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MOHINDER SINGH JARIAL
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External Examiner: Dr. S.H.P. Maddrell
Department of Zoology
University of Cambridge
England

Research Supervisor: G.G.E. Scudder
HISTOPHYSIOLOGICAL AND ULTRASTRUCTURAL STUDIES ON THE HINDGUT AND BRAIN OF CENOCORIXA BIFIDA (HEMIPTERA-INSECTA)

ABSTRACT

This study primarily concerns the cellular failure in the organs associated with osmotic regulation in the water bug Cenocorixa bifida (Hung.), when this insect is placed in highly saline media. This species which normally lives in fresh to moderately saline waters dies in high salinity media. However, experiments show that it can survive for long periods in low saline waters and unfed adults live up to three weeks in unreplaced distilled water at 5°C.

In C. bifida the organs associated with osmotic regulation are the Malpighian tubules, the hindgut and the protocerebrum. It is shown that there are four Malpighian tubules, each of which has four morphologically distinct regions. The ileum has a distinct iliac pad, but no such structure is present in the rectum.

A light and electron microscope study of the Malpighian tubules and the iliac pad in insects in natural hypoosmotic medium show that these organs exhibit structural specialization associated with directional movement of material across the walls.

The presence of numerous infoldings of the basal plasma membrane, the presence of mitochondria and a large number of vesicles on the basal (haemocoel) side of the distal three regions of the Malpighian tubules, plus the direction of movement of neutral red in experimental solutions, suggest that these three regions are concerned with active transport of material from the haemocoel to the
The lumen of the tubule. The presence of mitochondria in the lumen border microvilli and the lumen border, the large pinocytic vesicles, and the path of neutral red in the first segment of the Malpighian tubules suggest that this proximal capillary-like region is concerned with the absorption of physiologically important solutes from the tubule fluid. The iliac pad shows infoldings of both the luminal and basal plasma membrane, numerous elongated mitochondria and a rich tracheole supply. The direction of passage of neutral red in this region, together with determinations made on the contents of the gut, suggests that this pad is concerned with solute uptake; the rectum is shown to be a simple storage chamber.

A study of haemolymph osmotic pressure changes when insects are in various media, together with simultaneous determinations of osmotic pressure of the urine, shows that *C. bifida* is able to hyperregulate its haemolymph over a range of media with a freezing-point depression of between 0 and −0.7°C. In media having a freezing-point depression of between −0.72 and −1.1°C the insects tend toward conformity, while the haemolymph becomes hypoosmotic to the media in higher external concentrations. The insect produces a urine which is always hypoosmotic to the haemolymph, but the urine osmolarity increases with the increase in external salinity.

The electron microscope study of the Malpighian tubules and iliac pad showed no visible ultrastructural changes when insects were placed in various hypoosmotic media (including distilled water). However, in isosmotic media, when there is a tendency for osmotic conformity, and in the hyperosmotic media, when it can be shown that the insects' hyperregulatory capacity fails, definite
ultrastructural changes were found. In both isomotic and hyperosmotic media, the mitochondria of the Malpighian tubules and iliac pad showed structural breakdown. Further the plasma membrane infoldings towards the lumen of the iliac pad cells, became separate from the intima. These changes being consistent with failure of hyperregulation, indicate an inability of the tissue to function properly under isosmotic and hyperosmotic conditions.

The study of the protocerebrum showed that there are six to eight neurosecretory cells therein which can be classed as 'A' cells and which undergo changes when the insects are placed in different salinities. A relationship between osmotic regulation and the neurosecretion of these cells is indicated.
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HISTOPHYSIOLOGICAL AND ULTRASTRUCTURAL
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A thesis submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

in the Department of Zoology

We accept this thesis as conforming to the required
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THE UNIVERSITY OF BRITISH COLUMBIA
August, 1967
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Department of Zoology

The University of British Columbia
Vancouver 8, Canada

Date September 8, 1967
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This study primarily concerns the cellular failure in the organs associated with osmotic regulation in the water-bug *Cenocorixa bifida* (Hung.), when this insect is placed in highly saline media. This species which normally lives in fresh to moderately saline waters dies in high salinity media. However, experiments show that it can survive for long periods in low saline waters and unfed adults live up to three weeks in unreplaced distilled water at 5°C.

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INTRODUCTION

The corixid *Cenocorixa bifida* (Hungerford) is a common insect in small alkaline lakes of the B.C. Interior, but is absent from those of high salinity (Scudder, 1965). Preliminary studies (Jarial, 1964; Teraguchi, 1964) suggested that absence from these highly saline waters was due to the insects inability to regulate its internal milieu. Almost all insects died within six hours in natural lake water having a freezing point depression of -1.40°C. However, the exact cause of death of these insects in such concentrated media is not known. It is presumed that failure of one or more of the organ systems of the body has taken place.

In the majority of insects, the Malpighian tubules and the hindgut are the main organs through which uptake and loss of solutes and water takes place (Wigglesworth, 1965). It has also been shown in *Iphita limbata* Stal. (Nayar, 1960) and *Schistocerca gregaria* Forskal (Highnam et al. 1966) that the medial neurosecretory cells of the pars intercerebralis are intimately associated with osmoregulatory function in that they appear to elaborate a principle which directly or indirectly affects the water balance of these insects.

It is apparent that a knowledge of ultrastructure of a tissue is of utmost importance in elucidating the physiological process occurring in that tissue. The study of cells involved in active transport of ions and water provides a clear instance of the importance of correlating structure with function. For example recent studies of vertebrate tissues (Peachy and Rasmussen, 1961; Diamond and Tormey, 1966; Kaye et al. 1966) reveal that, besides
the cells, extra- and intercellular spaces in the epithelia play an important role in the movement of ions and water against an osmotic gradient.

From what we currently know of functions at the cellular level, it should be possible to detect changes and failure in organ systems by studying their histology and ultrastructure. Sohal and Copeland (1966) have noted marked changes in the basal plasma membrane infoldings of the anal papillae cells of mosquito larvae, *Aedes aegypti* (L.) in isosmotic and hyperosmotic media. Further Berridge and Gupta (1967) working on the blowfly *Calliphora erythrocephala* (Meig.), have shown that the complex system of intercellular spaces formed by the infolding of the lateral plasma membrane of the rectal papillae cells shows a direct response to the conditions of minimal and maximal transport of fluid. These spaces are grossly distended in the flies injected with hypotonic media, highly dilated under normal conditions, and completely collapsed in the fasting and starved flies.

The present investigation has as its main aim, the histological and ultrastructural description of the organs associated with osmotic regulation in *C. bifida* in its normal environment, and the detection and description of any cellular changes in such organs, when insects are placed in various concentrations of natural lake water.

One of the important parameters that has to be measured prior to the study of such ultrastructural changes is the osmolarity of the tissues being fixed. In any investigation like the present one it is important to know that any changes observed in tissues are not the cause of the fixation process.
Sjostrand (1956) has pointed out that the slow penetrating osmium tetroxide solution of Palade is strongly hypoosmotic to most cells and can cause marked swelling. Rhodin (1954) working on the proximal convoluted tubule of the mouse kidney showed that the mitochondria swell with hypoosmotic fixatives and shrink with hyperosmotic solutions. He has thus stressed that it is essential to use isosmotic (to the tissues) fixatives for ultrastructural studies, especially when these involve experimental conditions as in the present thesis. This being the case, it is necessary to determine the osmotic pressure of the cells to be fixed. To date no one has measured the osmotic pressure of cells, but it is generally assumed that they are isosmotic with the body fluids (Robinson, 1965) although there may be local osmotic gradient across the cells. The determination of the osmotic pressure of the haemolymph in the insects to be studied is thus a prerequisite to the formulation of the solution for tissue fixation.

In order to relate the ultrastructural details with the physiological processes going on in the insect and in order to establish reference points for comparison of this work with other research reported in the literature, other parameters such as mortality in different experimental media, acclimation, osmotic pressure of fluids from different parts of the gut and the uptake of vital dyes by the excretory systems also have to be studied.

We know that various cell based processes can vary considerably in response to the physiological requirements of the organism as a whole. However, we have little information to date on the exact site of such cellular responses. Only when there is a detailed study of ultrastructure along with physiology will many of
the processes going on in the living organism be clearly understood. While some progress has been made in this line in the study of vertebrate and some insect tissues, to date no study of this sort has been undertaken in aquatic insects and it is these which most easily lend themselves to experiments concerning environmental factors. The insect C. bifida provides an ideal animal for concomitant studies of physiology and ultrastructure since it naturally occurs in lakes of varying salinity.

The work of Claus (1937) showed that Corixidae are able to actively regulate the osmoconcentration of their body fluids. The mixohaline Sigara lugubris Fieber was able to regulate better than the fresh water species S. fossarum Leach. However, in view of the short duration of Claus' experiments (two hours), Krogh (1939) and Shaw and Stobbart (1963) doubt the validity of this conclusion, since it is possible that the values of the osmotic pressure of the haemolymph given by Claus may not represent the true steady state. Holgate (1956) considers that acclimation periods of less than three days may be too short in insects with low cuticular permeability. A study of osmotic regulation of the haemolymph in C. bifida after acclimation is thus a further prerequisite for this study.
MATERIALS AND METHODS

Corixidae in the lakes in the Cariboo district of British Columbia have been collected over a 7 year period (1959-1966) and their distribution determined with respect to the salinity of the natural lake water (Scudder, unpublished).

The insect Cenocorixa bifida (Hung.) used in this study was obtained from one of these lakes, White Lake, on the Green Timbers Plateau. Insects were transported to the laboratory in one gallon thermos jugs and held at 5°C or 10°C in constant temperature cabinets without food until needed.

Gross morphological dissections were carried out in insect Ringer solution (Hoyle, 1954). Insects used for general histology were fixed in Bouin's fluid and sections stained with Ehrlich's acid haematoxylin and eosin.

Neurosecretory changes in the brain were studied exclusively in the male C. bifida (flying form) since in the female insect any changes in different environmental salinities could be obscured by the differences which occur in the medial neurosecretory cells during the reproductive cycle. The insects were fixed in modified Bouin's fluid (Ewen, 1962) and sections stained with chrom-haematoxylin-phloxin stain (Gomori, 1941).

The green granules found in the Malpighian tubules were histochemically stained for magnesium using Titan yellow (Glick, 1949; Bowling and Wertlake, 1966). The Periodic Acid-Schiff (PAS) test for mucoproteins and mucopolysaccharides was done on the sections of the rectal pad and the first region of the
Malpighian tubule.

For ultrastructural studies the organs were dissected out in and fixed for 15 minutes at room temperature in the following mixtures:- 1 part 5% Osmium tetroxide; 1 part 10% glutaraldehyde; 2 parts 0.2M phosphate buffer (Millonig, 1961) at pH 7.2; and 4-8% sucrose to adjust the osmotic pressure to that of the haemolymph. After fixation the material was washed in a few changes of phosphate buffer, cut into small pieces, dehydrated and finally embedded in Epon (Luft, 1961). Polymerization was effected at 60°C for 48 hours. Sections were cut on a Porter-Blum MT1 ultramicrotome, stained for 5 minutes in a saturated solution of uranyl acetate in 50% alcohol (Watson, 1958), washed with distilled water and post stained for five minutes in lead citrate (Reynolds, 1963). The sections were examined with a Hitachi HU 11A electron microscope.

