Urine
$Y=1.606-0.056X$	$Y=3.189-0.067X$
$P<.001$ (39)	$P<.100$ (40)
$s_{y.x}$ 0.222	$s_{y.x}$ 0.650

All blood concentrations

Blood
$Y=2.285-0.097X$
$P<.001$ (211)
$s_{y.x}$ 0.401

the same rate with increase in weight at, for example, 24, 18 and 12 hours after injection as does blood concentration at 12, 18 and 24 hours respectively. Thus, it is felt that the relationship between weight and amount of inulin in the medium is biologically, though not statistically, significant.

Inulin concentration in the urine tended to be greater than that in the blood of the same crab (data not shown for individual crabs). This fact is biologically important in that it indicates that water is being resorbed from the blood filtrate, assuming filtration is the mode of urine formation. The relative concentrations of inulin in urine and blood are expressed as a urine/blood (U/B) ratio which is 1 if the two concentrations are equal. Urine/blood ratios were taken from the regression equations (Table VIII to XI) for a 6 gram crab and a 16 gram crab at the four time periods after injection, in two seasons and salinities and are shown in Figure 9 A, B. For example, in winter, 25% salinity (Table VIII), urine concentration for a 6 gram crab at 6 hours is 4.105-0.212x6 and blood concentration is 3.406-0.154x6 giving a U/B ratio at this time of 2.833 ÷ 2.482 = 1.141. These arbitrary weights, 6 and 16 grams, bracket the range of weights used experimentally. Figure 9 A, B shows that in both seasons and salinities U/B ratios tend to be greater than 1 and to generally increase with time. This increase with time is attributable to the fact that blood inulin concentrations (Fig. 8) for a particular weight of crab, e.g. 10 grams,

decreased more rapidly with time than did urine concentrations at the same weight. This will be more evident in Fig. 10 A, B. Table XIII shows that urine inulin concentrations as a function of weight of crabs are significantly higher than blood inulin concentrations after 12 hours in virtually every season and salinity. Thus, U/B ratios considered for all weights of crabs as a group are significantly higher than 1 after 12 hours. The fact that three U/B ratios (Fig. 9 A, B) at 18 hours for the 16 gram crab are below 1 does not contradict this statement. The reason will be explained below. In both seasons (Fig. 9 A, B) the 6 gram crab at nearly each time after injection has a U/B ratio greater than that of the 16 gram animal in the same salinity, except in winter and summer 25% in which at 12 hours the 16 gram crab has greater U/B ratios than the 6 gram crab. Larger U/B ratios exhibited by small animals results from the fact that urine inulin concentration decreases at a greater rate with increasing weight than does blood inulin concentration at any time (Fig. 8). This difference in rate of decrease with increase in weight is not statistically significant. However, urine concentrations are still generally higher than blood concentrations for extremely large animals, e. g. 16 grams. In some seasons and salinities, at certain times such as summer, 25%, 18 hours, urine concentration decrease with increasing weight resulted in urine concentrations lower than those in the blood for large crabs, e. g. 16 grams (Fig. 9B). It appears, then, that small crabs tend to absorb more water from the urine filtrate than do larger animals. Reference to Figure 9A shows

Figure 9. Ratio of inulin concentration in the urine to that in the blood (U/B) as a function of time after injection, for two weights of crabs, 6 grams and 16 grams, during winter (A) and summer (B) seasons, in 25% and 75% salinities.

