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STUDIES ON THE EXCRETORY AND NEUROENDOCRINE SYSTEMS  
OF SOME ORTHOPTERAN INSECTS, WITH PARTICULAR  
REFERENCE TO JAMAICANA FLAVA (CAUDELL)

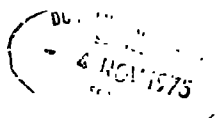
by

A. J. Peacock B.Sc. (Dunelm)

being a thesis submitted for the degree of Doctor of  
Philosophy at the University of Durham.

GREY COLLEGE  
UNIVERSITY OF DURHAM

AUGUST 1975



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\*\*\*\*\*

## GLOSSARY

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumen
C.N.S.	central nervous system
xg	centrifugal force
Mg <sup>2+</sup> ATPase	magnesium activated ATPase
Na <sup>+</sup> - K <sup>+</sup> ATPase	magnesium dependent ATPase, synergistically stimulated by sodium and potassium ions
NCC I	nervus corporis cardiaci I
NCC II	" " " II
NCA I	nervus corporis allati I
PI	inorganic phosphate
RNA	ribonucleic acid
rpm	revolutions per minute
S.G.N.S.	stomatogastric nervous system
Tris	Tris (hydroxymethyl) aminomethane

\*\*\*\*\*

## ABSTRACT

Some aspects of the general physiology, structure and biochemistry of Jamaicana flava were examined. Measurements of growth and oxygen consumption were made, throughout the stadium, for individuals of the sixth instar. The anatomy and histology of the stomatogastric nervous, neurosecretory and retrocerebral endocrine systems of this species are described. The effect of temperature on these systems was examined in insects of the sixth instar. Temperature was found to markedly influence the rate of larval development. These findings are discussed in relation to the present knowledge of neurosecretory activity and protein synthesis.

A detailed account is given of the structure of the excretory system of Jamaicana flava and a brief description of the Malpighian tubules and rectum of Locusta migratoria. In both species the electron microscopic structure of the Malpighian tubules and rectum were found to be similar to that reported for other terrestrial insects. The ultrastructure of these tissues is discussed in relation to current theories of water transport across epithelia. The ultrastructure of the ileum is discussed in relation to its possible role in the excretory process.

The excretory system of Jamaicana flava and several other Orthopteran species, was examined for  $\text{Na}^+ - \text{K}^+$ , and  $\text{Mg}^{2+}$  ATPase activity. Two biochemical methods of obtaining microsomal preparations with ATPase activity were used and are described. Only one, involving homogenization in mannitol and deoxycholate medium, followed by extraction with sodium iodide, provided preparations with measurable  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  activity. In all species examined,  $\text{Na}^+ - \text{K}^+$  and  $\text{Mg}^{2+}$  ATPase activity was present in microsomal preparations obtained from the Malpighian tubules, ileum and rectum. The properties of  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  were determined and were generally found to be similar to those reported by other workers. Experiments to determine the distribution of  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  within the excretory epithelia of Locusta migratoria, using histochemical methods were unsuccessful. The possible role(s) of  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  in the functioning of the excretory system is discussed.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	i
GLOSSARY	ii
ABSTRACT	iii
TABLE OF CONTENTS	iv
CHAPTER ONE INTRODUCTION	1
CHAPTER TWO GENERAL MATERIALS AND METHODS	14
CHAPTER THREE STUDIES ON THE STRUCTURE OF THE NEUROENDOCRINE SYSTEM OF <u>JAMAICANA</u> <u>FLAVA</u> .	
INTRODUCTION	16
MATERIALS AND METHODS	
1. Anatomical study of the stomatogastric nervous system and retrocerebral endocrine glands.	19
2. Histological study of the stomatogastric nervous neurosecretory and retrocerebral gland systems.	20
RESULTS	
1. Stomatogastric nervous system	21
2. Neurosecretory and retrocerebral endocrine systems	35
DISCUSSION	40
CHAPTER FOUR STUDIES ON MALE JAMAICANA FLAVA THROUGHOUT THE <u>SIXTH INSTAR</u> , GROWTH, OXYGEN CONSUMPTION AND THE EFFECT OF TEMPERATURE ON DEVELOPMENT AND THE NEUROENDOCRINE SYSTEM.	
INTRODUCTION	46
MATERIALS AND METHODS	
1. Sampling of stock population	47
2. Maintenance of experimental insects	48
3. Measurement of growth and oxygen consumption	48
4. Temperature and the sixth instar	49
RESULTS	52
DISCUSSION	64

	Page
CHAPTER FIVE <u>STUDIES ON THE STRUCTURE OF THE EXCRETORY SYSTEM OF JAMAICANA FLAVA AND LOCUSTA MIGRATORIA.</u>	
INTRODUCTION	67
MATERIALS AND METHODS	
1. Anatomical and histological studies of the excretory system	69
2. Electron microscopic study of the excretory system	70
3. Histochemical localization of Na <sup>+</sup> - K <sup>+</sup> ATPase in the rectum of <u>Locusta migratoria</u>	70
RESULTS	
1. Structure of the excretory system	71
2. Histochemical localization of Na <sup>+</sup> - K <sup>+</sup> ATPase in the rectum of <u>Locusta migratoria</u>	112
DISCUSSION	117
CHAPTER SIX <u>BIOCHEMICAL STUDIES ON THE EXCRETORY SYSTEM OF SEVERAL ORTHOPTERAN INSECTS.</u>	
INTRODUCTION	136
MATERIALS AND METHODS	
1. Dissection and isolation of excretory system	141
2. Homogenization and centrifugation procedures	142
3. Reaction media and incubation conditions	146
4. Assay of inorganic phosphate	149
5. Assay of protein	149
6. Preparation of the Tris salt of adenosine triphosphate (ATP)	152
7. Electron microscopic study of membrane fractions	155
8. Surgical interference with the stomatogastric nervous system: its effect on Na <sup>+</sup> - K <sup>+</sup> and Mg <sup>2+</sup> ATPase activity of Malpighian tubule, ileal and rectal preparations	156
RESULTS	
1. Extraction and distribution of Na <sup>+</sup> - K <sup>+</sup> and Mg <sup>2+</sup> ATPases	160
2. Properties of Na <sup>+</sup> - K <sup>+</sup> ATPase	169
3. Electron microscopic examination of membrane fractions	200
4. The effect of frontal connective severance on Na <sup>+</sup> - K <sup>+</sup> and Mg <sup>2+</sup> ATPase activity of Malpighian tubule, ileal and rectal preparations	205
DISCUSSION	212
CHAPTER SEVEN    DISCUSSION	218
BIBLIOGRAPHY	225

## CHAPTER ONE

## INTRODUCTION

The major portion of the insect excretory system is composed of the Malpighian tubules, ileum and rectum. This system is shown diagrammatically in Figure 1. (In some insects, the hindgut preceding the rectum consists of an anterior ileum and a posterior colon, e.g. Schistocerca gregaria ). The fundamental roles that these structures play in excretion and maintenance of both haemolymph osmotic pressure and ionic concentration, have been well described (Maddrell, 1971; Stobbart and Shaw, 1973). These parts of the excretory system have long been known to be involved in the transport of monovalent cations and water (Wigglesworth, 1931, 1932; Shaw, 1955). The physiological characteristics of ion movements have been well documented for several species of insect, including Carausius morosus (Ramsay, 1955b, 1956) Locusta migratoria (Ramsay, 1953) Calliphora erythrocephala (Berridge, 1968, 1969) Schistocerca gregaria (Phillips 1964a,b,c) and Rhodnius prolixus (Maddrell, 1969).

Although the Malpighian tubule fluid has been shown to contain many constituents of the haemolymph (see reviews by Maddrell, 1971; Stobbart and Shaw, 1973 for details), it is the transport of potassium ions into the lumen of the tubule which is, as first suggested by Ramsay (1953), the 'prime mover' in urine production. The concentration of this ion in the Malpighian tubule fluid is four to twelve times higher than that of the haemolymph and this is true of the chloride concentration in two species Carausius and Schistocerca but not in Rhodnius (Stobbart and Shaw, 1973). In addition, the tubules of Calliphora (Berridge, 1969) and Carausius (Ramsay, 1956) secrete a fluid rich in phosphate ions,



Fig.1 Diagrammatic representation of the insect excretory system and the course of water circulation in alimentary and excretory systems. Arrows signify direction of water movements. Fluid is probably both secreted and absorbed in the midgut. It is absorbed in the hindgut, especially in the rectum and returned to the gut by the Malpighian tubules. During antidiuresis water is absorbed from the rectal lumen against an increasing osmotic gradient as the concentrated faecal pellet is formed (redrawn from Wigglesworth, 1932; Fig.1).

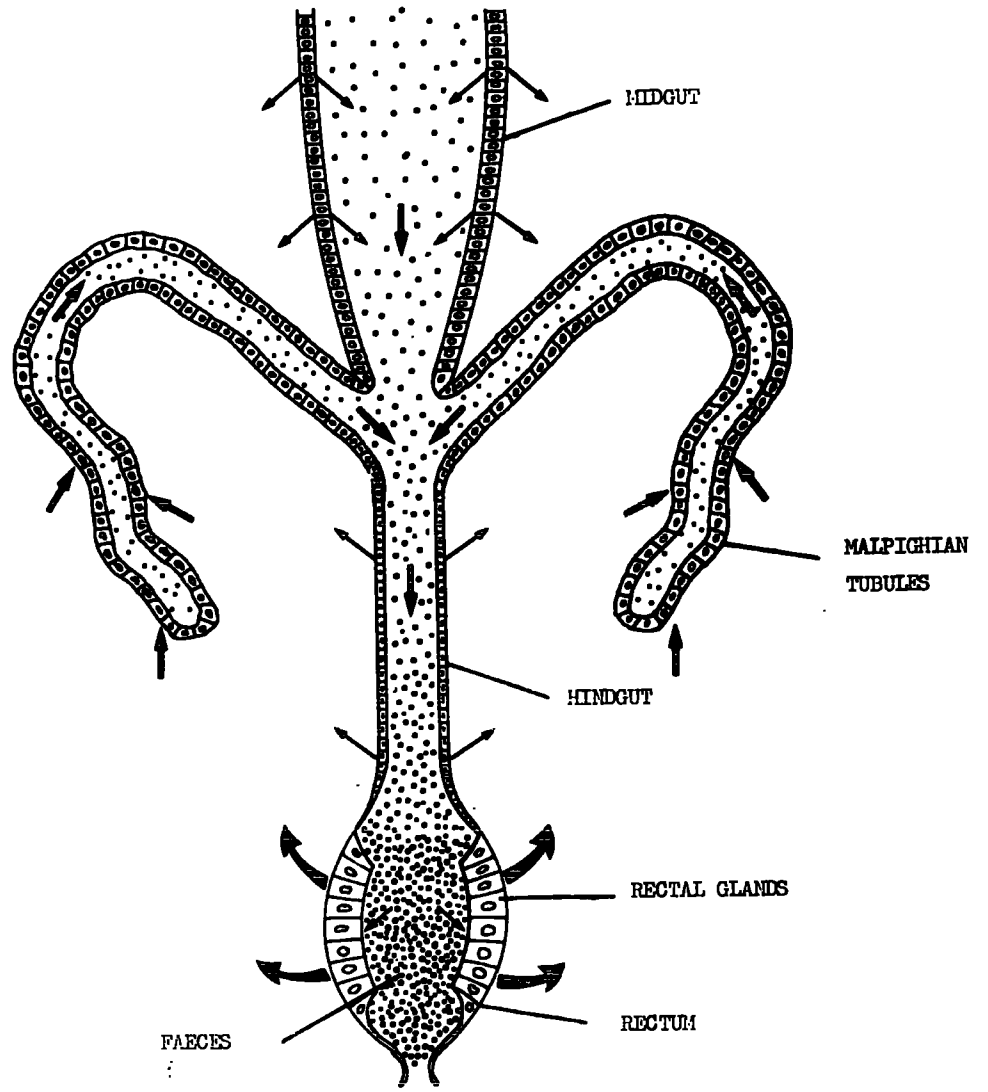


Fig. 1

whilst in all insects examined, the osmotic concentration of the fluid is very close to being isomotic with the bathing fluid over a wide range of osmotic concentrations (Maddrell, 1971). Moreover, it has been shown that small changes in the potassium concentration of the bathing fluid from the normal level, results in substantial changes in that of the tubule fluid (Maddrell, 1971). In the few cases in which measurements have been made, the transepithelial potential shows the lumen to be about 30 mV positive to the bathing solution. This suggests that potassium is being moved against an electropotential chemical gradient. (This is not the case in Rhodnius and Locusta where the transwall potential has been shown to be negative with respect to the bathing solution (Ramsay, 1953)). It has also been shown in a number of insects that sodium ions, when present in low concentrations, greatly accelerate the transwall movement of potassium (Maddrell, 1971). From their work on the tubules of Calliphora erythrocephala and Carausius morosus, Berridge (1968) and Pilcher (1970b) respectively, have independently suggested models explaining the transwall movement of potassium ions. Both authors propose that in the cells of the Malpighian tubules, there is an apical potassium pump and two basal mechanisms for potassium entry from the haemolymph. The apical pump might be an electrogenic potassium pump or an electrically neutral potassium chloride pump. Basally, in the absence of sodium ions, potassium would enter the cells slowly at a rate dependent on the potassium concentration of the external medium. In the presence of sodium ions, potassium entry into the cell would be enhanced by a sodium dependent mechanism.

A second model explaining the transwall movement of potassium ions has been suggested by Kafatos (1968). Briefly, this model involves potassium and hydrogen ion exchange between mitochondria and the

extracellular fluid. In the basal region of the cell, potassium ions are transferred from the extracellular fluid into the mitochondria in return for hydrogen ions which pass in the opposite direction. In the apical region, the reverse process operates. Here, extracellular potassium combines with bicarbonate to form potassium bicarbonate which raises the osmotic pressure of the extracellular fluid, in response to which, water flows out of the cells.

In the majority of insects, the fluid produced by the Malpighian tubules, passes into the intestine at the junction of the mid- and hindguts where it mixes with the residues of midgut digestion. This mixture then flows into the rectum. Knowledge of the physiological role of the intestine anterior to the rectum is limited, although Wigglesworth (1932) observed that in several terrestrial insects some resorption of water takes place in this region of the gut, whilst Wall (1970) considers solute to be resorbed by the ileum of Periplaneta americana.

Although the Malpighian tubule fluid is probably altered in a minor way in the ileum, the most dramatic modification of this fluid occurs in the rectum, where selective resorption of solutes and water occurs in relation to the needs of the insect. In many cases the rectum is composed of a single layer of cells, e.g. Schistocerca gregaria (Phillips, 1964a) but where the need to conserve water is more acute, the rectum is more complicated, e.g. Tenebrio molitor (Ramsay, 1964; Grimstone, Mullinger and Ramsay, 1968). Although the exact way in which the rectum functions in the resorption of solutes and water is not understood, the extensive studies of Phillips (1961, 1964a,b,c, 1969) on the

recta of Calliphora erythrocephala and Schistocerca gregaria, have shown that in the resorption of sodium, potassium and chloride, chloride is taken up actively from the start, whilst sodium and potassium ions may leave the rectum passively at first but as their concentration falls, their uptake is active occurring via active transport mechanisms. The rate of uptake of these ions is dependent on their respective concentration in the rectum, although potassium is absorbed ten times more rapidly than sodium at a similar concentration. Furthermore, Phillips (1969) has shown that water is absorbed from the rectal lumen (a) in the absence of active or passive solute transport from the lumen (b) against an osmotic and in the absence of significant hydrostatic pressure difference (c) with an increase in the osmotic pressure of the lumen fluid.

Electron microscope investigation of the rectum of several insect species (see Maddrell, 1971) has revealed a system of intercellular spaces formed by the infoldings of the lateral cell membrane. Berridge and Gupta (1967) have suggested that water absorption by the rectum of Calliphora erythrocephala is achieved by the pumping of ions ( $K^+$ ,  $Cl^-$ ) into the intercellular spaces. This produces a local osmotic gradient across the cell membrane causing water to flow out of the cells into the intercellular spaces. The hydrostatic pressure within the spaces is supposed to force water and ions along the spaces towards the haemolymph. This scheme is very similar to the double membrane model of water transport proposed by Curran and MacIntosh (1962) (see below).

The precise details of how water movement across the Malpighian tubule and rectal epithelia is accomplished remains

unclear, although such movements are now thought to be passive and secondary to the transport of ions and solute (Maddrell, 1971). It is now widely accepted that the mechanisms advanced in the past to explain water movements across epithelia, notably classical osmosis, filtration, electrosmosis, active transport of water and pinocytosis are not applicable to all types of epithelia. Each method has its merits in individual cases, for example filtration occurs in the proximal tubule of the kidney (Stein, 1967) and pinocytosis in the corneal endothelium (Levin, 1969), but neither can be considered as the basis of water movements in the majority of epithelia.