For light microscope study of the Malpighian tubules and hindgut, 1.5-2,μ thick sections of osmium-glutaraldehyde fixed and Epon embedded material were obtained and stained with basic fuchsin-crystal violet stain (Lee, 1965).

Mortality experiments were carried out at 5°C in a constant temperature cabinet. Twenty insects were put in 250 ml. glass beakers containing 100 ml. of the medium. The number of dead insects were recorded each day until the whole population died and an average survival period in days was obtained.

Osmotic pressure of haemolymph, urine and the media was measured with a Ramsay and Brown (1955) osmometer. Haemolymph was collected in a glass capillary from a drop which formed on detachment of a fore wing. Urine was collected by securing the insect
between fingers and gently pressing the abdomen. By doing so a clear drop of urine oozes out of the anus which could be easily collected in a glass capillary.

Fluid from different parts of the gut was obtained by dissecting the insect in insect Ringer and ligating midgut, hindgut and rectum with sira wax drawn into threads. The gut was removed and placed under liquid paraffin, the different parts being then cut with fine scissors. A capillary was then inserted into the cut end and the fluid in the gut drawn up into the tube after an initial drop of liquid paraffin which was also sealed with liquid paraffin. Some experimental error may have been incurred in the process of obtaining fluids from the midgut and the ileum. Such a source of error might occur through cell injury and/or contamination by the medium in which the insects were dissected.

In the study of the pathway of excretory products, small quantities of 0.005% neutral red in the insect Ringer were injected into the haemocoele and within five minutes insects were dissected and the location of the indicator noted. In addition, uptake of neutral red was studied by injecting the solution into the hindgut through the anus and also by immersing dissected Malpighian tubules in insect haemolymph containing a drop of 0.005% neutral red in insect Ringer.

All media used in this study were various dilutions of natural water from a single lake (Long Lake) on the Green Timbers Plateau. Long Lake water collected on September 8th, 1965 was evaporated by an electric fan to four times concentration, millipore filtered and diluted to required concentrations. Chloride measurements were
made on a Buchler Cotlove Chloridometer. A Zeiss PF 5 flame photometer was used to estimate sodium and potassium, the potassium values being obtained with a constant background of sodium. The media used are listed in Table I.

Statistics for morality and osmotic pressure data were computed by the IBM computer at the U.B.C. Computing Center; a S3-6 (simple regression) program was used. The difference between the means was tested by students' "t" test (Steel & Torri, 1957). The difference in the neurosecretory activity between the treatments was tested by Friedman's two way analysis of variance (Siegal, 1956). In all statistical tests the significance was considered at $P \leq 0.01$ level of probability. All means of osmotic pressure values are based on five measurements from different insects.
TABLE I

MEDIA USED IN EXPERIMENTS

<table>
<thead>
<tr>
<th>MEDIA</th>
<th>Distl. water</th>
<th>Hypoosmotic</th>
<th>Itsosmotic</th>
<th>Hyperosmotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotic Pressure</td>
<td>0</td>
<td>0.18±0.01</td>
<td>0.28±0.01</td>
<td>0.44±0.01</td>
</tr>
<tr>
<td>(\Delta ^\circ C) (\bar{x}) ± S.E.</td>
<td>0</td>
<td>4,720</td>
<td>6,500</td>
<td>11,180</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0</td>
<td>59</td>
<td>114</td>
<td>227</td>
</tr>
<tr>
<td>Na⁺ mEq/l</td>
<td>0</td>
<td>8.10</td>
<td>15.81</td>
<td>31.78</td>
</tr>
<tr>
<td>Cl⁻ mEq/l</td>
<td>0</td>
<td>9.92</td>
<td>3.40</td>
<td>7.50</td>
</tr>
<tr>
<td>K⁺ MEq/l</td>
<td>0</td>
<td>* White Lake water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\Delta ^\circ C\) = Freezing-point depression
\(\bar{x}\) ± S.E. = Mean ± Standard error
RESULTS

I. Mortality of *C. bifida* adults in the experimental media

In order to establish the time at which the osmotic pressure of body fluids, histology and ultrastructure was to be compared under different salinities, it was necessary to determine the average time an insect would live in the various media of a known volume. Further, this information was essential to show that insects could not live in all media. Table II gives the results of a mortality experiment carried out on unfed insects at 5°C. The upper salinities are shown to be too extreme for the insects and it is seen that in these highly saline waters, they lived an average of only one or two days as compared to a long survival in the lower salinities as well as in unreplaced distilled water.

II. Osmotic pressure of the haemolymph

It was noted in the introduction that a measure of the osmotic pressure of the haemolymph was necessary in order to determine the correct formulation of the fixative to be used in ultrastructural studies and to know whether this species was able to regulate its internal milieu over a range of external concentration. However, previous studies (Teraguchi, 1964) showed that there were marked fluctuations in the osmotic pressure of the haemolymph when insects were placed in a new medium. Insects used in such studies should be acclimated to the factor i.e. salinity being investigated. Once the period of acclimation is known it is then possible to compare the osmotic pressure of the
### TABLE II

**MORTALITY IN THE EXPERIMENTAL MEDIA**

<table>
<thead>
<tr>
<th>Osmotic Pressure</th>
<th>Distl. water</th>
<th>HYPOOSMOTIC</th>
<th>Isosmotic</th>
<th>Hyperosmotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Δ °C) = Freezing-point depression</td>
<td>0</td>
<td>± 0.18</td>
<td>± 0.28</td>
<td>± 0.44</td>
</tr>
<tr>
<td>(Δ °C) Mean ± S.E.</td>
<td>0</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.01</td>
</tr>
<tr>
<td>Conductivity, micromhos/l</td>
<td>0</td>
<td>4,720</td>
<td>6,500</td>
<td>11,180</td>
</tr>
<tr>
<td>Mean Survival ± S.E.</td>
<td>22.65</td>
<td>± 3.98</td>
<td>30.60</td>
<td>± 3.99</td>
</tr>
</tbody>
</table>

*White Lake water

Δ °C = Freezing-point depression

* = Mean

S.E. = Standard error
haemolymph of acclimated animals to see if different fixatives are required and whether the insects can regulate their internal osmo-concentration.

a) Acclimation

Acclimation to different media at 5°C was measured by observing the changes in the osmotic pressure of the haemolymph of C. bifida adults. Insects were placed in a more dilute and a more concentrated medium than the water from which they were taken in the field, and measurements of the depression of freezing point of the haemolymph made at regular intervals. The results of the acclimation experiments are shown in Table III.

It is seen that the freezing point depression of the haemolymph, while changing somewhat in the more dilute medium in the first 24 hours, had stabilized in both media by 72 hours.

Determination of the osmotic pressure of haemolymph of insects in the various media should thus be carried out at 72 hours, or after. The mortality experiment showed that a short time period would have to be selected but these acclimation experiments show that the period should not be less than 72 hours. The time 72 hours was thus selected as the experimental period.

b) Osmotic pressure of haemolymph

Adjustment of the fixative

The osmotic pressure of haemolymph of C. bifida in its natural hypoosmotic environment, in media isomotic and hyper-osmotic to its haemolymph, and in distilled water was measured at 72 hours when insects had become acclimated to the media.
# TABLE III

**ACCLIMATION EXPERIMENTS**

Haemolymph osmotic pressure (Δ°C) at different periods of acclimation

<table>
<thead>
<tr>
<th>Medium Δ°C (x ± S.E.)</th>
<th>0 hr.</th>
<th>6 hrs.</th>
<th>12 hrs.</th>
<th>24 hrs.</th>
<th>48 hrs.</th>
<th>72 hrs.</th>
<th>96 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.68 ± 0.02</td>
<td>0.62 ± 0.02</td>
<td>0.68 ± 0.02</td>
<td>0.69 ± 0.02</td>
<td>0.70 ± 0.02</td>
<td>0.73 ± 0.02</td>
<td>0.75 ± 0.02</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>0.07 ± 0.02</td>
<td>0.62 ± 0.02</td>
<td>0.55 ± 0.03</td>
<td>0.52 ± 0.03</td>
<td>0.56 ± 0.03</td>
<td>0.58 ± 0.05</td>
<td>0.62 ± 0.03</td>
<td>0.62 ± 0.03</td>
</tr>
</tbody>
</table>

* Insects from White Lake

(Δ°C = 0.27 ± 0.02)

\(\bar{x} ± S.E. = \text{Mean} ± \text{Standard error}\)

Δ°C = Freezing-point depression.
Table IV shows results of these determinations.

It is seen that the haemolymph values vary in the different media used. Thus, it is assumed that the osmotic pressure of the cells to be studied, likewise varies in the same manner. A single fixative formulation therefore could be used throughout this study.

The osmotic pressure of the fixative was thus adjusted using various amounts of sucrose, as suggested by Caulfield (1957). Table IV gives the adjustment made to the fixative \( \Delta^\circ C = 0.68 \pm 0.02 \) to correspond to the haemolymph and hence presumably tissue osmotic pressure. Plate I shows a comparison of the effects of hypoosmotic and isosmotic to the haemolymph fixative on the cell organelles of region IV of the Malpighian tubule.

c) Osmotic pressure values of haemolymph and urine in different media

Measurements of haemolymph and urine osmotic pressure (\( \Delta^\circ C \)) were obtained after the insects had been in different experimental media for 72 hours. The results are given in Table V and Figure I. The differences between the mean points of the haemolymph and urine osmotic pressure graphs from those of the isosmotic line were found highly significant \( (P \leq 0.01) \) except those on the broken line which were not significantly different from the isosmotic line.

These results clearly indicate that \textit{C. bifida} is able to hyperregulate its haemolymph osmococoncentration over a moderate range of media hypoosmotic to its haemolymph and in distilled water. However, in media with \( \Delta = -0.72 \) to \(-1.1^\circ C \) the insects tend to conform to the environment and finally become hypoosmotic on transfer to more concentrated media (Fig. I).
### TABLE IV

**ADJUSTMENT OF FIXATIVE'S OSMOTIC PRESSURE**

<table>
<thead>
<tr>
<th></th>
<th>Distl. water</th>
<th>Hypo-osmotic</th>
<th>Iso-osmotic</th>
<th>Hyper-osmotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta^\circ C$ of Medium $x \pm S.E.$</td>
<td>0 $\pm 0$</td>
<td>$0.29 \pm 0.01$</td>
<td>$0.93 \pm 0.01$</td>
<td>$1.30 \pm 0.01$</td>
</tr>
<tr>
<td>$\Delta^\circ C$ haemolymph $x \pm S.E.$</td>
<td>$0.62 \pm 0.02$</td>
<td>$0.65 \pm 0.01$</td>
<td>$0.90 \pm 0.01$</td>
<td>$1.11 \pm 0.02$</td>
</tr>
<tr>
<td>Sucrose added to the fixative</td>
<td>-</td>
<td>-</td>
<td>$4.07%$</td>
<td>$7.96%$</td>
</tr>
</tbody>
</table>

* White Lake water

$X \pm S.E.$ = Mean + Standard error

$\Delta^\circ C$ = Freezing point depression

N.B. All means based on 5 measurements from different insects
Plate I

Effect of fixative osmolarity on the plasma membrane infoldings and mitochondria of region IV of the Malpighian tubule. x 32,000

Figures I and 2 show effect of a fixative hypoosmotic to the haemolymph. Note the highly expanded plasma membrane infoldings and poorly fixed mitochondria. x 42,500