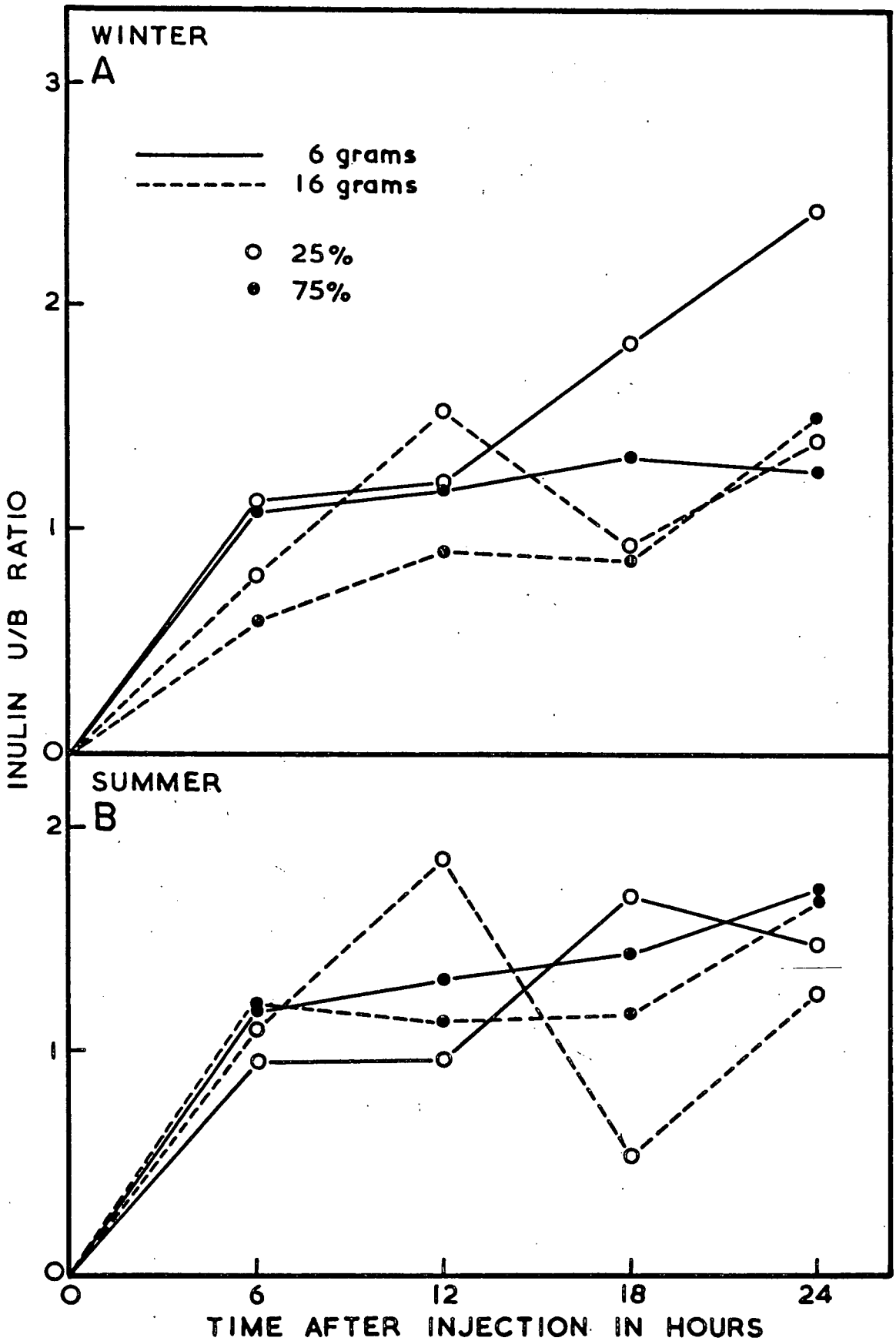

WINTER
A
6 grams
16 grams
25%
75%
SUMMER
B
INULIN U/B RATIO
0
1
2
3
0
6
12
18
24
TIME AFTER INJECTION IN HOURS

Table XIII. Results of Welch test, on Y-intercepts, and sign test to determine if urine lines lie above corresponding blood lines for four time periods, 6, 12, 18 and 24 hours, during two seasons, winter and summer, in two salinities, 25% and 75%. P values from the Welch test are the probability that the Y-intercepts of blood and urine inulin regressions as a function of weight are the same. P values from the sign test indicate the probability that urine inulin concentrations lie equally above and below blood concentrations from the same set of crabs.

Season	Salinity	Time Period							
		6 Hours		12 Hours		18 Hours		24 Hours	
		P value from Welch test	P value from sign test	P value from Welch test	P value from sign test	P value from Welch test	P value from sign test	P value from Welch test	P value from sign test
winter	25%	>0.100	>0.100	<0.050	<0.010	<0.001	<0.010	<0.001	<0.010
winter	75%	>0.100	>0.100	>0.100	>0.100	<0.005	<0.010	<0.010	<0.010
summer	25%	>0.100	>0.100	>0.100	>0.100	<0.050	<0.050	<0.005	<0.010
summer	75%	>0.100	<0.050	>0.100	<0.050	<0.005	<0.010	<0.001	<0.010

