OSMOREGULATION IN THE AQUATIC HEMIPTERA: *CORISELLA EDULIS* CHAMPION AND *BUENOA MARGARITACEA* TORRE-BUENO

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PREFACE

Little is known about the dynamic processes of osmoregulation in adult aquatic insects whose sclerotized body wall and respiratory plastrons present barriers to the movements of fluids and solutes between the insect and its environment. In addition, the growing problem of saline pollution present a potential hazard to these important aquatic fauna. There is much concern over improving the quality of our freshwater, and as a result, federal agencies have established criteria necessary to measure and evaluate different environmental pollutants so that knowledgeable economic tolerant limits can be established. These standards are being improved but attempts to develop them further, especially as they relate to physicochemical criteria, without knowledge of the effects on the basic functions of life therein could present problems in the future.

The present research utilized two adult aquatic hemipterans, Corisella edulis and Buenoa margaritacea, to investigate basic functional aspects associated with the stress stimuli presented to the animals by different concentrations of sodium chloride, a common pollutant of freshwater in Oklahoma. Concentrations of these ions in the haemolymph of both species were shown to be regulated over the full range of experimental salinities (0 to 0.9% NaCl) while above this level, mortality quickly ensued. The total water content and blood volume were regulated in C. edulis but the excretory system could not physiologically adjust to the high external salinities as indicated by

the ratio of the haemolymph and rectal fluid osmotic pressures approaching unity. As a result of the changes in the osmotic stress caused by variations in the environmental salinity, the rates of movement of water through the body wall of the insect were altered, oral imbibition was affected and the apparent rate of fluid secretion by the Malpighian tubules was changed. This information becomes valuable in view of its potential use for the future improvement of water quality criteria and supplements the scarcity of knowledge which has accumulated in this field of physiological ecology.

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CHAPTER I

INTRODUCTION

The problem of salt and water balance in insects is of special interest in view of the broad physiological diversity and superb adaptations of these cosmopolitan arthropods to a wide variety of environments. Most adult insects live a terrestrial existence while many of the larval stages are aquatic or semiaquatic. Some are restricted to saline water habitats while others spend parts or all of their life cycle in brackish or freshwater habitats. A critical physiological problem encountered by these insects is one of maintaining an internal (haemolymph) medium relatively constant while living in an external environment that is quite different.

Insects of terrestrial and saline environments are faced with the continual problem of water loss (dehydration) and must therefore rely upon different means for conserving water and maintaining solute levels within the limits required for the function of tissues and internal organs. On the other hand, freshwater insects must overcome the problem of blood flooding (dilution) by the development of an impermeable body wall (evasion) or an effective means of eliminating copious amounts of fluid (correction) without upsetting the delicate solute to fluid ratio of the haemolymph.

One of the chief difficulties encountered in the research of osmotic and ionic regulation in insects has been their small size.

However, in recent years, the development of ultra-micro analytical techniques together with radioactive tracers have contributed greatly to the advances in this field. Already, numerous workers have contributed to this field and many authors have reviewed aspects of this subject (Krogh, 1939; Wigglesworth, 1953; Roeder, 1953; Shaw and Stobbart, 1963; Stobbart and Shaw, 1964; Craig, 1960; Barton-Browne, 1964; Beament, 1961, 1964; Edney, 1957; Wyatt, 1961; Sutcliffe, 1962). These investigations were limited to only a few species; most of which were terrestrial insects and aquatic larvae. The adult aquatic insects were largely ignored. Work with the larger, more common and ultimately the more easily handled insects progressed into studies designed to elucidate and locate the specific mechanism by which regulation was accomplished.

Most advancements with adult aquatic insects have been achieved through the use of the hemipterans and more specifically, members of the family Corixidae. Staddon has reported on the aspects of water balance in Notonecta glauca L. and Notonecta marmorea Fabr. (1963), Corixa dentipes (Thomas.) (1964), Ilycoris cimicodies (L.) (1969a, b) and the permeability of the cuticle to water in each species save N. marmorea (1966). Oloffs and Scudder (1965) reported on the transitional phenomenon of the lipid layer in relation to the permeability of water and detailed electron microphotography of the Malpighian tubules and hindgut tissues were investigated by Jarial and Scudder (1970). The osmotic and ionic balance in the haemolymph (Scudder et al., 1972) and evidence for a relationship between water balance and neurosecretion (Jarial and Scudder, 1971) in the corixid, Cenocorixa bifida have recently emerged. The corixids used in these

investigations belong to a different genera than the ones reported on here and each genera inhabitated greatly different geographical regions of the North American continent; i.e., the southwestern region of the United States for Corisella as compared to the British Columbia region of Canada for Cenocorixa. Cenocroixa were collected from the inland lakes of British Columbia while Corisella were collected from the quiet waters of ponds in and around Stillwater, Oklahoma. A complete model for the movements of water and ions has not yet emerged and a generalized scheme is yet forth coming.

The salt content of the natural water from which C. edulis and B. margaritacea was taken was within the lower limits defined as freshwater by Prosser and Brown (1961) yet many of the streams and lakes are laden with salts. Saline pollution is a common occurrence in the streams and lakes of the Southwestern United States. Streams flowing over the relatively arid Great Plains region where vast amounts of salts are stored carry large amounts of dissolved solutes. Oil-drilling and refinery effluents contribute additional solutes which occassionally result in "kills" of aquatic biota; usually fish, however. Accumulation of these salts in reservoirs often cause chemical stratification and density flows which compound the problems of water quality by producing anoxic waters in the deeper strata (Eley et al., 1963). Physicochemical studies in relation to the community structure of benthic macroinvertebrates have received attention and a typical approach to such a study was carried out on the Cimarron arm of Lake Keystone, one of the north central Oklahoma's recently constructed reservoirs (Ranson, 1969). Species diversity and redundancy analysis were applied to a total of twenty-five individual benthic

macroinvertebrates. Variations in the community structure due to seasons, depths, and stages in the life histories were related to various water quality parameters such as turbidity, dissolved oxygen and conductivity. However, the lack of basic physiological knowledge of these macroinvertebrates did not permit a direct evaluation of the effects of these environmental parameters or what was directly responsible for the diversity indicies acquired. The diversity indices utilized (Wilhm et al., 1966) are limited in that they indicate the presence, or absence, of certain species relative to a given set of parameters, and degree of pollution but they provide no indication of the casual phenomena or the physiological effects of the different pollutants. Without knowledge of the tolerance limits of important aquatic organisms or of the basic physiological control mechanisms which allow them to cope with some of the more common pollutants, we limit our capabilities to more accurately standardize the quality of the water for the far-reaching future. New methods and techniques need to be provided which will directly relate the various water quality parameters to specific physiological malfunctions. At the cellular and tissue levels the methods of energy utilization and production begin to converge into a few common pathways and when the energy flow is blocked (possibly by some pollutant) the system ceases to function and the animal utimately succumbs to death. Knowledge of these critical energy consuming functions is important in view of the relationship of virtually all animals to variations in the different factors of the environment; e.g., the ability of C. edulis to regulate its specific body ions and water is vital and it is believed to be directly related to the concentrations of specific environmental

solutes (NaCl) and the energetics of specialized tissues probably associated with the excretory system. Nerve transmission, muscular activity, and many other normal body functions are dependent upon the proper ionic and water balance of the body (Threherne, 1970) in spite of the differences encountered with the surrounding medium. Critical dilutions and regulations of essential blood ions are buffered by the organism's energy-consuming, regulatory mechanisms.

In the past aquatic insects have scarcely been used in studies relating to water quality management because of the lack of accumulated knowledge upon which critera can be established. Today, however, knowledge concerning the physiology of insects is being accumulated. The role of the cuticle, the involvement of hormones, nutritional requirements, excretion and osmoregulation and others are now being utilized to more naturally control those species that are in competition with man in the environment. The aquatic hemipterans are truly an integral part of the freshwater environment and it seems that knowledge of this organism as well as its physiology lends itself well to an intense study on the function of osmotic and ionic regulation which relates to the saline problems of water quality.

CHAPTER II

SODIUM AND CHLORIDE REGULATION IN TWO ADULT AQUATIC HEMIPTERANS, *CORISELLA EDULIS* CHAMPION AND *BUENOA MARGARITACES* TORRE-BUENO

It is well established that the mechanisms involved in maintaining salt and water balance in aquatic insects are closely related to the salinity of the environment (Shaw and Stobbart, 1963; Stobbart and Shaw, 1964). Numerous investigators have provided valuable information about this function in aquatic larvae but only a few have done the same for the aquatic imagines (Staddon, 1963; 1964; 1966; 1969a; 1969b; Claus, 1937).

The internal composition of numerous insects has been assayed (Buck, 1953; Duchateau et al., 1953; Clark, 1958; Wyatt, 1961; Sutcliffe, 1962) and from this information comes an important generality concerning the exoptergotes. Approximately 70% of their total haemolymph osmotic pressure is accounted for by the concentration of inorganic ions, most of which are sodium and chloride (Stobbart and Shaw, 1964). Furthermore, the assumption that the cuticle of adult aquatic insects is a relatively unimportant route for the entry of water (Holdgate, 1956; Beament, 1961) is not true in the adult waterbugs (Staddon, 1963; 1964; 1966; 1969a). Typically, these animals exhibit a freshwater behaviour in the sense that their excretory

system must work continuously to eliminate water that is gained unavoidably by the osmotic uptake through the cuticle (Staddon, 1969a).

The following experiments are the first in a series designed to elucidate the mechanisms involved in maintaining salt and water balance in two adult waterbugs: Corisella edulis Champion and Buenoa margaritacea Torre-Bueno. Both are common inhabitants of Oklahoma (Schaefer, 1968, 1969) and are under the constant threat of salt pollution from oil well operations (Clemens and Jones, 1955). An assessment has been made on their ability to maintain haemolymph sodium and chloride ion concentrations over wide ranges of environmental salinity. Attempts have also been made to correlate these data and mortality rates to models that have been designed to help provide an explanation for the possible mechanisms concerned with the maintenance of ion and water balance.

Materials and Methods

C. edulis and B. margaritacea were collected as needed from ponds (water temperature 24-30^{o}C) near Stillwater, Oklahoma. Careful sweeps with an aquatic net in shallow water were made to minimize injury to the insects. Specimens were transferred to the laboratory in glass containers with water from their natural habitats and allowed to adjust to laboratory temperatures (25 ± 2^{o}C). Only freshly collected specimens acclimated in this manner were used. Experimental insects were taken directly from these containers and placed in 10 ml test tubes (one insect per tube) containing different amounts of saline. To obtain media with the fewest possible number of foreign unknown substances, only sodium chloride and deionized water were used. The

Na and Cl concentrations ranged from 0 (deionized water) to 550 mM/l. Each specimen was left unattended in an uncapped tube on a shelf receiving natural daylight for 48 hrs. The maximum change noted in the concentration of Na and Cl after this time was no more than 4% of the initial concentration. Following acclimation, mortality rates were determined.

Ion assays were made on 1 µℓ quantities of clear haemolymph collected with a micropipette from a puncture in the thorax at the base of the wing. All insects used for these assays were acclimated as previously outlined. A dissecting microscope was used to examine closely all samples collected. Occasionally, a sample appeared contaminated and was discarded. The concentrations of chloride and sodium were determined to the nearest mM/l with a Fiske/Marius micro-chlor-o-counter and a Beckman 440 atomic absorption spectrophotometer, respectively.

Results

The Concentrations of Haemolymph Sodium and Chloride in Freshly Collected *C. edulis* and *B. margaritacea*

To establish the "normal" levels of internal sodium and chloride, experiments were conducted on specimens living in and associated with water from the natural habitat. The level of chloride was lower in *B. margaritacea* (106 mM/l) than *C. edulis* (125 mM/l) but the concentration of sodium was virtually the same for both insects (167 and 168 mM/l, respectively) (Table I).

TABLE I

CONCENTRATION OF HAEMOLYMPH OF INSECTS TAKEN DIRECTLY FROM THEIR "NATURAL" HABITAT

	Sodium	Chloride
Corisella edulis (Water Boatman)	168 ± 9	125 ± 8
Buenoa margaritacea (Back Swimmer)	167 ± 14	106 ± 6

Values are expressed as mM/l Na and Cl and represent means of 20 assays with respective standard deviations.

The Effect of Salinity on the Mortality Rates of *C. edulis* and *B. margaritacea*

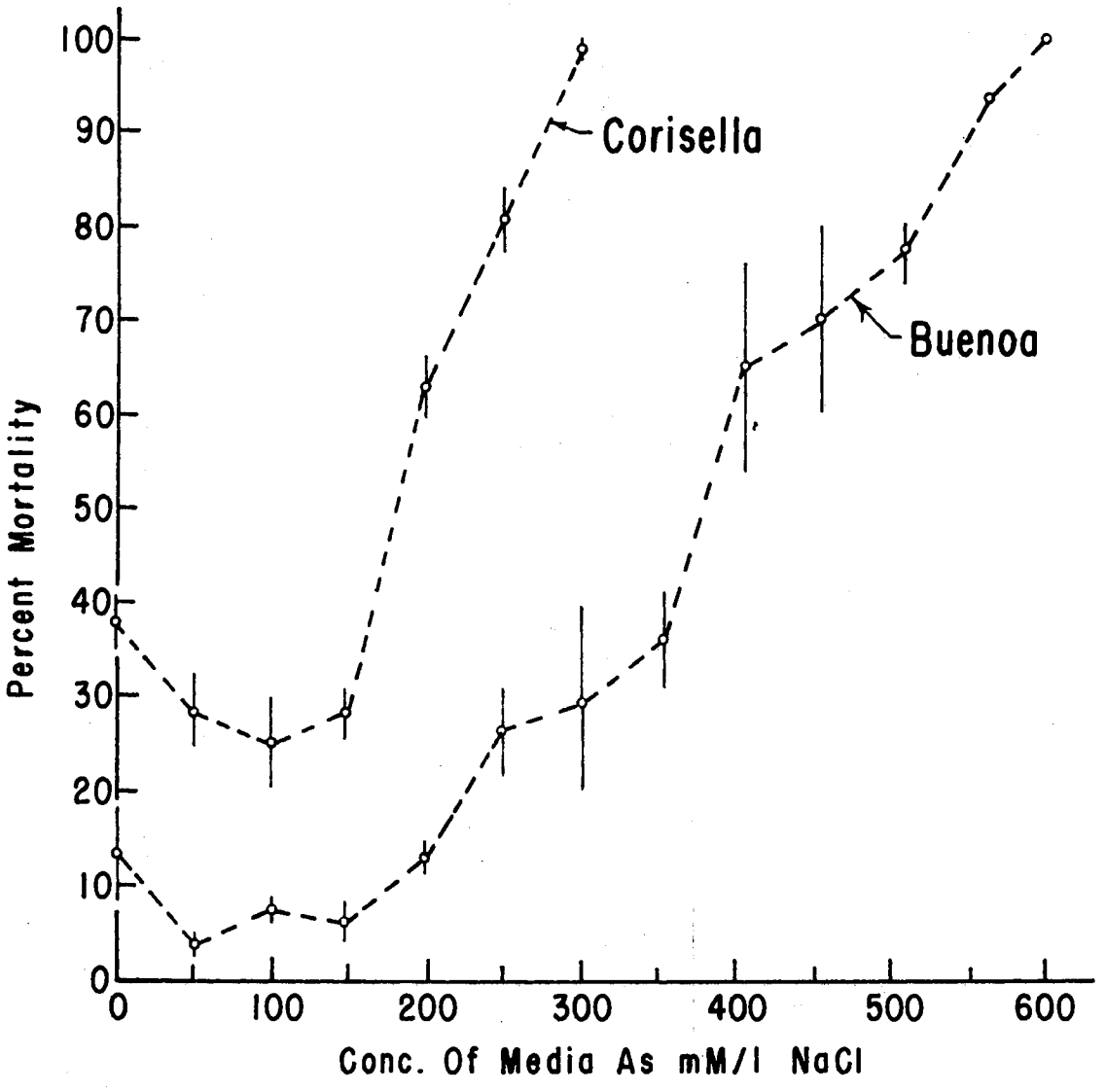
Animals held in various salines revealed an increase in mortality with an increase in both environmental concentration (NaCl) and time. Therefore, to establish an acclimation period, it was necessary to select a holding time long enough to assure acclimation and yet provide a percent survival that was high enough to supply unbiased results. This was arbitrarily done by determining the optimum survival conditions for the least tolerant insect (*C. edulis*) and noting the time (48 hrs) that produced a maximum average of 25% mortality. The same holding time was used for all subsequent experiments to provide information that would allow comparisons to be made between *C. edulis* and *B. margaritacea*.