Figure 3 indicate properly fixed organelles in isosmotic fixative. x 54,000

Key to lettering of Plates

am abnormal mitochondria
bm basement membrane
c electron dense coat
cm cell membrane
crt cristae
er endoplasmic reticulum
f filaments
imm inner mitochondrial membrane
int  cuticular intima
ipm  inner plasma membrane infoldings
ln  lumen
ls  laminated sphere
m  mitochondria
ml  muscle layer
mt  microtubules
mv  microvilli
n  nucleus
nm  nuclear membrane
nsg  neurosecretory granules
omn  outer mitochondrial membrane
opm  outer plasma membrane infoldings
pi  pars intercerebralis
pm  plasma membrane infoldings
s  sphere
sg  secretory granules
tr  tracheoles
um  unit membrane
v  double membrane bound space
vs  vesicles

N.B.  This lettering is followed in all plates.
### TABLE V

**OSMOTIC PRESSURE MEASUREMENTS (Δ °C) HAEMOLYMPH AND URINE IN DIFFERENT MEDIA**

<table>
<thead>
<tr>
<th></th>
<th><strong>MEDIAN</strong></th>
<th><strong>HYPOOSMOTIC</strong></th>
<th><strong>ITSOOSMOTIC</strong></th>
<th><strong>HYPEROSMOTIC</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distl. water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fall 1966</strong></td>
<td>0 ± 0</td>
<td>0.25 ± 0.01</td>
<td>0.45 ± 0.01</td>
<td>0.71 ± 0.01</td>
</tr>
<tr>
<td><strong>C. bifida</strong></td>
<td>0.62 ± 0.02</td>
<td>0.67 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td><strong>Spring 1966</strong></td>
<td>0 ± 0</td>
<td>0.27 ± 0.02</td>
<td>0.38 ± 0.02</td>
<td>0.62 ± 0.02</td>
</tr>
</tbody>
</table>

- **Δ °C Mean ± S.E.**
- **Mean ± S.E.**
- **Mean ± S.E.**

*White Lake water
S.E. = Standard error
Δ °C = Freezing-point depression

N.B. All means based on 5 measurements from different insects.
Haemolymph and urine freezing-point depression plotted against that of external media. Each point represents mean Δ °C value of haemolymph and urine obtained from five different insects after 72 hours of acclimation. Vertical bars indicate the extent of Standard deviation. Open circle (o) indicates fall 1965 insects and closed circles (●) Spring 1966 insects. The dotted part of the haemolymph and urine graph is not significantly different from the isosmotic line.
The insects can only produce urine hypoosmotic to the haemolymph, although its osmoconcentration increases with the increase in the external salinity. The urine is hyperosmotic to unreplaced distilled water, isosmotic to media with $\Delta = -0.27$ to $-0.65^\circ C$, and in media with $\Delta = -0.65^\circ C$ or above the urine becomes strongly hypoosmotic.

d) Osmotic pressure measurements of haemolymph, midgut fluid, iliac fluid and rectal fluid (urine)

Osmotic pressure measurements of the gut contents after the insects had been in distilled water, hypoosmotic or isosmotic medium for 72 hours are given in Table VI and Figure 2.

The results suggest that the ileum is site of osmotic work. The fluid in the ileum is more dilute than that in midgut. The osmotic pressure measurements of the midgut fluid show that it is hyperosmotic to the haemolymph and to the medium which the insects normally take in. It is possible that the high osmotic pressure of the midgut fluid is due to the secretion of osmotically active substance (like the digestive enzymes) from the midgut epithelium and/or the absorption of water from the lumen into the haemocoel.

III. General description of the gut and excretory organs

a) Gut

The gut of Cenocorixa bifida is divisible into three main regions, viz. foregut (stomodaeum), midgut (mesenteron) and hind-gut (proctodaeum). The foregut consists of a long narrow tubular pharynx which opens into [slightly wider oesophagus:
<table>
<thead>
<tr>
<th>MEDIA</th>
<th>Distl. water</th>
<th>Hypoosmotic</th>
<th>Isoosmotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium $\Delta^\circ C$</td>
<td>0</td>
<td>0.29 ± 0.01</td>
<td>0.93 ± 0.01</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemolymph $\Delta^\circ C$</td>
<td>0.59 ± 0.01</td>
<td>0.65 ± 0.01</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midgut $\Delta^\circ C$</td>
<td>0.60 ± 0.02</td>
<td>0.70 ± 0.02</td>
<td>1.07 ± 0.03</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iliac fluid $\Delta^\circ C$</td>
<td>0.35 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal fluid (urine) $\Delta^\circ C$</td>
<td>0.24 ± 0.01</td>
<td>0.30 ± 0.02</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* White Lake water
S.E. = Standard error
$\Delta^\circ C$ = Freezing-point depression
Upper part of the figure shows a diagrammatic representation of the alimentary canal of *C. bifida*. Lower part of the figure shows a plot of data from Table VI. Each point represents the mean value of freezing-point depression of one region of the gut in one particular medium. Solid vertical bars indicate the extent of Standard deviation. All values were obtained from Spring 1966 insects.
The oesophagus in turn opens into the midgut. The midgut and the hindgut are separated by a small pyloric chamber which receives the openings of the four Malpighian tubules. The ileum which is the first part of the hindgut is a straight tube with a thick dorsolateral and a thin ventral wall. The ileum ends in a thin walled sac, the rectum, which opens to the exterior at the anus.

b) Malpighian tubules

The four Malpighian tubules in *C. bifida* are composed of four morphologically distinct regions. The first part which opens into the pyloric chamber, is the shortest and narrowest, being 0.9mm in length and 19μ in diameter.

The second region is longer and wider than the first with a diameter of 38.4μ and length of 2.2mm. The third region, which with a length of 3.7mm and a diameter of 58μ, is the longest and widest of the four regions and also most distinct in appearance. The surface of this segment is rather "globular" and the whole region appears green in contrast to the hyaline appearance of the rest of the tubule. This green coloration is evidently due to the presence of pigment granules in the cells.

The fourth and blind end segment of the tubule is shorter than either the second or third region, being 1.3mm long and 38μ in diameter. This fourth region of the four Malpighian tubules is applied to the wall of the rectum but is not intimately bound to the rectal wall.

IV. Histology of the Malpighian tubules and hindgut
a) Malpighian tubules

The capillary-like first region of the Malpighian tubule which opens in the pyloric chamber is transparent. It has a thin wall and a wide lumen and usually shows two cells in the transverse section. The inner border of the tubule appears smooth (Plate II Fig. 3)

The second and fourth regions are hyaline in appearance and show 3-4 cells in cross section. The cells have large oval nuclei, eosinophilic cytoplasm and the luminal margin has a brush border (Plate II Figs. 4 and 7)

The third region shows 3-4 cells in cross section. The cells show numerous dark magnesium positive granules in the cytoplasm which gives this region a green appearance in the living condition. The luminal border of the cells has a brush border appearing similar to that of the cells in the second and fourth region (Plate II Figs 5 and 6)

b) Hindgut

Light microscope sections of the ileum and the rectum show these regions to be covered with layers of longitudinal and circular muscles on the haemocoele side. In the wall of the hindgut there are two main epithelium types (Plate II Figs. 1 and 2). The one type, restricted to the ventral wall of the ileum and the rectum, consists of a thin 17μ tall syncytium with small (4.8μ x 2.4μ) scattered nuclei, scanty granular cytoplasm and is lined by a cuticular intima. The other type of epithelium forming the dorsal and lateral walls of the ileum, is composed of large 84μ tall
Plate II

Transverse sections of the iliac pad, the rectum, and four regions of the Malpighian tubule of C. bifida in its normal hypoosmotic medium. Figure 1 is a stained transverse section of the ilium showing a well developed iliac pad. Figure 2 is a transverse section of the rectum. Note the reduced epithelium of the rectum and the ventral wall of the iliac pad. Figures 3; 4; 5; 6 and 7 are transverse sections through the tubule regions I, II, III and IV respectively. Figures 1 - 7 x 960.
columnar cells with indistinct lateral walls, large (24µ x 12µ) coarsely granular nuclei and eosinophilic PAS positive cytoplasmic striations running from the intima to the interior of the cells. The latter epithelium with these large cells constitutes the "iliac pad". The pad is richly supplied with tracheoles.

V. Ultrastructure of the Malpighian tubules and hindgut

a) Malpighian tubules

The cells of all regions of the Malpighian tubules are divisible into three regions. From the outside of the tube (haemocoel) towards the lumen these regions are: the basal zone, an intermediate zone with the nucleus and an apical zone. The outer (basal) zone and the inner (apical) zone show the most interesting structure. The basal zone has an elaborate infolding of the cell membrane, these folds forming intracellular compartments characteristically filled with vacuoles, mitochondria, pigment and mineral granules. The apical zone of parts II, III, and IV of the Malpighian tubule has a brush border observed in normal light microscope study and its filaments are made up of vertically arranged protoplasmic processes.

Segment 1

The plasma membrane infoldings of the basal zone extend about one third of the distance from the base to the lumen surface (Plate III Fig. 1; Plate IV Fig.1). Many microtubules are evident throughout the cells and a few mitochondria are associated with the basal infoldings. The lumen border of the apical zone has
Plate III

Figure 1 - An electron micrograph of a cross section of region 1 of the Malpighian tubule in hypoosmotic medium. Note the presence of large vesicles and a few small microvilli. x 67,500

Figure 2 - The luminal (inner) border of two cells of region 1 of the Malpighian tubules. Note the inner border and microvilli covered with a fringe of fine filaments. x 60,000
Plate IV

Figure 1 - An electron micrograph of the outer border of a cell of region 1 of the Malpighian tubule. Note the large vesicle lined by electron dense material. x 42,500

Figure 2 - A high magnification electron micrograph of the inner border of a cell of region 1 of the Malpighian tubule. Note mitochondria protruding into the microvilli and a coat of thin wavy filaments covering the inner border and microvilli. x 90,000
Plate V

Low power electron micrographs of regions II and IV of the Malpighian tubule in the hyposmotic medium.

Figure 1 - Transverse section of a cell portion of region II of the Malpighian tubule. x 18,400

Figure 2 - Transverse section of a cell portion of region IV of the Malpighian tubule. x 18,800

Note in the II and IV regions a close similarity in the overall ultrastructure.
microvilli which are small and well separated (Plate III Fig. 2) and in some preparations have elongated mitochondria protruding into them (Plate IV Fig. 2). At high magnifications wavy filaments extend from the intervillar membrane and beyond the tips of the microvilli (Plate IV Fig. 2). At the base these filaments appear to be continuous with the outer leaflets of the unit membrane. Within the cells many single membrane-bound vesicles are evident in preparations. These vesicles are lined on the interior side with an electron dense granular coat and in their lumen contain a material similar in appearance to that found in the lumen of the tubule.

Segments 2 and 4

The ultrastructure of these segments is very similar and so these regions can be considered together (Plate V Figs. 1 and 2). The space between the plasma membrane infoldings of the basal zone is filled with conspicuous oval mitochondria (Plate VI Fig. 1; Plate VII Fig. 1a). The apical zone of both regions is elaborated as a brush border consisting of many closely packed microvilli devoid of mitochondria (Plate VI Fig. 2; Plate VII Fig. 2). The cytoplasm in the intermediate zone of the cells contains mitochondria, microtubules and many small vesicles with their walls coated with electron dense material. The cells of the fourth segment contain in addition some mineralized granules which, when well cut, show concentric rings (Plate VII Fig. 1c). Among the normal mitochondria in the fourth segment also are seen amorphous deposits which appear similar to the x-granules described in the
Plate VI

High magnification electron micrographs of region II of the Malpighian tubule.