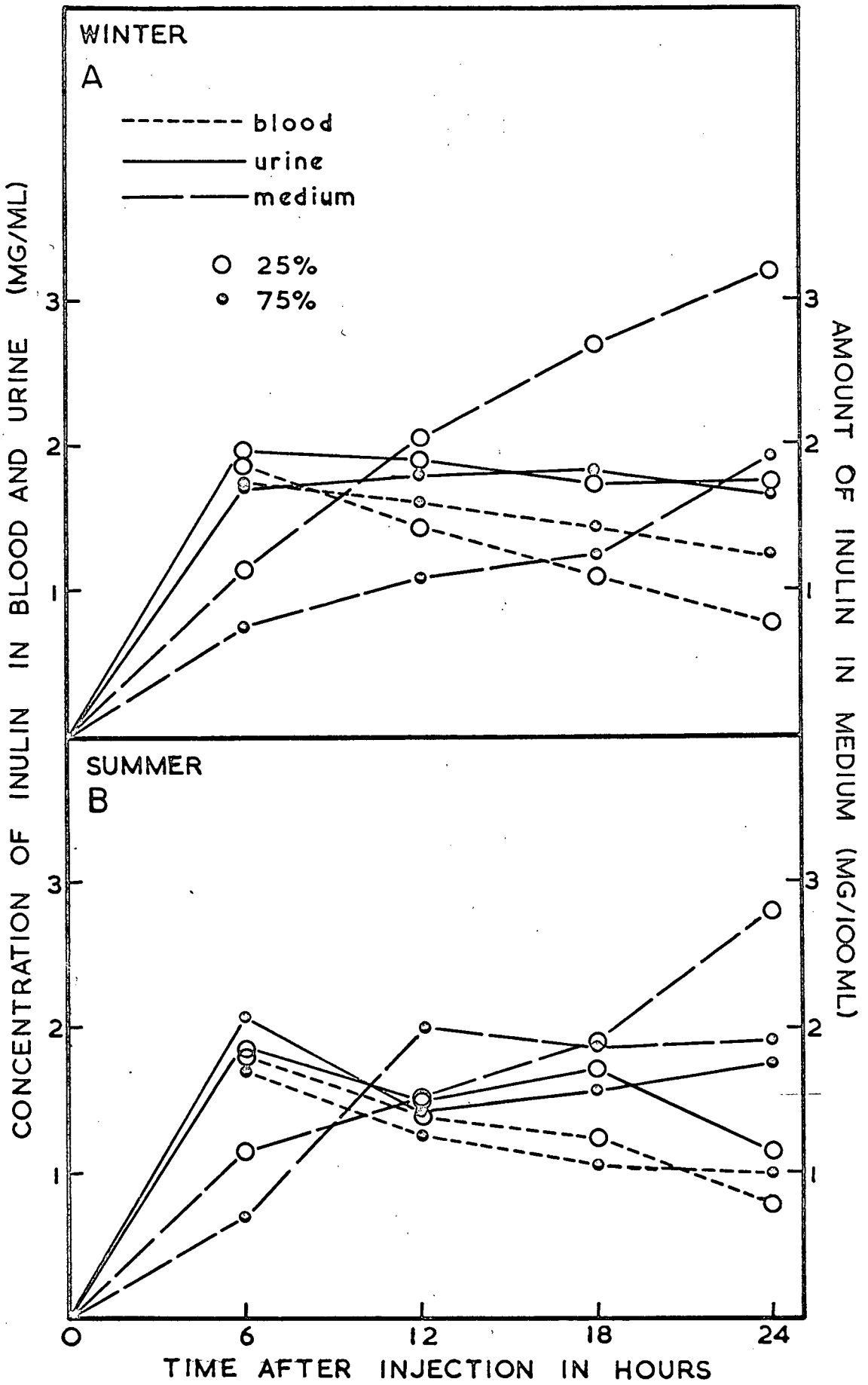
that in the winter season, the U/B ratios for each weight of crab tend to be greater in 25% than in 75% salinity. In summer, (Fig. 9B), U/B ratios for each weight tended to be greater in 75% than in 25%, excepting those of the 16 gram crab at 12 hours and the 6 gram crab at 18 hours. Differences were not tested statistically. It would seem, then, that winter crabs resorb more water from the urine in low salinity and summer crabs resorb more in the higher salinity.

Figure 8 illustrates a further trend. It appears that blood and urine inulin concentrations become less pronounced functions of weight as time progresses from 6 to 24 hours after injection, i.e. the slopes of these lines decreases with time. The amount of inulin in the medium becomes a more pronounced function of weight as time progresses, i.e. the slopes of these lines tends to increase with time. In only one case, for blood, in winter, 25% salinity (Fig. 8) was this change with time of the inulin concentration relationship with weight statistically significant. The biological significance of this trend will be considered later.

Inulin concentration changes in blood, urine and medium with time are shown for a 10 gram crab (Fig. 10). This weight is close to 10.234 grams, the average weight of 243 crabs sampled, and thus, is considered to be the most representative. Concentrations at each time were determined from regression equations in Tables VIII to XI at X=10 grams. For example, in Table VIII, blood inulin concentration at 12 hours is 2.486-1.020 = 1.466 mg/ml and amount of inulin in the

medium at 12 hours is 2.600-0.520=2.080 mg/100 ml. Figure 10 A, B shows that blood inulin concentration decreases with time in both salinities. Urine inulin concentration in 25% salinity reaches a peak at 6 hours, then tends to decrease with time, slowly for winter crabs (Fig. 10A) and with fluctuations for summer crabs (Fig. 10B). In 75% salinity, urine concentrations may be interpreted to show slight increase with time (Fig. 10, A, B) although, in summer, concentrations after 6 hours do not exceed that at 6 hours. Thus, blood inulin concentration falls more rapidly than that of the urine. This accounts for the increase in U/B ratio with time shown in Fig. 9 A, B. The amount of inulin in the medium is seen to increase with time in both seasons and salinities (Fig. 10 A, B). The effect of salinity is that blood concentration decrease with time and increase in the amount of inulin in the medium occur at a greater rate in 25% than in 75% salinity (Fig. 10 A, B). Thus, crabs in the lower salinity are excreting more inulin per unit time than those in the higher salinity. Since urine concentration shows only slight changes with time in this salinity, described above, greater inulin excretion can be accounted for only as greater urine production in this salinity compared with 75%. The effect of season on changes in inulin concentration with time are less obvious. Winter and summer concentration in the blood are very similar in 25% salinity, but decreases at a faster rate in summer than in winter in 75% salinity, (Fig. 10 A, B). In both salinities, urine inulin concentrations may be slightly higher in winter compared with summer (Fig. 10 A, B). In 25%

Figure 10. Concentration of inulin in blood and urine (mg/ml) and amount of inulin in medium (mg/100 ml) as a function of time for a 10 gram crab, during winter (A) and summer (B) seasons, in 25% and 75% salinities. The left-hand ordinate indicates blood and urine concentrations, the right-hand gives amount of inulin in the medium.