Experiments were designed to assess the effects of various environmental salines (NaCl) on the mortality rates of *C. edulis* and *B. margaritacea*. As can be seen from Figure 1, both animals possess a similar range for optimum survival, i.e., 50 to 150 mM/l with *B. margaritacea* being slightly more tolerant than *C. edulis*. However, as the concentrations of the media were raised above 150 mM/l, the differences in tolerance levels increased sharply. After 48 hr, in 300 mM/l NaCl, nearly all *C. edulis* had died; whereas, the mortality rate of *B. margaritacea* was only 30% (Fig 1).

The Effect of Environmental Salinity on the Haemolymph Concentration of Sodium and Chloride in *C. edulis*

In deionized water, *C. edulis* maintained its haemolymph

Figure 1. Effect of environmental saline on mortality rates of *C. edulis* and *B. margaritacea* after 48 hr. Each point represents two experiments using 100 insects each. Vertical lines represent limits.


Percent Mortality
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
Conc. Of Media As mM/l NaCl
Corisella
Buenoa

concentrations of sodium and chloride well above that of the environment (125 mM/l and 100 mM/l, respectively). Both values were only slightly lower than the concentrations previously noted in insects taken from the "natural" habitat (Table I and Fig 2). At increasing environmental salinities, the haemolymph concentrations of chloride increased slowly but steadily up to 150 mM/l, and then increased sharply to become almost identical with environmental concentrations of 150 mM/l and above. Steadily increasing levels of haemolymph sodium were recorded as the environmental concentration of sodium was increased. The concentration of sodium was always maintained higher than the environmental concentration (Fig 2).

The Effect of Environmental Salinity on the Haemolymph Concentration of Sodium and Chloride in B. margaritacea

After 48 hr in deionized water, the chloride concentration in B. margaritacea was 110 mM/l (Fig 3). This represented little change from that recorded in insects from the "natural" habitat (Table I). At higher environmental salinities, however, the concentration of chloride in the haemolymph was higher (Fig 3). In contrast to the results achieved with C. edulis, the haemolymph concentration of chloride was maintained lower than environmental concentrations above 200 mM/l. After 48 hr, in deionized water, the sodium concentration in B. margaritacea (125 mM/l) was considerably less than that observed in insects from the "natural" habitat (Table I). As the environmental salinity was increased, the haemolymph concentration increased, reaching a "plateau" at environmental concentrations between 100 to

Figure 2. Effect of environmental saline on the concentration of haemolymph sodium and chloride in C. edulis. Vertical lines represent ± S.D. of the means. Numbers indicate the number of animals used. The solid unbroken line corresponds to a haemolymph to media concentration ratio of 1:1.

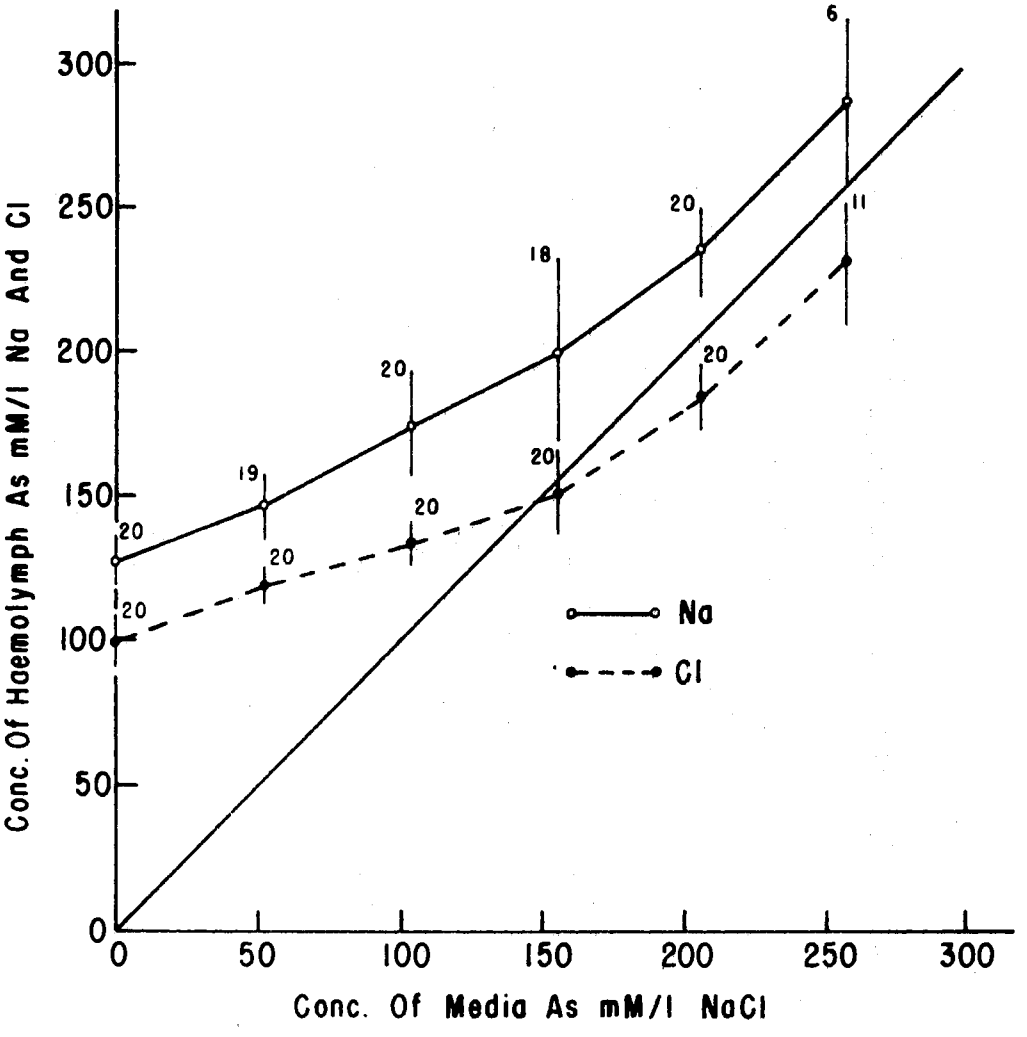
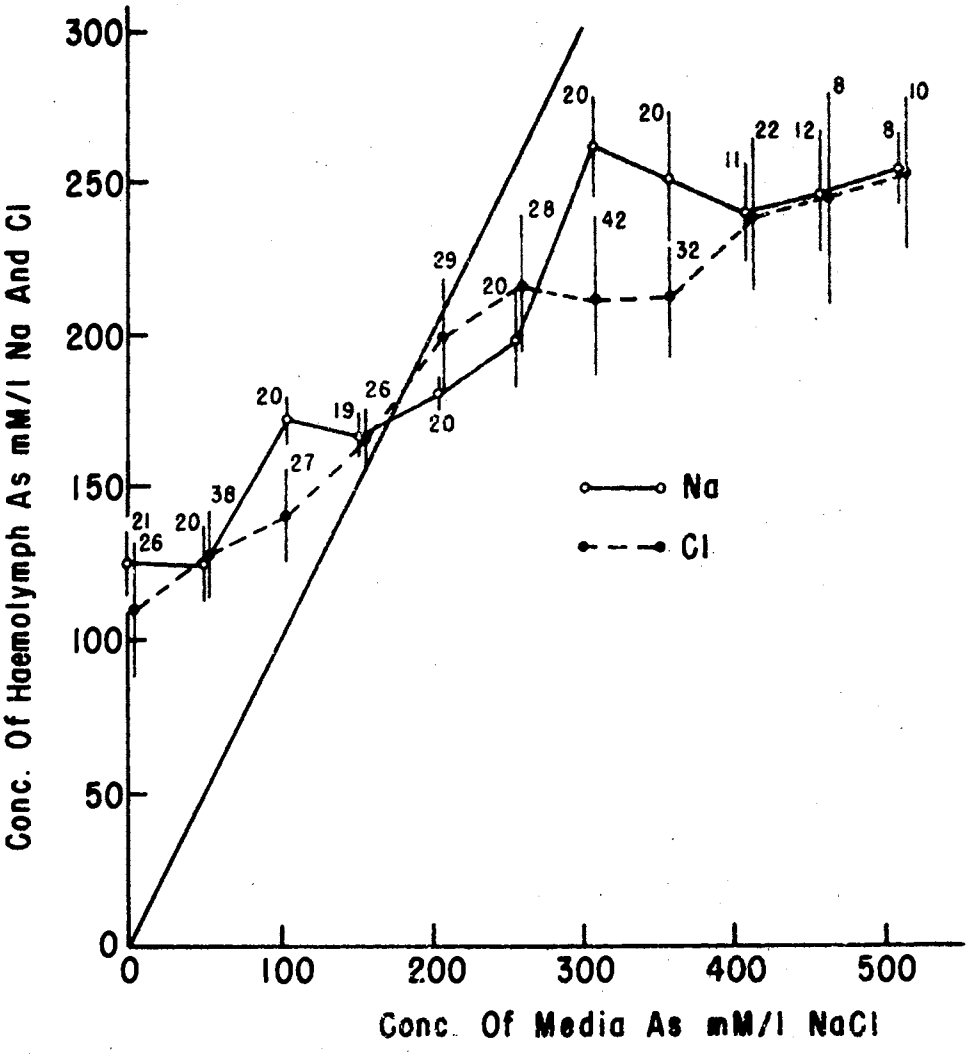

Conc. Of Haemolymph As mM/l Na And Cl
300
250
200
150
100
50
0
0
50
100
150
200
250
300
Conc. Of Media As mM/l NaCl
Na
Cl
20
19
20
18
20
6
20
20
20
20
20
11

Figure 3. Effect of environmental saline on the concentration of haemolymph sodium and chloride in B. margaritacea. Vertical lines represent ± S.D. of the means. Numbers indicate the number of animals used. The solid unbroken line corresponds to a haemolymph to media concentration ratio of 1:1.


300
250
200
150
100
50
0
0
100
200
300
400
500
Conc. Of Haemolymph As mM/l Na And Cl
Conc. Of Media As mM/l NaCl
Na
Cl
21
26
20
38
20
27
19
26
29
20
20
28
42
20
32
20
11
22
12
8
8
10

250 mM/l (Fig 3). A sharp increase in the haemolymph concentration of sodium was noted when the environmental concentration was raised to 300 mM/l. Further increases in environmental sodium caused little change. As with the level of chloride and in contrast to the results obtained with C. edulis, at environmental concentrations above 150 mM/l, the level of sodium in the haemolymph of B. margaritacea was maintained below that of the environment.

The Levels of Na and Cl in C. edulis and B. margaritacea After 48 Hours of Experimental Conditions in Pond Water

To investigate the possibility that experimental conditions (other than those presented by the laboratory prepared environments) might be partially or wholly responsible for some of the preceding results, specimens of both C. edulis and B. margaritacea were subjected to the identical experimental conditions as outlined above except that pond water served as the experimental medium. After 48 hr there was little change (as compared to the values obtained from insects taken directly from the natural habitats) in the level of sodium (167 to 172 mM/l) in B. margaritacea (Table II) and only a slight change (106 to 118 mM/l) in the level of chloride. There was a slight change, in similar assays, in the level of sodium in C. edulis (168 to 145 mM/l), but no difference in the level of chloride (125 mM/l) (Table II). These results suggest that there are no large fluctuations in the levels of haemolymph Na and Cl which can be attributed to the experimental design other than the laboratory prepared environmental concentrations of NaCl or deionized water.

TABLE II

HAEMOLYMPH CONCENTRATION OF INSECTS FOLLOWING 48 HOURS ACCLIMATION IN WATER FROM THEIR NATURAL HABITAT*

	Sodium	Chloride
Corisella edulis (Water Boatman)	145 ± 6	125 ± 11
Buenoa margaritacea (Back Swimmer)	172 ± 9	118 ± 14

*Values are expressed as mM/l Na and Cl and represent means of 20 assays with respective standard deviations.

Discussion

In regard to the regulation of salt and water balance in aquatic insects, Shaw and Stobbart (1963) state that: "Another feature, which has important consequences for the mechanisms of osmoregulation and is in contrast with the situation found in many terrestrial insects, is that the haemolymph osmotic pressure is made up largely of electrolytes. Thus, as with most other animals, osmoregulation must depend upon the underlying process of regulation of the ionic composition." It was felt that by obtaining information on the composition and tolerance levels of Na and Cl at least some information could be offered concerning the possible mechanisms involved in the ionic regulation of these adult aquatic insects.

Stobbart and Shaw (1964) have reviewed the composition of insect haemolymph and a generalized scheme reveals high Na and Cl concentrations for Hemiptera. Sutcliffe (1962) has reported more specifically on aquatic Hemiptera and the "normal" Na and Cl concentrations reported here are in close agreement with these reports.

The regulation curves for both insects were quite similar in dilute solutions but revealed substantial differences in the more concentrated media. B. margaritacea regulated their Na and Cl closely over the entire external range while at higher salinities Na and Cl of C. edulis were regulated quite differently. For example, Na remains hyperconcentrated to the media while Cl is hypoconcentrated. Shaw and Stobbart (1963) indicate that this is not uncommon among aquatic larvae and suggest that the two ions may be regulated separately, at least over part of the external range. Therefore, one might expect that B. margaritacea is regulating its Na and Cl simultaneously since the

two curves so closely parallel each other. In most studies, however, Cl is regulated below and roughly parallel to Na and/or the osmotic pressure, whichever has been measured (Sutcliffe, 1960, 1961a, 1961b) and a similar hypothesis has been made.

One of the major sources of pollution in the southwestern United States is brine from oil wells, and as a result, presents an osmotic problem to the aquatic fauna by producing occasional sharp fluctuations in the concentrations of various salts, most of which are Na and Cl (Clemens and Jones, 1955). Observations made on mortality differences in the laboratory testing solutions indicate that B. margaritacea is more tolerant to these changes than C. edulis. When considering these data simultaneously with the maintenance of Na and Cl, it is felt that B. margaritacea's ability to maintain its internal composition at the higher concentrations is sufficient to account for the differences in tolerance limits. However, it is not known if such parameters as pH and oxygen tension are differentially affecting mortality rates. The effects of possible differences in the pH between the experimental media and the natural habitats are presently under investigation. Oxygen tension may not be too important because insects that gain air at the surface of the water can counteract low oxygen tensions with more frequent visits to the surface (de Ruiter et al., 1952).

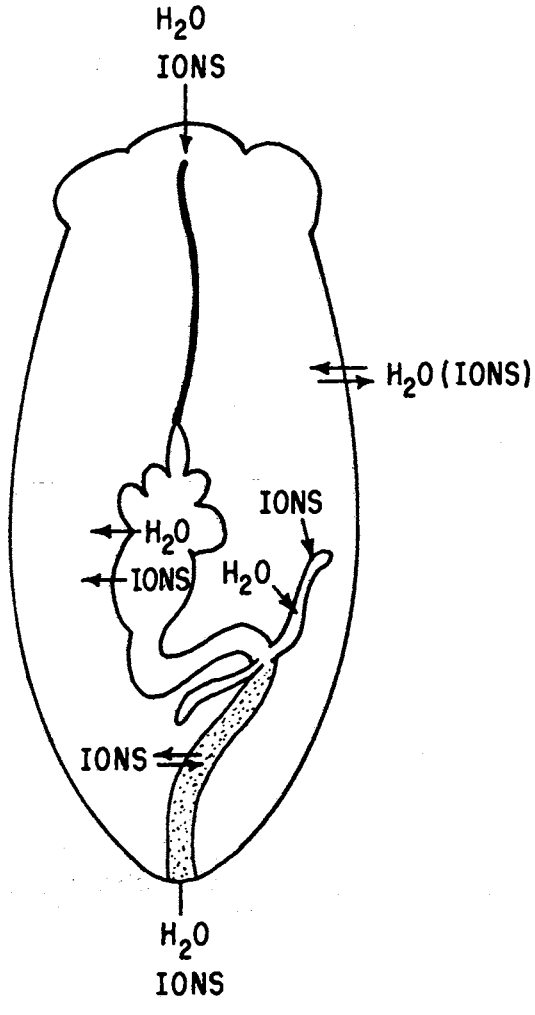
From this information arise important questions as to the possible physicochemical mechanisms involved in the maintenance of different levels of Na and Cl in these adult water bugs. The elimination of water in addition to the separation and retention of "normal" salt levels in the haemolymph is one of the major problems most often confronted by these two aquatic insects. The reported rate of osmotic

water uptake through the cuticles of representatives of Notonectidae and Corixidae range with the limits of 1.8 and 7% of the body weight per day (Staddon, 1963, 1964, 1966) and 10% was later reported in Ilyocoris cimicoides (L.) (Staddon, 1969a). The mouth has also been shown to be an important route for the uptake of water (Staddon, 1969a). Regulation of water in these bugs whose need is to eliminate water continuously may be under neurohormonal control (Staddon, 1969b) in much the same manner as the terrestrial insect Rhodnius prolixus Stal. (Maddrell, 1963, 1964a, 1964b).