An important step regarding water transport in epithelia was made when Curran and Solomon (1957) suggested that intestinal water absorption in the rat ileum was a passive process, achieved by the active transport of salt. In order to explain these findings, Curran and MacIntosh (1962) proposed a model (Fig.2) in which two membranes (Fig.2. 1,2) of differing permeabilities to solute and solvent molecules separated three compartments (Fig.2. L,M,R). Solutes are actively transported from (L) across an impermeable membrane (1) into an intercellular space or compartment (M). The osmolarity of this space (M) increases and water flows from (L) in response to the osmotic gradient. Fluid flow across the epithelium is achieved when increases in the hydrostatic pressure within the inelastic intercellular compartment (M), causes fluid to flow through the relatively permeable second membrane (2) into compartment (R).

Diamond and Bossert (1967) modified this model and suggested osmotic gradients develop locally within the extracellular spaces

Figs. 2 and 3

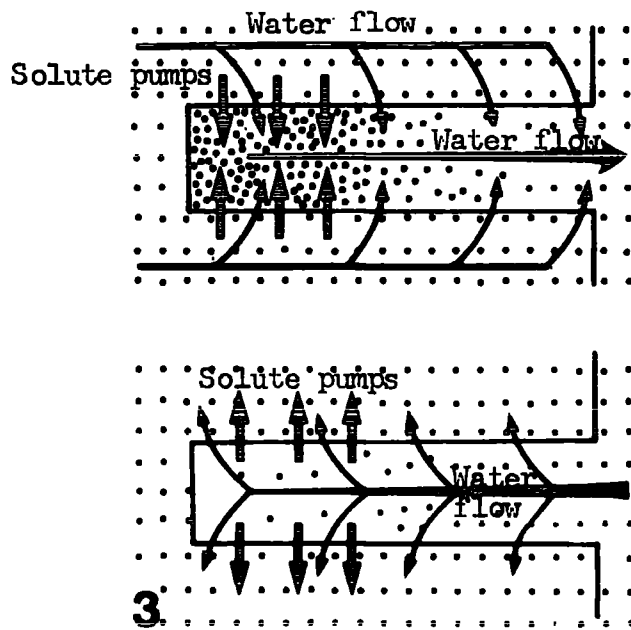
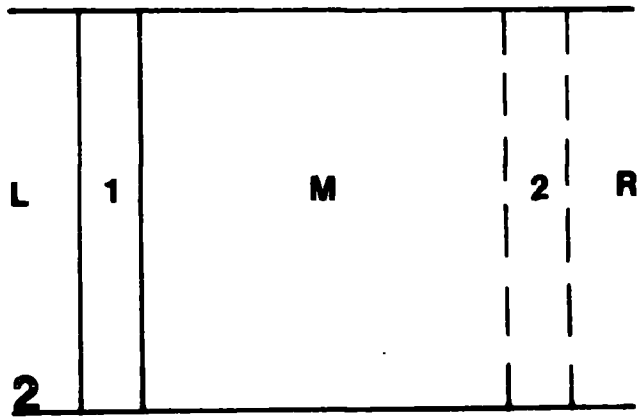
Models explaining water transfer across epithelia.

Fig.2.

The 'double membrane' model of Curran and MacIntosh (1962). For explanation of model see text.

Fig.3.

The 'standing gradient osmotic flow system' of Diamond and Bossert (1968). The density of dots indicates the solute concentration. For explanation of model see text. (Taken from Diamond and Bossert, 1968 Figure 1).



Figs. 2 and 3



of tissues. These authors have noted that the membranes forming the extracellular spaces are organised such that the spaces are usually in the form of extracellular channels. Figure 3 shows the Diamond and Bossert (1968) model for fluid flow into and out of forward (top) and backward (bottom) facing channels. Forward facing channels face in the direction of fluid flow and are to be found in the microvilli of secretory and basal infoldings of absorptive epithelia, for example the salivary glands and gall bladder respectively. Backward facing channels face in the opposite direction to that of fluid transport and occur in avian salt glands, microvilli and the apical infoldings of absorptive epithelia. Diamond and Bossert point out that<sup>11</sup> in forward facing channels solute is actively transported into the channel across its walls, making the channel fluid hypertonic. As solute diffuses down its concentration gradient towards the open mouth, more and more water enters the channel across its walls due to the osmotic gradient. In the steady state a standing osmotic gradient will be maintained in the channel by active solute transport, with the osmolarity decreasing progressively from the closed end to the open end: and a fluid of fixed osmolarity (isotonic or hypertonic, depending upon the values of such parameters as radius, length and water permeability) will constantly emerge from the mouth. Backward operation (bottom); solute is actively transported out of the channel across its walls making the channel fluid hypotonic. As solute diffuses down its concentration gradient towards the closed end, more and more water leaves the channel across its walls owing to the osmotic gradient. In the steady state a standing osmotic gradient will be maintained in the channel by active solute transport, with the osmolarity decreasing progressively from the open end to

the closed end: and a fluid of fixed osmolarity (isotonic or hypertonic, depending upon the parameters of the system) will constantly enter the channel mouth and be secreted across its walls. Solute pumps are depicted only at the bottom of the channels for illustrative purposes but may have different distributions along the channel."

Apart from water absorption by the insect cuticle (Beament, 1964), solvent movement in animals is usually associated with an active sodium pump (Keynes, 1969). House (1974) has noted that in a wide variety of vertebrate epithelia, for example, amphibian bladder, mammalian gall bladder and proximal kidney tubule, sodium chloride is the solute actively transported. There is now a large body of evidence implicating  $\text{Na}^+ - \text{K}^+$  ATPase in the active transport of sodium (Siegel and Albers, 1970). Furthermore, this enzyme has been identified in a variety of tissues noted for the active transport of sodium and passive movements of water. Urinary bladder of the teleost (Fossat, Lahlou and Bornancin, 1974) amphibian epidermis Farquhar and Palade, 1966) mammalian cornea (Leuenberger and Novikoff, 1974) and kidney (Skou, 1962) have all been shown to possess a  $\text{Na}^+ - \text{K}^+$  ATPase. In addition translocation of fluid by these epithelia, ceases or is reduced if ouabain, a potent inhibitor of  $\text{Na}^+ - \text{K}^+$  ATPase activity is present in, or if potassium ions are removed from, the serosal fluid, suggesting a casual relationship between transport and  $\text{Na}^+ - \text{K}^+$  ATPase activity.

House (1974) also points out that the fluids produced by insect Malpighian tubules, salivary and labial glands and midgut are all rich in potassium ions. Whilst the active transport of potassium is well known in insects, the transport mechanism(s) is not.

A hormonal control of ion and water balance would be predicted for insects since, except for potassium, normal variations in haemolymph composition would not have a significant effect on the rate of fluid production by the Malpighian tubules. A hormonal control of insect excretion has been demonstrated by the direct monitoring of fluid production by the Malpighian tubules in the presence of nervous system extracts. For example, the mesothoracic ganglion of Rhodnius prolixus (Maddrell, 1969), the neurosecretory cells of Dysdercus fasciatus (Berridge, 1966) and the corpora cardiaca of Carausius morosus (Pilcher, 1970a) have all in this way been shown to exert an effect on secretion by the Malpighian tubules. According to Mordue (1969) excretion in locusts is most probably controlled by neurosecretory factors which exert their control in two distinct ways, either by accelerating excretion through the Malpighian tubules or by increasing absorption through the rectal wall. The release of endocrine factors from the neuroendocrine system has been shown in a variety of insect species, to be brought about by feeding stimuli (Clarke and Langley, 1963d; Berridge, 1966; Mordue, 1967; Strong, 1967). In this respect, the stomatogastric nervous system (S.G.N.S.) located for the most part on the dorsal surface of the gut, probably acts as a relay, passing nervous information, derived from foregut movements, to the brain. The S.G.N.S. is found in one form or another in all insects that have been examined and, in conjunction with the neurosecretory system and retrocerebral endocrine glands, forms the neuroendocrine system. The latter has been implicated in the control of a wide variety of physiological processes including normal growth and development (Clark and Langley, 1963b,c,d) and water balance (Mordue, 1969).

Although the physiology of excretion is known in detail for a

limited number of insect species, there is however, comparatively little information regarding the mechanisms involved in the transfer and control of ions and water across the excretory tissues. The identification and distribution of these mechanisms would seem to be important in explaining how ion and water balance is achieved by insect excretory systems. The identification, distribution and control of the possible mechanisms involved formed part of the present work.

The remainder of the study arose as a result of using the tettigoniid Jamaicana flava as an experimental animal. In comparison with other Orthopteran insects e.g. Locusta migratoria, Schistocerca gregaria, this species has not been used extensively in the laboratory. Consequently, very little information is available regarding the general biology, anatomy and physiology of this insect. Larval growth, oxygen consumption, the effect of temperature on larval development, as well as the structure of the excretory and neuro-endocrine systems were examined, to provide such information. The majority of experiments were carried out using Jamaicana flava but owing to the difficulty experienced in maintaining adequate stocks of this insect, other Orthopteran species were also employed.

## CHAPTER TWO

## GENERAL MATERIALS AND METHODS

The following insects were used in this study:

Homocoryphus nitidulus vicinus (Wlk.), Jamaicana flava (Caudell),  
Locusta migratoria migratorioides (R. and F.) and Schistocerca  
gregaria (Forsk.).

1. Maintenance of insect populations.

(a) Jamaicana flava

Stock populations of Jamaicana flava were maintained in an insectary at 24°C and relative humidity ca. 50%. Insects were reared in cages measuring 1.2 x 0.45 x 0.45 m, constructed out of aluminium, clear perspex and stiffened muslin. The photoperiod was 12 h light, 12 h dark. Insects were fed daily on a diet of fresh cabbage, carrot and banana, supplemented with a commercially available cereal preparation (Beemax). Pieces of corrugated cardboard were present on the floor of the cages and provided cover under which the insects reside during the periods of light. A metal tray (17.5 x 2.5 x 10 cm) containing a mixture of sand and moderately coarse gravel, was present in each cage. Prior to use, the sand and gravel were sterilized by heating, overnight in an oven at 100°C. The contents of the trays were kept moist by spraying with tap water and provided material in which the females could deposit their eggs. The trays were examined weekly, the eggs removed, rubbed gently with damp cotton wool, to remove adhering sand and placed in petri dishes containing moist filter paper. The lids of the petri dishes were perforated to allow the circulation of air. The eggs were examined daily for hatchings and newly emerged insects transferred to small cages. The latter step was taken as a precaution since young

stages can escape through the holes and slits of the larger cages.

b) Locusta migratoria migratorioides, Homorocoryphus nitidulus vicinus, Schistocerca gregaria

Stock populations of each species were maintained in an insectary at 28°C and relative humidity ca. 50 %. Insects were reared in cages of similar size and construction to those described above. The photoperiod was 12 h light, 12 h dark. Insects were fed daily on a diet of fresh grass and Beemax. Water was supplied by means of a drinking fountain.

## 2. Reagents

All chemicals were the purest grade available and obtained from British Drug House Chemicals Ltd., Poole, Dorset. All salts were present as chlorides. ATP (disodium and barium salts), ouabain, histidine, imidazole and Tris were obtained from Sigma Chemical Company, St. Louis, Missouri. Chemicals used in electron microscopy were obtained from TAAB Laboratories Ltd.

## 3. Glassware.

All glassware was soaked overnight in a 1 % solution of quadralene, rinsed three times in tap water followed by four rinses in glass distilled water.

CHAPTER THREE  
STUDIES ON THE STRUCTURE OF THE NEUROENDOCRINE SYSTEM  
OF JAMAICANA FLAVA

Introduction

There is now considerable evidence implicating the stomatogastric nervous, neurosecretory and retrocerebral gland systems in the control of normal growth and metabolism in a variety of insects (Clarke and Langley, 1963a-d; Highnam, Hill and Mordue, 1966; Clarke and Gillot, 1967a,b; Clarke and Anstee, 1971; Dogra and Ewen, 1971). Interference with the stomatogastric nervous system produces a variety of effects including cessation of growth and development (Clarke and Langley, 1963b-d), inhibition of egg development (Highnam, 1962; Highnam, Hill and Mordue, 1966; Dogra and Ewen, 1971), reduction in corpus allatum activity (Highnam, Hill and Mordue, 1966; Strong, 1966a). These effects are mediated via the endocrine system, since interference with the stomatogastric nervous system, reduces or prevents release of neurosecretory material from the corpora cardiaca (Clarke and Langley, 1963d).

Furthermore, neuroendocrine factors have been shown to influence excretion and water balance in a variety of insects (Nunez, 1956; Maddrell, 1963, 1964; Wall and Ralph, 1964; Wall, 1965, 1967; Berridge 1966; Casal and Girardie, 1968; Mordue, 1969, 1971). There is now considerable evidence indicating a diuretic hormone in insects, although the source of the hormone depends on the species. For example, extracts of the protocerebrum and corpora cardiaca have been shown to have a marked diuretic effect on the Malpighian tubules of Anisotarsus cupripennis (Nunez, 1956), Schistocerca gregaria (Highnam, Hill and Gingell, 1965), Dysdercus fasciatus, (Berridge, 1966), and Carausius morosus (Pilcher, 1970a) whilst diuretic activity has been demonstrated

in extracts of the mesothoracic ganglion in Rhodnius prolixus (Maddrell, 1963, 1964) and Corethra (Gersch, 1967).

Antidiuretic factors have been demonstrated in several species of insect including Locusta migratoria, Gryllus domesticus (de Bessé and Cazal, 1968) and Periplaneta americana (Wall and Ralph, 1964; Wall, 1965, 1967). In the first two species antidiuretic activity has been demonstrated in extracts of the corpora cardiaca, whilst in Periplaneta, both the brain and corpora cardiaca have been shown to contain an anti-diuretic factor. Moreover in Periplaneta, the corpora allata possess both diuretic and antidiuretic hormones (Wall and Ralph, 1964; Mills, 1967).

The physiological mode of action of the hormones involved in water balance has been proposed for several species of insect including Rhodnius prolixus (Maddrell, 1963, 1964), Dysdercus fasciatus, (Berridge, 1966), Schistocerca gregaria (Mordue, 1969, 1971) and Carausius morosus (Pilcher, 1970a). It appears, in the majority of cases, that water balance, as far as the excretory system is concerned, is a balance between urine production by the Malpighian tubules and resorption by the rectum. The effect of the diuretic hormone is to accelerate excretion through the Malpighian tubules and in some cases e.g. locusts, to reduce resorption from the rectal lumen (Mordue, 1969). In this way, excess water, for example taken when feeding, is removed from the haemolymph. In the absence of diuretic hormone, the rate of excretion falls and, again in locusts, more water is absorbed through the rectal wall (Mordue, 1969). Where antidiuretic factors are involved, their effect is to increase the rate of absorption by the rectum, thereby reducing water loss from the hindgut (Mordue, 1971).



Thus a study of the stomatogastric nervous system and its relation to the neurosecretory and retrocerebral gland systems is necessary before investigating whether neurohormones control ion and water balance in Jamaicana flava. Furthermore such a study would also be of interest, since with the exception of Cazal (1948) and Nesbitts' (1941) work, very little is known of these systems of tettigoniids, although descriptions of these structures are available for other Orthoptera.

Nesbitt (1941) described the anatomy of the stomatogastric nervous and retrocerebral gland systems in three orders of insect with particular reference to Orthoptera including the tettigoniid Conocephalus fasciatus, whilst Bickley (1942) and Cazal (1948) presented anatomical descriptions of both systems of Schistocera americana (Drury).

The anatomical studies have been supported by extensive histological investigations so that among Orthoptera, comprehensive accounts of the S.G.N.S., neurosecretory system and retrocerebral glands, are available for Locusta migratoria through the work of Albrecht (1953), Clarke and Langley (1963b,d), Roome (1968), Clarke and Anstee (1971) and Allum (1973), Periplaneta americana (Willey, 1961), Schizodactylus monstrosus (Khatter, 1968b), Schistocera gregaria (Dando, Chanussot and Dando, 1968) and Melanoplus sanguinipes (Dogra and Ewen, 1970).

## Materials and Methods

### 1. Anatomical study of the stomatogastric nervous system and retro-cerebral endocrine glands.

#### i. Preparation of reduced methylene blue (Stark, Smalley and Rowe, 1969)

##### a. 0.5% ( $W/V$ ) Methylene blue.

The suspension was warmed and stirred continuously until all the solid had dissolved. It was then filtered and allowed to cool before use.