Figure 1 - Outer border of region II of the Malpighian tubule. Note the plasma membrane infoldings and mitochondria with well-defined cristae. x 36,300

Figure 2 - Inner border of region II of the Malpighian tubule. Mitochondria do not protrude into the microvilli. x 14,500
Plate VII

High magnification electron micrographs of region IV of the Malpighian tubule.

Figure 1a - Outer border of region IV of the Malpighian tubule with well-developed plasma membrane infoldings and mitochondria. x 54,000

Figure 1b-c - Middle region of the cell showing a few abnormal mitochondria and a laminated sphere (insert). 1b x 46,900; 1c x 50,800

Figure 2 - Inner border of region IV of the Malpighian tubule. Note the presence of large number of small vesicles and the microvilli devoid of mitochondria. x 37,000
Plate VIII

Figure 1 - A high magnification electron micrograph of the outer border of region III of the Malpighian tubule. Note large number of vesicles among the plasma membrane infoldings and large mitochondria. x 78,000

Figure 2 - Low power electron micrographs of the inner border of region III of the tubule. Note closely packed microvilli devoid of mitochondria. x 7,200
Plate IX

Low power electron micrographs of region III of the Malpighian tubule in the hypoosmotic medium.

Figure 1a - Outer border and middle part of a cell of region III. Note large membrane bound spaces. x 8,800

Figure 1b - Portion of a cell showing spaces full of secretory granules. x 20,600
Malpighian tubules of *Macrosteles* (Smith and Littau, 1961) and *Rhodnius* (Wigglesworth and Salpeter, 1962). Finally in the fourth segment there occur mitochondria with concentric lamination, and spherical granules also showing concentric lamination (Plate VII Fig. 1b)

**Segment 3**

The plasma membrane infoldings of the basal zone of third segment cells are similar to those of the second and fourth region (Plate VIII Fig.1). The apical brush border consists of regular compactly arranged clavate microvilli devoid of mitochondria but having many small vesicles throughout their length (Plate VIII Fig.2). The most noticeable feature of the cells of this part is the presence of membrane limited spherical granules in the intermediate zone which appear green in the dissections and in the light microscope (Plate II Fig. 5). These granules are very difficult to section and usually fall out leaving large holes in the tissue (Plate IX Fig. 1a). When soft they are easily cut and appear as round spheres (Plate IX Fig. 1b). Many small vesicles and mitochondria are dispersed in the basal and intermediate zones of the cells. A large number of vesicles are also present among the basal plasma membrane infoldings.

**b) Hindgut**

The inner surface of the iliac pad cells is lined by a very thin PAS positive cuticular intima and has a plasma
Plate X

Electron micrographs of the outer and middle regions of an iliac pad cell in hypoosmotic medium.

Figure 1 - Outer border of an iliac pad cell. Note extensive outer plasma membrane infoldings and the mitochondria associated with them. x 11,300

Figures 2 and 3 - Middle regions of an iliac pad cell. Note the outer plasma membrane infoldings associated with large mitochondria extending deep into the cell and the tracheolar endings.
Fig. 2. x 44,600
Fig. 3. x 35,600
Plate XI

Figure 1 - A low magnification field from the luminal (inner) region of an iliac pad cell in the hypoosmotic medium. Note the inner plasma membrane infoldings enclosing mitochondria between them and closely associated with the cuticular intima. x 38,400

Figure 2 - A high magnification field showing a few inner plasma membrane infoldings with unit membranes uniformly covered on the cytoplasmic side by a regular electron dense coat. x 72,800

Figure 3 - A low power electron micrograph showing the luminal side of a rectal cell. Note a few small mitochondria and complete absence of outer and inner plasma membrane infoldings in the rectal cell. x 15,600
membrane which is highly infolded (Plate XI Fig 1). The infolded plasma membrane to the inside of the cell is uniformly covered with a continuous layer of granular material. Furthermore, at higher magnifications the extracellular coat of the infoldings show electron dense granular material (Plate XI Fig.2). Between the infoldings are numerous microtubules and finger-shaped mitochondria both of which are grouped in rows parallel to the plasma membrane infoldings throughout the pad cells. At the basal margin to the inside of the circular muscle layer the plasma membrane has numerous infoldings as well (Plate X Figs 1-3). These foldings penetrating deep into the cells, interdigitate with the inner margin infoldings, but are devoid of any electron dense granular coating. The striated appearance of the pad cells seen in the light microscope is evidently then due to the presence of these numerous interdigitating inner and outer plasma membrane infoldings. Tracheoles are seen to penetrate the pad cells, not being confined to the surface only.

Ultrathin sections of the rectum and the ventral wall of ileum show a granular cytoplasm, very few small mitochondria and no plasma membrane infoldings (Plate XI Fig.3). Internally the rectum and ventral wall of the ileum are lined by a thick cuticular intima.

VI. Studies with Neutral Red

Excretion of the vital dye neutral red was used as an index of the flow of excretory products through the excretory system. The results of this study are shown in Table VII.
a) Malpighian tubules

Five minutes after the injection of 0.005% neutral red into the haemocoele the cells and lumen of the fourth, third and second segment of Malpighian tubules were found to be red on dissection; further, the concentration of dye in the lumen appeared greater than in the original medium. The cells and lumen of the first segment remained colourless during the first five minutes. After ten minutes, dye started appearing also in the lumen of the first segment and then in the cells. By fifteen minutes the cells of the first segment became intensely red while the cells and lumen of other segments became colourless. Similar results were obtained when the dissected Malpighian tubules were suspended in insects blood and a drop of insect Ringer containing 0.005% dye.

These observations suggest that there is a flow of material into the Malpighian tubule lumen through the fourth, third and second segments, while the first segment has a reabsorptive function.

b) Hindgut

The ilium and the rectum remained colourless when 0.005% neutral red was injected into the haemocoele. However, when the dye was injected into the lumen of the hindgut through the anus, the iliac pad cells became intensely red and the dye granules tended to lie in longitudinal rows within the cells. The tendency of the neutral red granules to lie in longitudinal rows is perhaps significant since the histological observations show the cytoplasm of the iliac pad cells to consist of longitudinal striations. Retroperistaltic movements were seen to move the dye solution into the ilium from the rectum.
## TABLE VII

**NEUTRAL RED UPTAKE BY MALPIGHIAN TUBULE SEGMENTS**

<table>
<thead>
<tr>
<th>Malpighian tubule segments</th>
<th>In dye 5 mins.</th>
<th>In dye 10 mins.</th>
<th>In dye 10 mins. followed by Ringer 5 mins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ coloured red with dye

- not coloured red with dye
VII. Functional Interpretation of Structural Features

It is convenient at this point to briefly consider the function of the iliac pad and the parts of the Malpighian tubules, since an idea on this function is essential for understanding the various structural changes to be considered in the following section. A full consideration of the function of the parts is however reserved for the discussion.

a) Malpighian tubules

The numerous infoldings of the plasma membrane in the basal zone of cells in the second, third and fourth regions of the tubule plus their association with mitochondria suggests that the basal margin of these regions is relatively more important in solute transport than the luminal border. It would seem from this that the direction of material flow, in the distal three parts of the Malpighian tubules is largely from haemolymph to lumen. This is further substantiated by studies with neutral red (see above).

The presence of numerous small vesicles lined with electron dense material in all cells of the third segment suggests transport by pinocytosis of pigment granules (precipitated catalytic bolites!) to the large spaces bounded by membrane and on maturation through microvilli into the lumen of the tubule. That there is maturation (hardening) process involved seems evident from the fact that sometimes the contents of the large membrane bound spaces can be cut, while at others this is not possible and the contents of the spaces drop out leaving large holes in the tissue.
Small coated vesicles are also evident in photomicrographs of the basal regions of the cells of the second and fourth segment of the tubule suggesting their involvement in transport of excretory products from the haemocoele into the lumen of the tubule.

In the first part of the tubule, the presence of mitochondria in the apical microvilli the large vesicles which are bounded by single membrane and internally coated with electron dense granules, and the wavy filaments extending beyond the tips of microvilli into the lumen from the intervillar apical membrane all suggest transport of material from the lumen to the haemocoele. Again, this can be documented by the studies with neutral red.

b) Hindgut

The iliac pad of C. bifida is characterized by large cells whose basal and apical surfaces present numerous plasma membrane infoldings, the latter uniformly covered with an electron dense granular coat. Associated with the infoldings throughout the cells are numerous finger shaped mitochondria having well developed cristae.

Scattered in the circular muscle layer of the iliac pad are found membrane bounded vesicles with dense granular contents.

The elaborate structure of the iliac pad of C. bifida suggests that this organ is associated with the absorption of physiologically important solutes from the fluid passing through it. This is further substantiated by the change in the osmotic pressure of the iliac fluid mentioned earlier, and the studies with neutral red solutions.
The rectum having few mitochondria and no plasma membrane infoldings, evidently serves as a storage chamber for excretory fluid before its release to the exterior.

VIII. Structural changes in experimental media

a) Malpighian tubules

No changes were detected when the insects were transferred from the normal hypoosmotic media to distilled water. When the insects were placed in isomotic and hyperosmotic media all segments of the Malpighian tubules showed marked changes in the mitochondria. The latter become enlarged, with a less condensed appearance and their cristae become irregular in regard to spacing and configuration. In the hyperosmotic medium the cristae of the mitochondria seem to break away from the mitochondrial membrane (Plate XIII Fig.1). The microvilli of the second, third and fourth segment became less regularly packed than when in hypo-osmotic medium or distilled water (Plate XIII Fig. 2). The size of the coated vesicles in the first segment also became reduced when in these media (Plate XII Fig. 1).

b) Hindgut

The cells of the iliac pad remained unchanged when insects were placed in distilled water. On placing the insects in isosmotic and hyperosmotic media the infoldings of the plasma membrane at the luminal border became separate from the cuticular intima and there appeared a regular layer of vacuoles inside the
Plate XII

Figure 1 - A portion of a cell of region I of the Malpighian tubule in hyperosmotic medium. Note reduction in the size of the vesicles as compared to those in the hypoosmotic medium. Nucleus appears normal, but the mitochondrial cristae are not clearly visible. x 22,100
Plate XIII

Portion of regions of the Malpighian tubules in hyperosmotic medium.

Figure 1 - A high magnification electron micrograph of the inner border of region IV of the tubule. Note a decrease in the size and number of the cristae as compared to those of hypoosmotic medium and separation of these from the inner mitochondrial membrane. Mitochondria of all regions of the Malpighian tubule exhibit similar changes in hyperosmotic medium. x 79,500

Figure 2 - A portion of the luminal border of a cell of region II of the Malpighian tubule. Note the disorientation of microvilli and their separation from the inner border membrane. Microvilli of of region IV and III exhibit similar changes in hyperosmotic medium. x 47,900

Note: Similar though less pronounced ultrastructural changes occur in all regions of the Malpighian tubules in isosmotic medium.
intima (Plate XIV Fig. 2). In the hyperosmotic medium particularly, the intima separated completely from the iliac pad ethelium (Plate XV Fig. 2). The mitochondria lost their normal configuration and became contracted (Plate XIV Figs. 1 and 2).