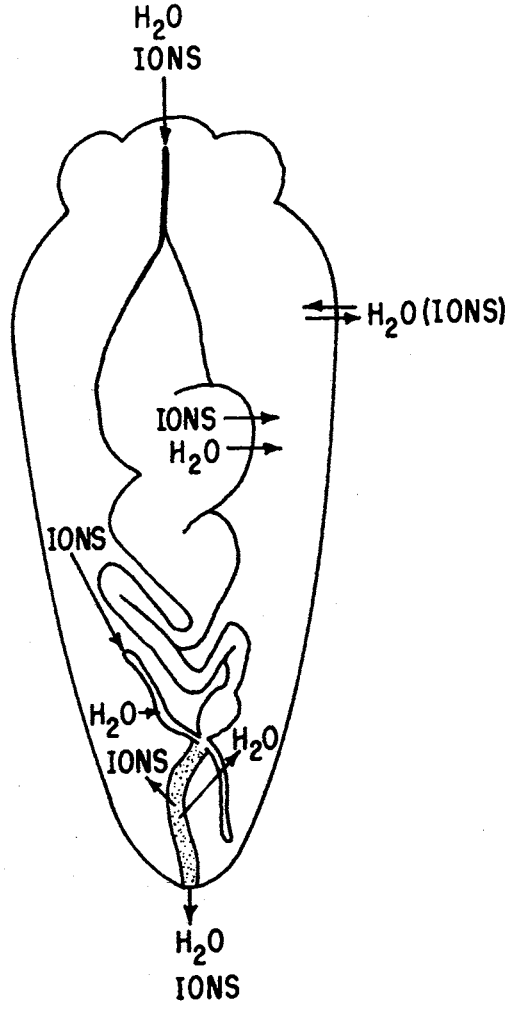
It is likely that the mechanisms involved in overcoming these problems are similar to those in aquatic larvae (Shaw and Stobbart, 1963; Stobbart and Shaw, 1964) and by using references cited for these larvae and a few Hemiptera that have been studied, one might hypothesize possible schemes which could account for the differences observed in the regulation of Na and Cl in these adult water bugs. In dilute media, the regulation of both insects appears to be quite similar (Fig 4), i.e., water and ions probably enter the haemolymph by following the osmotic gradient through the cuticle and/or gut if drinking occurs. At the same time, the Malpighian tubules may be actively engaged in the removal of ions to establish an osmotic gradient within the tubule lumen. The urine may be passively removed to the rectum where ammonium bicarbonate could be exchanged for the necessary haemolymph ions followed by the excretion of hypo-osmotic rectal fluid. When the medium is more concentrated than the haemolymph, the osmotic response reverses and water from the haemolymph moves into the surrounding medium. It is under this condition that B. margaritacea appears to have an advantage over C. edulis. To replenish the supply of continuous

Figure 4. Diagrammatic representation of the possible movements of water and ions necessary for the maintenance of haemolymph sodium and chloride in C. edulis (Water Boatman) and B. margaritacea (Back Swimmer). Details are in the text.

CORISELLA EDULIS



BUENOA MARGARITACEA



water loss, drinking is probably induced in both specimens. However, C. edulis was unable to tolerate these high concentrations of saline. Under these conditions the haemolymph samples of moribund C. edulis almost always appeared as cloudy homogenous mixtures (Frick, unpublished data) which are indicative of tissue deterioration. B. margaritacea, on the other hand, offered clear haemolymph, except for a few specimens in the maximum salinities (Frick, unpublished data). Following these observations it is felt that the gut is at least partially involved in maintaining the levels of Na and Cl in the haemolymph. In B. margaritacea these excess ions are either tolerated by the gut or drinking does not occur. More than likely, the latter is not the case and the animal must work continuously to remove these ions from the haemolymph via the Malpighian tubules to the rectum where water can be reabsorbed in much the same manner as reported in the terrestrial locust Schistocera gregaria Forskal (Phillips, 1965). In this case, however, a hyperosmotic rectal fluid would be expected.

Another advantage B. margaritacea may have over C. edulis when subjected to high saline environments is the degree of permeability. A less permeable cuticle could prevent the rapid loss of water in concentrated media and as a result maintain the levels of Na and Cl more effectively.

The mechanisms involved in the osmotic and ionic balance are of extreme importance to the survival of these invertebrates, and the elucidation of these functions may have wide spread consequences in regions where brine pollution is so prevalent and often detrimental to the aquatic fauna.

CHAPTER III

EXAMINATION OF A BIOLOGICAL CRYOSTAT/NANOLITER OSMOMETER FOR USE IN DETERMINING THE FREEZING-POINT OF INSECT HAEMOLYMPH

The preparation, fixing and embedding of tissue for ultrastructural examination and the preparation of physiological bathing media for use in *in vitro* studies of tissues and cells required knowledge of the osmotic pressure of the animal's normal *in vivo* bathing medium. Because of size, this has been particularly challenging with many insects. Ramsey and Brown (1955) designed an instrument that could measure the freezing-points of nanoliter volumes of fluid with ±0.2 - 0.8% accuracy. The chief disadvantage of this method is the time and trouble required to make the measurement. Recently, a biological cryostat/nanoliter osmometer[1] with a stable all solid state compact temperature controller and a direct digital readout has been employed for accurately and more conveniently determining the freezing-point of nanoliter quantities of fluid. This unit, independently developed by Clifton Technical Physics, is virtually a small solid state version of the device developed earlier by Prager and Bowman (1963) at the National Heart Institute. The purpose of this

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[1]Clifton Technical Physics, 48 Horatio Street, New York, New York 10014.

investigation was to explore and present evidence for the usefulness, convenience and accuracy of this instrument for making osmotic pressure determinations of small amounts of tissue, in particular haemolymph taken from small insects and related arthropods.

Materials and Methods

This instrument utilizes in combination a thermoelectric cooling module, a wheatstone bridge, and a variable output power supply. The thermoelectric cooling module is equipped with fittings for the attachment to a dry air source which prevents the specimens from frosting over and obscuring the crystals and a water source which aids in stabilizing the temperature of the cooling module. The temperature of the cooling module is virtually perfectly controlled by the variable output power supply and equilibrates quickly with the sample holder which is fastened to its upper surface. The complete cooling module is small enough to rest on the stage of a steromicroscope where light can be directed up through a centrally located aperture. The base of the aperture is equipped with an achromatic condenser for focusing the light on the crystals contained within the oil filled holes of the sample holder.

Fluid specimens may be collected and introduced into the oil filled holes of the sample holder in different ways, but one must keep in mind that error is not due to the size of the sample (Prager and Bowman, 1963) or the difficulty of observing the thawing end-point (Ramsay and Brown, 1955). Rather, the technique used in collecting and preparing the sample is normally the main source of error. Therefore, it would be wise to review carefully the operations of Ramsay and Brown

(1955) for collecting and handling samples and the procedures of Prager and Bowman (1963) for introducing the small samples.

For our use, we have found that finely tapered capillary tubes can be prepared quickly and easily by gently pulling the tubes over a small open flame and breaking off the tips at the desired diameter. The tube is then fitted onto a short piece of plastic tubing for oral aspiration. When collecting specimens a small amount of Cargilles 'A' immersion oil is drawn up into the tube followed by the specimen and another droplet or oil. Evaporation is retarded and contamination is reduced due to the presence of a coat of oil which envelopes the specimen. Samples that can be collected and introduced within 5-10 seconds do not require the aspiration of oil. Next, each specimen is aspirated directly into the holes filled with Cargilles 'B' immersion oil to the desired size (1/2 - 3/4 the diameter of the hole), from 2.0 to 5.5 nanoliters under a magnification of 80X provided the most desirable specimen size to assay. A standard curve is not necessary since the instrument is designed with a direct digital readout. However, two standard solutions must be prepared to calibrate the readout scale. These standard solutions are generally run concomitantly with the unknown samples and determinations made by interpolation.

The samples can be frozen and thawed either manually or automatically. We prefer controlling the thermoelectric cooling unit manually since a much faster assay can be made. With a little practice the sample holder with holes for six specimens can be filled within two minutes. All specimens are then frozen to about $-45^{o}C$ and warmed to the thawing-point automatically within another minute. Approaching the final crystal is extremely convenient but does require time for the

formation of a consistent crystal size. In the past, we have been able to determine the freezing-point of all six specimens within 20-30 minutes.

Results and Discussion

To provide evidence for the accuracy of this instrument, variations on the freezing-points of five standard NaCl solutions were measured (Table III). Refreezings and rethawings of the samples did not significantly alter the original values on any of the previous tests.

The usefulness of this instrument is exhibited by the fact that the body fluids of numerous invertebrates can be quickly and conveniently assayed. The assay is limited only by the transparency of the specimen and the available amount of body fluid. Measurement made on the haemolymph of several small arthropods collected in and around Stillwater, Oklahoma, in relation to their size is shown in Table IV.

Selected arthropods were collected during August and September of 1972 and all samples were assayed on the same day of collection. Schizaphis graminum (Rondani) and Chironomus attenuatus Walker were laboratory reared while all others were field collected. Criteria for selecting most specimens were based on their availability, small size and difficulty of obtaining body fluid. Sizes ranged from 0.21 mg (S. graminum) to 65.23 mg (Gyrinus sp.). Most specimens did not exceed 16.00 mg and four weighed less than 5.0 mg each. Heavier specimens that produced copious amounts of fluid were assayed for use in comparing variation to those whose body fluids were difficult to collect. Amblyomma americanum (L.), S. graminum, and Pogonomyrmex barbatus

TABLE III

COMPARISON OF CALCULATED AND OBSERVED FREEZING-POINT DEPRESSIONS OF STANDARD NaCl SOLUTIONS

$-\Delta^{o}C$ calc.[a]	$-\Delta^{o}C \pm$ S.D. obser.[b]
5.000	5.061 ± 0.060
4.000	4.015 ± 0.052
3.000	3.046 ± 0.043
2.000	2.021 ± 0.054
1.000	0.994 ± 0.067

[a]Values calculated from International Critical Tables, Vol. 4, p. 258.

[b]Values are means of 10 separate assays each.

TABLE IV

FREEZING-POINT DEPRESSIONS OF THE HAEMOLYMPH OF SELECTED ARTHROPODS USING A BIOLOGICAL CRYOSTAT/NANOLITER OSMOMETER

Species	Family	Stage[a]	No. tested	Avg. wt. (mg)	$-\Delta^{o}C \pm$ S.D.
Gyrinus sp.	Gyrinidae	A	14	65.23	0.683 ± .031
Acilius sp.	Dytiscidae	L	13	61.25	0.657 ± .037
Myrmeleon sp.	Myrmeleontidae	L	9	22.11	0.691 ± .038
Buenoa margaritacea Torre-Bueno	Notonectidae	A	13	15.80	0.621 ± .029
Corisella edulis Champion	Corixidae	A	10	15.34	0.636 ± .075
Pogonomyrmex barbatus (F. Smith)	Myrmicidae	A	10	11.00	0.974 ± .049
Amblyomma americanum (L.)	Ixodidae	A	10	4.27	0.653 ± .112
Chironomus attenuatus Walker	Chironomidae	L	13	3.82	0.345 ± .044
Culex pipiens quinquefasciatus Say	Culicidae	L	10	2.61	0.477 ± .030
Schizaphis graminum (Rondani)	Aphidae	N	9	0.21	0.743 ± .034

[a]A = Adult, L = Larvae and N = Nymph

(F. Smith) presented the greatest difficulty when attempting to collect body fluids. Fluid volume was very low in spite of the larger sizes of the flat unengorged tick, A. americanum, and the red ant, P. barbatus, while the small body size of S. graminum set the limit on the available amount of fluid necessary for assay. Haemolymph samples from greenbugs, S. graminum, were very difficult to obtain and the samples were probably contaminated with gut fluids. Differences in variation of the freezing-point depression were small except for that of the flat unengorged tick, A. americanum. Since the physiology of this tick is known to respond to differences in atmospheric relative humidities (Sauer and Hair, 1971; Shih et al., 1972), it is possible that differences in the osmolarity of these field collected specimens is normally quite great. Standard deviations ranged from $\pm 0.029^{o}C$ (Buenoa margaritacea Torre-Bueno) to a high of $\pm 0.049^{o}C$ (P. barbatus) for all other specimens and virtually no significant differences were noted between those whose body fluids were difficult to collect and those that exuded copious amounts.

When absolute values are necessary, additional inherent variations must be taken into account. Variation within the instrument was tested by observing the freezing-points of standard NaCl solutions after repeated freezings and thawings of the same samples. Variations were not significantly different. Virtually the same variations were found among the insect samples tested (Table IV).

Slight contamination and/or evaporation are difficult to avoid when only small samples are available, but this error may be minimized by closely following the procedures of Ramsay and Brown (1955). The procedures for obtaining the values in Table III did not follow exactly

that of Ramsay and Brown. Instead, soft glass capillaries were utilized for collecting the samples and this may have contributed somewhat to the variations observed.

One definite limitation of most osmometers is the amount of fluid required for assay. This instrument alleviates this problem without introducing a large margin of error in the measurements. Advantage can also be taken of the fact that only single specimens are required. In some instances, samples can be taken from large insects without noticeable injury. This becomes important when subsequent experiments on the same specimen is desired and when the specimens are rare or difficult to obtain. In physiological studies where only minute quantities of secretions or excretions are available, important measurements of the osmotic pressure can be made easily. Work with isolated, in vitro, tissues and their function is aided by the facile use of the cryoscopic principles utilized by this instrument. However, the chief advantage of the instrument is the convenience and expedience with which the measurements can be made. At present, as far as we know, no other method of assay of the freezing-point of very small samples so rapidly and conveniently is known. Therefore, we believe this biological cryostat/nanoliter osmometer could greatly facilitate the work of researchers employing small arthropods.

CHAPTER IV

OSMOREGULATION IN THE ADULT WATER BOATMAN *CORISELLA EDULIS* CHAMPION

Studies of osmoregulation in aquatic Corixidae have been stimulated by discoveries showing that various species exhibit marked differences in habitat preference, i.e., either fresh or saline water (Usinger, 1968; Claus, 1937; Scudder, 1969).

Experiments have been performed on the ability of *Corixa distincta* Frieb., *Corixa fossarum* Leach, (Claus, 1937), and *Cenocorixa bifida* (Hung.) and *Cenocorixa expleta* (Uhler) (Scudder, 1968) to regulate their internal osmolarity after exposure to wide ranging environmental salinities. More recently the osmotic and ionic balance in the latter two species has been investigated in greater detail (Scudder et al., 1972). Staddon (1964) studied water balance in *Corixa dentipes* (Thoms.) and the regulation of haemolymph sodium and chloride after exposure to saline environments were investigated in *Corisella edulis* Champion (Frick et al., 1972). Studies of the morphology and ultrastructure of the Malpighian tubules and hindgut in *Cenocorixa bifidae* (Hung.) (Jarial and Scudder, 1970) have indicated the presence of structures similar to those found in other insect fluid transporting epithelia. It has been suggested that the excretory system of Corixidae plays a fundamental role in the regulation of the haemolymph osmolarity (Jarial and Scudder, 1970) as in terrestrial insects (Shaw & Stobbart, 1963;

Stobbart and Shaw, 1964).

The purpose of this study was to assess the ability of C. edulis to regulate its haemolymph osmotic pressure after exposure to different environmental salines and to investigate the possible role of the excretory system in overall homeostasis.