##### b. 12.0% ( $W/V$ ) Sodium formaldehyde sulfoxate (rongalite, G.T.Gurr).

##### c. 8.0% ( $W/V$ ) Ammonium molybdate.

Reduced methylene blue was prepared by adding 6 ml of sodium formaldehyde sulfoxate to 30 ml of methylene blue stock solution. The mixture was heated and stirred continuously until it turned from the original blue to the dirty green colour of the reduced dye. Stirring was continued without further heating until the solution was colourless. It was then allowed to cool, filtered and stored in an appropriately sized bottle so as to prevent reoxidation by air. The unadjusted pH of the solution was between 4.0 - 5.0.

#### ii. Procedure

Anatomical work was carried out with sexually mature male and female individuals, using simple dissection. Specimens were decapitated and heads secured in beeswax. The cuticle, muscle and trachea of the head were removed to leave the S.G.N.S. and retrocerebral endocrine glands exposed on the foregut. A second method of studying the arrangement of the nerves involved *intra vitam* injections of reduced methylene blue (Stark, Smalley and Rowe, 1969.). This technique, rendered visible the finer nerves and proved to be very useful. As a result of preliminary experiments, 0.2 - 0.3 ml of reduced stain were injected into the back of the head through the neck membrane, using a 1 ml Gillete Scimitar syringe. During this operation the needle of the syringe pointed in a forward direction

parallel to the cuticle of the vertex. Experience revealed that firm continuous pressure, applied to the plunger of the syringe, ensured penetration of the reduced stain to most parts of the head. After injection, individuals were placed in small plastic containers where they remained for 1 - 2 h. After the 'staining' period individuals were decapitated and the heads secured in beeswax. The cuticle and muscle of the head region were then removed. Dissections were carried out under a cold solution of ammonium molybdate. Dissection was further facilitated by carefully placing in the foregut, via the mouth, small pieces of cotton wool or white tissue paper. This provided an adequately light background (white/grey colour) with which to contrast the dark blue of the nerves. It was also found that a partially completed dissection could be placed overnight at 0°-4°C and continued the next day without fear of loss of stain from the nerves and tissues.

## 2. Histological study of the stomatogastric nervous, neurosecretory and retrocerebral gland systems.

### Preparation of material.

Initially, all specimens underwent similar procedures irrespective of the subsequent staining reaction. The individuals were decapitated and the heads placed in Bouin's fluid at room temperature. A 20 min period at a reduced pressure of 28 p.s.i. ensured rapid penetration of the fixative into the tissues. The mouthparts and cuticle were then removed and the heads transferred to fresh fixative and left to stand for a further 2½ h at room temperature. In order to examine the ingluvial ganglia, the crop and midgut were dissected out under Bouin's fluid, transferred to fresh fixative and left for 2½ h. The tissues were processed in an Elliot Tissue Processor, embedded in paraffin wax and serially sectioned at 10 µm on a Spencer rotary microtome. Sections were

stained for neurosecretion using paraldehyde fuchsin (PAF) after Ewen (1962) or chrome haematoxylin phloxine (CHP) after Gomori (1941), dehydrated through a graded series of alcohols and mounted in Canada balsam.

### Results

The general organization of the stomatogastric nervous system and retrocerebral endocrine complex of Jamaicana flava is shown in Figures 1, 2. The stomatogastric nervous system lies on the dorsal surface of the foregut and innervates this organ. It comprises a frontal, hypocerebral and paired ingluvial ganglia. Nerve pathways link this system to the brain. The retrocerebral endocrine system is composed of the paired corpora cardiaca and corpora allata.

#### 1. Stomatogastric nervous system

##### Frontal ganglion and associated nerves

Figure 3 shows in detail the arrangement of the frontal ganglion and associated nerves. The ganglion is approximately pear-shaped and lies along the midline, dorsal to the pharynx, in the region where the alimentary canal bends ventrally to form the buccal cavity. It consists of large cells arranged dorsal and lateral to a ventral mass of neuropile (Fig.5). Typically, the cytoplasm of these cells stains light green with the PAF technique and pink/purple with the CHP method. Occasionally, in some specimens blue/purple granules are seen in the cytoplasm after PAF staining (Fig.5). Such granules were not observed in the axons of these cells. Nerve axons intermingle in the neuropile, making it difficult to follow them for any distance. The ganglion is enveloped by an acellular neurilemma that is continuous over the whole of the nervous system, and a cellular, perineurium. The cells and nuclei of the perineurium are oval and flattened (Fig.5). As in other insects several nerves were observed to leave the frontal ganglion.

Fig. 1. Stomatogastric nervous system and retrocerebral endocrine glands, right lateral view excluding the ingluvial and suboesophageal ganglia.

Abbreviations,

BC, buccal cavity; CA, corpus allatum; CC, corpus cardiacum; DA, dorsal aorta; D, deutocerebrum; E, eye; FC, frontal connective; FCN 1, frontal connective nerve one; FCN 2, frontal connective nerve two; FG, frontal ganglion; HG, hypocerebral ganglion; LFN, labrofrontal nerve; LN, labral nerve; MN, median nerve; NC, nervus connectivus; NCA I, II, nervus corporis allati one and two; NCC I, II, nervus corporis cardiaci one and two; NWC, nerve to wall of crop; ON, optic nerve; OPN, oesophageal nerve; PC, protocerebrum; PN, pharyngeal nerve; RCN, recurrent nerve; T, tritocerebrum.

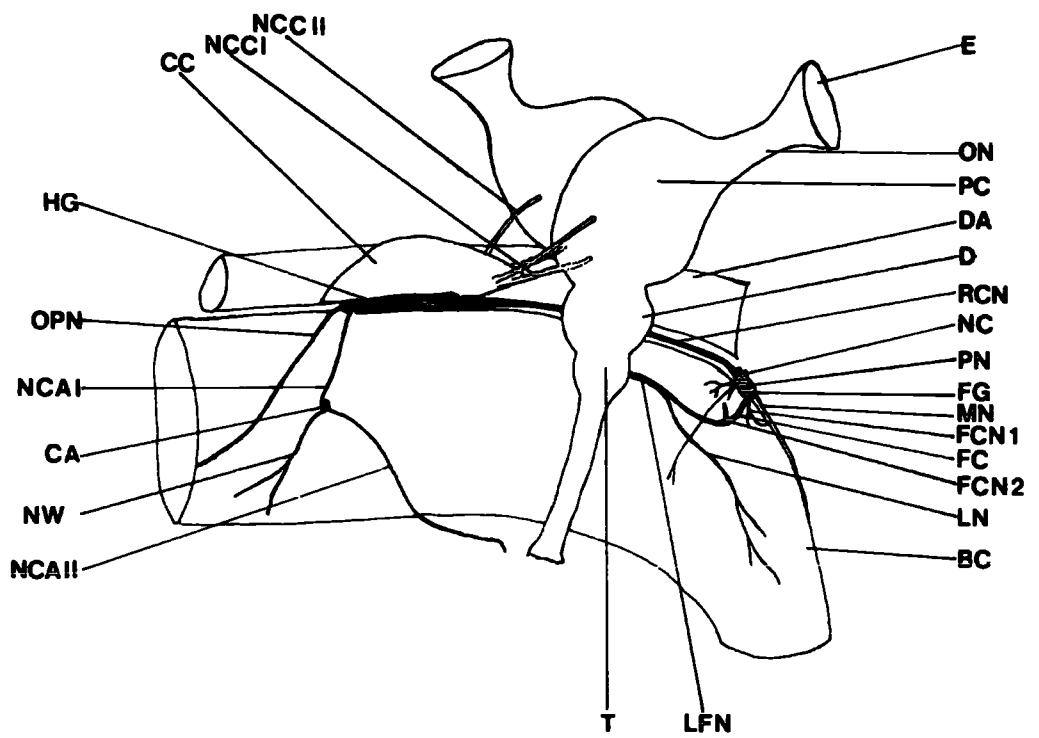


Fig.1

Fig. 2. Stomatogastric nervous system and retrocerebral endocrine glands, dorsal view, brain and circumoesophageal commissure removed.

Abbreviations,

BC, buccal cavity, C, crop; CA, corpus allatum; CC, corpus cardiacum. FC, frontal connective; FG, frontal ganglion; IG, ingluvial ganglion; LN, labral nerve; M, mouth; MG, midgut; MGC, midgut caecum; NCA I, nervus corporis allati one; NCC I, II, nervus corporis cardiaci one and two; O, oesophagus; OPN, oesophageal nerve; P, pharynx; PV, proventriculus; RCN, recurrent nerve.

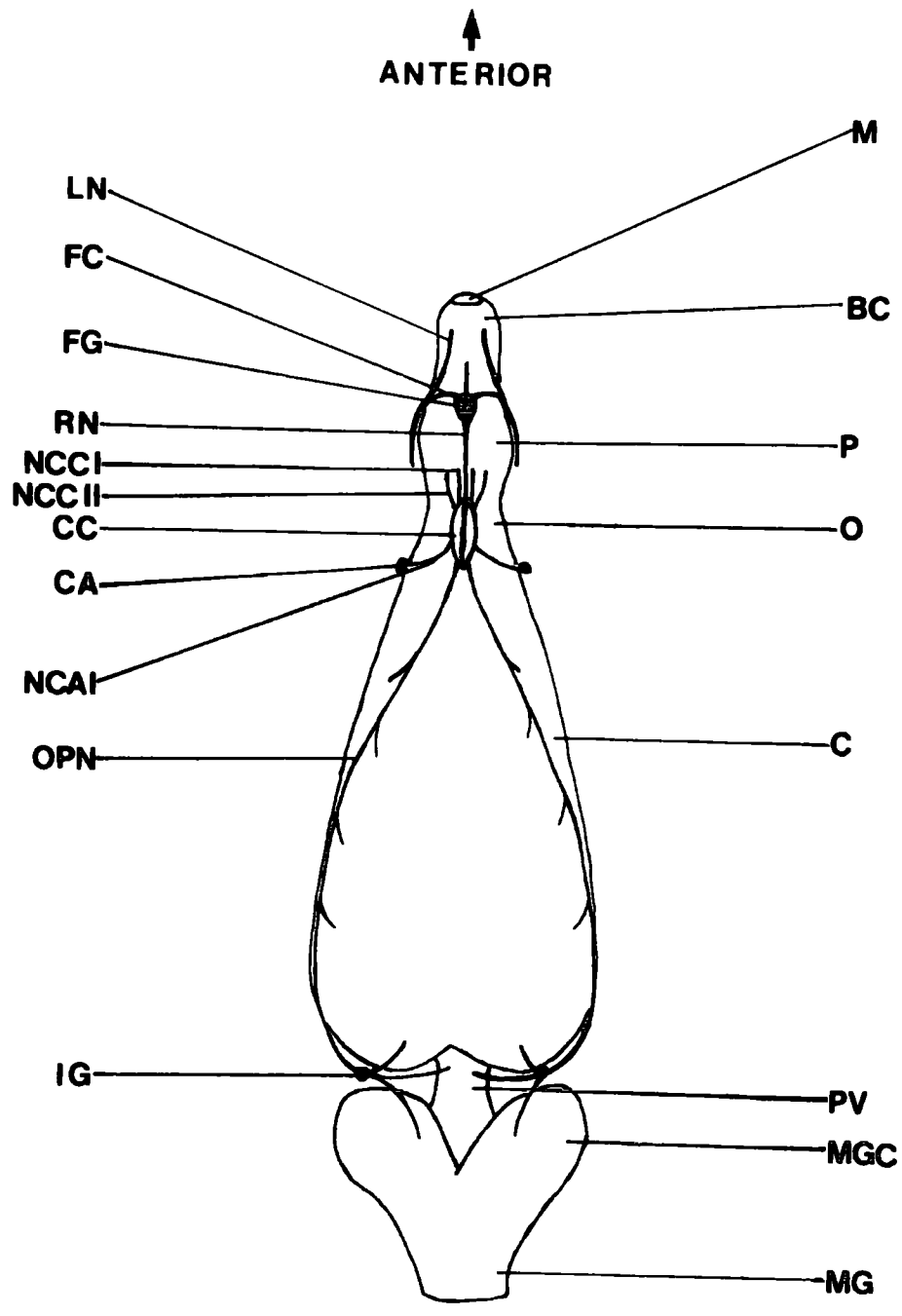


Fig.2



Fig.3. Frontal ganglion and associated nerves, dorsal view.

Abbreviations.

ARML, anterior retractor muscles of labrum; BLN, branches of labral nerve; C, clypeus; DDMP, dorsal dilator muscle of pharynx; DMC, dilator muscles of cibarium; F, frons; FC, frontal connective; FCN 1,2, frontal connective nerves one and two; FG, frontal ganglion; L, labrum; LN, labral nerve; M, mandible; MN, median nerve; NC, nervus connectivus; P, pharynx; PN, pharyngeal nerve; PN 1,2,3, branches 1-3 of pharyngeal nerve; PRML, posterior retractor muscle of labrum; RCN, recurrent nerve; RMA, retractor muscle of mouth angle.

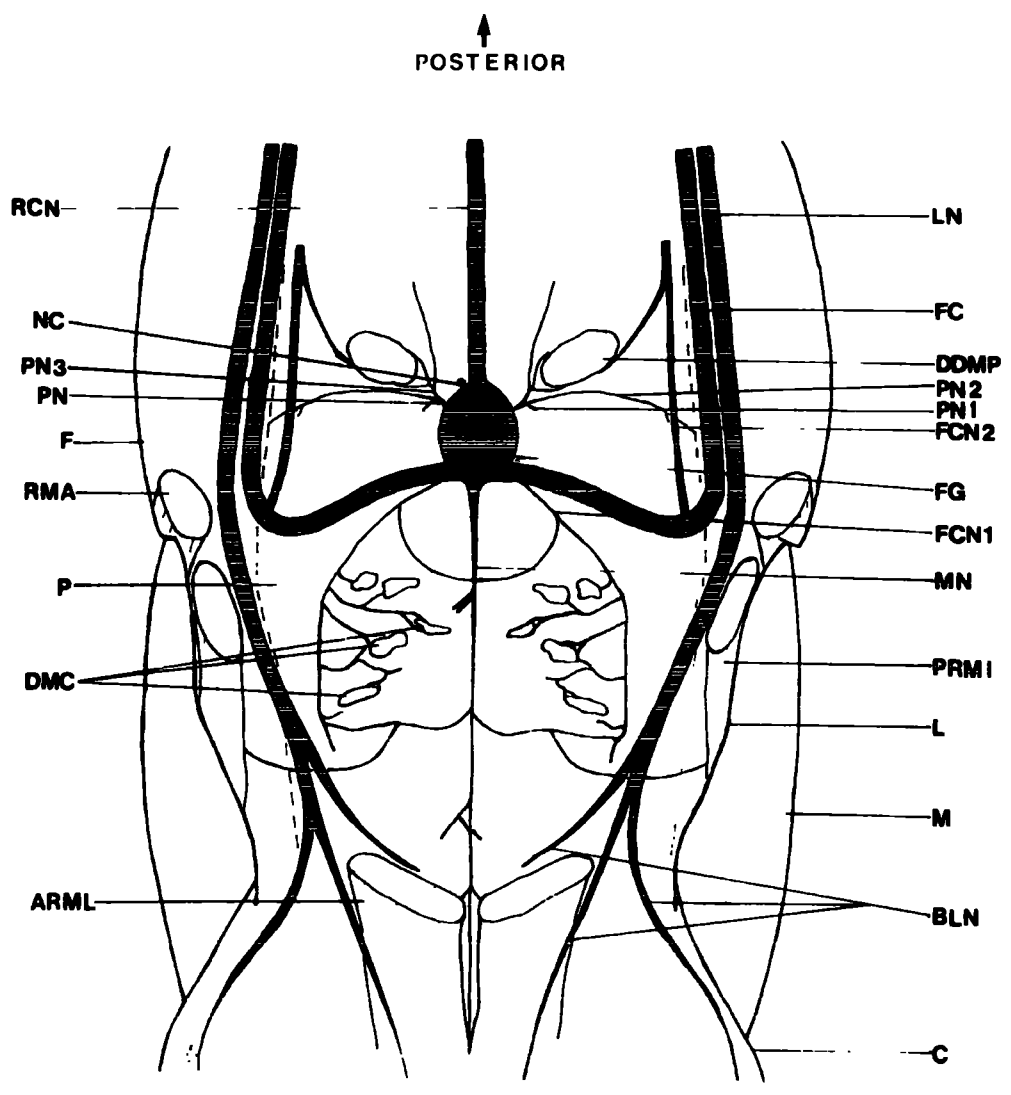


Fig.3

A very fine nerve, the nervus connectivus, leaves the mid dorsal surface of the frontal ganglion and serves to link it to the protocerebrum of the brain. Figure 6 shows a portion of this nerve leaving the frontal ganglion. A pair of pharyngeal nerves, one on either side, leave from the posterolateral margin of the frontal ganglion and immediately divide into three finer nerves (Fig.3). Of these, the most anterior branch(PN 1) passes immediately to the musculature of the pharynx whilst the posterior branch(PN 3) divides into three fine nerves (Fig.3). Of these, the middle and anterior nerves pass to the dorsal dilator muscles of the pharynx, whilst the posterior branch innervates the surface of the gut (Fig.3). The third and middle branch(PN 2) of the pharyngeal nerve, runs laterally over the pharynx, giving off branches to the musculature of the foregut and eventually dichotomises a little way short of the suboesophageal ganglion (Figs. 1, 3).