The rectum and the ventral thin wall of the ileum appeared unchanged in all media.

IX. Interpretation of the structural changes in experimental media

The adverse effect on the mitochondria in the Malpighian tubules and the iliac pad when insects are placed in isosmotic and hyperosmotic media, plus the separation of the cellular infoldings of the plasma membrane from the intima in the iliac pad cells, strongly suggest that the above media directly or indirectly produce changes in these organs, making them unable to function. The changes in the mitochondria would not be due to fixation, assuming that the intracellular fluid is isosmotic with the haemolymph: an isosmotic fixative was used in order to avoid such changes.

The shrinkage of the microvilli on the luminal border of the Malpighian tubule cells is quite possible due to the fixative used. In all cases the fixative used was hyperosmotic to the contents of the ileum and rectum, and so one would not expect vacuoles to form through the fixation process. The changes observed in the apical border of the iliac pad thus appear to be a result of the experimentation; tissue failure is indicated.
Plate XIV

Low power electron micrographs of a portion of an iliac pad cell in hyperosmotic medium.

Figure 1 - Outer and middle regions. x 11,200

Figure 2 - Middle and luminal regions. x 17,500

Note the contracted state of the mitochondria and the infoldings of the inner plasma membrane breaking away from the intima (marked by asterisks).
Plate XV

Figure 1 - Electron micrograph of the luminal border of a portion of an iliac pad cell in hyperosmotic medium. x 39,600

Breakage of the inner plasma membrane infoldings from the intima is clearly seen. (asterisks)
X. Neurosecretion

a) Changes in medial neurosecretory cells of brain

In the pars intercerebralis of the brain in *C. bifida* there were found to be six to eight cells which when the insects are in hypooosmotic medium, stain deep blue with Gomori's chrom-haematoxylin-phloxin stain: they may thus be designated as "A" cells in accordance with the scheme of Nayar (1955). In the hypooosmotic medium (White Lake water) in which the insects normally live, these neurosecretory cells contain large clumps of neurosecretory material (Plate XVI Fig. 2). On transfer to distilled water the "A" cells in insects were found to contain a greater amount of stainable granules, so much so that the nuclei became obscured in 8μ thick sections (Plate XVI Fig 1). However, there was a considerable reduction of neurosecretory material in these cells when the insects were placed in media isosmotic or hyperosmotic to the insects' haemolymph (Plate XVI Figs. 3 and 4). The results indicated a corresponding reduction of neurosecretory material from the "A" cells of the pars intercerebralis with the increasing external salinity.

The results of the experiments are given in Table VIII.

The difference between the treatments tested by the Friedman two-way analysis of variance gave a calculated $\chi^2_r$ of 24.11 and a $\chi^2_r$ from tables, for 5 degrees of freedom of 20.52. It can thus be concluded that the difference between the six treatments is highly significant (P ≤ 0.001).
Plate XVI

Neurosecretory changes in the 'A' cells of the pars intercerebralis in different salinities.

Figure 1 - Distilled water, (++++).
Figure 2 - Normal hypoosmotic medium, (+++).
Figure 3 - Isosmotic water, (++)
Figure 4 - Hyperosmotic medium, (+).

Note a gradual reduction in the neurosecretory material with increasing external salinity.

Figures 1 - 4 x 960.
TABLE VIII

NUMBER OF INSECTS SHOWING VARYING AMOUNTS OF NEUROSECRETORY MATERIAL IN DIFFERENT ENVIRONMENTAL MEDIA

N.B. Neurosecretory activity of medial "A" cells in different media was quantified by + mark, based primarily on the staining criteria of the cells, i.e. amount of neurosecretory granulation (+; ++; +++; ++++). Refer to Plate XVI

<table>
<thead>
<tr>
<th>Amount of neurosecretory granules in &quot;A&quot; cells</th>
<th>MEDIA ((\bar{x} \pm S.E.))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distl. water</td>
</tr>
<tr>
<td>(+)</td>
<td>0</td>
</tr>
<tr>
<td>(++)</td>
<td>1</td>
</tr>
<tr>
<td>(+++)</td>
<td>4</td>
</tr>
<tr>
<td>(+++)</td>
<td>9</td>
</tr>
<tr>
<td>TOTAL</td>
<td>14</td>
</tr>
</tbody>
</table>

* White Lake water (Spring, 1966)
\(\bar{x} \pm S.E.\) = Mean ± Standard error
\(\Delta ^{\circ} C\) = Freezing-point depression
Diagrammatic representation of changes of neurosecretory material in the 'A' cells of the pars intercerebralis with the change in the external salinity. The amount of neurosecretory colloid is quantified by ++++; +++; ++ and + signs. Note a gradual reduction of neurosecretory material with the corresponding increase in the urine osmoconcentration with the increasing external salinity.
b) Relationship between neurosecretory changes and the urine production in different environmental media

The osmotic pressure measurements of the haemolymph and urine indicate the *C. bifida* is able to hyper-regulate in low salinities producing a urine hypoosmotic to its haemolymph. However, the insects are incapable of producing a urine more concentrated than the haemolymph, i.e. the urine always remains hypoosmotic although its osmoconcentration arises with the increase in external salinity. Correlated with these haemolymph and urine changes there is a reduction of neurosecretory colloid in the "A" cells of the pars intercerebralis, so much so that the cells become virtually empty of neurosecretory granules in hyperosmotic medium (Fig. 3). This reduction of neurosecretory material when the insects are placed in increasingly more concentrated media, can be interpreted in two ways: 1) that the cells stop producing neurosecretory material in higher salinities, or 2) that the neurosecretory colloid is being utilized as soon as it is produced. The latter seems most probable since the granules can be seen in the axons of the nerve cells (Plate XVI Fig. 4).
DISCUSSION

Osmotic regulation of the haemolymph of C. bifida

Shaw and Stobbart (1963) have reviewed extensively the subject of osmotic regulation in insects. Recent researches have shown that the larvae of Aedes aegypti (L.), A. detritus (EDW), Culex pipiens (L), Anabolia nervosa Leach, Limnephilus stigma Curtis and Chironomus thummi Kieffer exert a fine control over their internal osmotic pressure through a range of external concentrations which extend far beyond the normal limits of the fresh-water environment (Wigglesworth, 1938; Ramsay, 1950; Sutcliff, 1961b, and Neumann, 1961).

Little is known about the regulatory ability of adult fresh water insects. Claus (1937) found in two freshwater species of Sigara )S. distincta Fieber and S. fossarum Leach, that there was a general increase in the osmotic pressure of the haemolymph as the external salinity was increased from 0.1 to 1.9% sodium chloride, but in view of the short (two hours) duration of the experiments the measured value of the osmotic pressure of the haemolymph may not represent a true steady state (Krogh, 1939; Shaw and Stobbart, 1963). In fact Holgate (1956) has suggested that acclimation period of less than three days may be too short in insects with a low cuticular permeability.

However, the present acclimation experiments on C. bifida showed that the freezing point depression of the haemolymph in both dilute and concentrated media had stabilized by 72 hours.
C. bifida has a highly permeable cuticle and this might account for the speed of acclimation observed. Certainly an equilibrium was not reached in less than six hours (see Table III) and so the criticism by Krogh (1939) and Shaw and Stobbart (1963) of Claus' (1935) results seems valid.

The regulatory capacity of aquatic animals is usually assessed by acclimating the animals to the widest range of saline solutions which they will tolerate, followed by a comparison of the osmotic pressure conveniently represented by the freezing-point depression of the medium (Δ₀) with that of the plasma or the haemolymph (Δ₁) after a new steady state has been reached. Good regulation is indicated where (Δ₁/Δ₀) reaches zero (Shaw and Stobbart, 1963). This method also shows the animal's tolerance limits and the external concentration at which the regulation starts to break down.

The present experiments clearly show that when adult C. bifida are placed in a range of external concentrations, they show good hyperregulation of their haemolymph osmoconcentration over a moderate range of media which are hypoosmotic to its internal milieu (including distilled water). However in media having a freezing-point depression of -0.72 to -1.10°C, the insects tend towards conformity and finally become hypoosmotic in more concentrated media.

Fresh water organisms which maintain a hyperosmotic condition in their body fluids usually live in environments which are osmotically less concentrated (hypoosmotic) to their body fluids and are consequently faced with continual flooding of water and a
leaching of their electrolytes. One of the physiological necessities of insects living in a hypoosmotic medium is the ability to retain essential ions while excreting a dilute (hypoosmotic) urine. In the larvae of the mosquito (*Aedes aegypti*) for example, the urine is nearly isosmotic with the haemolymph as it leaves the Malpighian tubules to enter the rectum, but is modified during the passage down the rectum, probably by ion absorption, so that it becomes strongly hypoosmotic to the haemolymph (Ramsay, 1950).

*C. bifida* which normally lives in dilute media, produce a urine which is always hypoosmotic to the haemolymph, although its concentration increases with the corresponding increase in the external salinity. Furthermore the urine is strongly hypoosmotic to media in the upper range of concentration; it becomes isosmotic to media with \(-0.27\) to \(-0.65°C\) and hyperosmotic in distilled water. These results indicate that *C. bifida* is thus similar to *A. aegypti* with regard to osmoregulatory capacity.

Osmolarity of fixatives

For a long time there has been among electron microscopists a diversity of opinion on the importance of the osmolarity of fixatives. Sjöstrand (1956) has stressed that the osmolarity was the most important part of the fixation vehicle and the slow penetrating osmium tetroxide solution of Palade (1952) being strongly hypoosmotic to most cells can result in marked swelling effects. Rhodin (1954) working on the proximal convoluted tubule of the mouse kidney showed that by changing the osmolar concentration of the fixative, there seemed to be a corresponding change
of the size of mitochondria. His analysis showed that divergence of the osmolar concentration far from that of the blood plasma of the animal used for the experiment may bring about structural changes in the mitochondria. He thus stressed that it is essential to use isosmotic fixative especially when this involves experimental conditions as in the present thesis.

Preliminary experiments on fixation of the Malpighian tubules of *C. bifida* with Palade's (1952) fixative showed that the osmolality of the fixative is important in this tissue. The usual narrow outer plasma membrane infoldings of the tubule (IV region) were found drastically changed into wide inpushings. The mitochondrial cristae and the microvilli were disoriented. Similar effects are evident in the photomicrographs of Malpighian tubules of insects where authors have used Palade's fixative (Beams et al. 1955; Tsubo and Brandt, 1962 and Baccetti, et al, 1963). In all studies on the ultrastructure of the Malpighian tubules and the iliac pad of *C. bifida*, the osmotic pressure of the fixative was adjusted to that of the haemolymph using varying amounts of sucrose as suggested by Caulfield (1957). One would assume that such a procedure is obligatory in similar studies on insects and other animals.

Excretory system

In the majority of insects the excretory system comprises a number of Malpighian tubules which lie free in the haemocoele and open into the alimentary canal at the junction of the midgut and hindgut. These tubules discharge fluid which passes to the hindgut and there, may be extensively modified by specialized cells in the
rectum which form the so-called rectal "papillae" or "pads" (Wigglesworth, 1932; Palm, 1949). Excretion in insects cannot therefore be considered solely in terms the Malpighian tubule secretion but as the net result of the secretion after modification by the epithelium of the hindgut.