Materials and Methods

Specimens of C. edulis were collected and acclimated prior to all experiments in the manner as previously described (Frick et al., 1972). Following an adjustment of the insects to natural pond water and the laboratory temperature (23 ± 2°C), insects were transferred to 3 liters of each experimental environment (deionized water, 0.3%, 0.6% and 0.9% NaCl) and left unattended on a shelf receiving natural daylight for 48 to 60 hr. All containers were equipped with rubber nets to provide attachment sites for the bouyant insects.

Freezing-point depressions of fresh untreated haemolymph and urine were measured with a Clifton Technical Physics biological cryostat/ nanoliter osmometer according to Frick and Sauer (1973a).

The total body water percent was determined by placing insects in a drying oven at 105°C and weighing every 8 hr until a constant weight was achieved. The difference between wet and dry weights was taken as the total water content of the insects. Wet weights were obtained after removing the surface water adhering to the cuticle by causing the insects to flutter about on a slightly moistened paper towel in a chamber maintained at 100% relative humidity. Weight changes in the relative humidity chamber were measured after different time intervals and minimal losses of 0.1% per min for the first 30 min were noted.

Blood volumes were determined by the ^{14}C - carboxyl inulin dilution technique of Wharton et al. (1965). Successful injection of the isotope was accomplished by puncturing the membrane between the 5th and 6th ventral abdominal sclerites with a precalibrated capillary tube filled with saline containing the isotope. Following the injection, the capillary was quickly withdrawn to prevent loss of fluid from the puncture site. Specimens that occasionally lost a droplet of fluid were discarded. After injection, the insects were placed in glass stoppered capsules containing a saturated cotton swab to maintain the relative humidity near 100%. After 30 min, 0.2 µl of clear haemolymph was withdrawn at the site of the injection and placed into 15 ml of the liquid scintillant of Wharton et al. (1965). The t test for determining whether two sample means are from the same population was utilized in all cases where significance was calculated. The possible movements of fluid in the lumen of the Malpighian tubules and intestine were observed visually after injection into the abdomenal haemocoel of 0.3 µl of 1.0% indigo carmine in a physiological saline modified from Maddrell (1971a). The assumption was made that the transfer of dye required the movement of fluid in the lumen. Locations where dye became concentrated were taken as the sites of fluid and dye segregation. No attempt was made to quantitate the amount of fluid secreted by the Malpighian tubules but only to visualize the path which the fluid follows and to note the sites where fluid is possible reabsorbed.

Results

To establish the "normal" levels of the experimental paramenters being investigated, measurements were made immediately after insects

were taken from their natural pond water habitat. The haemolymph osmotic pressure (expressed as $-\Delta^{o}C$) was $-0.636 \pm 0.075^{o}C$ and the urine or fluid collected from the region of the hindgut was $-0.475 \pm 0.120^{o}C$. The total body water percent was $71.4 \pm 3.62\%$ and blood volume percent was $29.82 \pm 8.91\%$. Each respective variance represents $\pm$ S.D.

To initially determine a period of time needed for C. edulis to equilibrate with the different laboratory experimental media, measurements of the freezing-point of the haemolymph of insects placed in solutions more concentrated and more dilute than the pond water were made after regular time intervals for up to 72 hr (Fig. 5). Changes occurred quickly after the insects were placed in either solution. After 48 hr and thereafter the changes were insignificant. An insect holding time of 48 to 60 hr in all experimental media was therefore selected for subsequent experiments.

Osmotic Pressure of the Haemolymph

The haemolymph osmotic pressure of C. edulis was maintained above that of all media used in the experiments (Fig. 6). After acclimation to deionized water, the average freezing-point ($-0.545^{o}C$) was significantly reduced ($P < .05$) below that of insects taken directly from the natural pond water ($-0.636^{o}C$). The freezing-point rose steadily as the salinity of the medium was increased. A value of $-0.725^{o}C$ was recorded in insects placed in solutions having a freezing-point of $-0.540^{o}C$.

Osmotic Pressure of the Urine

The urine of insects placed in deionized water for 48 hr was considerably hypo-osmotic to the haemolymph but increased rapidly when

Figure 5. Changes in the haemolymph osmotic pressure ($-\Delta^{o}C$) of specimens of C. edulis over time after placement in either media of 0.9% NaCl or deionized water. Each point represents the mean of at least 10 insects and vertical lines correspond to ± S.E.

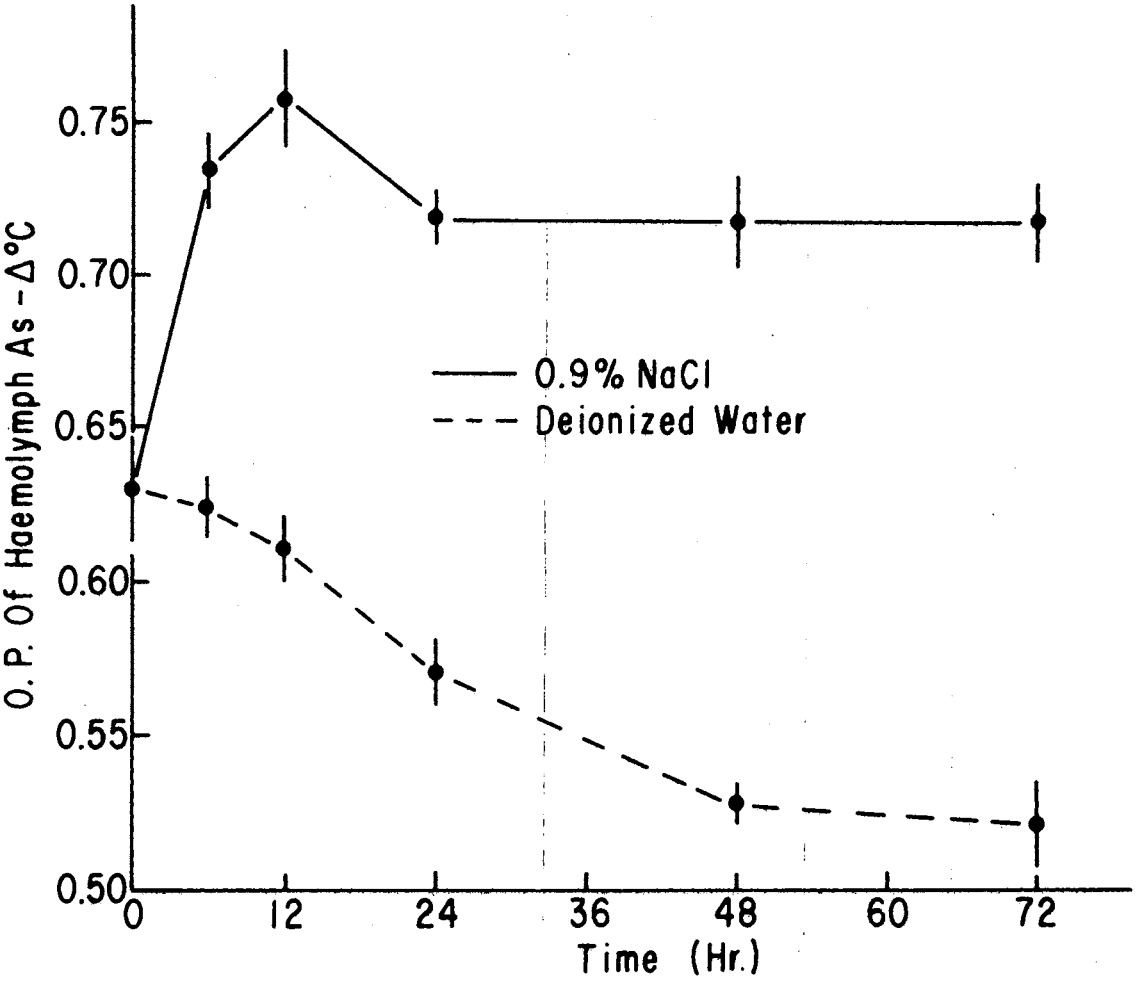
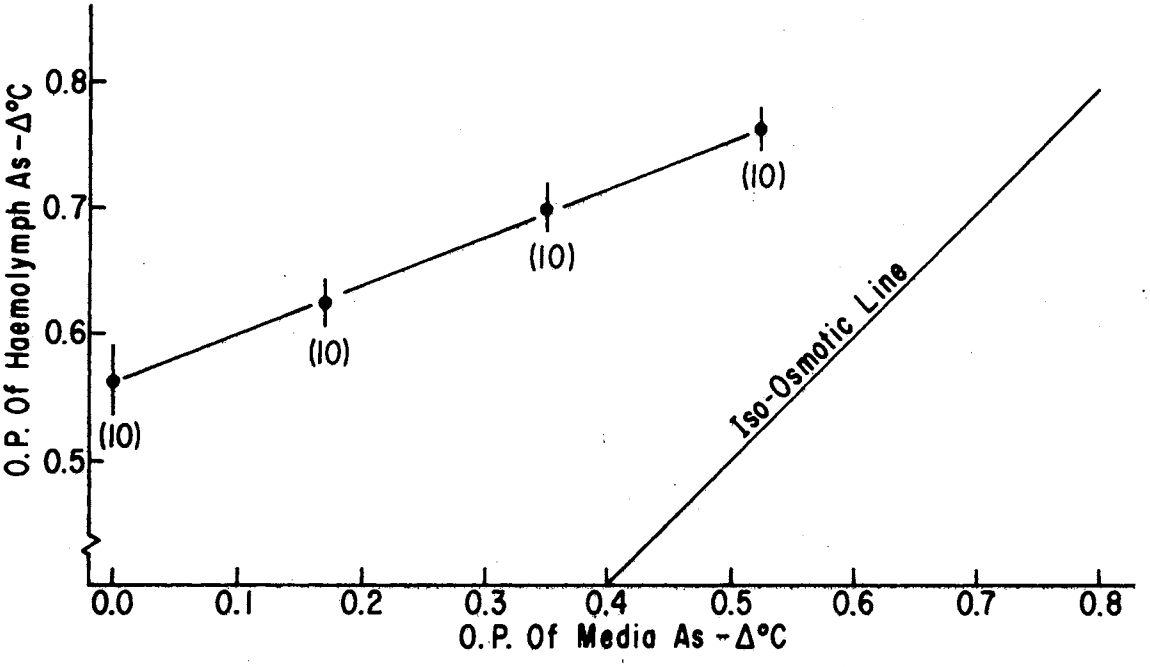

0.75
0.70
0.65
0.60
0.55
0.50
O. P. Of Haemolymph As - Δ°C
0.9% NaCl
Deionized Water
0
12
24
36
48
60
72
Time (Hr.)

Figure 6. Osmotic pressure of the haemolymph of specimens of C. edulis after placement in media of different salinities. Vertical lines correspond to ± S.E. of the mean.


O.P. Of Haemolymph As -Δ°C
0.8
0.7
0.6
0.5
(10)
(10)
(10)
(10)
Iso-Osmotic Line
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
O.P. Of Media As -Δ°C

the salinity of the medium was raised (Fig 7). At a medium osmotic pressure of -0.540°C, the osmolarity of the urine was only slightly below that of the haemolymph (Fig 8). The changes in the freezing-point of the urine paralleled the changes of the surrounding medium but was maintained approximately -0.250°C higher (Fig 7). Copius amounts of urine could be obtained from insects acclimated to dilute solutions while little was obtainable in insects acclimated to the higher salinities.

Total Body Water

The total body water percent of insects acclimated to all experimental media was near or slightly above 70%. We were unable to show any significant difference in this parameter in insects acclimated to the different experimental media (Fig 9).

Blood Volume

Measurements of the blood volume of C. edulis also indicated considerable abilities of regulation (Fig 10). Although there was a slight indication of an increased blood volume in insects acclimated to the higher salinities we were unable to show significance between any of the experimental means.

Fluid Movements

Following the injection of the indigo carmine, the dye was taken up and concentrated in the lumen of the Malpighian tubules. Shortly thereafter it was seen flowing proximally to the intestine. After reaching the intestine it passed anteriorally to the region of the

Figure 7. Osmotic pressure of the urine of specimens of C. edulis after placement in media of different salinities. Vertical lines correspond to ± S.E. of the mean.

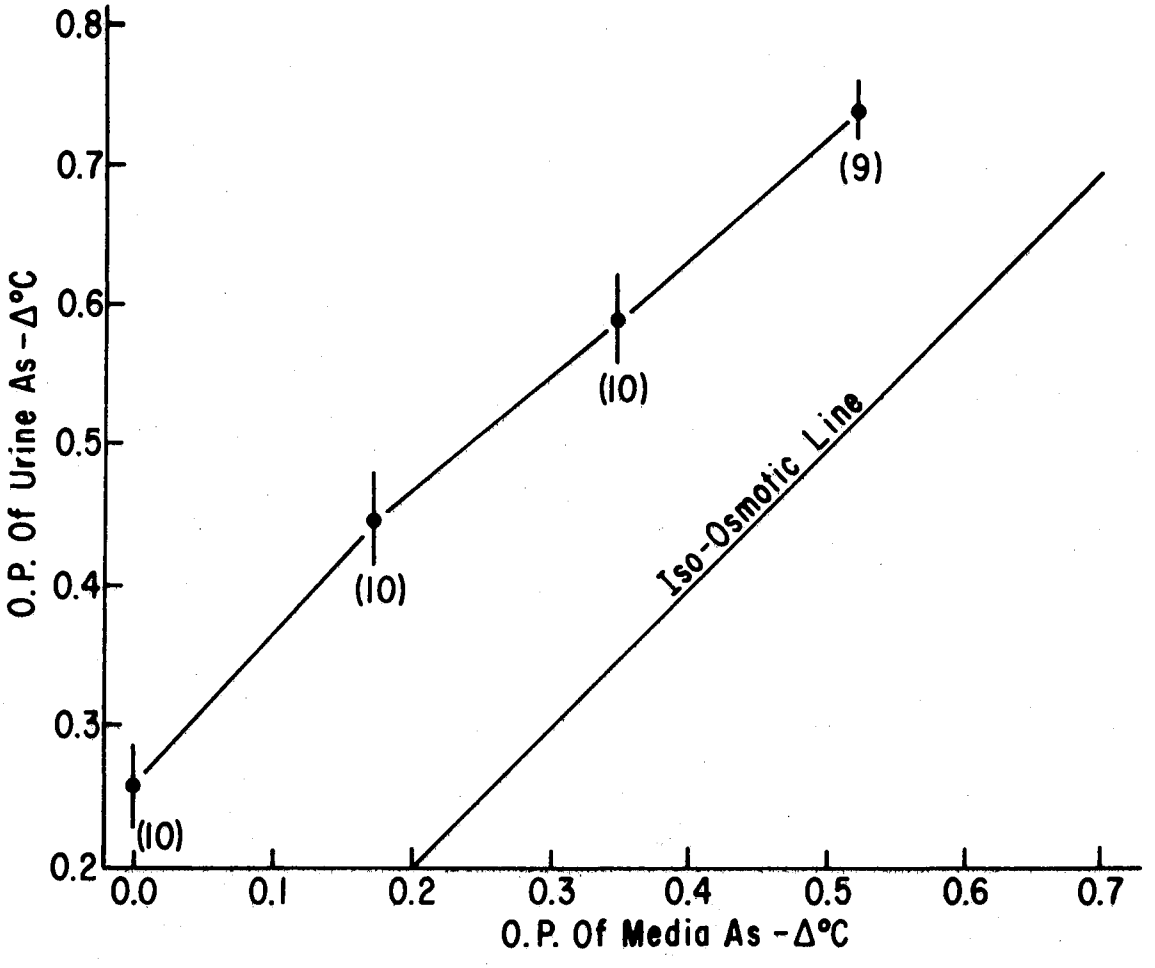

0.8
0.7
0.6
0.5
0.4
0.3
0.2
O.P. Of Urine As -Δ°C
(9)
(10)
(10)
(10)
Iso-Osmotic Line
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
O.P. Of Media As -Δ°C

Figure 8. The relationship between the osmotic pressure of the urine and that of the haemolymph of C. edulis after placement in media of different salinities. Variations correspond to ± S.E. of the mean.