#### Frontal connectives and their branches

Two large nerves, the frontal connectives, emerge from the anterolateral edge of the frontal ganglion (Figs.3,7) and pass back on either side of the gut to the tritocerebral lobes of the brain. Just prior to entering the tritocerebrum, each frontal connective fuses with the labral nerve on that side to form a short labrofrontal trunk (Fig.1). At the point where each frontal connective turns posteriorly towards the tritocerebrum, a pair of nerves (FCN 2), one on each side, leave the frontal connective and run in a dorsal direction before disappearing among the fibres of the anterior and posterior retractor muscles of the labrum, as well as the retractor muscles of the mouth angle (Fig.3). Shortly after leaving the frontal connectives, fine nerves, one on either side, leave the main nerve(FCN 2) and run ventrally to

innervate the dorsal dilator muscles of the pharynx (Fig.3). The labral nerve emerges from the labrofrontal root and passes ventrally on either side of the foregut and innervates the tissues of the labrum (Fig.1). On either side, the labral nerves give off branches that ramify extensively throughout the tissues of the lower clypeus (Fig.3).

A fine pair of nerves leave, one on either side, from the anterior region of each frontal connective (Fig.3, FCN 1). Each nerve travels a short distance along the dorsal surface of the pharynx before dividing into an inner and outer nerve (Fig.3). The inner nerve describes a half circle over the dorsal surface of the pharynx and joins with its counterpart from the opposite side in the midline (Fig.3). The outer nerve runs anteriorly over the surface of the pharynx and joins with a fine branch of the median nerve (see below). The outer nerve also supplies branches to the dilator muscles of the cibarium, as well as the posterior retractor muscles of the labrum (Fig.3).

A fine nerve, the median nerve, emerges from an anterior region of the frontal ganglion in the midline (Figs.3,7). This nerve runs along the dorsal surface of the pharynx and cibarium giving off six branches that innervate the tissues of these regions. The first branch, a single nerve, leaves the main nerve and divides into two finer nerves both branches of which innervate the anterior retractor muscles. Anteriorly, in the clypeus, a very fine pair of nerves, next leave the median nerve and join up, on either side, with the fine outer branch of the frontal connective nerve FCN 1 (Fig.3). The fourth, a single nerve leaves shortly after the latter pair, and innervates the tissues of the clypeus. Finally a fine pair of nerves leave the median nerve and innervate the basal region of

the anterior retractor muscles of the labrum. The median nerve continues anteriorly and eventually anastomoses with the tissues of the lower clypeus and labrum (Fig.3).

#### Recurrent nerve

A recurrent nerve leaves the posterior ventral surface of the frontal ganglion and passes back along the mid dorsal line of the foregut to the hypocerebral ganglion (Figs.1,8). Several nerves leave the recurrent nerve and pass to the tunica muscularis of the pharynx. One such nerve is shown in Figure 9.

#### Hypocerebral ganglion

The hypocerebral ganglion lies posterior to the brain along the midline of the oesophagus (Fig.1). The recurrent nerve enters the ganglion at its anterior end and a single pair of oesophageal nerves emerge from the posterior margin (Fig.1). The latter nerves run posteriorly over the surface of the crop to the proventriculus where they join the ingluvial ganglion (Fig.2). Two short connectives, one of which is shown in Figure 10, leave the anterolateral regions of the ganglion and link it to the overlying corpora cardiaca. A pair of fine nerves leave the ventral surface of the ganglion and pass to the dorsal musculature of the foregut. One of these nerves is shown in Figure 10.

In transverse section, the hypocerebral ganglion consists of a central neuropile, surrounded by the large cell bodies of the neurons (Fig.10). Neurosecretory material was never observed in the cells or axons within the ganglion, nor in the axons of the recurrent and oesophageal nerves.

#### Ingluvial ganglion

The ingluvial ganglia are a pair of small bodies that lie on either side of the crop in the region of the proventriculus (Figs.2,4). Each

Fig.4. Ingluvial ganglion and associated nerves, right lateral view.

Abbreviations,

C, crop; IG, ingluvial ganglion; MG, midgut; MGC, midgut caecum; MT, Malpighian tubule; OPN, oesophageal nerve; PV, proventriculus.

Numbers (1-5) refer to nerves leaving the ganglion and innervating the crop (1), proventriculus (2), midgut caeca (3), (4) and midgut (5).

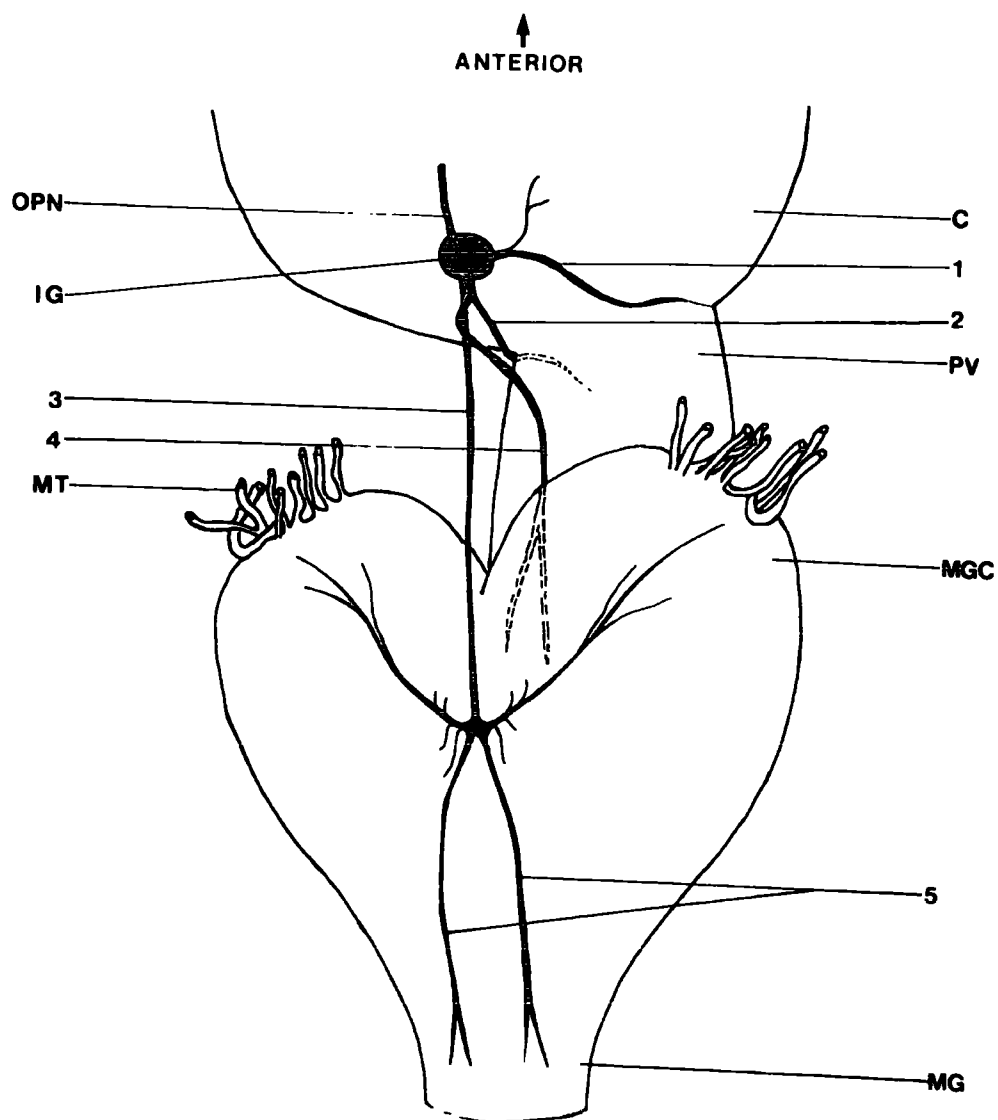


Fig. 4

## Figs. 5-13.

Photomicrographs of sections through various parts of the stomatogastric nervous and retrocerebral endocrine systems of Jamaicana flava. All tissues fixed in Bouin's fluid and stained with paraldehyde fuchsin (Ewen, 1962).

## Fig. 5.

Transverse section through the frontal ganglion. Note the dorsal and lateral arrangement of the cell bodies (CB) around the ventral neuropile (NP), as well as the stainable granules (G) in the cytoplasm of the cells.

NL, neurilemma; P, perineurium; Scale 50  $\mu$ m.

## Fig. 6.

An oblique longitudinal section through the frontal ganglion (FG) showing part of the nervus connectivus (NC). Scale 100  $\mu$ m.

## Fig. 7.

Slightly oblique, longitudinal section through the frontal ganglion (FG) showing one of the frontal connectives (FC) and median nerve (MN) leaving the anterior margin of the frontal ganglion as well as a fine nerve (FCN 1). Scale 100  $\mu$ m.

## Fig. 8.

Transverse section through the posterior region of the frontal ganglion (FG) in a region where the recurrent nerve (RCN) lies beneath the ganglion. M, muscle of pharynx. Scale 50  $\mu$ m.

## Fig. 9.

Transverse section through the recurrent nerve (RCN) to show one of the fine nerves (N) leaving it. Scale 50  $\mu$ m.

## Fig. 10.

Transverse section through the hypocerebral ganglion (HG) showing one of the short connectives (C) that link it to the corpora cardiaca (CC). A, aorta; M, muscle of pharynx. Scale 50  $\mu$ m.

## Fig. 11.

Section through the equator of an ingluvial ganglion. Note central neuropile (NP) surrounded by the cell bodies (CB). NL, neurilemma. Scale 50  $\mu$ m.

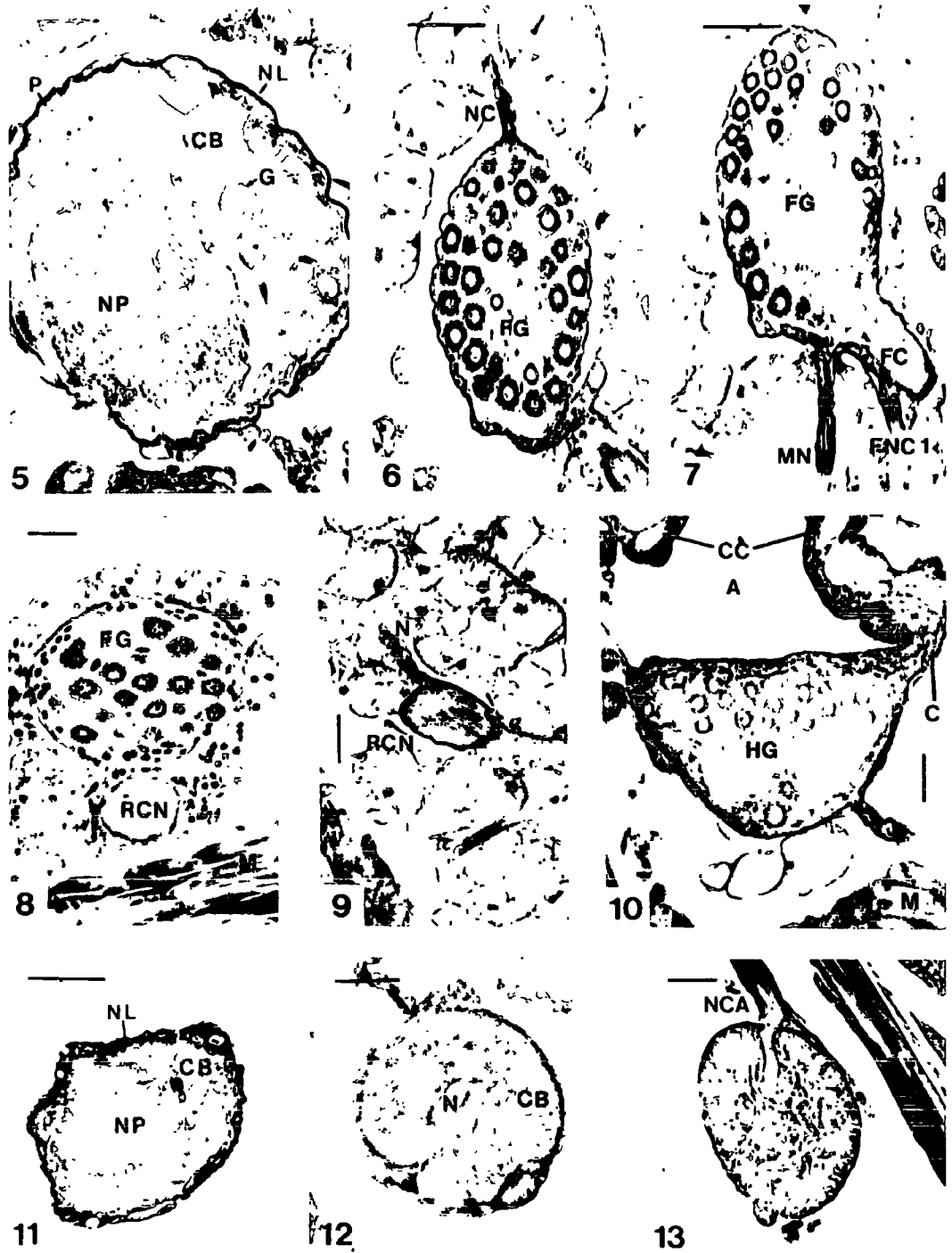
## Fig. 12.

Section through the equator of a corpus allatum. N, nerve axons; CB, cell bodies. Scale 50  $\mu$ m.

## Fig. 13.

Section through a corpus allatum in a region where the nervus corporis allati I (NCA) leaves the gland. Scale 50  $\mu$ m.





Figs. 5-13

ganglion consists of a small number of large neurons which are devoid of neurosecretory material (Fig.11). Four nerves leave each ganglion and innervate the posterior regions of the crop, proventriculus and midgut caeca (Fig.4, nerves 1,2,3,4 respectively). One of the nerves (3) supplying the midgut caeca divides into four, of which one pair (5) innervates the midgut. (Fig.4).

## 2. Neurosecretory and retrocerebral endocrine systems

Neurons that show cytological evidence of secretion are present in the pars intercerebralis of the protocerebral lobes of the brain, where they are located in two groups on either side of the midline (Fig.14). Three types of cell can be identified in each group in the basis of size and staining reaction (Fig.14). A large proportion of the cells stain deep purple with PAF and blue/black with CHP (Fig.14, (1), (1a)). Whether they are the same cells was undetermined, although Highnam (1961) reported this to be the case in Schistocerca gregaria. In Jamaicana flava the quantity of stainable material varied both in the perikarya and the axons of these cells. Where the cells are large, the inclusions are also large and completely fill the cells (Fig.14,(1)). In others the amount of stainable material is small with the inclusions in particulate form and evenly dispersed throughout the cytoplasm (Fig.14, (1a)). Both these types of cell are similar in their staining reactions to the 'A' type of neurosecretory cell described for Schistocerca gregaria (Highnam, 1961) and Locusta migratoria (Clarke and Langley, 1963d). A second less frequent, smaller type of cell intermingles with those above (Fig.14, (2)). The cytoplasm of these cells stains bluish green with PAF and pink/red with CHP. Stainable material was never observed in these cells.

## Figs. 14-23.

Photomicrographs of sections through various parts of the neurosecretory and retrocerebral endocrine systems of Jamaicana flava. Tissues treated as in Figures 5-13, except that Figure 15 was stained with chrome haematoxylin phloxine.

## Fig. 14.

Section through the pars intercerebralis of the brain to show the two groups of neurosecretory cells on either side of the midline. The numbers 1-3 refer to the types of cell observed in this region. Scale 50  $\mu\text{m}$ .

## Fig. 15.

Photomicrograph similar to Figure 14 except that neurosecretory material (NSM) can be seen in the proximal parts of the nerve tracts. Scale 50  $\mu\text{m}$ .

## Fig. 16.

Section through the protocerebrum of the brain to show the lateral group of cells (LC). Scale 100  $\mu\text{m}$ .

## Fig. 17.

Transverse section through the ventral part of the protocerebrum (P) showing one of the nervi corporis cardiaci I (NCC I) emerging from the brain. Scale 50  $\mu\text{m}$ .

## Fig. 18.

Low magnification photomicrograph showing the medial neurosecretory cells (NSC) of the protocerebrum (P), corpora cardiaca (CC) containing neurosecretory material (NSM) and regions of this gland where such material is absent (SC). The hypocerebral ganglion (HG), aorta (A), corpus allatum (CA) and gut (G) are also visible. Scale 100  $\mu\text{m}$ .