Malpighian tubules

The Malpighian tubules vary both in number and structure. In all species the distal end is closed and the tubules are made of a single layer of epithelial cells. In the locust *Schistocerca gregaria* there are about 250 Malpighian tubules with no visible difference in their appearance (Patton, 1963). In the grasshopper *Dissosteira carolina* (L.) the observed difference in the ultrastructure of the cells along the whole tubule are negligible (Tsubo and Brandt, 1962) and thus the tubules appear to be similar in structure throughout their entire length. Likewise in the tubules of *Aedes aegypti* larvae there is no clear evidence of difference in histological appearance or of sodium concentration of the tubule fluid between the distal and proximal regions (Ramsay, 1951).

In some insects the tubules are not alike structurally. In *Dixippus morosus* Brunner von Wattenwyl, for example, there are three types of Malpighian tubule: the superior, the inferior and the 'appendices of the midgut' (Ramsay, 1955a). The superior tubules differ from the inferior ones in that the latter have a conspicuous distal end. After dividing the superior tubules into three roughly equal parts, Ramsay (1955b) analyzed fluid produced
by each part. The ratio of potassium to sodium in the fluid secreted by the distal portion is greater than from the proximal regions. All parts of the tubule were found to be secretory, the rate being greatest in the middle portion and thus the difference in the potassium/sodium ratio is due to the relative rates of secretion of potassium and sodium in different portions. The inferior tubules are similar except that no secretion occurs in the distal dilation and a small amount of potassium reabsorption takes place.

In *Rhodnius prolixus* Stål, where there are four Malpighian tubules, each is composed of two distinct regions, a long distal part and a smaller proximal part. The distal region has a honeycomb (microvilli closely packed) border whereas in the proximal third the cells have a brush (microvilli well separated) border (Wigglesworth, 1931b; Wigglesworth and Salpeter, 1962). The regional differentiation of physiological properties is more marked in *Rhodnius* tubules as compared to those of *Dixippus* in that uric acid is precipitated in the proximal portion of the tubule (Wigglesworth, 1931b). Wigglesworth (1931c) showed both by ligating experiments and by the fact that certain dyes are rapidly taken up by the distal portion and transferred to the lumen, that the distal portion is secretory while the proximal part is probably concerned with reabsorption of required constituents. Analysis of the luminal contents in the two regions shows differences in the ionic composition (Ramsay, 1952). The distal region has, as usual, a high potassium concentration and a sodium concentration below that of the haemolymph. Also the
fluid in the distal region is significantly hyperosmotic to the haemolymph. The fluid samples taken from the proximal region show a greater concentration of potassium and a smaller concentration of sodium than the haemolymph but the differences are much less than in the case of the distal region. This suggests that the differences in concentration of potassium and sodium established by the activity of the distal region are decreased as the fluid passes through the proximal region. The osmotic pressure of fluid from the proximal region falls due probably to the passive reabsorption of potassium and an active uptake of sodium from the proximal part of the tubule (Ramsay, 1952).

A more marked regional differentiation occurs in the Malpighian tubules of the leafhopper Macrosteles fasciatus Stal. Here the tubules are divided into four distinct regions (Smith and Littau, 1960). In the first region (adjacent to the gut) the ultrastructure of the cells bear close resemblance to that of the proximal part of the Rhodnius tubule. The basal cell membrane is folded, with some mitochondria between the folds, and the microvilli form a brush border. In addition large mitochondria are present mainly in the apical region. In the second and the fourth regions the luminal border lacks microvilli but has a large number of flask-shaped cavities, each connecting with the tubule lumen through a narrow neck lined internally by numerous interdigitating membrane-bound leaflets of cytoplasm. The third segment which is the longest and the widest is specially adapted for producing and secreting special lipoprotein-rich secretory granules called "brochosomes".
In the larvae of the fruitfly *Dacus oleae* Gmel. (Trypetidae - Diptera) which live in succulent fruits, are found four Malpighian tubules, two long anterior and two short posterior ones (Mazzi and Baccetti, 1962). Each of these pairs enter into a ureter which in turn opens into the hindgut through an ampulla. The anterior tubules are divided into four regions: distal, transitional, middle and proximal, while in the posterior tubules only two regions are recognizable, these corresponding to the middle and proximal of the anterior tubule.

The Malpighian tubules of adult aquatic Hemiptera have been shown previously by Bahadur (1961) to consist of four different regions. However, this author did not give a detailed description of these regions and could not distinguish their various functions. The present study is the first to present a detailed description of the Malpighian tubule in the Corixidae.

The study of the four-segmented Malpighian tubules of *C. bifida* shows marked structural and functional differentiation between the regions. The ultrastructure and the selective uptake of neutral red suggest that the fourth, third and second regions of the tubule transport material from the haemocoele to the tubule lumen, while the first region (adjacent to the hindgut) is concerned with the reabsorption of useful dissolved constituents from the fluid passing through it down to the hindgut.

The structure and the probable function of the first region of the Malpighian tubule in *C. bifida* appears to be quite similar to that of the distal tubule of the vertebrate nephron. Neither has a brush border and both have only a few microvilli with
elongated mitochondria which are restricted to the basal plasma membrane infoldings and to the luminal border (Schmidt-Nielsen, 1965). The first region of the tubule of *C. bifida* also bears structural resemblance in many ways to the first region of the *Macrosteles* tubule and the proximal region of the *Rhodnius* tubule except they having fewer and shorter microvilli.

The uniform distribution of fine wavy filaments on the luminal border of the first region of the *C. bifida* tubule suggests that the relationship of this material to the underlying membrane is more intimate than would be expected if it simply represented an adherent layer of extraneous mucus. A corresponding filamentous coat on the amoeba *Chaos* has been found to bind particles of thorium dioxide (thorotrast), or ferritin, which are subsequently taken into the cell by pinocytosis (Brandt and Pappas, 1962). Transport of substances by vesiculation and membrane flow as proposed by Bennett (1956) in his hypothesis of pinocytosis, must in some way involve selective binding in or near the cell surface of pinocytosis-inducing agents like simple salts, amino acids, and proteins (Marshall, 1965). It has been proposed by Marshall (1965) that the PAS positive filamentous coat on the epithelial cells acts as a cationic exchanger, binding ions that are then taken into the cells by pinocytosis, as the first step in the vesicular transport. Alternatively Pinter (1967) has suggested that the PAS positive acid mucopolysaccharides of the renal medulla of mammalian kidney may function as a selective binding site for water. The presence of a filamentous layer on the laminal border, and the associated vesicles which are
coated internally with electron-dense material, and contain in their lumen a material similar in appearance to that found in the tubule lumen, strongly suggests that reabsorption in the first region of *C. bifida* tubules takes place by pinocytosis.

The second and fourth regions of the *C. bifida* tubule appear structurally similar to the proximal tubule of the vertebrate kidney, the distal region of the *Rhodnius* tubule and the tubules of the grasshoppers *Melanoplus differentialis* (thomas) (Beams et al. 1955) and *Dissosteira carolina* (Tsubo and Brandt, 1962), but are unlike those of other insects since there are no mitochondria in the microvilli. A large number of small vesicles observed in the basal region among the plasma membrane infoldings and mitochondria of all but the first segment of the tubules in *C. bifida* leaves little doubt that the serosal or haemocoel side of the distal three fourths of the tube is the site of intense metabolic activity, presumably active pinocytotic transport of physiologically important solutes from haemocoel to the tubule lumen.

Although the second, third and fourth regions of the *C. bifida* tubules exhibit similar affinity for neutral red, it is possible that a gradation of physiological properties resembling those in *Dixippus* superior tubules exists in them.

The cells of the third region of the *C. bifida* tubules, although quite different in structure from the third region of the *Macrosteles* tubule, appear to perform a similar function, i.e. formation, storage and discharge of excretory granules. In the *Rhodnius* tubule where vesicles containing granules have been
reported in distal region (Wigglesworth and Salpeter, 1962) such a distinct regional specialization is absent. In C. bifida it is the only region which, due to excretory granules, appears green and on treatment with titian yellow gives an intense brick-red magnesium positive test. Metcalf (1945) has shown that the green pigment in the fat body and the pericardial tissue of the squash bug Anasa tristis DeGeer, is a product of chlorophyll breakdown resulting in a tetrapyrrolic chlorophyll derivative. This pigment is perhaps analagous to the green coloured biliverdin (a resultant of haemoglobin breakdown) deposits in the gut and body wall of Rhodnius and Chironomus respectively (Wigglesworth, 1965). At present no reliable information on the food and feeding habits of corixids is available. Hence the true nature of these magnesium rich green granules remains uncertain. It appears though that they may be breakdown products of complex food molecules.

Hindgut

The cuticle lined hindgut of most insects is divisible into a wide terminal part called the "rectum" and narrower "ileum" which precedes it (Wigglesworth, 1965). In the rectum there is usually a number of specialized cells forming distinct rectal ridges, papillae, or pads. The rectal pads have been shown to be responsible for the modification of the hindgut contents (Wigglesworth, 1932; Palm, 1949; Phillips, 1964a). In many adult Coleoptera and the larvae of Lepidoptera, the distal ends of the Malpighian tubules may be closely applied to the rectal tissue.
This "cryptonephridial" condition evidently facilitates the reabsorption of water and some salts from the rectal contents (Saini, 1964; Ramsay, 1964).

In the hindgut of the Corixidae where a distinct ileum and rectum can be recognized, it is found that the latter lacks specialized areas corresponding to the rectal pads of other insects. Instead the ileum has the cells on the dorsolateral sides modified to form a longitudinal iliac pad. Observation on the histology and the uptake of neutral red by the iliac pad cells in *C. bifida* suggests that the iliac pad has a function similar to that of the rectal pads of other insects, in that they serve to modify the contents of the hindgut.

The significant ultrastructural features of the iliac pad cells, associated with fluid transport from the hindgut lumen to the haemocoele, include a) the intimate structural relationship between the apical surface of the pad cells and the thin (1.5 μ) intima; b) the plasma membrane infoldings interdigitating with the deep infoldings of the plasma membrane of the basal (haemocoele) surface of the cell. Such a system of continuous plasma-membrane infoldings throughout the length of pad cells, seems not reported in insects previously.

The close association of mitochondria with the infoldings of the plasma membrane forming a structure called by Copeland (1964) a "mitochondrial pump" has been described in the epithelial cells of the anal papillae of mosquito larvae (Copeland, 1964), the recital papillae of the termite *Anoplotermes sanctus* Silv. (Noirot and Noirot Timothee, 1960), the ileum of leafhopper *Macrosteles fascifrons* (Smith and Littau, 1960), The rectal pads of the desert locust *Schistocerca gregaria* (Irvine, 1966).
and the rectal papillae of the adult blowfly *Calliphora erythrocepha* (Gupta and Berridge, 1966a; 1966b). The anal papillae of mosquito larvae can absorb inorganic ions, i.e. sodium, potassium and chloride against a concentration gradient (Wigglesworth, 1938; Koch, 1938). The rectal papillae of the desert locust and the adult blowfly are involved in the uptake of water and ions from the rectum resulting in the production of hyperosmotic urine (Phillips, 1961, 1964a). Thus there is circumstantial evidence to show that in insects a close association of mitochondria with a greatly infolded plasma membrane is indicative of specialization in active ion and water transport.