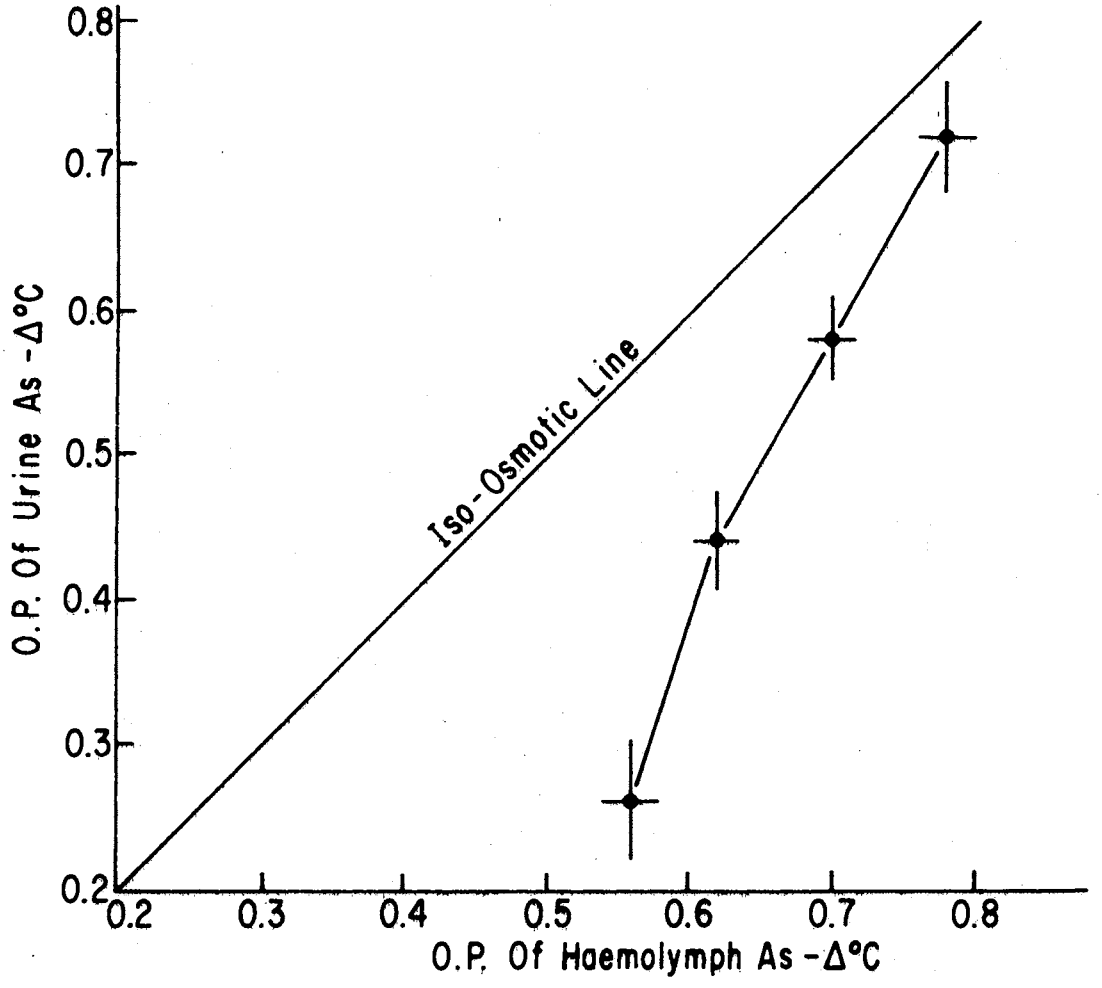

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.2
0.3
0.4
0.5
0.6
0.7
0.8
O.P. Of Urine As -Δ°C
O.P. Of Haemolymph As -Δ°C
Iso-Osmotic Line

Figure 9. Effect of environmental saline on the total body water percent of C. edulis. Vertical lines represent ± S.D. of the means. Numbers indicate the number of insects used.

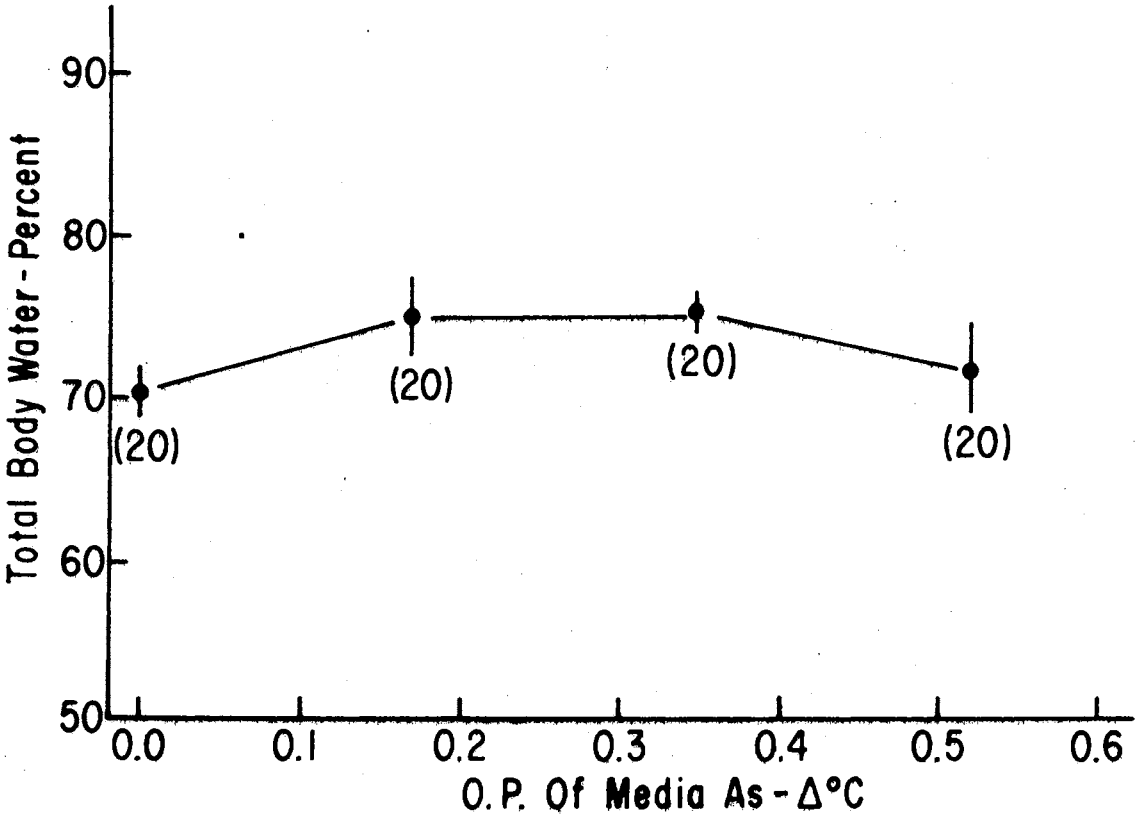
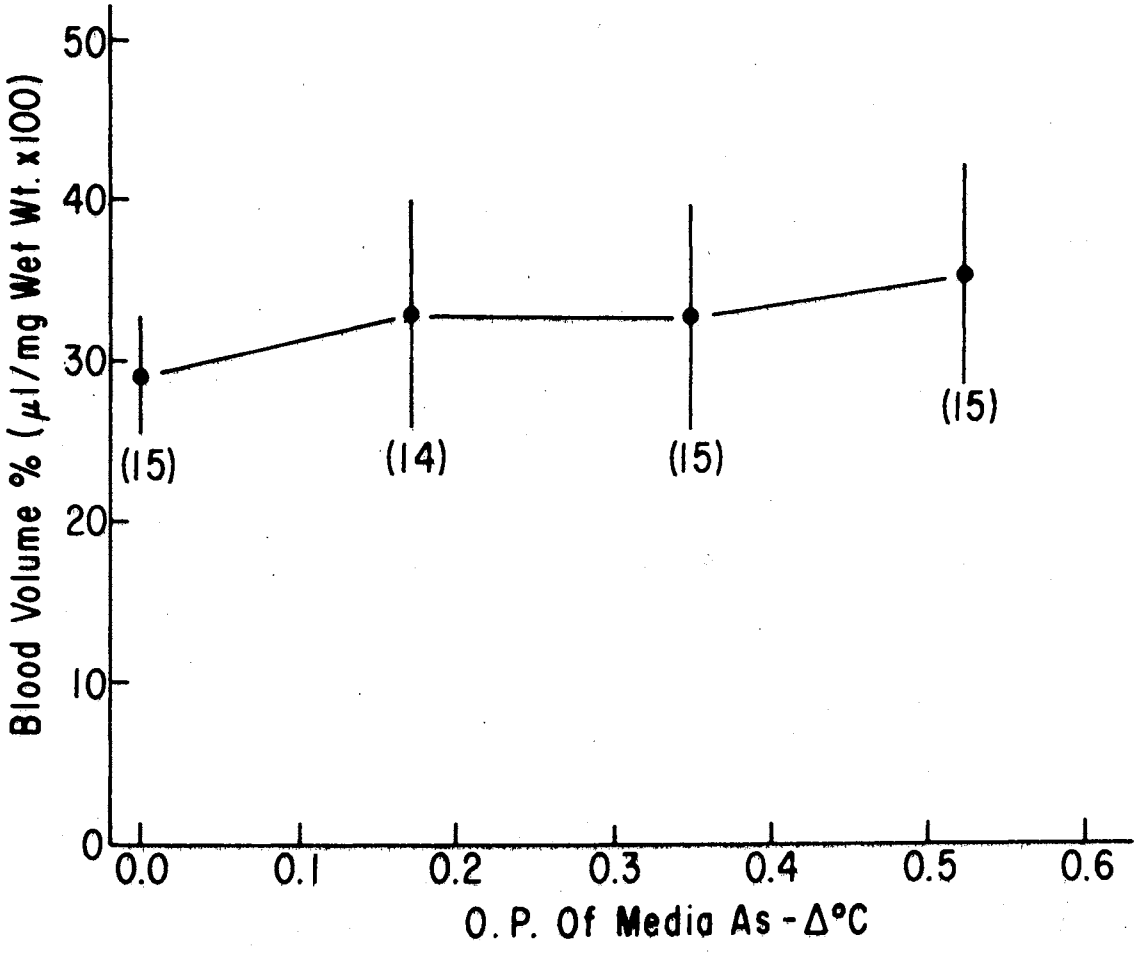

Total Body Water - Percent
90
80
70
60
50
(20)
(20)
(20)
(20)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
O. P. Of Media As - Δ°C

Figure 10. Effect of environmental saline on the blood volume percent of C. edulis. Vertical lines represent ± S.D. of the means. Numbers indicate the number of insects used.


Blood Volume % (µl/mg Wet Wt. x100)
50
40
30
20
10
0
(15)
(14)
(15)
(15)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
O. P. Of Media As - Δ°C

midgut II where it soon became concentrated. Later the dye concentrate was forced through the ileum in tiny particles to the rectal region where it was again diluted by the collection of rectal fluid prior to its elimination from the body.

Discussion

Insects that inhabit freshwaters are continually threatened by the potential hazard of dilution of essential blood solutes. The corixid, C. edulis, is found in ponds, lagoons and the shallow back-water arms of lakes in Oklahoma and are incapable of tolerating salinities of sodium chloride much greater than 1.0% (Frick et al., 1972).

The capacity of osmoregulation in C. edulis was assessed following acclimation to external salinities ranging from deionized water to 0.9% NaCl. The relationship between the haemolymph osmolarity and the medium resembles that of earlier reports on freshwater insects (Shaw and Stobbart, 1963), i.e., the haemolymph is maintained above the external salinity and approaches iso-osmotic conditions with the bathing medium as the concentration is increased. Although we observed a significant difference ($P < .05$) between the haemolymph freezing-points in insects taken from deionized water and those taken from the highest saline ($-0.540^{o}C$) a high degree of regulation was apparent because no significant difference could be demonstrated when comparing all other freezing-point measurements (Fig 6). The medium was always more dilute than the haemolymph and the urine was always more concentrated than the surrounding medium. To achieve such a condition, solutes must accumulate against the concentration gradient or there must be an increase in the movement of water away from the haemolymph.

The regulation of the ionic fraction is often achieved by the control of the fluid fraction (Shaw and Stobbart, 1963; Stobbart and Shaw, 1964) and to determine if the movements of water from the blood were involved in the regulation of the haemolymph osmolarity, the total body water percent was measured in insects from the different salinities. The total body water percent was not greatly affected by changes in the external salinity and no significant difference could be attributed to the different treatments. To test the possibility that water displacement within the body between different compartments was operable, the blood volume percent was measured. Again no significant difference was evident in insects following placement in the different experimental media. Apparently, movements of water are not a significant factor in regulating the osmolarity. In agreement with previous reports, osmoregulation in these aquatic insects apparently depends on the regulation of the ionic composition of the haemolymph which is probably handled largely by the excretory system (Shaw and Stobbart, 1963). Earlier results (Frick et al., 1972), indicate the presence of high concentrations of Na and Cl in the haemolymph of C. edulis. Both ions are regulated in relation to the external salinity. Because the changes in concentration of these two ions closely parallel the changes in the osmolarity, it is probable that these two ions play a major role in maintaining the osmolarity of the haemolymph.

An assessment of the function of the excretory system was made and the relative roles of the Malpighian tubules and hindgut in the regulation of the osmolarity were revealed by comparing this parameter in the urine to that in the haemolymph. When the freezing-point of haemolymph was at its lowest level, the urine osmotic pressure was reduced to less

than half that of the haemolymph while at the highest concentration the haemolymph and urine were not significantly different. The freezing-point of the urine never exceeded that of the haemolymph. Dilution of the urine may have been accomplished by the mixing of the fluids from the gut but even more likely from the reabsorption of ions in the ileum. Solutes are probably reclaimed from the primary secretory fluid (Jarial and Scudder, 1970) which is usually slightly hypotonic to the blood (Ramsay, 1950; 1951; 1953). However, as the blood concentration increases, a greater portion of the secreted water in the gut may be reclaimed in an attempt to regulate the haemolymph osmolarity by preventing it from increasing to the same extent as the external medium. Overall body conservation and regulation of solutes is not 100% efficient since the urine concentration is consistently higher than the external medium. Essential solutes which are excreted must be replenished by tissue or organs from the surrounding medium. The labium has shown this potential in C. bifida (Jarial et al., 1969) and the midgut cannot be discounted since drinking is a noted feature of C. edulis.

By observing the movements of dye in the intestines of Adult insects placed in media containing .01% indigo carmine, we noted that drinking was common but not a continuous process. In addition, drinking was significantly greater in deionized water as opposed to the saline (Frick and Sauer, 1973c). Insects, whose guts were distended with air, were also a common occurrence. Whether or not this helped to control drinking is not known but it did present insuperable technical problems in obtaining sufficient quantities for determing the osmolarity of the midgut fluid. Therefore, changes in the osmolarity

of this fluid brought about by the action of the Malpighian tubules and the iliac pad could not be determined by the conventional method (Shaw and Stobbart, 1963). Instead, attempts to qualitate fluid movements using indigo carmine dye were sought. The fluid fraction appeared to separate from the indigo carmine in the region of the midgut II as indicated by visual changes in the concentration of the dye. Either the fluid was reabsorbed at this point or moved separately into the iliac lumen. The midgut II region apparently acts as a temporary store for the primary secretions of the Malpighian tubules prior to its movement through the iliac region.

Sarcophaga bullata Parker larvae transport ammonium, chloride, Na and K ions across the isolated hindgut (Prusch, 1971) and the exchange of ammonium ions for sodium ions has been suggested in the crayfish, Astacus pallipes Lereboullet (Shaw, 1960). Corixa dentipes eliminate ammonium and bicarbonate ions in amounts almost great enough to account for the total osmolarity of the excreted urine (Staddon, 1964). Ammonium, chloride and bicarbonate ions may normally exchange for sodium and/or potassium in the region of the iliac pad and the increased absorption of water from the primary secretory fluid due to the release of an antidiuretic factor at the higher salinities as suggested by Jarial and Scudder (1971), may be a possibility in C. edulis.

In addition, the regulation of ions with the surrounding medium may be associated with either or both the labium and/or the midgut as a result of drinking water as has been shown for C. bifida (Jarial et al., 1969), and Sialis (Shaw, 1955).

Apparently C. edulis is well adapted for obtaining and conserving

essential haemolymph solutes from its normally dilute surrounding medium and is an excellent regulator of its internal water and haemolymph volume but when subjected to salinities equivalent to or greater than its own haemolymph, regulation breaks down and mortality ensues shortly thereafter.