## Fig. 19.

Oblique transverse section through the oesophageal region showing the nervi corporis cardiaci II (NCC II) dorsal and lateral to the nervi corporis cardiaci I (NCC I). RCN, recurrent nerve. Scale 100  $\mu\text{m}$ .

## Fig. 20.

Section through the protocerebrum of the brain to show the chiasma (C) of the axon tracts within the brain. Scale 100  $\mu\text{m}$ .

## Fig. 21.

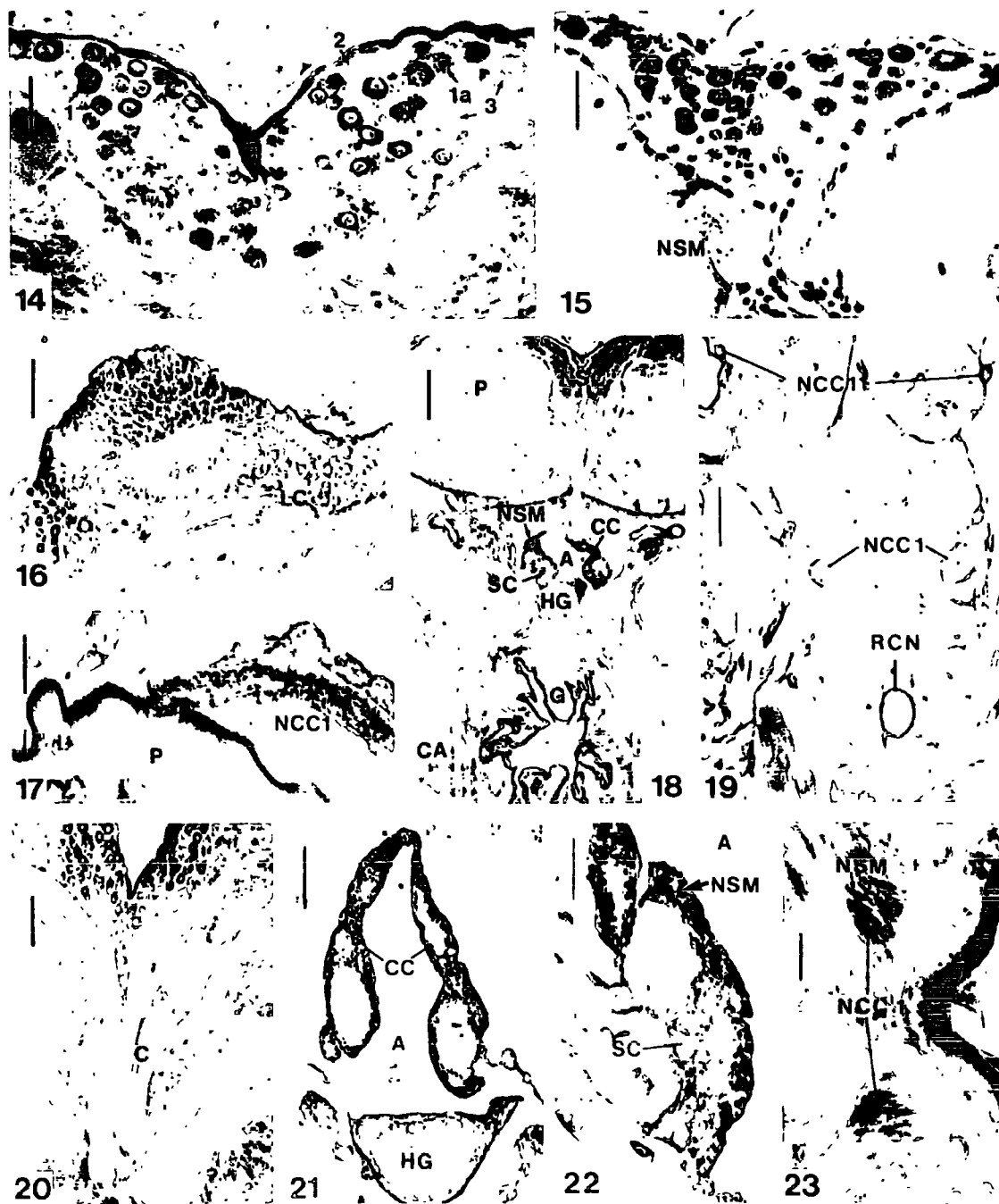
Transverse section through the middle of the corpora cardiaca (CC) A, aorta; HG, hypocerebral ganglion. Scale 100  $\mu\text{m}$

## Fig. 22.

Section through the corpora cardiaca to show the histologically distinct regions within the gland. A, aorta; NSM, neurosecretory material; SC, cells in which stainable material is absent. Scale 50  $\mu\text{m}$ .

## Fig. 23.

Transverse section through the ventral part of the protocerebrum showing neurosecretory material (NSM) in the axons of the nervi corporis cardiaci I (NCC I). Scale 50  $\mu\text{m}$ .



Figs. 14-23

The third type of cell is found near the periphery of each cell group. They are larger and fewer in number than either of those described above and their cytoplasm stains pale green with PAF and pink with CHP (Fig.14,(3)). These cells resemble the 'C' cell type of Schistocerca gregaria (Highnam, 1961) and Locusta migratoria (Clarke, 1966). Another group of cells is distinguishable in each half of the brain. These cells lie posterior and lateral to the medial groups. Stainable material was most easily observed in the cytoplasm of these cells after PAF staining (Fig.16). The material was in the form of small particles and although evenly distributed throughout the cytoplasm was not observed in the axons of these cells. Hence their path through the brain remained undetermined. The axons of the medial neurosecretory cells can be followed within the brain owing to the presence of stainable material within them (Figs.20,23). The axons from each cell group converge to form two nerve tracts that run in a posterior and ventral direction keeping almost parallel with the anterior surface of the protocerebrum, before crossing one another so that the axons from the left hand group of neurosecretory cells, emerge from the posterior and ventral surface of the protocerebrum as the right nervus corporis cardiaca (NCC I) and vice-versa (Figs.17, 20). The NCC I, ensheathed by extensions of the neurilemma of the brain, proceed to the dorsal medial surface of the anterior regions of the corpora cardiaca. Throughout their passage in the brain, the tracts appear as discrete bundles without interconnections (Fig.23). The stainable material within these tracts and the NCC I, is identical in its staining properties with the material in the cells of the pars intercerebralis.

In the axons, the stainable material is most noticeable in a region ventral to each group of medial neurosecretory cells (Fig.15).

This region may correspond to the point where, after leaving the cells, the axons converge to form the tracts.

#### Corpora cardiaca

The corpora cardiaca are white, translucent, bilobed structures that lie posterior to the brain and overlie the hypocerebral ganglion (Figs. 1,2). The dorsal aorta passes between the lobes, such that the walls of the corpora cardiaca are effectively those of the aorta (Figs.18,26). The lobes are separate ventrally and dorsally except for a short distance in the intermediate dorsal region (Fig.21). As has been described for other insects (Highnam, 1961), the corpora cardiaca are composed of two histologically distinct regions. The major portion of the gland consists of axons of the NCC I, cells with flattened or spherical nuclei that stain orange with the orange G component of PAF, and stainable material that has been transported from the brain (Fig.21). Stainable material is absent from the ~~posterior~~ ventral regions of the gland (Fig.22). In addition to the NCC I, another finer pair of nerves emerge from the mid posterior surface of the protocerebrum, dorsal and lateral to the NCC I and join the corpora cardiaca on their anterior surfaces (Figs.1,19). These nerves are the nervi corporis cardiaci II (NCC II). The corpora cardiaca are connected to the hypocerebral ganglion by two, short thick connectives (Fig.10) and to the corpora allata by a fine pair of nerves the nervi corporis allati I (Fig.1, NCA I). These nerves run parallel with the corpora cardiaca for a short distance before turning ventrally to unite with the corpora allata.

#### Corpora allata

The corpora allata are a pair of small, white spheres, located on either side of the lateral line of the oesophagus and posterior to the circumoesophageal commissure (Figs.1,2). They are connected

by fine nerves to the corpora cardiaca (NCA I), suboesophageal ganglion (NCA II) and the muscles of the pharyngeal wall. The histological appearance of a corpus allatum is shown in Figure 12. Sections through the equator of these bodies show a small central region of axons, surrounded by the cells of the corpus allatum (Fig.12). The nuclei of these cells are large, irregular in shape and stain orange with the orange G component of PAF. The axons of the NCA I can be followed into the central regions of the corpora allata (Fig.13). Stainable material was evident in the corpora cardiaca cells accompanying the NCA I, but was never observed in the axons of these nerves in the cells of the corpora allata.

### Discussion

The stomatogastric nervous system of Jamaicana flava is similar in general organization to the descriptions given for other Orthoptera (Nesbitt, 1941; Willey, 1961; Clarke and Langley, 1963c; Khatter, 1968a; Allum, 1973).

Morphologically and histologically, the frontal ganglion of Jamaicana flava, very closely resembles its counterpart in Periplaneta americana (Willey, 1961), Locusta migratoria (Clarke and Langley, 1963b,c) and Melanoplus sanguinipes (Dogra and Ewen, 1970). Clarke and Langley (1963c) described granules in the frontal ganglion cells of Locusta, but did not consider them to be neurosecretory, since the material was not observed in the axons of these cells. On the other hand, Van der Kloot (1959) found evidence of neurosecretory material, at the light microscope level, in the frontal ganglion of Bombyx mori, whilst Anstee (1968) and Casal, Holy and Porte (1971) have observed neurosecretory material in the frontal ganglion of Locusta migratoria at the electron microscope level. Electron microscopic examination of the frontal ganglion cells of Jamaicana flava would determine whether or not neurosecretory material is present.

Fine nerves have been observed to leave the frontal connectives in several insect species including Naucoria cimicoides (Cazal, 1948), Periplaneta americana (Willey, 1961), Schizodactylus monstrosus (Khatter, 1968a) and Blabera fusca (Brousse - Gaury, 1971) and Locusta migratoria (Roome, 1968; Allum, 1973). In Locusta, three fine nerves leave each frontal connective, in the region where the latter nerves turn back towards the tritocerebrum (Allum, 1973). These fine nerves form a complex system, the various branches of which, innervate the muscles of the cibarium, pharynx and labrum. One of these fine nerves (FCN 1, of Allum, 1973) has a branch that unites with the anterior and posterior pharyngeal nerves. In contrast to Locusta, a single nerve leaves from this region of the frontal connective of Jamaicana flava. Moreover, the situation in Jamaicana flava resembles that found in Periplaneta americana (Willey, 1961), where a single nerve ( $N_1 + N_2$  of Willey, 1961) leaves each frontal connective and, as in Jamaicana flava, innervates the dilator muscles of the pharynx as well as the retractor muscles of the labrum.

The fine nerves (FCN 1), arising just in front of the point of origin of the frontal connectives, have also been described for other species. Cazal (1948) described a similar pair of nerves in the Dermaptera whilst Willey (1961) observed two nerves ( $N_3 + N_4$ ) leaving each frontal connective in Periplaneta americana. On the other hand, Allum (1973) reports a fine nerve leaving from this region in Locusta migratoria and noted that it either rejoins the frontal connective lower down or passes to the musculature of the pharynx.

The median nerve that leaves the anterior margin of the frontal ganglion of Jamaicana flava has also been reported for other insects (Imms, 1957; Willey, 1961; Clarke and Langley, 1963c). The present work, together with the studies of Allum (1973), confirm the earlier suggestion of Nesbitt (1961) that this nerve innervates the clypeus and



epipharynx. In Jamaicana flava it also supplies branches to the anterior retractor muscles of the labrum as well as forming part of a nerve complex with the fine nerves (FCN 1) that leave the frontal connectives. The ramifications of the median nerve with other nerves of this region has been reported for Locusta migratoria (Allum, 1973) and was observed occasionally in Periplaneta americana (Willey, 1961).

Fine nerves have been reported leaving the frontal ganglion between the frontal connective and the recurrent nerve in Carausius morosus (Dupont - Raabe, 1957), Periplaneta americana (Willey, 1961; Davey and Treherne, 1963), Schizodactylus monstrosus (Khatter, 1968a) and Blabera fusca (Brousse and Gaury, 1971). Clarke and Langley (1963b) first described the anterior and posterior pharyngeal nerves in Locusta migratoria whilst Roome (1968) and later Allum (1973), observed a third pair, the median pharyngeal nerves, leaving the ganglion between the anterior and posterior pairs. Only a single pair of pharyngeal nerves were observed in Jamaicana flava and these ramify over the surface of the gut forming a complex network.

Serial sections through the foregut region revealed fine nerves linking the recurrent nerve and the dorsal musculature of the pharynx and between this muscle layer and the hypocerebral ganglion. In Periplaneta americana (Willey, 1961), Schizodactylus monstrosus (Khatter 1968a) and Actias (Roome, 1968) branches of the recurrent nerve also innervate the muscle coat of the pharynx and oesophagus whilst in Dytiscus marginalis (Raabe, 1963) fine nerves leave the recurrent nerve and innervate the lateral dilator muscles of the pharynx. In Gryllus, Aeschna and Carausius (Raabe, 1963), similar branches, unite with the NCC II, whereas in Locusta migratoria (Allum, 1973) fine nerves leave the recurrent nerve and unite with branches of the posterior pharyngeal nerves before innervating the tunica muscularis of the pharynx and oesophagus. The fine nerves,

leaving the hypocerebral ganglion of Jamaicana flava and passing to the surface of the oesophagus, have also been reported for Dixippus morosus (Nyst, 1942), Periplaneta americana (Willey, 1961), Schizodactylus monstruosus (Khatter, 1968a), Locusta migratoria (Allum, 1973). In Grylloblatta campodeiformis, Nesbitt (1956) observed nerve fibres passing from the hypocerebral ganglion and innervating the aorta. The nervus connectivus that links the frontal ganglion to the brain was first named by Baldus (1924) in the dragon fly Aeschna. Casal (1948) reviews the occurrence of this nerve through the insects and finds that it is present in most primitive orders, whilst Willey (1961) found it in all Orthoptera except the Saltatoria.

In Jamaicana flava, as in other insects, stainable material was most abundant in the neurons of the pars intercerebralis medialis of the protocerebrum and in the corpora cardiaca (Highnam, 1961; Clarke and Langley, 1963d; Thomsen, 1965; Dogra and Ewen, 1970). Although three types of cell were observed in this region of the brain of Jamaicana flava, only one type is recognized as neurosecretory and is similar to the classical Type 'A' cell of Hagadorn, (1958) and Highnam (1961). The other two types of cell are not considered to be neurosecretory, since granules were never seen in the cytoplasm or axons of these cells. Whilst these findings are similar to those reported for Calliphora erythrocephala (Thomsen, 1965) and Melanoplus sanguinipes (Dogra and Ewen, 1970) they differ from those of Oncopeltus fasciatus (Johannson, 1958), Schistocerca gregaria (Highnam, 1961) and Locusta migratoria (Clarke, 1966). In these species, the types of cell in this region of the brain varies, being four in Oncopeltus (Johannson, 1958) and Schistocerca (Highnam, 1961) and three in Locusta (Clarke, 1966), of which the 'A' and 'B' types of cell are recognized, by these authors, as being neurosecretory. The 'B' cells have been thought to be stages in the secretory cycle of the 'A'

cells (Thomsen, 1954), although Johannson (1958) and Highnam (1961) present evidence favouring the view that the 'A' and 'B' cells are distinct cell types. On the other hand Dupont and Raabe (1956) have shown that the choice of fixative and degree of overstaining can influence the end product of neurosecretory staining methods, whilst Scharrer and Brown (1962) report that electron microscopic observations suggested that the different stainability of neurosecretory cells in the earthworm Lumbricus terrestris, indicated only functional states of one cell type. In Jamaicana flava, aggregations of stainable product were observed in the proximal regions of the NCC I, where the axons of the neurosecretory cells converge to form the tracts. Similar deposits were reported for Oncopeltus fasciatus (Johannson, 1958), Melanoplus sanguinipes (Dogra and Ewen, 1970) and Locusta migratoria (Highnam and West, 1971). The latter authors describe this region as being a neuropilar neurosecretory reservoir. However Mason (1973) found little evidence of stores or reservoirs of such material in Schistocerca vaga.

Although the corpora cardiaca are structurally different to their counterparts in Locusta migratoria and Schistocerca gregaria (Highnam, 1961) and Melanoplus sanguinipes (Dogra and Ewen, 1970), they are similar to these species in that they are histologically divisible into two regions. In these species, the anterior portion of the gland is a storage region for the material passing from the brain. This is most probably true of Jamaicana flava, since material with similar staining properties was observed in the cells of the protocerebrum, the NCC I and within the corpora cardiaca. The posterior/ventral parts of the gland, where stainable material was absent, may produce a secretion of its own, as has been suggested for other species (Highnam, 1961; Mordue and Goldsworthy, 1969).