In the high power electron micrographs of the iliac pad cells of *C. bifida* it is seen that the cytoplasmic surface of the plasma membrane infoldings originating from inside the cuticular intima are uniformly covered with an extraneous coat of finely particulate material. This coat is probably responsible for the more intense periodic acid - Schiff reaction of the cytoplasmic striations on the inner (luminal) border of the pad cells when examined under the light microscope. The presence of a similar coat of electron-dense material has been demonstrated in the termite rectal pads (Noirot and Noirot-Timothee, 1966) and in the rectal papillae of the adult blowfly (Gupta and Berridge, 1966a). A mantle of this kind is regularly observed on the sarcolemma of skeletal and cardiac muscles, smooth muscles, Schwann cell of nerves, fibroblast and adventitial cells of blood vessels (Fawcett, 1964). According to Fawcett (1964) it appears to be a glycoprotein material and is probably a product of the cell itself
and not simply a condensation of ground substances of the surrounding tissue. It has been suggested that this so-called "glycocalyx" is the site of selective filtration of molecules and acts as a carrier in the ion pumps (Bennett, 1963). Noirot and Noirot-Timothee (1966) have localized sodium on the granular coating of the apical (luminal) plasma membrane infoldings in the rectal papillae of the termite and have suggested that these apical lamellae play a role in the sodium ion absorption and that the coating of the membranes has something to do with this phenomenon. If true, this would provide an increased surface for binding sites in ion transport.

The plasma membrane infoldings observed at the basal (Haemocoele) surface of the iliac pad are devoid of a granular extraneous coat, but they usually penetrate deep into the cell cytoplasm and interdigitate with the coated apical (luminal) foldings. Their close association with mitochondria suggests that they too are important in transporting material from the lumen across the cell into the haemocoele.

Microtubules of unknown function are evident in all segments of the tubule and the iliac pad. Fawcett (1966) has suggested that microtubules functions as cytoskeletal elements which help maintain the extended form of cells. If true the microtubules would be useful in maintaining the increased surface for transport.

The presence of neurosecretory granules in the muscle layer of the iliac pad of C. bifida suggests that the iliac pad reabsorption may be regulated according to the need of the insect, by the hormonal secretion from the insect's nervous system. It is not clear whether these neurosecretory granules come from the cerebral
endocrine complex or the thoracic ganglionic mass. According to Maddrell (1964b) the quantity and the concentration of urine may be adjusted by the rectum according to the concentration of the haemolymph. Regulation of rectal reabsorption has been established for the desert locust (Phillips, a,b,c) and is probably common to most other insects. Although the exact nature of the regulatory mechanism is not clear, it may possibly involve a change in the permeability in the pad epithelium. Gupta and Berridge (1956b) have shown similar neurosecretory granules in the rectal papillae of the adult blowfly.

The ultrastructure of the rectum and also the ventral wall of the ileum of *C. bifida* show in contrast to the recta of other insects, a granular cytoplasm, very few small mitochondria and virtually no inner or outer plasma membrane infoldings. Also the rectal cuticular intima is approximately twice as thick (1.7 \( \mu \)) as that over the iliac pad (1\( \mu \)). This strongly indicates that these epithelia play no role in the active reabsorption of ions and water and the rectum simply serves as a storage chamber for excretory fluid before it is eliminated to the exterior.

The ultrastructure of the iliac pad of *C. bifida* (a hyposmotic urine producer) differs quite markedly from that of the desert locust and the adult blowfly in that the inner and outer plasma membrane infoldings are dispersed throughout the length of the cells, and *C. bifida* lacks the additional layer of secondary cells and a system of channels and spaces present in the locust and blowfly. According to Phillips (personal communication), the secondary cells in the locust and the blowfly may be responsible for producing a hyperosmotic urine.
In *Aedes aegypti* larvae which can only produce hypoosmotic urine, the rectal pad is thin, with a narrow epithelium while that of *A. detritus*, which can osmoregulate in both fresh and sea water and can form hyperosmotic urine, is thicker and divided into two distinct regions (Ramsay, 1950). The posterior region extending from the anal canal to about the middle of the rectum resembles that of *A. aegypti* in having a layer of dense cytoplasm on the side next to the lumen and the nuclei next to the haemocoele. The anterior region extending from the middle of the rectum to the intestine (ileum) is lined with a deeply folded epithelium showing the evidence of striations and in which the density of cytoplasm is greater on the outer (haemocoele) side than towards the lumen. Ramsay (1950) tentatively identified the anterior region as the site of water reabsorption resulting in the formation of hyperosmotic urine in *A. detritus*, since cells of this type were not found in *A. aegypti*. Although this is opposite to the conclusion reached from the hindgut histology and ultrastructure of *C. bifida*, it should be noted that final proof of the function of the pad epithelium is lacking. The two types of papillary epithelial cells reported in *A. detritus* do not occur together in any species of Hemiptera (Goodchild, 1966).

**Hindgut structure in relation to its function**

There can be no doubt that the structural organization of an organ reflects its metabolic and functional activities. Thus, once a structure-function relationship has been established it is possible to predict, tentatively at least, the major metabolic
activity or gross functional mechanism of an organ on the basis of its cytoarchitecture. In the discussion to follow an attempt will be made to correlate structural information obtained during the present study on the iliac pad of C. bifida and the physiological information obtained from the osmotic pressure measurements of gut fluids. In addition, some possible functional mechanisms of solute and water transport which seem to be permitted by the evidence will be examined in the light of other studies available on the function of rectal pads of other insects.

Wigglesworth's (1932) theory that the function of the rectal pad is to absorb water to enable the terrestrial insects to maintain their water-balance is now most generally accepted. In Dixippus morosus a high value of the rectal fluid osmotic pressure apparently shows water absorption (Ramsay, 1955a). In Rhodnius prolixus after the diuresis period following a blood meal, the discharge of the rectal fluid becomes infrequent and the osmotic pressure of the rectal fluid rises steadily until after two days it is roughly double that of the haemolymph (Ramsay, 1952). Although the increase in osmotic pressure might be explained by the secretion of potassium into the rectum, the low rate of rectal discharge strongly suggests that water is reabsorbed. It follows therefore that the water uptake takes place against an increasing osmotic gradient. In fasting locusts (Schistocerca gregaria) supplied with tap water the rectum absorbs in 5-9 hours about 69% of an injected solution of xylose which is initially about 40% more concentrated than the haemolymph (Phillips, 1964a). During the absorption the osmotic pressure of the solution increases to
about 230% that of the haemolymph. Some of the xylose is also absorbed but the water uptake is not associated with the simultaneous solute uptake, since the same result is obtained if a nonpenetrating solute such as trehalose is used. It follows, therefore, that an independent net uptake of water takes place against an osmotic gradient and that the gradient is increased during the process (Phillips, 1964a).

Although it appears rather evident that a reabsorption of water takes place in the hindgut of terrestrial insects it is not clear whether this function is performed by the papillary epithelium or the rectal epithelium. According to Wigglesworth (1932) the pad or papillary epithelium is the only part of the rectal wall which can do active work against the osmotic pressure of excreta; it is not surprising therefore that the papillae should be made up of such conspicuously large cells with a rich tracheal supply. This most likely holds true for the insects where the urine becomes hyperosmotic to the haemolymph. But in insects like C. bifida which produce a urine hypoosmotic to their haemolymph, it would seem rather natural that water diffuses towards the haemocoele through the thin and reduced rectal epithelium from the watery gut contents, if the haemolymph osmotic pressure increases. In the latter case there is no need to speak of an active water uptake, but only a passive one according to the physicochemical laws. In order to know the true physiological nature of the rectal epithelium of the hindgut a detailed study of water absorption from the rectum of C. bifida is required.
In the aquatic insects on the other hand, there cannot be any need of a water reabsorption; Wigglesworth (1932) has suggested that the rectal pads here function in a similar manner, but in the reverse direction: they should have the function of maintaining the osmotic pressure of the body fluids by eliminating water and reabsorbing electrolytes.

In the larvae of *Aedes aegypti*, Ramsay (1950; 1953a) found that the rectal epithelium carried out the osmotic work in rendering the intestinal fluid strongly hypoosmotic by absorbing sodium and potassium before it is eliminated to the exterior. The larvae of *Limnophilus flavicornis* Fabricius (Trichoptera) and *Chironomus plumosus* (L.) (Diptera) reabsorb chloride against a concentration gradient at the level of the rectal gland. (Bone and Koch, 1942). Chloride is completely removed from the rectal fluid of *Sialis lutaria* (L.) (Shaw, 1955b).

It appears then that most insects in which the rectal epithelium is developed into prominent pads are capable of transferring required inorganic ions as well as water from the rectum to the haemolymph.

The osmotic pressure measurement of midgut, iliac and rectal fluids of *C. bifida* in distilled water, hypoosmotic and isosmotic media suggest that the ileum is the site of osmotic work with the effective dilution of ososmotic or hyperosmotic fluid entering it from the Malpighian tubules and/or the midgut. Whether this change is brought about by the reabsorption of electrolytes or the secretion of water remains to be discovered.
The lower freezing-point depression of the rectal fluid does not necessarily mean that further dilution takes place in this thin-walled organ. It is possible that complete reabsorption of required materials may not have occurred in the iliac fluid samples. Retroperistaltic movements, which in dissected insects are observed to move fluid anteriorly from the rectum, might result in the prolonged retention of excreta in the ileum, thereby facilitating the reabsorption of substances. The elaborate ultrastructure of the iliac pad and the osmotic pressure values of the iliac fluid clearly indicate that in *C. bifida*, unlike other insects studies so far the reabsorptive function has shifted from the rectum to the ileum which precedes it; the rectum simply serves as a storage chamber for excreta before its elimination at the anus.

Goodchild (1966), in reviewing the evolution of the alimentary canal in Hemiptera, has suggested the possibility of solute absorption, in the iliac pad in the aquatic species. Bahadurs' (1963) interpretation of the function of rectal pads of aquatic bugs in water reabsorption is misleading in the sense that most of these insects live in a very dilute medium and are thus faced with the problem of water flooding and need to conserve ions rather than water. They most probably produce a urine hypoosmotic to their haemolymph, as does *C. bifida*. However in such corixid species as *Sigara lugubris* (Fieber) and *Cenocorixa expleta* (Uhler) which have been reported to occur in very high salinity waters (Claus, 1937 and Edmondson, 1963), the iliac pad might serve a different function. In saline media these insects are confronted with the problem of tissue dehydration in which case the pad would
serve to selectively conserve water rather than ions.

Ultrastructural changes in experimental media

When the insects are placed in isosmotic and hyperosmotic media, the mitochondria in different regions of the *C. bifida* tubules become enlarged, their cristae become disoriented and separate from the inner mitochondrial membrane. The apical (luminal border of the iliac pad of *C. bifida* resembles in structure that of the outer border of the anal papillae of mosquito larvae (Copeland, 1964; Sohal and Copeland, 1966) but differs from the latter in the changes it undergoes in different external concentrations. No visible change was detected in the iliac pad ultrastructure when the insects are transferred from their normal hypoosmotic environment to distilled water. Sohal and Copeland (1966) reported that in higher salinities there was a considerable decrease in the number and extent of the outer plasma membrane infoldings or lamellae and in number of mitochondria, while in the hypoosmotic medium they found an increase in these infoldings.

In *C. bifida*, in isosmotic and hyperosmotic media the luminal plasma membrane infoldings which form the cellular lamellae, become separate from the cuticular itima by a layer of vacuolar spaces and the adjacent mitochondria lose their normal configuration. Such degenerative ultrastructural changes in the iliac pad and the Malpighian tubules of *C. bifida* strongly suggest that these organs are unable to function properly in isosmotic and hyperosmotic media.