CHAPTER V

THE EFFECTS OF SALINITY ON THE UPTAKE OF INDIGO CARMINE BY THE MALPIGHIAN TUBULES OF *CORISELLA EDULIS* CHAMPION

The excretion of useless or toxic organic molecules by the Malpighian tubules of arthropods is largely unknown. To date, only indigo carmine and phenol red dyes have been studied in detail (reviewed by Maddrell, 1971b). From this review the following generalities were concluded concerning the excretion of indigo carmine by the Malpighian tubules: (1) excretion of the dye *in vitro* is independent of fluid secretion in *Rhodnius* and *Carausius*, (2) its rate of excretion is pH dependent, and (3) the dye molecules with strong acid groups and no basic groups like indigo carmine are actively transported.

The purpose of this investigation was to study another parameter affecting the rate of excretion of indigo carmine by insects, i.e., the concentration of sodium and chloride and/or osmotic pressure of the external medium. A possible relationship between dye excretory abilities and increased insect mortality at the higher environmental salinities is indicated.

Materials and Methods

Insects were collected from a local pond and acclimated to laboratory conditions as described earlier (Frick et al., 1972). Following a

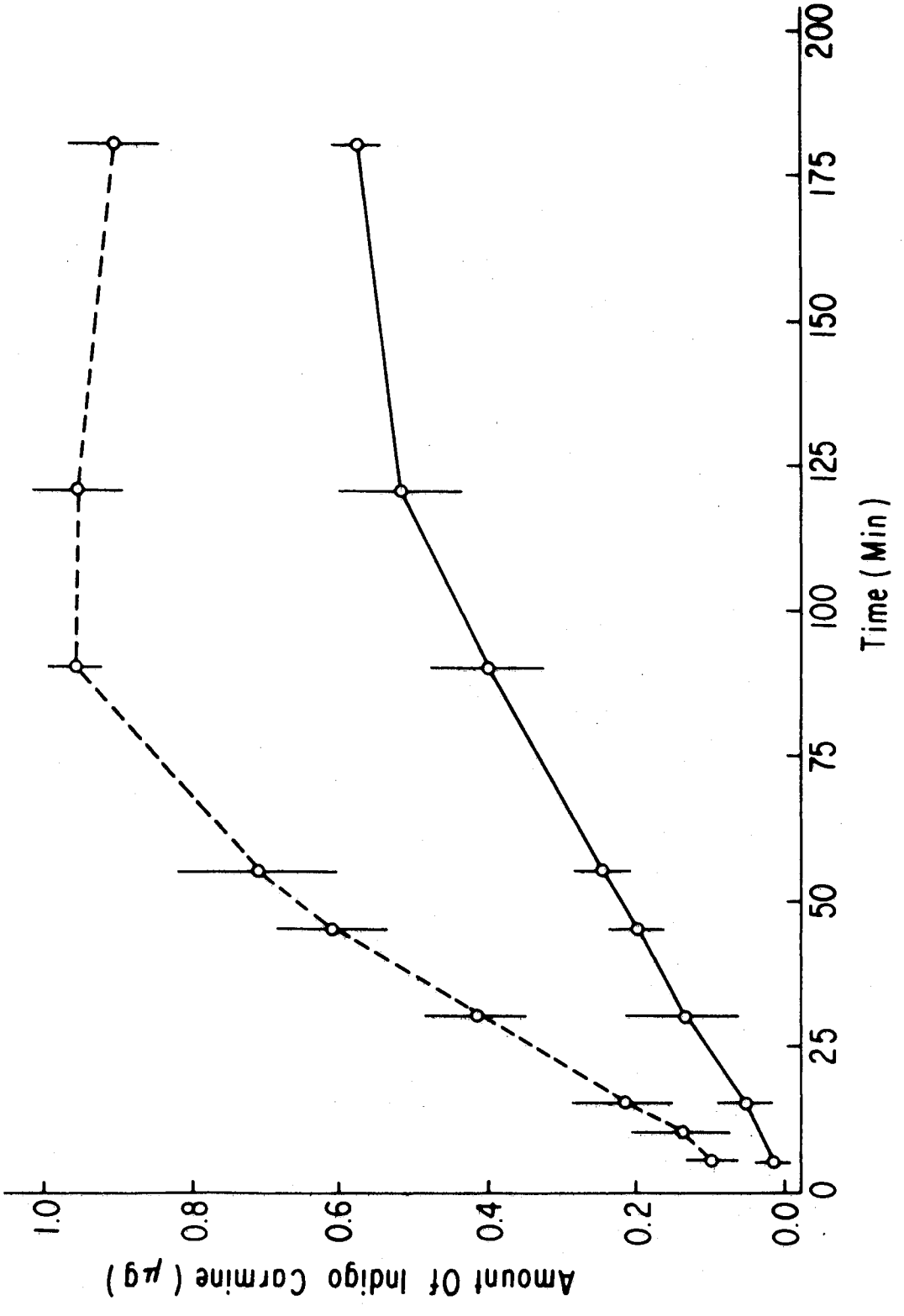
48 hr acclimation period in deionized water and 0.9% NaCl solutions, insects were surface dried on slightly moistened paper towels and their anal openings sealed with a fine droplet of Permount^{R} mounting media. The application was done with a finely tapered but blunt-tipped probe, and the liquid permount was allowed to solidify within one min. Only female insects were used, since sealing of the anus of males was much more difficult and usually unsuccessful. Three-tenths µℓ of a 1% solution of indigo carmine was injected directly into the haemocoel between the 5th and 6th ventral abdominal sclerites in the vicinity of the Malpighian tubules. Great care was taken to prevent damage to the insects. Injections were made under 60X magnification with a finely drawn glass capillary tube precalibrated to 0.3 µℓ. After each injection the insect was placed into a 15 ml glass vial with a saturated cotton swab to maintain the relative humidity near 100% and left unattended for 30 min. The average weight loss of 10 specimens during this time period was 3.2% of the original weight. After 30 min the insects were dissected open in Ringer's saline (Maddrell, 1971a) with the freezing point modified to -0.636^{o}C and the entire midgut, hindgut, and Malpighian tubule system containing fluid and dye were removed. Each gut was finely homogenized in a ground glass homogenizer and made up to a volume of 150 µℓ with deionized water. The solution was thoroughly mixed and centrifuged for 30 min at 4000 rpm. The supernatant was withdrawn and assayed colormetrically at 610nm with a Spectronic 20 spectrophotometer. Concentrations of the unknown dye solutions were determined by comparison to concentrations of known standard solutions.

Results and Discussion

When Corisella edulis is exposed to a wide range of laboratory solutions of deionized water and sodium chloride, most of the insects are able to survive only in solutions ranging from 0 to 0.9% NaCl. In salinities in excess of this range, the rate of mortality increases sharply (Frick and Sauer, 1972). In addition, this insect maintains its haemolymph concentrations of sodium and chloride parallel to the osmotic pressure and excretes a hypo-osmotic urine typical of other freshwater insects (Frick and Sauer, 1973b). These data suggest a correlation between the insect's salt and water balance capabilities and the increased mortality rates at the higher environmental salines. However, knowledge of the effects of environmental osmotic pressure and/or concentrations of saline on the excretion of useless or toxic substances of intermediate size is unknown. To investigate this functional aspect of the Malpighian tubules, indigo carmine dye was employed in both an in vitro and in vivo system. Using the Ringer's saline and indigo carmine made up to 0.01%, the dye was seen accumulating in the lumen of in vitro preparations of Malpighian tubules and flowing to the attached intestines. The movement of the dye was always inhibited by 7.7×10^{-5} M KCN, as indicated by the lack of visual accumulation in the Malpighian tubules.

To check the effects of external salinity (NaCl) on the excretion rate of indigo carmine in vivo, 3.0 µg of the dye in 0.3 µℓ of Ringer's saline was injected directly into the animal's haemolymph or 0.9% NaCl solution. The amount of dye taken up by the Malpighian tubules with time was determined (Fig 11). Within 3 hr, insects that were preacclimated to the saline medium excreted roughly 20% of the dye injected,

Figure 11. A comparison of the rate of uptake of indigo carmine dye *in vivo* by the Malpighian tubules of insects preacclimated to deionized water (.----.) and 0.9% NaCl (.____.). Each point represents the mean of 10 measurements ± S.E.


Amount Of Indigo Carmine (µg)
0.0
0.2
0.4
0.6
0.8
1.0
0
25
50
75
100
125
150
175
200
Time (Min)

while insects preacclimated to deionized water achieved a similar status within 1 hr. No significant difference (t-test) could be shown between the means of insects preacclimated to deionized water for 90 to 180 min, but all measured differences between the means of insects preacclimated to deionized water and saline water were significantly different ($P < .05$) after 15 min and thereafter. The maximum excretion of indigo carmine by the Malpighian tubules of C. edulis in these experiments was about 30% of that injected and occurred when the insects were preacclimated to dilute external media. In other insects the dye which is not excreted is initially taken up by pericardial tissues and blood cells; and, if the concentration is too great to be handled by these tissues, the remainder stays in the blood, possibly staining other organs (Palm, 1952). This seems to be a plausible explanation for the observed reduction in the percent of dye excreted by C. edulis, since the chorion of eggs and reproductive organs were often stained.

The total body water percent and the blood volume were each unchanged in insects taken from the two different preacclimating media (Frick and Sauer, 1973b) but the influx of tritiated water was greater while the insects were in deionized water (Frick and Sauer, 1973d). These data suggest that C. edulis eliminates a larger volume of excess water when placed in deionized water. However, the portion of this imbibed water that is eliminated via the excretory system is not known. Initial attempts to measure fluid secretion in this insect by in vitro preparations of the Malpighian tubules using the technique of Maddrell (1971a) were unsuccessful and the recent report of Pilcher (1969) and Maddrell and Reynolds in Maddrell (1971b) casts doubt on the validity

of using indigo carmine as a marker for measuring the movement of fluid across the Malpighian tubules in the terrestrial insects, Rhodnius prolixus and Carausius morosus. Regardless of the movement of water, however, the data show that the rate of excretion of indigo carmine in C. edulis is greater when preacclimated to dilute media as compared to the saline media. Coupling this information with the fact that C. edulis increases its rate of tritium uptake in dilute media while regulating its total body water and haemolymph volume within narrow limits suggests that there may be a closer relationship between dye and fluid movement into the Malpighian tubules in these freshwater insects than in the terrestrial insects studied by Maddrell (1971b). On the other hand, if these two activities are also unrelated in C. edulis and the excretion of indigo carmine reflects the excretion of other uncolored molecules as suggested by Maddrell (1971b), then the reduced dye excretion at the higher salinities may reflect an accumulation of non-metabolizable residues to levels detrimental to the insect and may in part be responsible for the increased insect mortality observed at the higher salt concentrations.

Various parameters such as dye metabolism, absorption by other tissues, phagocytosis by special cells, coating with free haemocytes, diffusion to regions of the body unexposed to the Malpighian tubules (Maddrell, 1971b) or endocrine factors (Jarial and Scudder, 1971) all present possibilities for affecting the internal dye concentrations and/or the uptake by the Malpighian tubules in this insect. The possibility also remains that any one of these factors could be affected by differences in the concentration of the external saline. The solution to these basic questions has great significance to the problem

of salt and water balance in aquatic insects.

CHAPTER VI

WATER BALANCE IN THE ADULT WATER BOATMAN, *CORISELLA EDULIS* CHAMPION

Movements of water in several different freshwater insects have been investigated (reviewed by Shaw and Stobbart, 1963; Stobbart and Shaw, 1964). Holdgate (1956) and Beament (1961) have measured the permeability constants for the cuticles of several different aquatic and terrestrial insects and accumulated evidence suggests that during evolution to the freshwaters, aquatic insects have gone the cuticle permeability in aquatic insects had increased (Staddon, 1969). The cuticle was shown to be an important route for the uptake of water in the adult aquatic, Hemiptera (Staddon, 1963, 1964, 1966, 1969). In spite of the high cuticle permeability suggested for the aquatic corixid, *Corixa dentipes* (Thomas.) (Staddon, 1969), the total water within the body is regulated within narrow limits by others of the same family; i.e., *Cenocorixa bifida* (Hung), *Cenocorixa expleta* (Uhler) (Scudder et al., 1972) and *Corisella edulis* Chamption (Frick and Sauer, 1973a). The relative importance of cuticle permeability, drinking, and excretion in dilute media, as compared to saline environments and the movements of water under such conditions, have not been investigated in this group.

This paper reports on the results of a study investigating the influence of dilute and saline environmental media on the relative

exchange rates of THO tritium oxide across the general body surface, oral ingestion, and anal excretion in the adult aquatic corixid, C. edulis.

Materials and Methods

Corisella edulis were collected from a local lagoon. All specimens were transferred to the laboratory in water from the lagoon, where they were allowed to adjust to laboratory temperatures. Only slight adjustments in the temperatures were required, since the natural water was always very near the temperatures of the laboratory. Insects were placed into the different saline solutions for 48 to 72 hr prior to each experiment. The handling of specimens was kept to a minimum and soft cotton and nylon nets were used during the transfers. Each experimental holding medium was equipped with rubber matting to provide support for the bouyant insects.

Permanent seals of thickened Permount^{R} over the mouth and anus were applied manually with a small, blunt-tipped probe. The application was aided by the use of a stereomicroscope set at 60X magnification. Numerous mouth-sealed insects were placed into 0.1% solutions of indigo carmine dye for 8 hr and subsequently dissected open to check for the presence of dye in the guts. Visual observations indicated that the process of oral uptake was effectively blocked.

The percent total water was determined by calculation from pre- and post-dessication weights. Complete dehydration of the tissue was accomplished by drying in an oven at 105^{o}C until a constant weight was achieved. Live specimens lost weight very rapidly in the laboratory when out of water. To avoid this unnecessary loss, insects were held

on paper towels moistened by the water from their holding medium in a chamber kept near 100% relative humidity.

Radioactive solutions were prepared by adding tritium oxide (THO) obtained from New England Nuclear to either deionized water or 0.9% NaCl media. Tritiated solutions were made up to an average of 4300 cpm per 100 µℓ. For each group of experimental assays, a concomitant measurement of the external holding solution was made. Counts resulting from the adhesion of THO to the surface of the cuticle was determined by dipping the insects in the experimental solution, blotting the insect on clean, absorbant towels, and homogenizing the whole insect in 1 ml of liquid scintillant (Wharton et al., 1965). The final homogenate was made up to 15 ml with the same "cocktail" and measured for radioactivity with a Beckman 100 Liquid Scintillation System^{R}. Contamination by the homogenizers was determined by following the same procedure except without insects. An average total contamination count was routinely subtracted from the experimental count. Residual counts were related to the amount of THO remaining in the experimental insects. Saturation was defined as unity of the ratio between the cpm/µℓ of the insect's total water content and the cpm/µℓ of the external medium. After 8 hr the uptake ratios in deionized water were very near unity. Counts for the external medium were determined from several experiments and the actual count of each individual specimen was related to this mean value. Variations were calculated as standard error and significances were determined to the $P < 0.5$ level by utilizing the standard t-test.