WINTER
A
blood
urine
medium
25%
75%
SUMMER
B
CONCENTRATION OF INULIN IN BLOOD AND URINE (MG/ML)
AMOUNT OF INULIN IN MEDIUM (MG/100 ML)
1
2
3
0
6
12
18
24
TIME AFTER INJECTION IN HOURS

salinity, winter medium inulin amount is higher at each time than in summer (Fig. 10 A, B) indicating that winter crabs excrete more inulin per unit time than summer crabs. In 75% salinity, however, summer medium inulin amount is higher than winter at two times, 12 and 18 hours, and equal at 6 and 24 hours. Concentration differences were not tested statistically. The greater urine excretion by winter crabs in 25% salinity could be due to greater urine production and to the slightly higher urine concentrations compared with those of summer crabs. Winter blood inulin concentrations, however, do not fall more rapidly than those of summer animals as would be expected. Inulin must be removed from the blood in order to appear in the medium. In 75% salinity, the somewhat larger summer excretion of inulin could be explained as greater urine production by these crabs compared with those in winter. In 75% salinity, blood concentration shows the expected slightly greater rate of decrease in summer. There is the suggestion, then, that greater urine production is occurring in 25% salinity than in 75%, in both seasons. In 25% salinity, urine production in winter crabs may exceed that of summer crabs in the same salinity. In 75%, the reverse may be occurring, that of greater urine production by summer crabs. Inulin concentration changes with time are not shown for crabs smaller and larger than 10 grams since these concentrations are merely functions of the regressions given in Tables VIII to XI. Decrease in blood inulin concentration and increase in amount of inulin in the medium with time tend to be greater for a crab smaller

than 10 grams and to be less pronounced for a crab larger.

Calculated urine production as per cent body weight/24 hours for crabs weighing 6 to 16 grams, in two seasons and salinities are given in Table XIV. At each of these integral weights (6, 7, 8 to 16 grams) the amount of inulin in the medium at 24 hours was divided by the average urine inulin concentration averaged over the 24 hours to calculate the volume of urine produced by that weight of crab. When points on one line (medium inulin amounts as a function of weight) are divided by points on a second line with different slope (average urine inulin concentration as a function of weight), the results lie on a curve. Thus, the apparent decrease then increase in urine production as weight of crab increases from 6 to 16 grams in winter and summer crabs in 75% and winter crabs in 25% (Table XIV) is believed to be an artifact of the calculations and not a biological phenomenon. Further, the weight at which minimum urine production occurs is not consistent in these four experimental situations. Lack of relationship between urine production and weight is indicated statistically by confidence intervals (Table XIV). The interval for the 6 gram crab, for example, brackets the urine production by every other weight of crab in each season and salinity. Thus, there is no significant effect of weight on urine production. The calculated urine productions for the 10 gram crab; 16.890% body weight/24 hours in winter, 25% salinity; 11.896% body weight/24 hours in winter, 75% salinity; 16.930% body weight/24 hours in summer, 25% salinity; and 11.440% in summer, 75% salinity, are considered to be the best measurements of urine production obtainable from these data

Table XIV. Urine production as per cent body weight per 24 hours for crabs weighing 6 to 16 grams, during two seasons, winter and summer, in two salinities, 25‰ and 75‰. Confidence intervals are given on the urine productions of 6, 10 and 16 gram crabs in each season and salinity.

Weight of Crab in grams	Urine Production as ‰ body weight/24 hrs.			
	Winter 25‰	Winter 75‰	Summer 25‰	Summer 75‰
6	22.533	18.050	28.233	16.267
7	20.271	15.771	24.200	14.414
8	18.688	14.100	21.175	13.088
9	17.600	12.844	18.822	12.122
10	16.890	11.896	16.930	11.440
11	16.472	11,182	15.391	10.964
12	16.358	10.658	14,100	10.692
13	16.523	10.315	13.008	10.615
14	17.021	10.150	12.071	10.750
15	17.920	10.187	11.260	11.140
16	19.406	10.488	10.538	11.919

99% Confidence Intervals on Urine Productions

6	Winter, 25‰	$6.150‰ < \mu < 38.917‰$
	Winter, 75‰	$7.383‰ < \mu < 28.717‰$
	Summer, 25‰	$-5.417‰ < \mu < 61.883‰$
	Summer, 75‰	$-4.233‰ < \mu < 36.867‰$
10	Winter, 25‰	$11.103‰ < \mu < 22.750‰$
	Winter, 75‰	$8.026‰ < \mu < 15,766‰$
	Summer, 25‰	$6.400‰ < \mu < 27,460‰$
	Summer, 75‰	$4.520‰ < \mu < 18.360‰$
16	Winter, 25‰	$5.344‰ < \mu < 33.469‰$
	Winter, 75‰	$0.388‰ < \mu < 20.588‰$
	Summer, 25‰	$-11.688‰ < \mu < 32.763‰$
	Summer, 75‰	$-5.650‰ < \mu < 29.488‰$

since they have the smallest confidence intervals. These urine productions are assumed to apply to all crabs in the weight range. It is clear that urine production is greater in 25% than in 75% salinity in both winter and summer. Overlap of confidence intervals indicates no statistical difference in urine production in the two salinities. Season apparently has no effect on urine production in 25% salinity. It appears, however, that urine production in 75% salinity, is slightly higher in winter than in summer. No seasonal effect was demonstrated statistically.