According to Malamed (1965) and Parsons et al. (1966) it is only the inner mitochondrial membrane which is susceptible to
osmotic pressure changes. The matrix space to the inside is regarded as an osmometer bounded by a selectively permeable inner membrane which is thrown into folds (the cristae). Experimental studies show that water but not sucrose penetrates this barrier; the outer mitochondrial membrane binds a space between it and the inner membrane (including the lumen of the cristae) and appears to be permeable to both water and sucrose. In hypoosmotic media, it is speculated that the volume of the matrix compartment increases, with little or no membrane stretch, but with unfolding of the cristae (Malamed, 1965); the outer membrane stretches or ruptures as a result of the distension of the inner compartment. A confirmation of this idea appears in the work of Weinbach et al. (1963) who showed that the reappearance of the inner membrane infoldings in mitochondria occurred when the colloid osmotic pressure was reversed. In hyperosmotic solutions Malamed (1965) showed that solutes enter into the membrane compartment, between the outer and inner membrane and withdraw water from the matrix compartment such that the inner membrane contracts probably by folding and invagination of the surface leading to shortening of the cristae.

The electronmicrographs of the Malpighian tubules and the iliac pad of C. bifida when placed in experimental media show that the inner membrane of the mitochondria is affected by osmotic pressure changes. After insects have been in isosmotic or hyperosmotic media for 72 hours. The metachondria of the Malpighian tubules exhibit decrease in the size and number of the cristae and show separation of these from the inner membrane. In the iliac pad of these same insects, the mitochondrial cristae appear
electron dense and the matrix space contracted. No such changes were detected in the insects kept in hypoosmotic media.

The presence of ultrastructural integrity of the cell organelles, e.g. microvilli of the tubules, membrane system, nuclei and most of the mitochondria in both the tubules and the iliac pad of C. bifida in isosmotic and hyperosmotic media indicate that the cells were alive at the time of fixation. The ultrastructural alterations of mitochondria of mouse hepatic parenchymal cells during the development of necrosis to 24 hours in vitro showed vesiculation, distortion and fragmentation of membrane and the appearance of large characteristic aggregation of dense material in the mitochondrial matrix (Trump et al. 1965). Although injury is evident in mitochondria of the C. bifida tubules and the iliac pad cells, the dense deposits in the matrix of the mitochondria which according to Trump et al. (1965) are the most characteristic change in the process of necrosis, are absent. Cook and others (1965) while studying electron microscopically the process of cell death to proximal, distal and collecting tubules of the rat renal cortex during autolysis in vitro for 24 hours showed that the rapid vacuolization of the cell cytoplasm is followed by the swelling and fragmentation of mitochondria, loss of the microvilli of the brush border and the margination of chromatin granules in the nucleus, followed by shrinkage of pycnotic chromatin away from the disintegration nuclear envelope. The fact that most of such changes cannot be detected in the cells of the tubules and the iliac pad of C. bifida indicate that these cells were not dead at the time of tissue fixation and the
ultrastructural alterations are most likely due not to cell death but to experimentation. From the ultrastructural change alone, it is difficult to say what functional state they were in. According to Hielbrunn (1956) "a cell may suffer irreparable injury and yet certain vital functions may linger on. Thus a cell may be completely disorganized, it may be ground up into bits, and yet the resultant mash may retain some of the metabolic attributes of the living system. Enzyme reactions of one sort or another may continue; respiration may remain. For this reason it may be wise to use the term 'Necrobiosis' to designate the series of changes which intervene between life and death."

Neurosecretion

The histological study of the neurosecretory changes in the protocerebrum and osmotic pressure measurements of urine in different external salinities suggest an influence of the cerebral endocrine complex on the water balance of male *C. bifida*. A neurosecretory supply to the iliac pad has already been noted (Plate X Fig.1).

The experimental results are consistent with the premise that the medial neurosecretory cells of the pars intercerebralis of *C. bifida* produce a chemical factor which controls water loss. The insect in its normal hypoosmotic environment seems to release a chemical factor slowly, since there is a recognizable accumulation of stainable colloid which extends throughout the system in all the 'A' cells in the pars intercerebralis. On the other hand transfer of insects to isosmotic or hyperosmotic media results in
a considerable reduction of stainable colloid in the medial neurosecretory cells, indicating that such transfer brings about a rapid release of the factor. The presence of some fine granules in axons suggests an increase in the utilization of neurosecretory colloids in the higher salinities rather than a cessation of material production. It seems that in higher salinities water needs to be conserved, as indicated by the production of a urine which has a higher osmolarity than that produced in less saline media. Unfortunately a satisfactory measure of urine volume produced in different environmental media was not obtained in the experiments, due to the small size of the insect (5-6 mm).

However, I found considerable difficulty in collecting sufficient microquantities of urine in isosmotic and hyperosmotic media for the osmotic pressure measurements. No such difficulty was encountered in obtaining urine from insects when in hypoosmotic media or distilled water. The impression is that the insect produces less urine in more concentrated media.

Neurosecretory changes similar to those here reported in *C. bifida* have been shown to occur in other insects. In the Hemipteran *Iphita limbata* (Nayar, 1960) showed that the neurosecretory material accumulates in the 'A' cells of the pars intercerebralis when the insects are hydrated, but is considerably depleted if they are dehydrated or fed on salt solution. It was inferred from these observations that the 'A' cells elaborate a chemical factor, which has an antidiuretic effect. Evidence of an antidiuretic hormone in *Blabera fusca* Brunn. was obtained by Stutinsky (1953) when he injected extracts from the pars intercerebralis or corpora cardiaca
or corpora allata into rats and noted a retardation of urinary products. Extracts of the corpus cardiacum of the cockroach *Periplaneta americana* (L.) cause a decrease in the rate of excretion of indigo carmine by the Malpighian tubules in vitro (Wall and Ralph, 1964). The 'A' cells of the third and all abdominal ganglia of the locust *Schistocerca gregaria* are depleted of neurosecretory material under experimental conditions of dehydration (Delphin, 1965).

Although there is a considerable evidence to show that a chemical factor is released from the cerebral endocrine complex in different insects, the term "diuretic" and "antidiuretic" have often been used rather loosely and this has resulted in misleading inferences. It is only in *Rhodnius prolixus* that conclusive evidence of a "diuretic" hormone has been demonstrated. By carefully planned experiments Maddrell (1963; 1964a, b, 1966) has shown that a factor which is released into the haemolymph from the fused mesothoracic gangliomeric mass, also increased the urine from the isolated Malpighian tubules.

The fact that the reduction of Gomori positive material in the 'A' cells of *C. bifida* as correlated with an evident production of more concentrated urine, would seem to indicate the medial neurosecretory cells in this insect produce a substance which has a urine concentrating effect. Further, since the volume of urine produced would appear to be less in the isosmotic and hyperosmotic media than in hypoosmotic media and distilled water, and since the insects are known to show about 5% reduction in total body water in the more concentrated media (Jarial, 1964), it appears that water must be conserved in the isosmotic and hyperosmotic media.
This would imply an antidiuretic function for the secretion of the cerebral endocrine complex. A failure of this complex would lead to an inability of the insect to regulate its internal milieu.

Ecological considerations

Collections of *C. bifida* from a large number of water bodies in central British Columbia show that it is usually absent from lakes having a conductivity of more than 12,000 (Δ = -0.46°C) micromhos (Scudder unpublished). Mortality experiments in the laboratory indicate that these insects do not live more than 2-3 days in media isomotic or hyperosmotic to its haemolymph. It can hyperregulate its haemolymph in the low external concentration: it tends to conform to media having freezing-point depression of -72°C to -1.1°C and becomes hypoosmotic in a more concentrated environment. *C. bifida* produces urine which is always significantly hypoosmotic to its haemolymph. The degenerative changes in mitochondria in the tubules and the separation of the intima from the plasma membrane infoldings in isomotic and hyperosmotic media indicate that *C. bifida* cannot cope with large quantities of salts which enter its body via the mouth. Also, the medial neurosecretory cells of the pars intercerebralis which through their secretion have been shown to control salt and water balance in other insects, become increasingly devoid of neurosecretory material with the corresponding increase in the external salinity. Thus the isosmotic and hypoosmotic conditions of the haemolymph of *C. bifida* seem to show the breakdown of hyperosmotic regulation
rather than its true osmoregulatory ability in isosmotic and hyperosmotic media.

From the results reported in this study of "C. bifida" it appears that there is a strong correlation between the distribution and its ability to regulate its internal milieu.
SUMMARY

1. *Cenocorixa bifida* cannot tolerate highly saline media. However, insects are able to live for long periods in hypoosmotic (to the haemolymph) media and unfed adults live up to three weeks in distilled water at 5°C.

2. Acclimation of insects to saline as well as fresh water at 5°C, determined by osmotic pressure measurements of the haemolymph, show acclimation at 72 hours.

3. *C. bifida* is able to hyperregulate its haemolymph over a range ($\Delta = -0.00$ to $-0.7^\circ$C) of hypoosmotic media. In media having $\Delta = -0.72$ to $-1.1^\circ$C on the other hand, the insects tend towards conformity, but finally become hypoosmotic in higher external concentrations.

4. The usual osmium tetroxide fixative is extremely hypoosmotic to the insects' haemolymph and caused marked swelling of the infoldings of plasma membrane and the mitochondria.

5. Osmolarity of the fixative was adjusted by the addition of sucrose to osmotic pressure of the haemolymph of *C. bifida* in each experimental medium. Such isosmotic fixatives preserved the ultrastructural details of the cell organelles.

6. The ultrastructure of the Malpighian tubules and uptake of dye suggest that the second, third and fourth segments are active in transport of material from the haemocoele
into the lumen of the tubule.

7. The presence of mitochondria in the microvilli, thin filaments extending into the lumen from the microvilli and the lumen border; the large vesicles with granular coats and the path of neutral red in the first segment of the Malpighian tubules suggest that this region is concerned with the absorption of physiologically important solutes.

8. Studies of the ultrastructure, passage of neutral red and the osmotic pressure measurements of midgut, iliac and rectal fluid suggest that the ileum which has a well developed pad is concerned with solute uptake resulting in the production of a hypoosmotic urine and that the rectum simply serves as a storage chamber.

9. In isosmotic and hyperosmotic media degenerative ultrastructural changes occur in the mitochondria of Malpighian tubules and iliac pad. The cellular infoldings of the plasma membrane in the iliac pad also become separated from the intima suggesting the failure of these tissues in concentrated media.

10. Gradual reduction of neurosecretory material from the "A" cells of the pars intercerebralis with increasing salinity and resultant increase in urine concentration indicate that water balance of C. bifida is under the influence of a chemical factor. The presence of some fine neurosecretory granules in the axons, may indicate that the factor is utilized during an osmotic stress in
isomotic and hyperosmotic media to conserve water.

11. *C. bifida* is able to survive and regulate its body fluids only in hypoosmotic media. Distribution pattern, mortality data, osmotic pressure measurements of haemolymph, ultrastructure of hindgut and the Malpighian tubules in hypoosmotic environment, the degenerative changes in the ultrastructure of excretory organs in isomotic and hyperosmotic media, and finally the depletion of almost all of the neurosecretory material from the 'A' cells of the pars intercerebralis in hyperosmotic media substantiate this conclusion.