Results

Oral Ingestion of Water

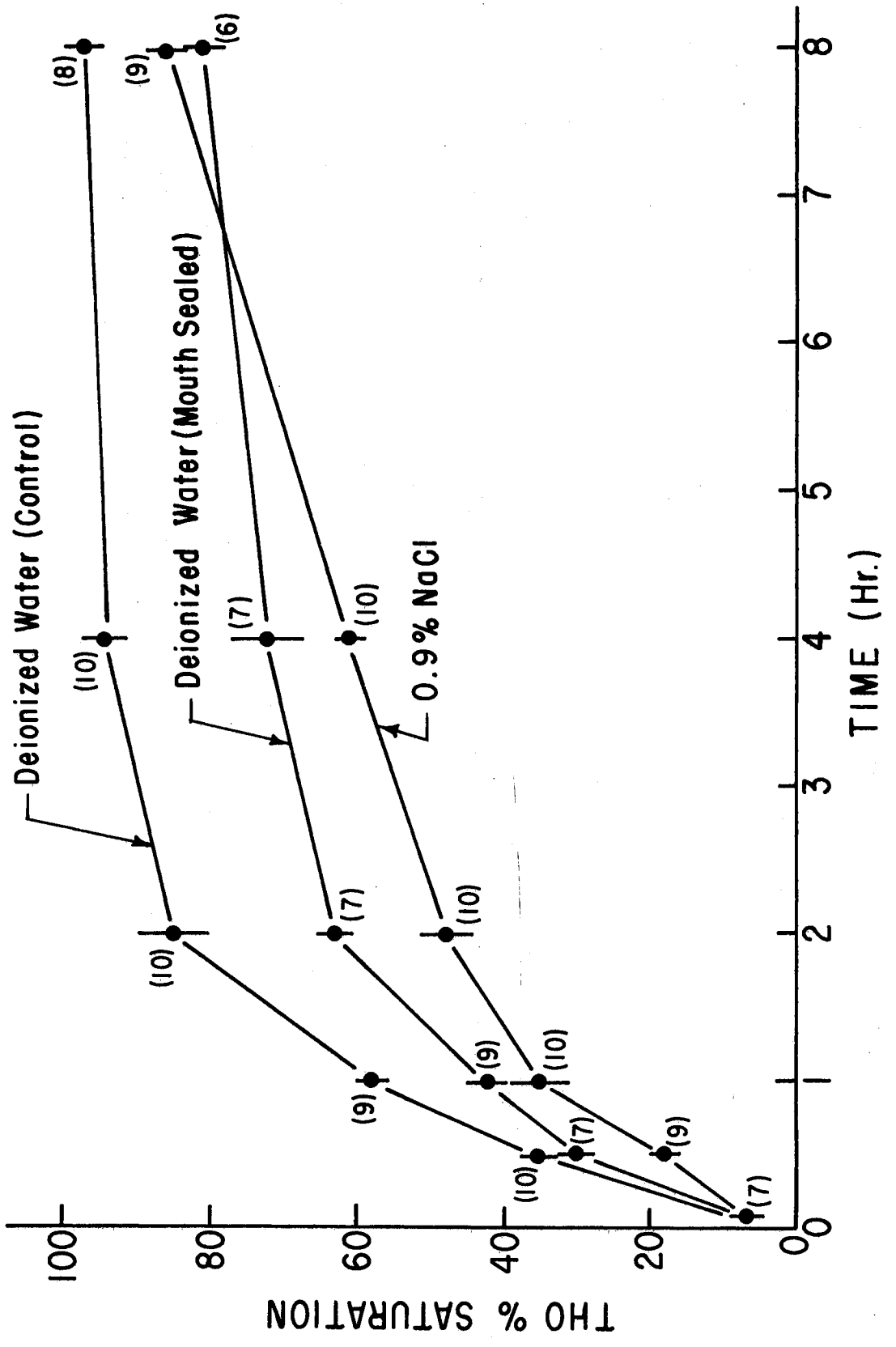
The total amount of water orally ingested after 8 hr was determined following placement of insects into 0.01% solutions of indigo carmine and deionized water or indigo carmine and 0.9% NaCl. The insects in the deionized water ingested an average of 1.6 µℓ, while insects in the saline solution ingested only about ½ this amount (0.8 µℓ). These mean values were significantly different ($P < 0.05$). Occasionally, however, the dissected guts contained a quantity of dye indicating an uptake as high as 4 µℓ of water, while at other times a gut would appear totally void of dye.

Dye uptake was equally prevalent in the guts of anus-sealed specimens. After 4 to 8 hrs the guts of these insects were taut with fluid. Apparently, this treatment prohibited the excretion of excess fluid but did not greatly affect drinking. On the other hand, sealing the mouth completely blocked the intake of dye.

Tritium Uptake by *C. edulis*

Following exposure of "cold" preacclimated insects to tritiated experimental media and measuring the rate of THO uptake with time, it was shown that "normal" untreated insects imbibed THO at a rate significantly greater ($P < 0.05$) in deionized water than either of the other groups which were exposed to 0.9% NaCl or to deionized water but with their mouths sealed (Fig 12). Within the initial 2 hr, "normal" insects imbibed up to 85% of the equilibrium count, while during the final 6 hr of the experiment a "plateau" was reached. A maximum uptake

Figure 12. The "normal" rate of tritium-deionized water uptake by *C. edulis* as compared to specimens of the same species in the same medium but with mouth-seals and when acclimated to 0.9% NaCl without mouth-seals. Vertical lines represent ± S.E. and numbers indicate the number of insects used.


Deionized Water (Control)
Deionized Water (Mouth Sealed)
0.9% NaCl
THO % SATURATION
TIME (Hr.)
0
20
40
60
80
100
1
2
3
4
5
6
7
8
(8)
(10)
(10)
(9)
(6)
(7)
(10)
(7)
(10)
(9)
(10)
(10)
(7)
(9)
(7)

of THO during the 8 hr period (97% saturation) was accomplished by the "normal" insects exposed to deionized water for 8 hr.

To compare the rates of uptake of water through the body surface to ingestion by the mouth, sealed and "normal" specimens of C. edulis were placed into tritiated solutions of deionized water and the percent saturation of THO in both kinds of treated insects were compared at different time intervals. When the mouths of "cold" insects preacclimated to deionized water were sealed and placed into "hot" deionized water, the rate of THO uptake was grossly reduced when compared to "normal" insects from the same experimental media, but whose mouths were left unsealed. A sharp increase in the rate of THO uptake was still apparent; however, it required almost 2 hr for these insects to imbibe a quantity of THO equivalent to that imbibed by "normal" insects within 1 hr. The maximum uptake attained by these insects within 8 hr was 81% saturation; 16% below that of the "normal" insects.

By placement of "cold" insects preacclimated to 0.9% NaCl into tritiated media of the same salinity and with mouths left unsealed, the overall rate curve of THO uptake was noticeably reduced below the curves of both groups of insects which were preacclimated to deionized water. After 8 hr, however, the mean value for the rate of THO uptake appeared to exceed that of the mouth-sealed insects, but was insignificantly lower ($P < 0.05$) than the group of "normal" insects. In fact, a 4 hr period in "hot" experimental saline was required to allow sufficient time for these specimens to achieve essentially the same saturation (60%) as that achieved by "normal" insects in deionized water within 1 hr.

Tritium Loss from 8 Hr Pre-equilibrated C. edulis

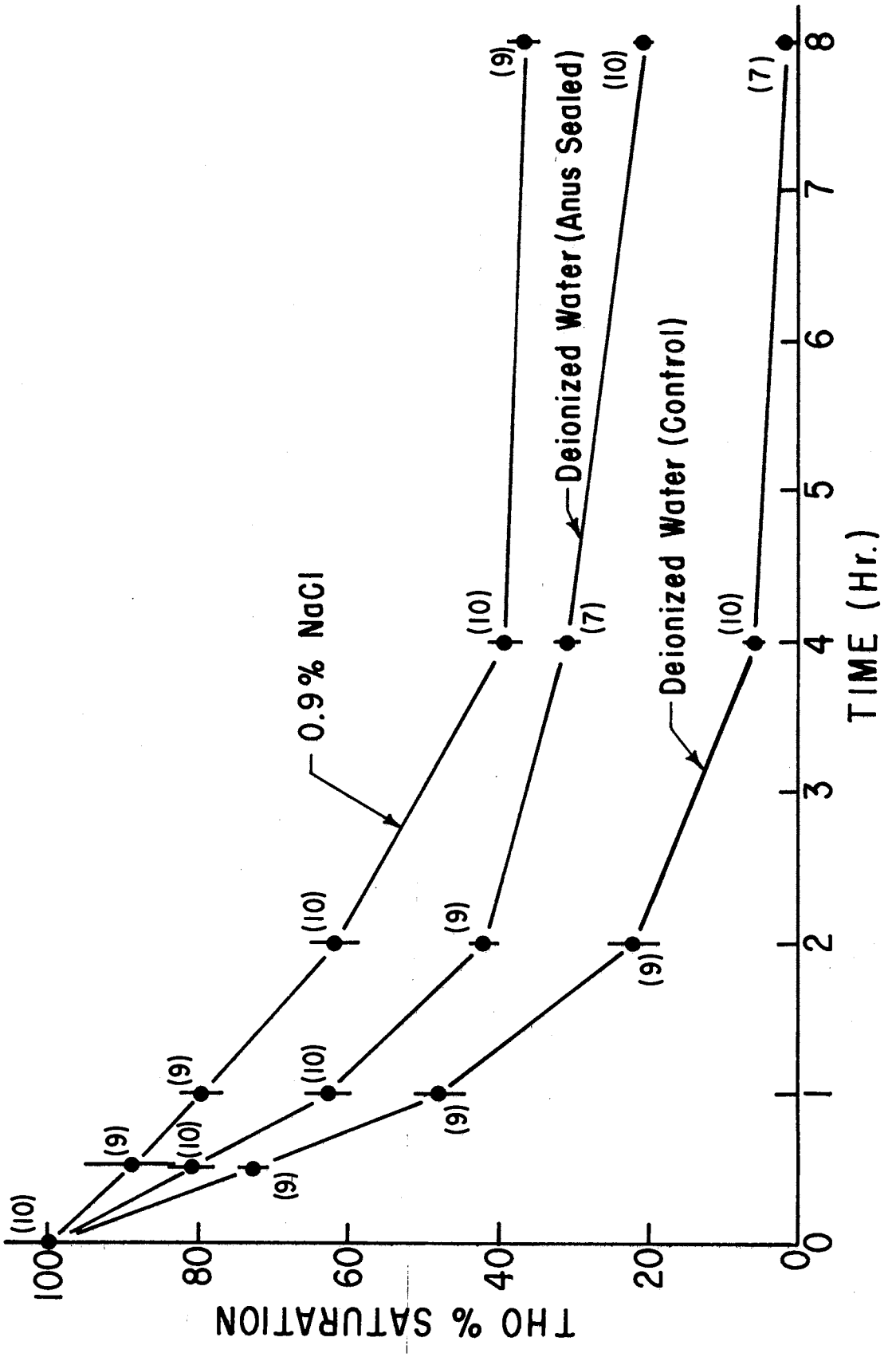
Following exposure of "hot" insects to "cold" deionized water at different time intervals, the rate of THO loss was shown to be approximately the reciprocal of the rate of THO uptake by "cold" insects (Fig 12 and Fig 13). Sealing the anus appeared to have an equivalent effect by delaying the loss of THO from the insect as compared to the delay of THO uptake when the mouth was sealed. After exposure for 8 hr, the maximum loss of THO from "hot" anus-sealed insects was nearly identical to the maximum uptake by "cold" mouth-sealed insects (80%).

However, when "hot" insects preacclimated to 0.9% NaCl were exposed to "cold" saline media with the same concentration, the rate curves were different as compared to the reciprocal rate curve for THO uptake in the same saline. Although the initial rates of THO loss and uptake by these insects were near reciprocals, the time interval between 4 to 8 hr demonstrated that "hot" insects in "cold" media (Fig 13) reached a "plateau", while a continuous rise in the % saturation of "cold" insects in "hot" saline was evident. The THO loss in percent saturation (63%) of 8 hr "hot" insects in "cold" media is considerably less than the THO uptake by "cold" insects in "hot" media (86%).

Effects of Sealing the External Openings of the Gut on the Percent Water

To determine the effects of blocking orally ingested and rectally eliminated fluids on the ability of C. edulis to regulate its total percent water, measurement of this parameter was made following 48 to 60 hr preacclimation in deionized water and then 8 hr of exposure to

Figure 13. The "normal" rate of tritium-deionized water loss by *C. edulis* as compared to specimens of the same species in the same medium but with anus-seals and when acclimated to 0.9% NaCl without anus-seals. Vertical lines represent ± S.E. and numbers indicate the number of insects used.


THO % SATURATION
TIME (Hr.)
0.9% NaCl
Deionized Water (Anus Sealed)
Deionized Water (Control)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
(10)
(9)
(10)
(9)
(10)
(9)
(10)
(9)
(10)
(7)
(10)
(7)

the same medium after sealing only the mouth or anus singly or both together (Table V). The selection of deionized water for this experiment was made on the assumption that this solution would provide a condition favorable for the greatest movement of water between the insect and its external environment. Sealing the mouth had no apparent effect on the total water as compared to the control; however, the variations of the recorded values were greatest among these experimental animals. Sealing the anus resulted in a significant increase ($P < 0.05$) in the total water, as did sealing both the mouth and anus, which suggests the necessity for a properly functioning excretory system to regulate the total water in C. edulis.

Effects of Sealing the Mouth and the Anus on the Total Water of Corisella as a Function of the Salinity of the External Medium

The total water was determined following placement of anus-sealed and mouth-sealed specimens into different concentrations of sodium chloride (Fig 14). The greatest volume of water was found in insects exposed to deionized water (75%). This parameter decreased steadily to 71% as the salinity of the external medium was increased (from deionized water -0.540°C). When placed in salinities having freezing-points near that of the freezing-point of the haemolymph, the total water was not greatly different than in the mouth-sealed insects. With the mouth sealed the regulation of total water was about the same as that found in "normal" unsealed insects (Fig 14).

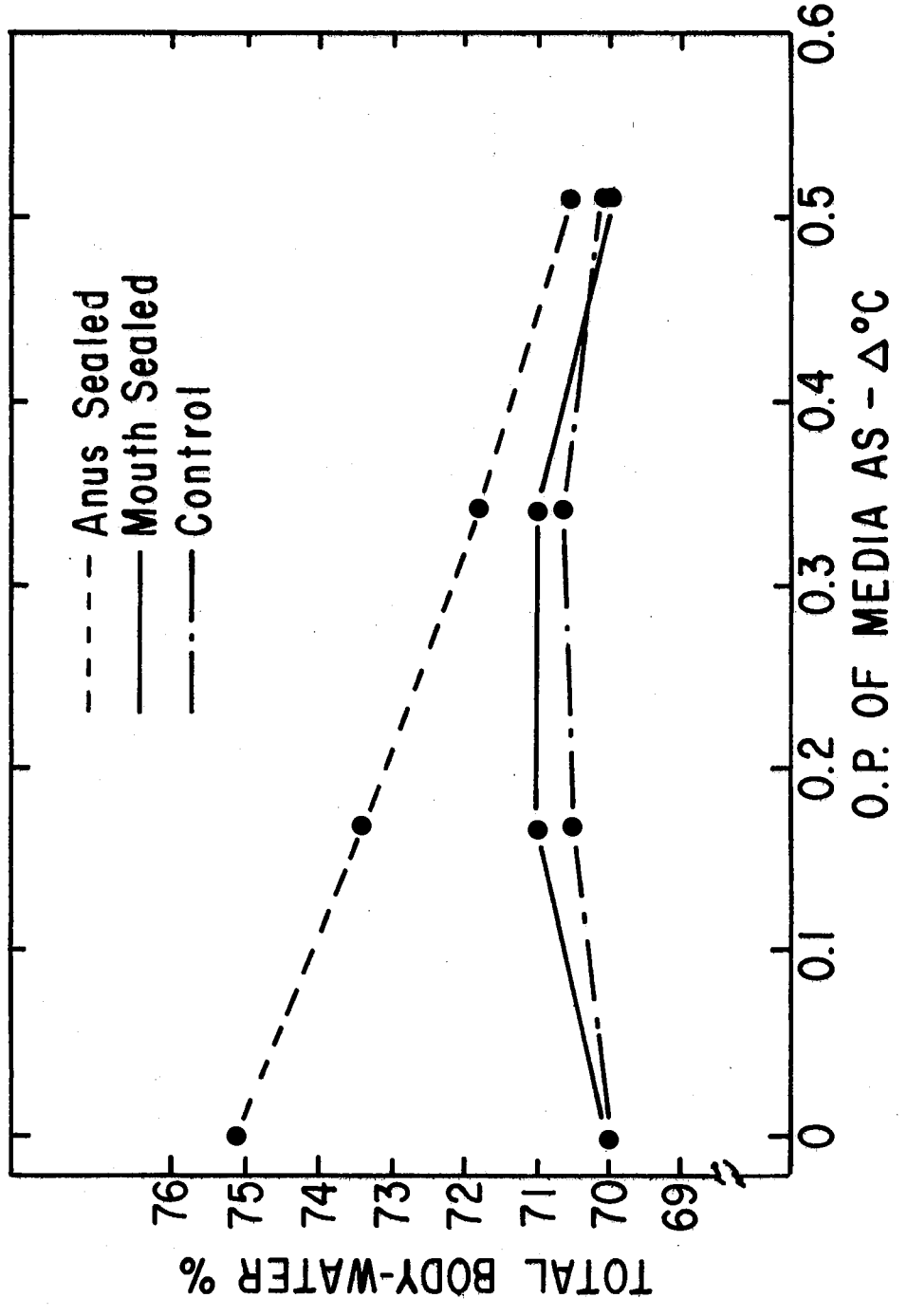
TABLE V

PERCENT TOTAL WATER OF *CORISELLA EDULIS* FOLLOWING ACCLIMATION TO DEIONIZED WATER FOR 10 HOURS*

No Treatment (control)	71.5 ± 1.1
Mouth Sealed	71.1 ± 1.7
Anus Sealed	75.4 ± 0.6
Mouth and Rectum Sealed	75.3 ± 1.0

* Values represent the mean of 10 specimens each with respective S.E.