The rate of inulin clearance from the blood of a 10 gram crab in winter was 23.79% body weight/24 hours in 25% salinity and 13.83% in 75% salinity. In summer crabs, inulin clearance was 19.55% body weight/24 hours in 25% salinity and 15.79% in 75% salinity. These values indicate that the blood is being cleared of inulin in both seasons more quickly in 25% than in 75% salinity. It appears that in 25% salinity, the blood is cleared more quickly in winter. In 75%, the reverse occurs and the blood is apparently cleared more quickly in summer crabs. Since a constant amount of inulin was injected into all crabs these results are consistent with the conclusions drawn from Figure 10 A, B. These were that in 25% salinity winter crabs excrete more inulin per unit time than summer crabs and in 75% salinity inulin excretion is apparently slightly higher by summer crabs.

In conclusion, urine production measurement by the inulin excretion method gave values for a 10 gram crab of about 17% body weight/24 hours in 25% salinity and 11% or 12% body weight/24 hours in 75% salinity. An effect of salinity on urine production is apparent but not statistically significant. The possible effect of season is not obvious.

These results indicate consistently, by blockage with dental cement, and with M2C-2, and by inulin excretion that urine production is markedly higher in 25% salinity compared with 75% salinity. The seasonal effect is neither marked nor consistent. The M2C-2 blockage data suggest strongly that urine production is higher in winter in 25% salinity and shows no seasonal effect in 75% salinity. It was reasoned, without statistical support, from rates of inulin excretion by a 10 gram crab that in 25% salinity urine production is higher in winter than in summer and further, in 75% salinity urine production may be higher in summer than in winter. Urine production calculated from the inulin injection data for the 10 gram crab were virtually identical in 25% salinity, while in 75% they were slightly higher in winter than in summer. The effect shown by the M2C-2 blocked crabs is the more consistent and the only one shown to be statistically significant. Thus, if a seasonl effect does, in fact, exist it appears to be that of higher urine production by winter crabs in low salinity.

DISCUSSION

Consideration of concentration of inulin in blood and urine and amount of inulin in the medium in relation to each other and as influenced by weight of crab and time leads to several conclusions. Concentrations of inulin in the urine greater than those in the blood are interpreted to be an indication of water resorption from the blood filtrate, since inulin itself has been shown to be neither resorbed nor secreted by the vertebrate kidney tubule (Richards, Bott, Westfall & Landis, 1938). It is possible that urine concentrations lag behind those in the blood as the latter falls accounting for at least a portion of the difference. Inulin U/B ratios greater than 1 were obtained by Riegel & Kirschner (1960); Riegel (1961) in crayfish and attributed to absorption of water from the blood filtrate. Riegel & Lockwood (1961) from ion studies concluded water resorption was occurring from the urine of Carcinus maenas in 100% sea water. Although inulin is assumed by virtually every worker concerned with it to not be actively resorbed or secreted, Maluf (1941c) interprets his results as indicating that inulin, as well as most other substances in the urine, is secreted by the antennary gland of crayfish. Since inulin U/B ratios appear to decrease to 1 at high blood inulin concentrations, he concludes that this is evidence of an active transport mechanism becoming saturated at these high levels. In opposition to this, Forster & Zia-Walrath (1941) concluded that inulin

is excreted solely by filtration in Homarus americanus. Riegel & Kirschner (1960), further, showed that the reasoning which led Maluf to his conclusion of inulin secretion is faulty. One direct piece of evidence for transport of inulin is the work of Scott, Maude, Shehadeh & Solomon (1964) which suggests that absorption by pinocytosis is occurring in Nectures proximal tubules. It is felt that, although inulin is not the ideal substance for these studies it was formerly thought to be (Cotlove, 1954; Bassir, 1956; Phelps, 1965), it may at least not be actively absorbed or secreted by the kidney.

Indication was given that blood inulin concentration became less a pronounced function of weight as time progressed (Fig. 8). Further, the amount of inulin in the medium became a more pronounced function of weight as time progressed (Fig. 8). It is interpreted from these trends, that small crabs excrete proportionately more inulin per unit time than large crabs. Since small crabs do not produce proportionately a greater urine volume, they must put out a more concentrated urine. More concentrated urine, relative to the blood, in small crabs was shown by greater U/B ratios in 6 gram than in 16 gram crabs. Presumably, small crabs pass proportionately more blood per unit time through the kidney than the larger animals, and absorb more water from the blood filtrate. It would be of interest to know the effect of crab weight on the rate of excretion of other substances handled by the antennary gland. Considerable evidence indicates that small members of

of a species maintain higher metabolic rates than larger animals (Prosser & Brown, 1961) and it is possible that the weight effect observed here on inulin excretion is simply a result of this differential metabolic rate.

The primary mode of urine formation in crustaceans is most commonly assumed to be filtration (Riegel & Kirschner, 1960; Riegel, 1961). On the other hand, Maluf (1941c) reasoned that secretion must be the primary mechanism since there is no glomerulus-like structure in the decapod kidney against which hydrostatic pressure could act, and further, the gland histologically shows abundant secretory activity (Maluf, 1941a; Flemister, 1959). However, the antennary gland is well supplied with arteries (Parry, 1955; Riegel & Kirschner, 1960) and the sinusoids surrounding the end sac may well maintain sufficient hydrostatic pressure to allow filtration. Picken (1936) has demonstrated that hydrostatic pressure of the blood of Carcinus maenas even in the large sternal sinus exceeds the difference between colloid osmotic pressures of blood and urine, and thus, that filtration is mechanically possible. There is evidence that the presumptive urine is altered by secretion and resorption (Nagel, 1934; Peters, 1935). The secretory appearance of the antennary gland histologically does not necessarily imply that secretion is the primary event in urine formation. Indeed, it is quite compatible with Picken's (1936) hypothesis of blood filtrate elaborated by secretion and absorption.