In the present work, the connection between the corpora cardiaca and the hypocerebral ganglion was not easily observed. In this respect

it is of interest to note the findings of Strong (1966b) and Mason (1973) for Locusta migratoria and Schistocerca vaga respectively. Both authors observed axons of the NCC I passing, via the corpora cardiaca, into the hypocerebral ganglion. In addition Mason (1973) was able to follow these same axons into the outer oesophageal nerves. Further study of this region of Jamaicana flava would determine if a similar situation exists in this species.

The corpora allata of Jamaicana flava are generally similar, histologically, to the corpora allata of other Orthoptera (Mendes, 1948; Odhiambo, 1966; Joly, Joly Porte and Girardie, 1968; Dogra and Ewen 1970). Stainable material, at least at the light microscope level, was never observed in the cells of the corpora allata or along the axons of the NCA I, although other authors have reported neurosecretory material in these regions of other species (Highnam, 1961; Scharrer, 1964).

## CHAPTER FOUR

STUDIES ON MALE JAMAICANA FLAVA THROUGHOUT THE SIXTH  
INSTAR. GROWTH, OXYGEN CONSUMPTION AND THE EFFECT OF  
TEMPERATURE ON DEVELOPMENT AND THE NEUROENDOCRINE SYSTEM.

Introduction

In insects, post embryonic development is interrupted by a series of moults or ecdyses, which are the means whereby individuals increase in size and grow (Wigglesworth, 1965). Successive moults are separated by longer intermolt periods which are usually characterised by an increase in cell size and deposition of food reserves (Bursell, 1970). These processes are reflected in the weight of individuals so that the latter may be used as an index of growth. Weight changes have been monitored throughout the entire post embryonic life of several species including Locusta migratoria (Clarke, 1957), Tenebrio molitor and Dixippus morosus (see Wigglesworth 1965 for references).

The rate of development of insects is greatly affected by temperature. The relationship between temperature and the duration of developmental stages has been discussed by Clarke (1967) and Bursell (1970) and studied in a variety of insects including Locusta migratoria (Hamilton, 1936), Oncopera fasciculata (Madge, 1956) and Aedes aegypti (Bar-Zeev, 1958). These investigations reveal that the duration of developmental stages falls steeply as temperature increases from 15° - 20°C to about 24°C. Further increase in temperature has little effect on the duration of development until temperatures near the upper critical limit are reached, when, the duration of development shows a marked increase.

From his studies on Locusta migratoria, Clarke (1960) has suggested that the effect of temperature on the rate of development and on

metabolism, is not merely a direct one on chemical processes but is also indirect and mediated through the endocrine system. Although there are very few observations of the effect of temperature on the endocrine system, those that have been made (see Clarke, 1967 for references) favour this suggestion. Moreover Clarke (1966) has shown that temperature can induce changes in the endocrine system of Locusta migratoria.

Details of oxygen consumption have been reported for many species and post embryonic stages of Orthopteran insects, including fifth instar and adult Schistocerca gregaria (Bodenheimer, 1929), adult Locusta migratoria (Clarke, 1957) and second - fourth Melanoplus differentialis first instar and adult Dixippus morosus, adult Periplaneta americana (see Keister and Buck, 1964 for references). With the exception of Clarke's study, very few of the investigations have monitored the oxygen consumption of the same individual throughout the instar.

The aim of the present work was to examine the weight changes and oxygen consumption of Jamaicana flava during the sixth instar. In addition the effect of temperature on the rate of development and on the neurosecretory and retrocerebral endocrine systems was also investigated throughout the sixth instar.

### Materials and Methods

#### 1. Sampling of Stock population.

Male insects of the sixth instar were used throughout this part of the study. Stock populations were prepared for sampling by removing all insects of the sixth instar. The populations were then examined at 24 h intervals and insects that had moulted to the sixth instar, removed at each observation. Thus the time of ecdysis to the

new instar must lie between the time the insect was removed and the previous observation. The mid-point of this period was taken as the time of ecdysis and the insect aged as  $0 \pm 12$  h at this time.

## 2. Maintenance of experimental insects

Experimental insects were maintained individually, in clear perspex containers. The lids were perforated to allow the circulation of air. Fresh food and water were supplied daily. (Maintaining the insects in this way enabled growth and oxygen uptake measurements (see below) to be made on the same individual during the instar. Since the time of ecdysis to the sixth instar was known, the age of insects at the time measurements were made, could be calculated). Insects were usually maintained in constant temperature cabinets, set at a temperature of  $25^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$  although in some experiments temperatures of  $28^{\circ}$ ,  $30^{\circ}$ ,  $32^{\circ}$  and  $35^{\circ}\text{C}$  were used. Background relative humidity was ca. 50 % and the photoperiod was 12 h light, 12 h dark.

## 3. Measurement of growth and oxygen consumption

The growth and oxygen consumption of insects were investigated throughout the instar. Weight measurements were used as an index of growth and insects weighed on a torsion balance. Oxygen consumption was measured by the direct method of  $\text{CO}_2$  absorption with potassium hydroxide, using the Warburg constant volume respirometer. 60 ml flasks were used and contained, in addition to the insect, 0.5 ml of 10 % potassium hydroxide in the centre well. Air was the gas phase throughout. Control flasks were run and contained only 0.5 ml of the potassium hydroxide solution. Both experimental and control flasks were thermoequilibrated for 15 min prior to reading the manometers. Flasks were not shaken during the experiment.

In the calculation of flask constants, no correction was made for the volume of the insect. Preliminary experiments indicated that the volume of an individual changed relatively little during the instar. Furthermore, the volume of an insect amounted to no more than 10 % of the flask volume and as such, was considered to lie within the limits of experimental error. During experiments, the manometers were read at 10 min intervals for 1 h, over which period oxygen consumption was linear (Fig.1).

#### 4. Temperature and the sixth instar.

Insects, aged  $0 \pm 12$  h, were transferred to experimental temperatures of either  $25^{\circ}$ ,  $28^{\circ}$ ,  $30^{\circ}$  or  $35^{\circ}\text{C}$  and maintained as described above. They were observed at the same time each day to determine whether metamorphosis had occurred. Where adults were present, the time at which metamorphosis occurred, was determined in a similar manner to that already described for ecdysis. The duration of the instar was then calculated for each experimental temperature.

In a second series of experiments the histology of the neurosecretory and retrocerebral endocrine systems was investigated as a function of three experimental temperatures  $25^{\circ}$ ,  $30^{\circ}$  and  $35^{\circ}\text{C}$ . Insects, aged  $0 \pm 12$  h were transferred to one of the experimental temperatures and decapitated at zero time and 24 h intervals throughout the instar. (Owing to the difficulty of maintaining adequate stocks of Jamaicana flava only one individual was sacrificed every 24 h at each temperature). Heads were fixed in Bouin's fluid at room temperature and the brain and retrocerebral endocrine system dissected out. The tissues were then treated as described in Chapter Three under Materials and Methods.



Fig. 1. Oxygen consumption as a function of time.

Insect, sixth instar male Jamaicana flava Age 11  
 $\pm 0.5$  days, temperature  $25^{\circ}\text{C}$ , 0.5 ml, 10 %  
KOH as  $\text{CO}_2$  absorbing agent. Air the gas  
phase.

Ordinate: oxygen consumption expressed as  
 $\mu\text{ls O}_2/\text{g}$ . Abscissa: time in minutes.

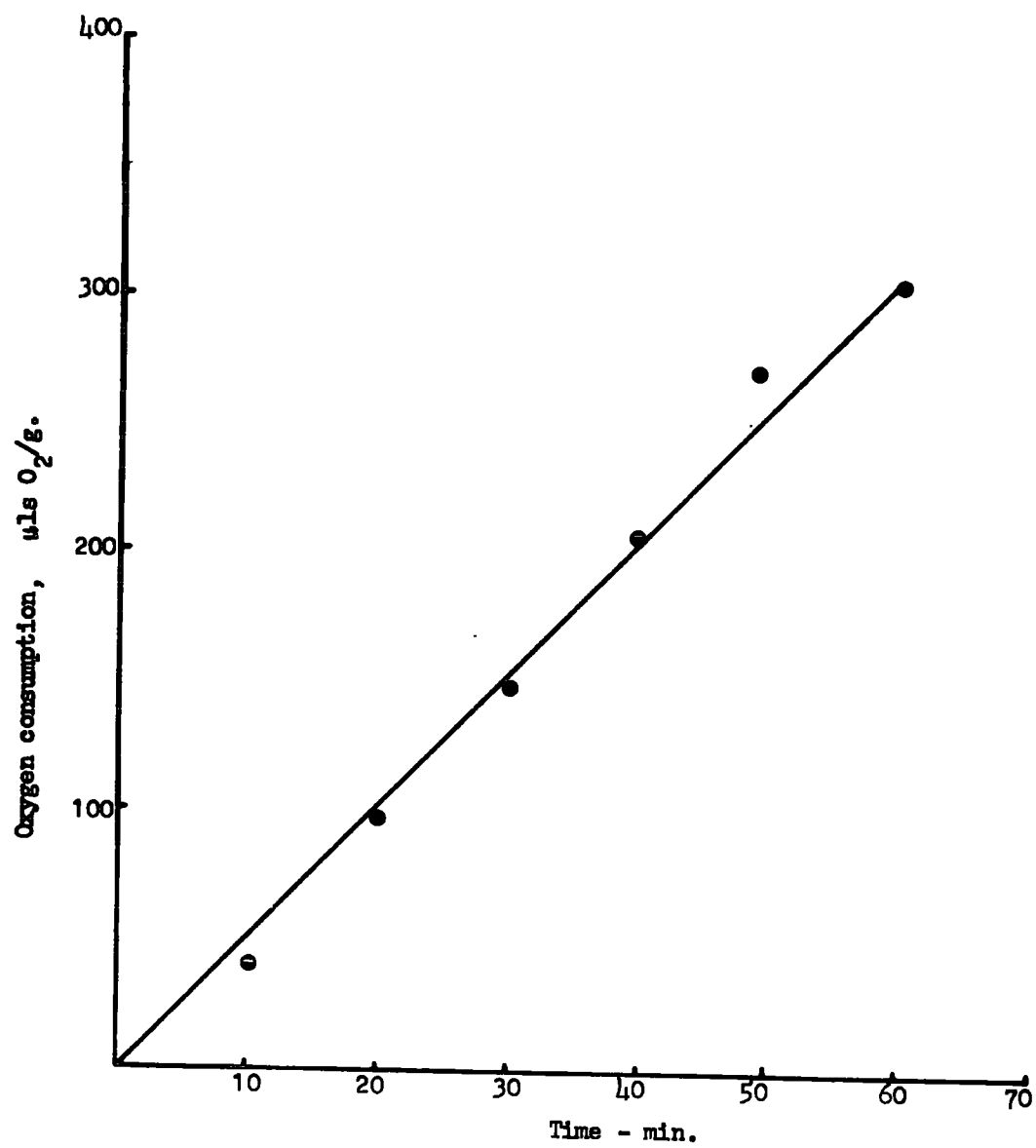


Fig. 1

## Results

Figure 2 shows the changes in mean weight and oxygen consumption of five insects during the sixth instar. It will be seen that during the instar, insects increase in weight by approximately 55 %. Although the data for oxygen consumption shows a degree of variation, there is generally a slight decrease in oxygen consumption throughout the instar.

Figure 3 shows the effect of temperature on the rate of development of insects from the sixth instar. (Rate of development expressed as the proportion of sixth stadium completed/day. For clarity, these units have been omitted below). Insects maintained at 25°C (25°C insects) developed at a mean rate of  $0.046 \pm 0.0008$  whilst at 28°C the mean rate of development was  $0.049 \pm 0.0027$ . Insects maintained at 30°C developed at a significantly higher mean ( $p=0.001$ ) rate of  $0.064 \pm 0.0021$  whereas insects reared at 32°C and 35°C had a mean rate of  $0.047 \pm 0.0018$  and  $0.041 \pm 0.0045$ , respectively.

The histology of the adult male neurosecretory and retrocerebral endocrine systems was described in Chapter Three. Similar studies of these systems, in insects of the sixth instar, reveal that they do not differ from that of the adult. As in the latter, neurosecretory 'A' material was present in the cells of the pars intercerebralis region of the protocerebrum throughout their tracts in the brain, the NCC I and the anterior and dorsal regions of the corpora cardiaca.

The neurosecretory cells of the pars intercerebralis of 25°C and 30°C insects contain similar amounts of material during the instar (Figs.6,7), although at the beginning, slightly more material was present in these cells of insect reared at 25°C (Figs.4,5). The pars intercerebralis region of 35°C insects contained many cells packed with 'A' material. Such cells occurred frequently in this region of the brain at all times during the instar (Figs.8,9). Similar types

Fig. 2 Changes in weight (●-●) and oxygen consumption (■-■) throughout the sixth instar. Each point is the mean of five observations. Vertical lines  $\pm$  one standard error of the mean. Insects maintained at 25°C.

Ordinate: weight in milligrams and oxygen consumption expressed as  $\mu\text{lsO}_2/\text{g/h}$ .

Abscissa: time in days.

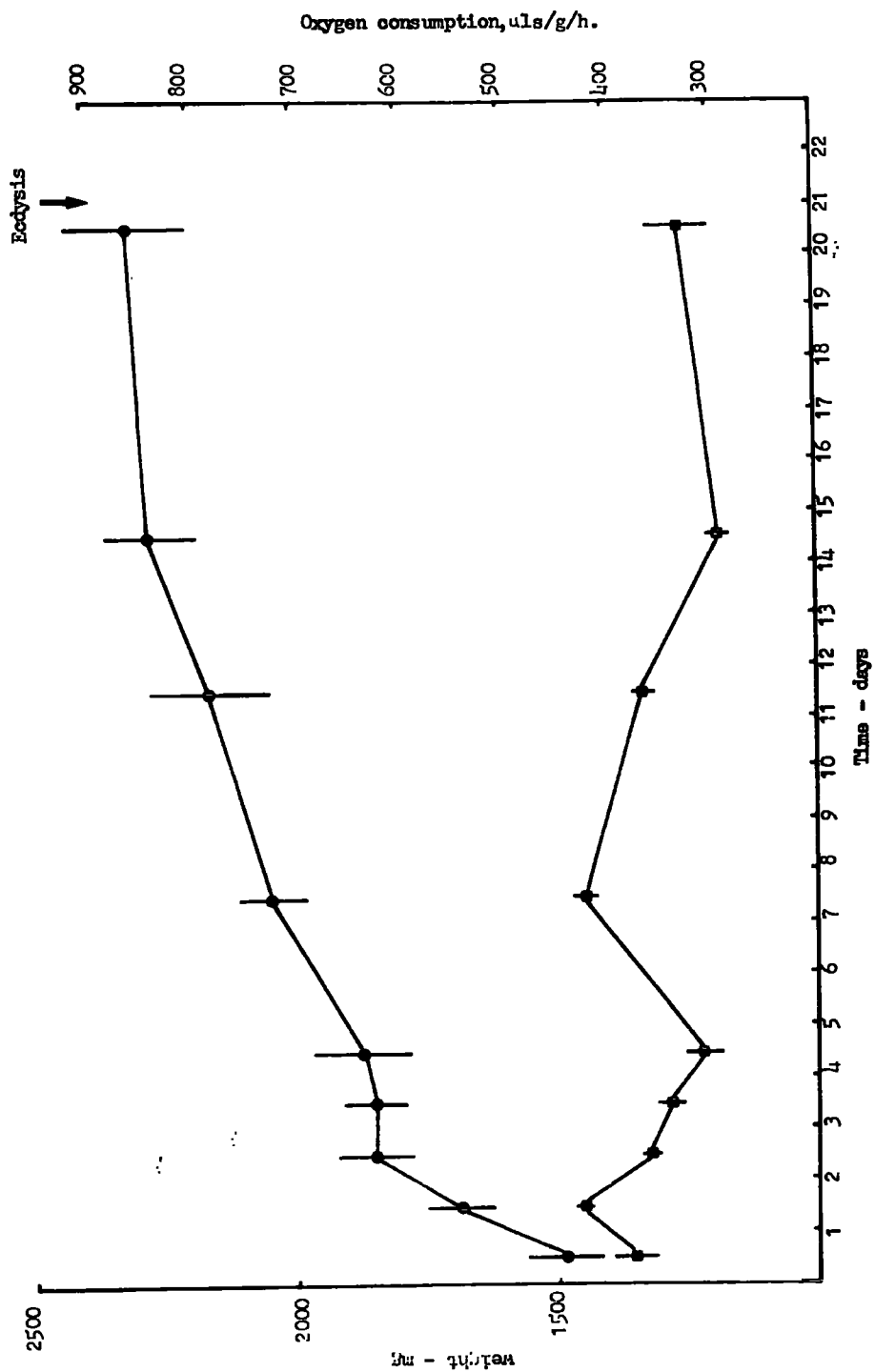


FIG. 2

Fig.3. Effect of temperature on the rate of development of insects of the sixth instar. Each point is the mean of ten observations. Vertical lines,  $\pm$  one standard error of the mean.