Figure 14. A comparison of the changes of the "normal" total water content in C. edulis to specimens of the same species with mouth-seals and anus-seals as a function of the salinity (NaCl) of the medium. Each value represents the mean total body water percent of 550 ± 10 mg of insects.


Anus Sealed
Mouth Sealed
Control
TOTAL BODY-WATER %
76
75
74
73
72
71
70
69
0
0.1
0.2
0.3
0.4
0.5
0.6
O.P. OF MEDIA AS - Δ°C

Discussion

"In the freshwater insects water balance is maintained by the excretion from the rectum of a volume of fluid which is equal to that which enters through the cuticle due to osmosis, and through the gut as a result of drinking" (Shaw and Stobbart, 1963). Numberous investigators have been concerned with permeability of water to the cuticle of insects (Holdgate, 1956; Beament, 1961; Oloffs and Scudder, 1965; Staddon, 1964, 1966). Most of the work dealing with cuticular permeability and water balance in the adult corixids has been a result of the excellent study of Staddon (1963), but a clear-cut scheme has not yet emerged. Attempts to give a complete picture of water balance in C. dentipes adults have left several important questions unanswered, including the role of the cuticle. Staddon (1969) believes that the rate of net water uptake by C. dentipes is about 13-16% of the body weight per day and states that this rate takes on added importance when considering the presence of a plastron.

Cenocorixa bifida, Cenocorixa expleta (Scudder et al., 1972) and Corisella edulis (Frick et al., 1972) have demonstrated an ability to regulate their total water content and Jarial and Scudder (1971) have suggested that an antidiuretic principle may be involved. Neurosecretory influence on water balance is believed to affect target organs associated with excretion. In the experiments reported here insects in 0.9% NaCl after 8 hr imbibed an average of one-half (0.8 µl) the quantity of fluid imbibed by insects in deionized water (1.6 µl). Differences were apparently influenced by the salinity of the environment, which suggests that drinking is controlled. The behaviour of C. edulis is opposite to that of Sialis, where drinking is greatest at

the highest salinities, evidently to replace water lost by reverse osmosis through the body surface (Shaw, 1955).

Because the isotope effect of THO for the cuticle of C. edulis was not determined, quantitation of the water permeability could not be calculated. However, the water exchange rate of the Atlantic green crab, Carcinus maenas (L.), using THO as a tracer (Rudy, 1967) underestimated by 20% the value obtained using DHO (deuterium oxide) as a tracer in a later experiment by Smith (1970). Results on water-exchange in the crab, Hemigrapsus nudus (Dana), revealed an isotope effect, THO:DHO:H_2O, of 91:95:100, respectively (Smith and Rudy, 1972). In the adult aquatic hemipterans, Ilyocoris cimicoides (L.), and Notonecta glauca L., the theoretical values derived from a knowledge of the rate of entry of heavy water (DHO) (Staddon, 1966) underestimates the rate of net water uptake by net flow methods (Staddon, 1969). Therefore, our data are very likely conservative estimates of the absolute movements of water.

The "normal" relative movements of THO was established by allowing insects, "hot" and "cold", to swim freely in the opposite medium; i.e., "cold" and "hot", respectively. At specified time intervals insects were removed and the total count with respect to the expected equilibrium count was determined. Within 4 hr a "plateau" very near equilibrium was established and in less than 1 hr both "hot" and "cold" insects became 50% saturated with THO. Sealing the mouth reduced the rate of THO uptake overall as compared to "normal" movements of THO. After 4 hr, mouth-sealed insects still imbibed up to 72% of the expected count, which was 23% below the total uptake by unsealed insects. When equating this reduction in radioactivity to the amount

of imbibition of the dye solution, the total uptake of water as indicated by THO would average about 9.6 μℓ per day. These data pose an interesting question as to how these insects handle such a large volume of water.

To determine the extent to which the excretory system is involved in the role of elimination of excess water, movements of THO were investigated following the abnormal treatment of sealing the anus of "hot" insects and placing them into deionized water. The percent saturation was determined and plotted as a function of time. The loss of THO was reduced in comparison to "hot-normal" insects. Under normal conditions the excretory system may exhibit a more dramatic role, since blocking the anus may increase the volume of fluid which flows through the body wall into the surrounding medium.

In "hot" solutions also containing 0.9% NaCl, the uptake of THO by "cold" preacclimated insects was more gradual as compared to insects in deionized water. The uptake after 4 hr was dampened 34% by the influence of the external salinity. The reduction of THO loss from "hot" insects in "cold" saline was about the same (33%). In both instances, however, the rate of THO movement did not attain a "plateau" after 4 hr, instead, the uptake of THO by "cold" insects continued to rise after 4 to 8 hr. Because the movements of water in these insects while in saline media is small and the influence of sealing the anus is equivalent to sealing the mouth, one is lead to believe that the forces allowing this insect to imbibe water through the cuticle under such treatment is an osmotic one.

Sealing the mouth did not significantly change the total water (compared to control insects) but sealing the anus appeared to reduce

the elimination of fluids by causing a net gain of water from 71.1% to 75.4%. Sealing both the mouth and anus had the same effect as sealing only the anus. Visual observations on the guts of dissected specimens reveal an accumulation of fluid which caused the alimentary canal to become distended from the esophagus to the anus. This volume of fluid may explain the net increase of total water in insects placed in deionized water after sealing the anus. If sealing the anus alters the total water in dilute media, what happens when the external salinity is increased? The data indicate a steady decline in the total water as the salinity was increased. At 0.9% NaCl media (where the osmotic pressure is near that of the haemolymph) the total water was equivalent to that of insects whose mouth was sealed and placed in the various salinities and whose gut was not taut with fluid. This suggests that regulation of body water is dependent upon a properly functioning excretory system but not necessarily drinking. The role of drinking may be important in relation to the uptake of essential solutes from the dilute media, since the oral intake is influenced by the reduction of salts in the external medium. The movement of solutes is probably regulated for the most part by tissues along the alimentary canal, i.e., midgut epithelium or ileum. In other words, oral uptake may reflect a need for specific solutes.

In summary, the volume of fluid orally imbibed while insects are in deionized water is closely related to the volume of fluid excreted; the general body surface is involved in the greatest portion of the total water movements. Although the amount of water entering through the body surface is large, it is counter-balanced by an equal volume returned to the surrounding medium and no net gain in the total water

content of the insect results. The reaction of C. edulis to the higher salinities is simply to reduce the rate of exchange of water across the body surface and concomitantly the volume of water orally imbibed. Of interest, the total water content can be regulated at "normal" levels when the anus is sealed as long as the insect is exposed to a saline medium with an osmotic pressure very near its own. It may be that when the haemolymph to urine osmotic pressure ratio approaches unity (Frick and Sauer, 1973a) the osmotic response is reduced, thereby lessening the movements of water through the body surface. In dilute solutions oral ingestion is increased possibly to obtain solutes from the surrounding medium and a greater volume of hypo-osmotic fluid is voided. Results from earlier experiments suggest that the Malpighian tubules of C. edulis secrete more fluid when the insect is exposed to dilute external media (Frick and Sauer, 1973b) and that the insect, when in dilute media, produces a very hypo-osmotic urine (Frick and Sauer, 1973a).

The importance of drinking is probably related to the regulation of internal ion and/or osmotic pressure levels, while the movements of water across the body surface appear to respond passively to the osmotic pressure ratios. No mechanism for correcting the problem of increasing haemolymph osmolarity at the higher salinities was evident. Therefore, when environmental concentration levels approach that of the internal haemolymph, solutes continue to be taken up by the insect. As a result, the osmotic pressure ratio approaches unity with a parallel reduction in the exchange of water across the general body surface. The volume of water necessary to dilute and safely void the toxic nitrogen waste, ammonia, may be insufficient for survival.

However, ammonia excretion has not been studied in this species and this suggestion is at best speculative. Other activities which are vital to the insect, such as muscle and nerve function, could also conceivably be affected. Additional work is required to further elucidate these phenomenon and the problems facing aquatic fauna in saline-polluted, freshwater environments.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Concentrations of sodium in C. edulis and B. margaritacea, when taken from "natural" habitats, were 168 and 167 mM/l while the concentrations of chloride were 125 and 106 mM/l respectively. Both insects were able to regulate sodium and chloride levels above the environment in deionized water. In media containing high concentrations of sodium and chloride, B. margaritacea exhibited a marked ability to regulate the levels of sodium and chloride below that of the environment, whereas, C. edulis did not with respect to sodium and only slightly so with respect to chloride. C. edulis was unable to survive in media containing more than 300 mM/l Na Cl. Specimens of B. margaritacea on the other hand, survived and tolerated environmental salines up to 550 mM/l NaCl.

The usefulness, convenience and accuracy for measuring freezing-points of small amounts of insect fluid were examined by utilizing a biological cryostat/nanoliter osmometer. A sufficient volume of fluid to make a measurement was obtained from insects weighing as little as 0.21 mg and no significance could be shown when comparing variations of measurements to size of the insect or to the amount of available body fluid. Variations of measurements of standard NaCl solutions having known freezing-points averaged ± 0.055^{o}C S.D. while arthropod haemolymph samples averaged ± 0.048^{o}C S.D. It was shown that the use

of this apparatus provided an accurate and facile method for determining the osmolarity as freezing-point depressing of small amounts of insect body fluids.

After determining the feasibility for measuring the freezing-point of small quantities of fluid, the ability of C. edulis to regulate its haemolymph osmotic pressure after exposure to various environmental salinities (NcCl) and the role of the excretory system was investigated. Following the placement of C. edulis into deionized water the average freezing-point of the haemolymph was reduced significantly below that of insects when taken directly from the natural pond water. The freezing-point rose steadily as the salinity of the medium was increased. The urine of C. edulis, after placement of the insect in deionized water, was hypo-osmotic to the haemolymph but increased rapidly in osmotic pressure when the salinity of the medium was raised. As the salinity of the medium was increased, the insect's haemolymph to urine osmotic pressure ratio approached unity and the freezing-points of the urine paralleled the changing concentrations of the surrounding medium but were maintained slightly higher. The total body water content of insects acclimated to all experimental media was maintained near or slightly higher. The total body water content of insects acclimated to all experimental media was maintained near or slightly above 70%. The haemolymph volume was also regulated over the full range of environmental media at about 30 to 35%.

This information, when related to data on ionic regulation, suggests that sodium and chloride regulation is, for the most part, responsible for the regulation of the osmotic pressure while the water content appears to be uninfluenced by the changes in the osmotic

pressure or the concentrations of sodium and/or chloride. The level of haemolymph osmotic pressure is regulated in dilute solutions near deionized water without a sinificant net loss of blood solutes in spite of the fact that the insect excretes a urine hyper-osmotic to the external medium. This implies that C. edulis is capable of maintaining the osmotic pressure by accumulating solutes from the surrounding medium or by mobilizing osmotically active solutes internally.

Information concerning the relative behavior of the osmotic pressures of the haemolymph and urine, provides evidence for the importance of the role of the excretory system. In dilute media C. edulis excretes a fluid hyper-osmotic to the external medium but hypo-osmotic to the internal medium and as the external salinity rises the osmotic pressure of the urine increases equivalently while the osmotic pressure of the haemolymph experiences less than one-half the changes noted for the urine. The fact that the osmotic pressure ratio between the blood and urine approaches unity reveals that tissues of the Malpighian tubules and/or the alimentary canal are concerned with the process of ion reabsorption. Since the problems associated with the measurement of the primary excretory fluid in small insects are usually too great by utilizing the presently known techniques, it is normally assumed that this fluid is slightly hypo-osmotic to the haemolymph as was discovered through the great pioneering efforts of Ramsay (1950, 1951, 1953) for the aquatic mosquito larvae. By following this assumption for C. edulis, then the reabsorptative capacity of the excretory system can most likely be attributed to the ileum, a tissue that has been shown to be morphologically similar to others known to perform reabsorptative functions (Jarial and Scudder, 1970).

One may speculate that the role of this tissue (ileum) is to eliminate the nitrogenous waste product ammonia while concomitantly recovering essential ions which are necessary for the formation of the primary excretory fluid. Ammonia is a common by-product of nitrogen catabolism in aquatic corixids (Staddon, 1964) and is toxic at low concentrations in the blood by virtue of its alkalinity. Continuous elimination of ammonia is therefore a necessity and the manner in which it is done may compare to the ammonium-sodium ion exchange mechanism suggested by Shaw (1960) for the crayfish, A. pallipes.

To investigate the role of the Malpighian tubules in relation to the overall osmotic regulation of C. edulis, the movements of indigo carmine dye relative to the movements of fluid were investigated. The rate of indigo carmine dye movement into the Malpighian tubules was significantly lower in insects preacclimated to 0.9% NaCl as compared to insects of the same species acclimated to deionized water. The rate of movement of dye into the Malpighian tubules after injection of indigo carmine directly into the insect's haemolymph was roughly twice as great as it was in in vitro preparations. This behaviour supports the possibility of the presence and influence of a blood borne factor. Also, the uptake of dye by in vitro Malpighian tubules is related to the energy requirements of the cell as was noted by the visual inhibition of uptake by 7.7×10^{-5} M KCN. Assuming that the rate of indigo carmine dye excretion in C. edulis is related to fluid secretion by the Malpighian tubules, then the effect of the increased environmental salinity is one of reduced fluid secretion. On the other hand, however, if these two activities are unrelated in C. edulis, then the reduced dye excretion at the higher salinities may reflect an

accumulation of non-metabolizable residues to levels detrimental to the insect and may in part be responsible for the increased insect mortality observed at the higher salt concentrations.

To regulate the osmotic pressure, it is necessary that both the ionic composition and water content are under control. C. edulis possesses the capacity to regulate its ionic levels without experiencing a change in the blood volume or total water content. The manner in which water is regulated becomes important in light of the fact that changes in the blood volume could dictate detrimental changes in the concentration of essential ions. To study water balance in C. edulis the relative rates of oral ingestion, excretion and exchange of water were investigated using indigo carmine dye and tritium oxide (THO). The volume of water orally imbibed was reduced to one-half when acclimated to 0.9% NaCl and when in deionized water the rate of fluid eliminated by the excretory system. The rate of net inward and outward movements of THO between the haemolymph and the external medium are near reciprocals in deionized water and 0.9% NaCl but were greatly reduced in the saline medium. The importance of this information to the physiology of salt and water balance is that C. edulis is faced with the movement of large volumes of water while regulating its osmotic pressure, sodium and chloride ions.

Of interest, however, the total water content can be regulated at "normal" levels when the anus is sealed as long as the insect is exposed to a saline medium with an osmotic pressure very near its own. It is plausible to assume that when the haemolymph to urine osmotic pressure ratio approaches unity, the osmotic response is lessened, thereby reducing the movements of water through the body surface. In

dilute solutions oral imbibition is increased and consequently the volume of water to be handled increases. The tissues and mechanisms involved in the movements of water, however, remain obscure.

In summary, C. edulis is restricted to freshwater habitats by virtue of the inability to hyper-concentrate its urine or alter its blood volume accordingly. There seems to be a great need to move large volumes of water through the body wall, the mouth and the anus. The Malpighian tubules and ileum appear to play major roles in the regulatory process by reabsorbing solutes from the primary excretory fluid but how these movements are controlled and what specific tissues are involved remains unanswered. The exact physiological lesion leading to death is yet unknown but it does appear to relate to either the failure to regulate the ionic composition of the blood or to the reduced movements of water at the higher salinities. The accumulation of ammonia by the reduced elimination of water may present a possible reason for death at the higher salinities but this is only speculative because nitrogen excretion in C. edulis has not yet been investigated. It is clear, however, that changes in the environmental salinity (NaCl) have a major effect on the physiology of salt and water balance in C. edulis. When the insect is attempting to correct for the stressful condition of high salinity it reveals an absence of specific mechanisms commonly exhibited by insects living in brackish- and salt-water environments.

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