Urine production as per cent body weight has been measured in Hemigrapsus nudus and is considered to be constant for all weights of crabs in the range sampled. Evidence suggests that urine flow is greatly influenced by salinity and perhaps to a lesser degree by season.

Virtually no attempt has been made in the literature to determine if urine production as per cent body weight changes with weight of individuals. Maluf (1941c) studying inulin excretion in the crayfish, Cambarus clarkii, deduced that urinary flow must be constant with weight since Herrmann (1931) suggests that urine flow is determined by the rate of diffusion of water into the crayfish.

The rate of urine production in Hemigrapsus nudus was found to increase with decrease in salinity, in both blocked and inulin-injected crabs, and to be greater the lower the salinity. The relationship between salinities hypotonic to the blood of regulating crabs and increased urine flow is well documented in the literature. Nagel (1934) found that the rate of urine flow in Carcinus increased from 10% body weight/day in 100% sea water to 17% body weight/day in 50% sea water. Carcinus blood is hypertonic to these salinities (Prosser & Brown, 1961). Shaw (1961) determined urine production by Carcinus maenas and found an increase in urine flow in salinities less than 100% sea water. For example, urine production in 100% sea water was 3.6% body weight/day and in 40% sea water was 31.0% body weight/day. Parry (1955) studied

the effect of salinity on urine production in the brackish water prawn, Palaemonetes varians. The pattern of excretion found by several methods was minimal flow in salinities which were isosmotic with the blood (60% to 70% sea water) and increased flow in salinities hypo- and hyperosmotic to the blood. Gross & Marshall (1960) reported the rate of urine production for Pachygrapsus crassipes as 1.5% body weight/day in 150% sea water, and 58% body weight/day in 50% sea water. According to Jones (1941) P. crassipes maintains blood hyperosmotic to salinities below about 80% sea water and hyposmotic to those above. In order to explain the occurrence of this increased urine flow in salinities hypos-motic to the blood, it is hypothesized that this phenomenon eliminates excess water which has entered the body osmotically. This serves, then, to regulate body volume.

To show that these regulating brackish-water decapods are indeed affect by osmotic gradients between blood and medium, evidence that the body surface of crustaceans is permeable to water is necessary. Experiments with heavy water suggest a rapid exchange of water in Palaemonetes regardless of external salinity (Parry, 1955). Evidence indicates that the exoskeleton and gills are most certainly permeable to water though to a lesser degree in the shore crabs, Cancer and Carcinus, than in the marine Portunus and Hyas (Prosser & Brown, 1961). Nagel (1934) demonstrated that several non-regulating decapod crustaceans have more permeable exoskeletons than those which regulate. Gross

(1957) confirmed this, finding that regulators, Cambarus, Pachygrapsus, and Hemigrapsus nudus, have a low but measurable permeability to salts and water. Presumably, the permeability of the exoskeleton and gills limits the rate at which water enters osmotically (Gross, 1957). It is expected that crustaceans when placed into salinities hyposmotic to the blood immediately gain water osmotically, in isomotic salinities will demonstrate no net exchange of water and in salinities hyperosmotic to the blood will lose water osmotically. This pattern of events has been found to occur in Maja (Schwabe, 1933) and in Palaemonetes (Parry, 1955).

A direct relationship between urine production and the osmotic gradient which exists between blood and medium has been suggested by Gross & Marshall (1960) and Potts & Parry (1964). This gradient has been defined as the difference in osmotic concentration between blood and medium expressed as per cent sea water (Dehnel, 1962). The possibility that urine production in Hemigrapsus nudus is proportional to the existing blood-medium gradients was examined. Blood-medium gradients were taken from Dehnel (1962, Fig. 5 and 7) for H. nudus in winter and summer seasons at 15°C. in 25%, 75% and 125% salinities. Data were chosen at 15°C. since this temperature is more equivalent in its effect on this species to 10°C., the temperature of the present study, than is 5°C. also documented by Dehnel (1962). Blood is always hypertonic to the medium in the salinities considered. The animal is,

thus, always subject to osmotic inflow of water. Urine production values for the three sets of determinations, dental cement blockage, M2C-2 blockage and inulin injections are shown in Table XV. The respective blood-medium osmotic gradients are indicated for the three salinities and two seasons. The gradient in winter shows an increase of 2.9 times in 25‰ salinity, where it is 57‰, compared with the gradient in 75‰ salinity, which is 20‰. In 75‰ salinity, urine production of M2C-2 blocked crabs is 6.0‰. The rate of urine production increases 2.8 times in 25‰ salinity, being 16.7‰ body weight/24 hours. Urine production determined by the inulin method gave values of 11.9‰ body weight/24 hours in 75‰ salinity and 16.9‰ in 25‰ salinity. This is a 1.4 times increase in urine production for a 2.9 times increase in the gradient. For summer crabs, the blood-medium gradient in 75‰ (17‰) is 1.06 times the gradient in 125‰ (16‰). Over the same salinity range urine production is shown to have increased 1.9 times by the dental cement blockage method. The gradient for summer crabs in 25‰ (58‰) is 3.4 times larger than that in 75‰ (17‰). The increase in urine production in 25‰ salinity compared with 75‰ salinity is 3.0 times for dental cement blockage, 2.3 times for M2C-2 blockage and 1.5 times for the inulin method. Thus, it appears that the urine production values of H. nudus obtained by the blockage technique are roughly proportional to the blood-medium gradients in the three salinities considered. Urine production values measured by inulin injection do not fit the hypothesis as well. The discrepancy is caused by the inulin urine