Ordinate: Rate of development expressed as proportion of sixth stadium completed/day.

Abscissa: temperature  $^{\circ}\text{C}$ .

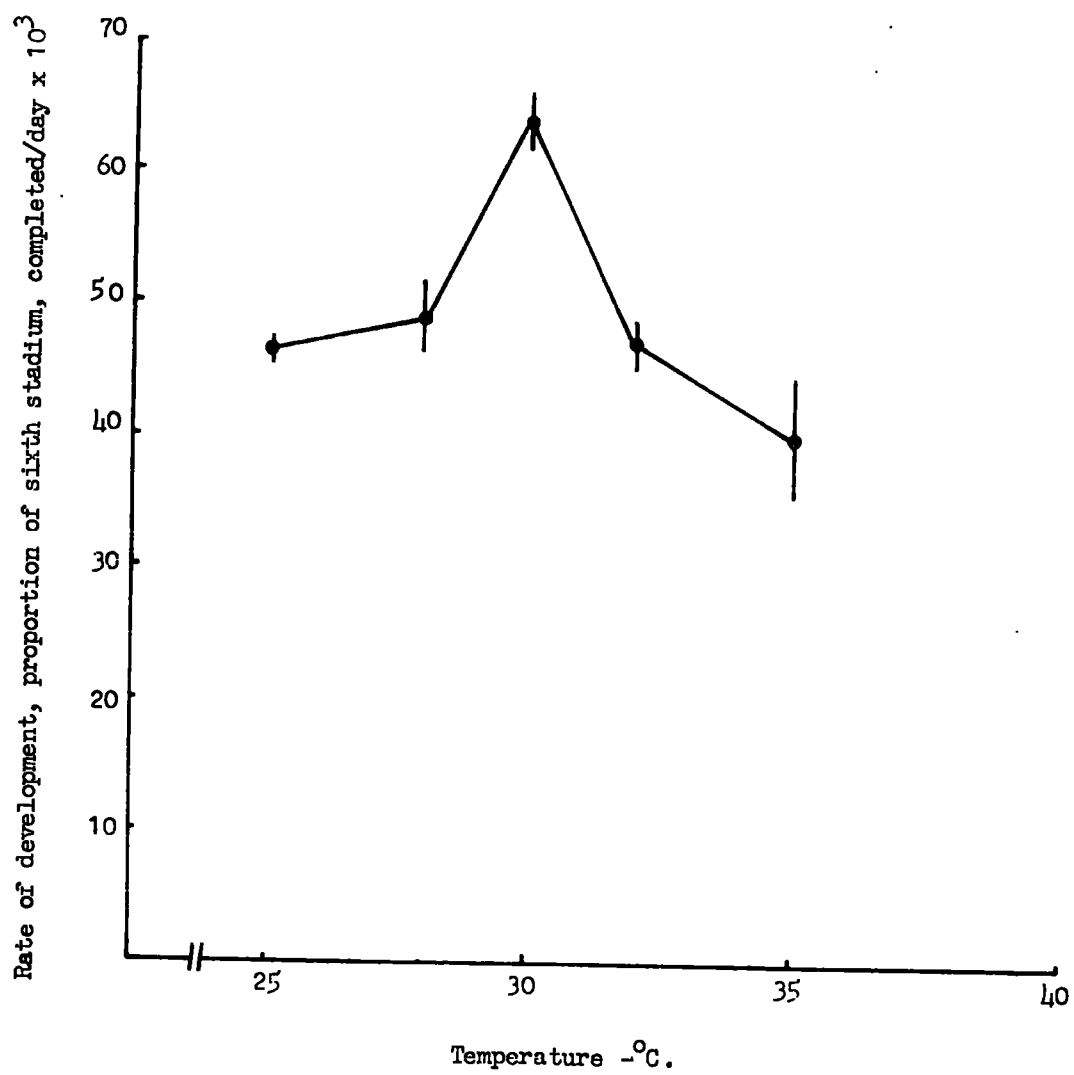


Fig. 3

## Figs. 4 - 24.

Photomicrographs of section through parts of the neurosecretory and retrocerebral endocrine systems of insects maintained at various temperatures. Insects sacrificed at 24 h intervals throughout the instar. All heads fixed in Bouin's fluid and sections stained with paraldehyde fuchsin after Ewen (1962).

## Figs. 4 - 9

Photomicrographs of sections through the pars intercerebralis medialis region of the protocerebrum.

## Figs. 4 and 5

Insects maintained at temperatures of 25°C (Fig. 4) and 30°C (Fig. 5) and sacrificed at  $3 \pm 0.5$  and  $2 \pm 0.5$  days respectively. Note the presence of cells (arrows) full of neurosecretory material. Scale 50  $\mu\text{m}$ .

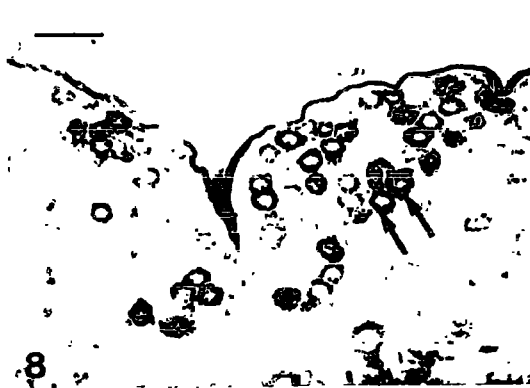
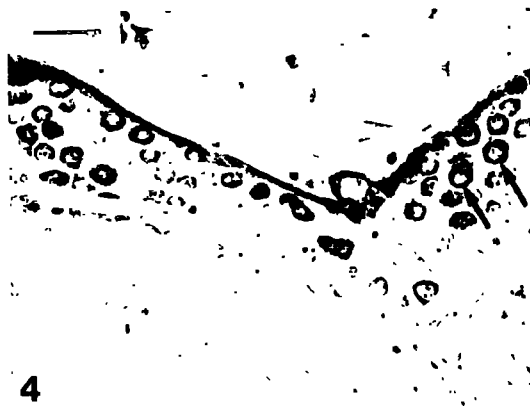
## Figs. 6 and 7

Insects maintained at temperatures of 25°C (Fig. 6) and 30°C (Fig. 7) and sacrificed at  $20 \pm 0.5$  and  $18 \pm 0.5$  days respectively. Note similar amounts of 'A' material in this region of the brain, of both types of insect. Scale 50  $\mu\text{m}$ .

## Figs. 8 and 9

Insects maintained at 35°C and sacrificed at  $10 \pm 0.5$  days (Fig. 8) and at  $22 \pm 0.5$  days (Fig. 9) of the instar. Note the large number of cells full of neurosecretory material (arrows) and the aggregation of 'A' material (NSM) in a region ventral to the groups of neurosecretory cells. Scale 50  $\mu\text{m}$ .





Figs. 4-9

## Figs.10 - 15

Photomicrographs of sections through the nervi corporis cardiaci (NCC I).

## Figs. 10 - 12

Insects maintained at 25°C and sacrificed at 3 ± 0.5 days (Fig.10), 11 ± 0.5 days (Fig.11) and 20 ± 0.5 days (Fig.12) of the instar. Note presence of 'A' material (arrows) at all times in the axons of the NCC I, but its prominence at the beginning and towards the end of the instar. Scale 25 μm.

## Fig. 13

Insect maintained at 30°C and sacrificed at 18 ± 0.5 days. Note the presence of neurosecretory material (arrows) in the axons of the NCC I. Scale 25 μm.

## Figs. 14 and 15

Insects maintained at 35°C and sacrificed at 2 ± 0.5 days (Fig.13) and 22 ± 0.5 days (Fig.14) of the instar. Note the abundance of 'A' material (arrows) in the axons at these times. Scale 25 μm.

## Figs. 16 - 18

Photomicrographs of sections through the corpora allata, of insects sacrificed towards the end of the instar.

## Fig. 16

Insect maintained at 25°C and sacrificed at 20 ± 0.5 days. Note that the gland is composed of small cells with spherical nuclei. Scale 50 μm.

## Fig. 17

Insect maintained at 30°C and sacrificed at 18 ± 0.5 days. The appearance of the corpus allatum is very similar to that shown in Figure 16. Scale 50 μm.

## Fig. 18

Insect maintained at 35°C and sacrificed at 22 ± 0.5 days. Note that the gland is composed of large cells with irregularly shaped nuclei. Scale 50 μm.



Figs. 10-18

of cell were observed at the beginning of the instar in insects maintained at the lower temperatures (Figs. 4,5). At all three temperatures, neurosecretory material was more abundant in the NCC I at the beginning and towards the end of the instar (Figs.10-15) and to a lesser degree those reared at 25°C. In 35°C insects 'A' material was particularly prominent towards the end of the instar, in a region just ventral to the medial groups of neurosecretory cells (Fig.9).

The corpora cardiaca of insects maintained at 30°C show variation during the instar, in the amount of neurosecretory material they contain. Similar quantities of 'A' material are present at the beginning and towards the end of the instar (Figs.19,21 respectively), whereas around the middle of the instar relatively little material is present in the gland (Fig.20). On the other hand, the corpora cardiaca of insects maintained at 25°C and 35°C contain large quantities of 'A' material at all times during the instar. Figures 22, 23 and 24 show the corpora cardiaca of 35°C insects at the beginning, middle and towards the end of the instar respectively. It will be seen that the glands are 'packed' with neurosecretory material. A similar appearance is observed in the corpora cardiaca of 25°C insects although the amount of 'A' material is slightly less.

The corpora allata of insects maintained at 25°C and 30°C have a similar histological appearance throughout the instar. The glands are composed of numerous small cells with spherical nuclei (Figs.16,17). The corpora allata of 35°C insects are similar to those of 25°C and 30°C insects at the beginning of the instar but thereafter present a different picture. The glands are composed of large cells with irregularly shaped nuclei (Fig. 18).

Figs. 19 - 24

Photomicrographs of sections through the corpora cardiaca.

Figs. 19 - 21

Insects maintained at 30°C.

Figs. 19

Insects sacrificed at  $2 \pm 0.5$  days. Note the presence of 'A' material within the gland. Scale 100  $\mu\text{m}$ .

Fig.20

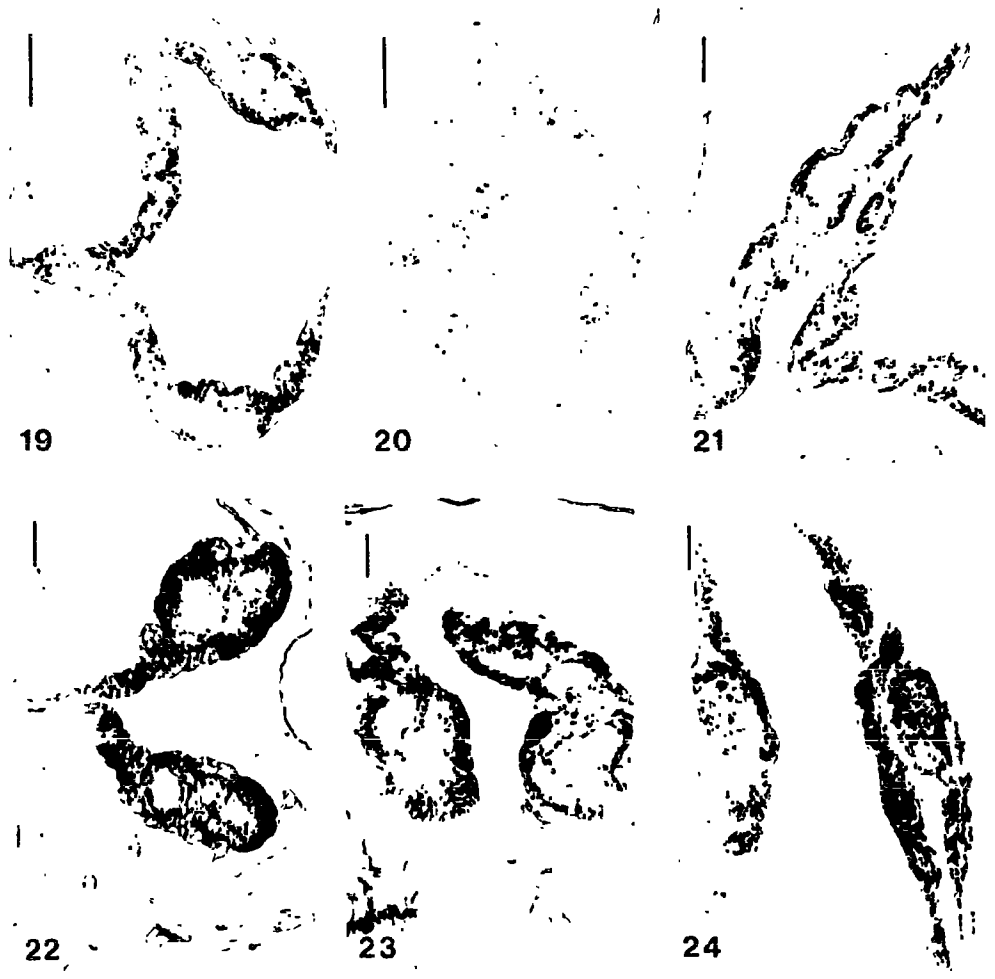
Insect sacrificed at  $8 \pm 0.5$  days. Note relative absence of 'A' material in the corpora cardiaca, when compared with Figures 19,21. Scale 100  $\mu\text{m}$ .

Fig.21

Insect sacrificed at  $18 \pm 0.5$  days. Note the presence of 'A' material within the gland. Scale 50  $\mu\text{m}$ .

Figs. 22 - 24

Insects maintained at 35°C and sacrificed at  $2 \pm 0.5$  days (Fig.22),  $10 \pm 0.5$  days (Fig.23) and  $22 \pm 0.5$  days (Fig.24). Note abundance of 'A' material within the gland at those times during the instar. (Compare with insects maintained at 30°C, Figs. 19-21). Scale 50  $\mu\text{m}$ .



Figs. 19-24

## Discussion

The growth pattern of Jamaicana flava during the sixth instar is in general terms similar to the descriptions reported for the larval stages of Locusta migratoria (Clarke, 1957). Although both species show an increase in weight during the instar, the rate of growth of Jamaicana flava is much slower than that of Locusta migratoria. The extensive studies of Clarke (1957) on Locusta show that for example during the third instar, oxygen consumption closely follows the pattern of weight increases. This is not the case in Jamaicana flava where oxygen consumption shows only a slight increase during the instar. This implies that the weight increases reflect the laying down of new cuticle and food reserves in preparation for metamorphosis. In this respect it is worth noting that both sixth instar individuals and adult insects have enormous quantities of fat body, when compared with locusts (personal observations of author). Thus it would be of interest to determine, for Jamaicana flava, the changes in the quantity and content of the fat body during the instar.

The effect of temperature on the rate of development of Jamaicana flava during the sixth instar is very similar to the descriptions given by Clarke (1966) and Bursell (1970). As these authors have pointed out, temperature increases the rate of development up to an optimum after which further increase produces very little acceleration of development. This situation continues until the upper lethal limit is approached when the rate of development decreases. It is interesting to note that the upper lethal limit of larval Jamaicana flava is  $37.5^{\circ}\text{C}$  (personal observation of author) and that the rate of development decreases at  $35^{\circ}\text{C}$ . The quantity of 'A' material in the neurosecretory cells of the protocerebrum is altered by maintaining insects at different experimental

temperatures. This region of the brain of 35°C insects always contained many cells 'packed' with neurosecretory material. On the other hand, this type of cell was limited to the beginning of the instar of insects maintained at 25°C and 30°C. Although much has been written concerning the physiological state of insect brain cells full of 'A' material (Dupont - Raabe, 1952; Arvy and Gabe, 1952; Formigoni, 1956; Highnam, 1961), it is interesting to note that in the present study cells full of 'A' material are associated with a low rate of development. This implies, at least in Jamaicana flava, that such cells are in a relatively inactive state. The only other work relevant to the present study is that of Clarke (1966), who reported that oscillating temperatures, even of large amplitude, were without effect on the neurosecretory cells of Locusta migratoria.