Table XV. Urine production by Hemigrapsus nudus as per cent body weight/24 hours determined by three techniques, dental cement blockage, M2C-2 blockage and inulin injection, in some, but not all of three salinities, 25‰, 75‰ and 125‰, and two seasons, winter and summer. Blood-medium osmotic gradients indicated for each season-salinity combination are taken from Dehnel (1962). The ratios 25‰:75‰ and 75‰:125‰ indicate the relative magnitude of urine production and osmotic gradient in the lower salinity compared with the higher salinity.

Salinity	Urine Production as % Body Weight			Blood-Medium Osmotic Gradient
	Dental Cement	M2C-2	Inulin Injection	
Winter				
25‰	--	16.672	16.890	57‰
75‰	--	6.012	11.896	20‰
25‰:75‰	--	2.8	1.4	2.9
Summer				
25‰	11.964	14.842	16.930	58‰
75‰	3.966	6.449	11.440	17‰
125‰	2.071	--	--	16‰
25‰:75‰	3.0	2.3	1.5	3.4
75‰:125‰	1.9	--	--	1.0

production values in 75% salinity which are about twice as great as those obtained by the blockage method in the same salinity. The reason for these high values is not clear.

A clear-cut seasonal effect on urine production is not evident from the data in Table XV. It is believed, however, that were such an effect present, it would act by altering blood-medium osmotic gradients. Thus, salinity and season presumably both affect the gradient which in turn determines the volume of urine produced.

If it can be shown that the volume of Hemigrapsus nudus is regulated, then it may be concluded that the kidney contributes to this regulation. Regulation of body volume has been demonstrated in H. nudus by Dehnel (1962). Winter crabs regardless of temperature (5° and 25°C.) or salinity (6% to 175%) maintained a mean body water content of 62% body weight. (Gross (1957) demonstrated that volume changes in P. crassipes in varying salinities are insignificant and that the solute space remains constant.

In summary, Hemigrapsus nudus in all salinities is considered to be subject to osmotic entry of water. Since urine production appears to be dependent on the osmotic gradient existing between blood and urine and the body volume is maintained constant in all salinities, it is believed that the antennary gland functions to maintain constant body water volume. It is possible that extra-renal mechanisms in addition perform this function.

It is suggested that together with the often-discussed terms,

osmoregulation and ionic regulation, a third should be given equal consideration. This is volume regulation or water balance. Water balance would include elimination of excess water from the body by the kidney, gut, gill and other possible routes when blood is hyperosmotic to the medium, and the uptake of water from the medium by gill (Burger, 1957) and gut (Burger, 1957; Green, Harsch, Barr & Prosser, 1959) when blood is isosmotic and hyposmotic to the external salinity. In contrast to the other terms, water balance considers osmotic gradients rather than absolute concentrations.

Water balance as an active process can be applied to poikilosmotic crustaceans as well as to those which regulate blood osmotic concentrations. The former species maintain no osmotic gradient between blood and medium since blood osmotic concentration approaches that of the external salinity. It is expected that urine flow be constant regardless of external salinity, and further, that it be low. Maja, isosmotic with the environment once equilibrated (Schwabe, 1933), has a rate of urine production of 3.0% body weight/24 hours (Bialaszewicz, 1932). Cancer in 100% sea water has a urine flow of 3.0% to 10.0% body weight/24 hours (Robertson, 1939). This crustacean similarly is isosmotic with the environment (Krogh, 1939). The requirement of ionic regulation should account solely for kidney activity since there is no osmotic water to eliminate. In this case, water balance involves only the net uptake of water needed to balance that lost via the urine.

Fresh-water crustaceans are in a similar situation as H. nudus in that they universally maintain blood hypertonic to the medium. Urine production values determined for fresh-water crustaceans in this environment are usually lower than those of brackish-water and marine species maintaining similar blood-medium osmotic gradients. Eriocheir in fresh-water puts out a urine volume 4.2% body weight/24 hours (Scholles, 1933). The crayfish, Cambarus clarkii, excretes 5.2% body weight/24 hours (Lienemann, 1938), 5.0% body weight/24 hours (Maluf, 1941c) in fresh water. Potamobius has a urine flow of 3.8% body weight/24 hours (Herrmann, 1931). The lower than expected urine flow in fresh water species can be attributed to low permeability of the body surface (Nagel, 1934; Maluf, 1941c) and perhaps to enhanced extra-renal elimination of water, advantageously conserving considerable salt which would be lost by copious flow.

A generalized scheme of ionic, osmotic and water regulation in brackish-water decapods is proposed. In salinities hyposmotic to the blood the antennary gland has not been shown to function in osmotic regulation (Jones, 1941; Prosser et al, 1955; Gross, 1957; Robertson, 1960) except in two species. Dehnel & Stone (1964) have shown that in Hemigrapsus nudus and H. oregonensis in winter the urine is significantly hypotonic to the blood, a clear demonstration that the antennary gland in brackish water crustaceans is capable of functioning in osmoregulation. The antennary gland has been shown to contribute to ionic

regulation (Prosser et al, 1955; Webb, 1940; Gross, 1959; Parry, 1960; Dehnel, 1965), a universal phenomenon in crustacea (Robertson, 1960), by absorption and secretion of ions. Primarily, Mg and SO_4 ions are regulated by the antennary glands. It has been demonstrated in the present study that the antennary gland, further, regulates body volume in hyposmotic salinities. Sites other than the antennary gland must function in osmoregulation and probably also in ionic regulation. By a matter of elimination, the gills have been elected as the probable sites for osmotic and ionic regulation (Gross, 1957; Flemister, 1959). There is some direct evidence to support this assumption (Koch et al, 1954; Webb, 1940). Thus, in hyposmotic salinities, gills are assumed to be the major site of osmoregulation (Robertson, 1960; Nagel, 1934). The gills, further, are very important in ionic regulation, selectively absorbing ions from the medium (Robertson, 1960). In addition to the antennary glands, other routes may exist for the elimination of excess water, for example, the gut.

In isosmotic salinities, the kidney functions solely for ionic regulation. It has been shown that in Homarus americanus (Burger, 1957) the activity of ionic regulation can control the volume of urine produced. The injection of magnesium sulfate or chloride into animals in 100% sea water, in which blood and medium are isosmotic, causes an increased magnesium excretion by increased volume of urine released per unit time. Similarly, high sulfate concentration in the blood has a

diuretic effect but in this case, the concentration of sulfate in the urine was increased also. The antennary glands do not function in volume regulation since no water enters osmotically. It is necessary for an amount of water to be taken up from the medium equal to that lost via the urine (Lockwood, 1962). It is considered that "most of the observed uptake is an active process" (Robertson, 1960; Webb, 1940). There is no evidence, however, for active transport of water. Thus, presumably "active uptake" of water may be interpreted to mean transport in association with an actively transported substance. Water uptake in the isosmotic situation may be linked with ionic regulation by the gills. For example, water may follow actively transported sodium and chloride as they are taken up by the gills (Lockwood, 1962). The intake of water through the gut contributes as a water balance mechanism in Homarus americanus, since this lobster drinks sea water intermittently and absorbs the stomach contents completely (Burger, 1957). Green, Harsch, Barr & Prosser (1959) also consider water absorption from swallowed sea water important in water balance.

In salinities hyperosmotic to the blood, there is no evidence in brackish water forms to suggest that the antennary gland functions in osmoregulation by producing hyperosmotic urine (Parry, 1960). The kidney maintains its usual ionic regulatory role and this presumably accounts for the volume of urine produced. Antennary gland excretion does not aid volume regulation since any urine flow tends to reduce body

water already stressed by osmotic outflow. Furthermore, since the urine is isosmotic and not hyperosmotic to the blood, the kidney does not resorb any water from the urine filtrate. Water resorption would tend to lessen the stress on body water. Unexpectedly, urine flow of Palaemonetes varians increased in hyperosmotic salinities (Parry, 1955) presumably to accommodate ionic regulation by the antennary gland (Potts & Parry, 1964). The gills in these salinities regulate ionically. Salts are secreted by gills and gut (Gross, 1955) for the purpose of osmoregulation. Water must enter the body to balance that lost in urine flow and osmotic outflow of water. This may occur in gills and gut by linkage with actively transported substances. Lockwood (1962) considers drinking of water and absorption from the gut the most likely pathway for water entry.

In brackish water species, it appears that in salinities hyposmotic to the blood, the kidney functions for ionic regulation, volume regulation and in some species for osmoregulation. In isosmotic salinities, ionic regulation alone accounts for antennary gland activity. In salinities hyperosmotic to the blood, the antennary gland again functions solely for ionic regulation, to which the gills contribute. Functions of osmotic and volume regulation in the latter situation are assumed by the gills and gut.

SUMMARY

1. Urine production by Hemigrapsus nudus was measured by blockage of nephropores with dental cement, by blockage with a physiological adhesive, methyl 2-cyanoacrylate monomer (M2C-2), and by excretion of inulin.

2. Dental cement proved somewhat ineffective giving urine production values 20% lower than those obtained by M2C-2 blockage, due to leakage of urine around the block.

3. Urine production determined by blockage with M2C-2 in winter crabs was 16.7% body weight/24 hours in 25% salinity and 6.0% in 75% salinity; in summer crabs, 14.8% in 25% salinity and 6.4% in 75% salinity. This method was judged to give the most reliable results.

4. Inulin excretion yielded urine production values for winter crabs of 16.9% body weight/24 hours in 25% salinity and 11.9% in 75% salinity; for summer crabs of 16.9% in 25% salinity and 11.5% in 75% salinity.

5. Evidence suggests that small crabs (6 grams) excrete inulin at a proportionately greater rate than large crabs (16 grams).

6. Urine production is independent of weight of crabs over the range sampled.

7. Urine production increases with decrease in salinity.

8. The effect of season is not clear but appears to cause greater urine flow in winter than in summer crabs in 25% salinity.

9. The antennary gland of H. nudus appears to regulate body volume be excreting excess water which enters osmotically.

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