The amount of 'A' material in the anterior and dorsal regions of the corpora cardiaca is reduced in insects maintained at 30°C, especially around the middle of the instar. At all other temperatures neurosecretory material is always abundant, although more is usually present in insects maintained at 35°C than at 25°C. Clarke (1966) also reported a reduction in the quantity of 'A' material in insects kept under temperature regimes in which there were large fluctuations. In addition this author has pointed out that the emptying of the anterior storage lobes of the corpora cardiaca in a variety of insects, always seems to be associated with the action of some stressor on the insect. This would not seem to be the case in the present work, for two reasons. Firstly, this effect is not observed in insects maintained at 35°C which, in terms of water balance might be considered to be under greater stress than insects maintained at 30°C. Secondly, the failure to store neurosecretory material in the corpora cardiaca appears to be correlated with



an increase in the rate of development. The last observation would seem to support the suggestion of Clarke (1960) that the effect of temperature on insect development and metabolism is mediated through the endocrine system. The corpora allata of insects maintained at temperatures of 25°C and 30°C have a similar histological appearance, although it is possible that at these experimental temperatures differences occur between the glands which pass undetected at the light microscope level. On the other hand, the corpora allata of insects maintained at 35°C are affected by temperature. Whether the changes observed in the corpora allata of these insects indicate an alteration in the physiological state of the glands is difficult to determine, although Clarke (1966) considers this to be the case for the corpora allata of Locusta migratoria, as does Scharrer (1964) for the corpora allata of Leucophaea maderae.

Clearly, further work, perhaps involving integrated physiological and histological studies, is required to clarify the effect of temperature on the synthesis and release of neurosecretory material from the cells of the protocerebrum and on the storage and release of this material from the corpora cardiaca. In addition studies on the corpora allata of Jamaicana flava should endeavour to correlate a particular histological appearance, with a physiological state of the glands.

## CHAPTER FIVE

## STUDIES ON THE STRUCTURE OF THE EXCRETORY SYSTEMS OF

JAMAICANA FLAVA AND LOCUSTA MIGRATORIAIntroduction

Extensive studies of the anatomy and histology of the excretory systems of a variety of insect species have been made since about 1924, and it is clear that there is an uniformity in the microanatomical and histological structure of Malpighian tubules (see Maddrell, 1971 for an extensive review of the literature). In contrast, the structure of the rectum is limited to a few species including Rhodnius prolixus (Wigglesworth, 1931), Locusta migratoria (Hodge, 1939), Melanoplus differentialis (Marshall, 1945), Dixippus morosus (Ramsay, 1955a), Calliphora erythrocephala (Graham-Smith, 1934; Berridge and Gupta, 1967), Cephalotermes rectangularis (Noirot and Noirot-Timothee, 1966), Schistocerca gregaria (Irvine, 1966), Aedes aegypti (Hopkins, 1967) and Periplaneta americana (Oschman and Wall, 1969), whilst information regarding the structure of the intestine anterior to the rectum is available for Dixippus morosus (Ramsay, 1955a), Thermobia (reported by Maddrell, 1971) and Aedes campestris (Meredith and Phillips, 1973).

More recent work, using electron microscope techniques has led to a detailed understanding of the secretory, excretory and resorptive functions of the several different cell types, identifiable in the Malpighian tubule, hindgut and rectum. Briefly, the findings of the electron microscope studies indicate that the excretory epithelia of insects are characterised by extensive invagination of the apical and basal or apical and lateral cell membranes, which create an extensive system of extracellular spaces and channels. Furthermore, the mitochondria of the cells are usually associated with the infolded membranes.

Features of this kind also occur in many other epithelia noted for ion and water transport, including mammalian gall bladder (Kaye, Wheeler, Whitlock and Lane, 1966), mammalian kidney (Rhodin, 1958) reptilian kidney (Schmidt-Nielsen and Davis, 1968), insect salivary gland (Berridge and Oschman, 1969) and vertebrate choroid plexus (Maxwell and Pease, 1956).

Curran and MacIntosh (1962) and Diamond and Bossert (1967, 1968) have proposed theoretical models of transporting epithelia, in which water movements are passive, occurring in response to local osmotic gradients, created by the pumping of solutes (ions) into confined spaces within the epithelium. (See Chapter One page 10 for details). In this respect the extracellular spaces and channels, characteristic of transporting epithelia, are thought to constitute the confined spaces of the models and the associated mitochondria, the energy source for the ion pumps.

A  $\text{Na}^+ - \text{K}^+$  ATPase, with similar properties to that identified in the crab nerve (Skou, 1957), has been isolated from the excretory tissues of several insect species (Chapter Six, Results). Furthermore, ATPases have been demonstrated histochemically in a variety of tissues noted for ion and fluid transport. Kaplan and Novikoff (1959) located ATPase activity in the plasma membrane of rat kidney tubule cells as did Bartosewics and Barnett (1964) in the urinary bladder of the toad. Kaye and Pappas (1965) showed ATPase activity in the ciliary epithelium whilst Berridge and Gupta (1968) demonstrated this type of enzyme in the lateral plasma membranes of the rectal papillae cells of Calliphora erythrocephala.

Thus, it was of interest to examine the structure of the excretory system of Jamaicana flava and to determine, whether or not the features,

found in the excretory epithelia of other insect species, are present in the excretory system of this insect. Moreover, this type of study would provide additional information of the structure of the Orthopteran excretory system.

The histochemical localization of  $\text{Na}^+ - \text{K}^+$  ATPase within the excretory epithelia was also of interest, since the presence of solute pumps is considered to be of prime importance in the functioning of transporting epithelia (Curran and MacIntosh, 1962; Diamond and Bossert, 1967, 1968). In view of the difficulty in maintaining adequate stocks of Jamaicana flava, the excretory tissues of Locusta migratoria were used in the histochemical study. Information regarding the ultrastructure of this insect's excretory system was lacking, consequently a preliminary electron microscopic study of the Malpighian tubules and rectum were carried out.

### Materials and Methods

#### 1. Anatomical and histological studies of the excretory system

Both sexually mature male and female individuals were used. The gross anatomy of the excretory system was studied by dissection. For histological work, insects were killed by decapitation and the abdomen removed and placed in Bouin's fixative. The Malpighian tubules and hindgut were dissected out under a binocular microscope, transferred to fresh fixative and left to stand for 48 h at room temperature. Prior to staining, tissues were treated as described in Chapter Three under Materials and Methods. Sections were stained with Erlich's haematoxylin and eosin, dehydrated through a graded series of alcohols and mounted in Canada balsam.

Whole amounts of Malpighian tubules were prepared by fixing them for 15 min in 2.5 % glutaraldehyde in 0.15 M cacodylate buffer (pH 7.3)

and 0.15 % lead nitrate. After a buffer wash of 1 h, the tubules were treated with ammonium sulphide and examined in buffer. Secondary cells retain lead, which is precipitated as brown/black lead sulphide. (Method attributed to M.Locke and cited in Berridge and Oschman, 1969).

## 2. Electron microscopic study of the excretory system

Adult male individuals only were used. Insects were killed by decapitation. Malpighian tubules, ileum and rectum were rapidly removed, placed in 2.5 % glutaraldehyde containing 0.15 M sucrose buffered with 0.05 M sodium cacodylate (pH 7.3). This was followed by post osmication using a 1.0 % solution of osmium tetroxide in 0.15 M sucrose in 0.05 M sodium cacodylate buffer (pH 7.3). The material was dehydrated through a graded series of alcohols to propylene oxide, followed by embedding in Epon 812 epoxy resin. Thin sections (silver/grey in colour) were cut on a Reichart Om<sub>2</sub> ultramicrotome, post stained in uranyl acetate and lead citrate (Reynolds, 1963) and examined in an AEI 801 electron microscope.

## 3. Histochemical localization of Na<sup>+</sup> - K<sup>+</sup> ATPase in the rectum of *Locusta migratoria* after McClurkin (1964)

### 1. Isolation and freezing of tissues

The rectum was rapidly dissected out from adult male individuals and placed on the end of a cardboard strip. Both were then plunged into a freezing mixture, composed of equal parts of 2 methyl butane and liquid nitrogen (approximate temperature - 100°C). They remained in this mixture for several minutes. The strip was withdrawn and the rectum carefully removed and one end placed in gumtragacanth on a Slee cryostat mounting block. Since transverse sections were required, the long axis of the rectum was orientated perpendicular to the flat surface of the block. The tissue was secured to the block by freezing the gumtragacanth

using solid carbon dioxide. The block and rectum were left in the cold compartment of the cryostat for several hours prior to sectioning.

ii. Sectioning and incubation of tissues

Sections were cut at 12  $\mu\text{m}$  on a Slee cryostat. In order to determine section integrity some sections were initially stained in Erlich's haematoxylin and eosin. Sections for histochemistry were placed on clean 76 x 25 mm glass slides and treated in the following manner:

1. fixed in 10% formalin, 1 min at 20°C,
2. rinsed in distilled water,
3. immersed in one of the incubation media described in Table 1;  
incubation 20 min at 37°C,
4. rinsed in distilled water,
5. treated with 10% ammonium sulphide, 30-40 sec at 20°C,
6. rinsed in distilled water,
7. excess water removed from slide and sections mounted in glycerol jelly,
8. sections examined using a Leitz Wetzlar dialux light microscope.

## Results

### 1. Structure of the excretory system

#### Malpighian tubules

The Malpighian tubules of Jamaicana flava are slender, blind ending yellow tubes that can either lie free in the haemolymph, where they ramify among the abdominal organs, or be attached at various points along their length to the midgut caeca. The tubules open into the alimentary canal at the junction of the mid and hind guts (Fig.1,A,C) where they are assembled into six groups or ampullae, containing approximately twenty four tubules each (Fig.1,B). The tubules are of similar

Incubation Medium	Concentration (mM)							
	Tris (pH7.8)	ATP (di- barium salt)	Pb(NO <sub>3</sub> ) <sub>2</sub>	MgSO <sub>4</sub>	NaCl	KCl	Ouabain	
1	24.0		3.0	3.0				
2	24.0m	3.3	3.0	3.0				
3	24.0	3.3	3.0	3.0	100.0	20.0		
4	24.0	3.3	3.0	3.0	100.0	20.0	1.0	

Table 1 Composition of incubation media used in the histochemical localization of Na<sup>+</sup> - K<sup>+</sup> ATPase in the rectum of Locusta migratoria migratorioides.

All incubation media were thermoequilibrated for 15 min. at 37°C. Incubation time 20 min. at 37°C.

Fig. 1

Diagrams showing the various parts of the alimentary tract of Jamaicana flava.

- A. Sketch showing the entire, uncoiled alimentary tract. The excretory system constitutes approximately one third of the tract and comprises the Malpighian tubules, ileum and rectum. (For clarity a group of Malpighian tubules have been removed from the gut at point X).
- B. Diagram showing, in external view, the insertion of the Malpighian tubules into the gut.
- C. Similar to diagram B from an internal aspect. Six single orifices are situated between the columnar epithelial tracts at the anterior end of the ileum.
- D. Sketch showing the rectum and posterior part of the ileum. The rectal pads are easily distinguishable by their shape and the extensive tracheation they receive. Bundles of longitudinal muscles are visible between the rectal pads.
- E. Diagram similar to D, but an internal view. Three rectal pads are visible which pass through the constriction at the anterior end of the rectum before entering the ileum.

Abbreviations:

A, anus; AM, ampulla; C, crop; CE, columnar epithelium; CR, cuticular ridges; I, ileum; LM, longitudinal muscle; M, mouth; MG, midgut; MGC midgut caecum; MPT, Malpighian tubule; O, oesophagus; P, pharynx; PV, proventriculus; R, rectum; RE, reduced epithelium; RP, rectal pads; TRP, tracheal supply to the rectal pads; X, point of insertion of Malpighian tubules into gut; Y, Malpighian tubule attached to the midgut caecum.



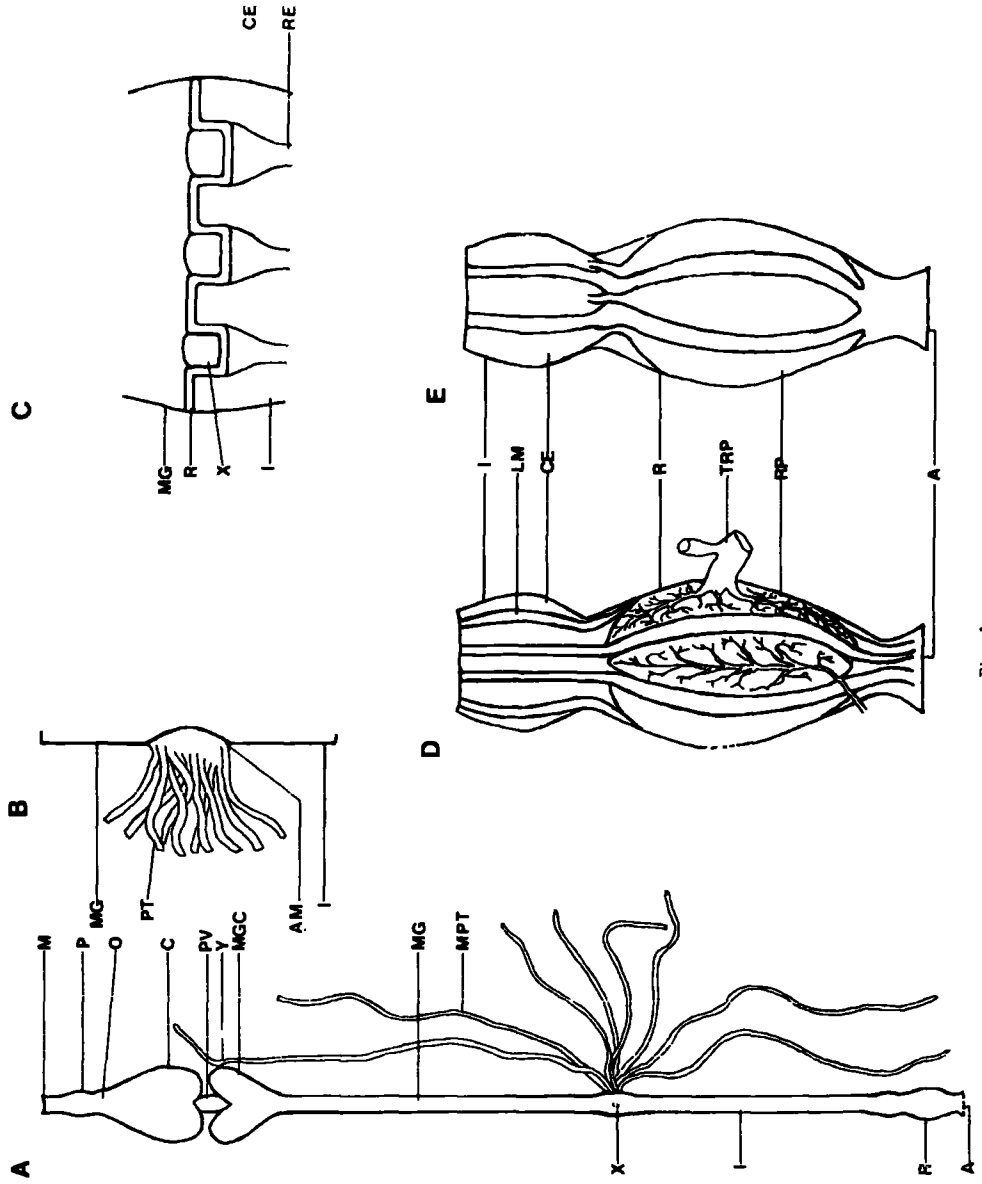


Fig. 1

morphological appearance throughout their length. They are composed of two types of cell, primary and secondary. (Figs. 10,11,12). The primary cells make up the bulk of tubule whilst the secondary cells occur less frequently and intermingle with the primary cells. Two similar types of cell were identified in the Malpighian tubules of Locusta migratoria (Figs. 13,14,15,16),

#### Primary cells

A transverse section through a primary cell is shown in Figure 2. The primary cells, which rest on a thick basement membrane, may be divided into basal, intermediate and apical regions. All three regions are clearly visible in Figure 2 and are denoted by B, I and A respectively.

#### Basal region

The basal cell membrane is invaginated to form a complex series of cytoplasmic processes and narrow, interconnecting, extracellular channels (Fig.4). The electron dense tips of the cytoplasmic processes rest on the basement membrane and there are clear openings between adjacent infoldings (Fig.4). The electron dense tips are similar to the hemidesmosome type of junction described by Berridge and Oschman (1969) in the Malpighian tubules of Calliphora erythrocephala and by Taylor (1971a) in the tubules of Carausius morosus. The cytoplasmic processes are between 5-8 $\mu$ m in length, 0.07-1.0 $\mu$ m in width and are separated from one another by a space of 0.015 $\mu$ m (Fig.4). Rod and oval shaped mitochondria, as well as small vesicles 300-500 $\text{\AA}$  in diameter are present in the cytoplasmic processes (Fig.4).

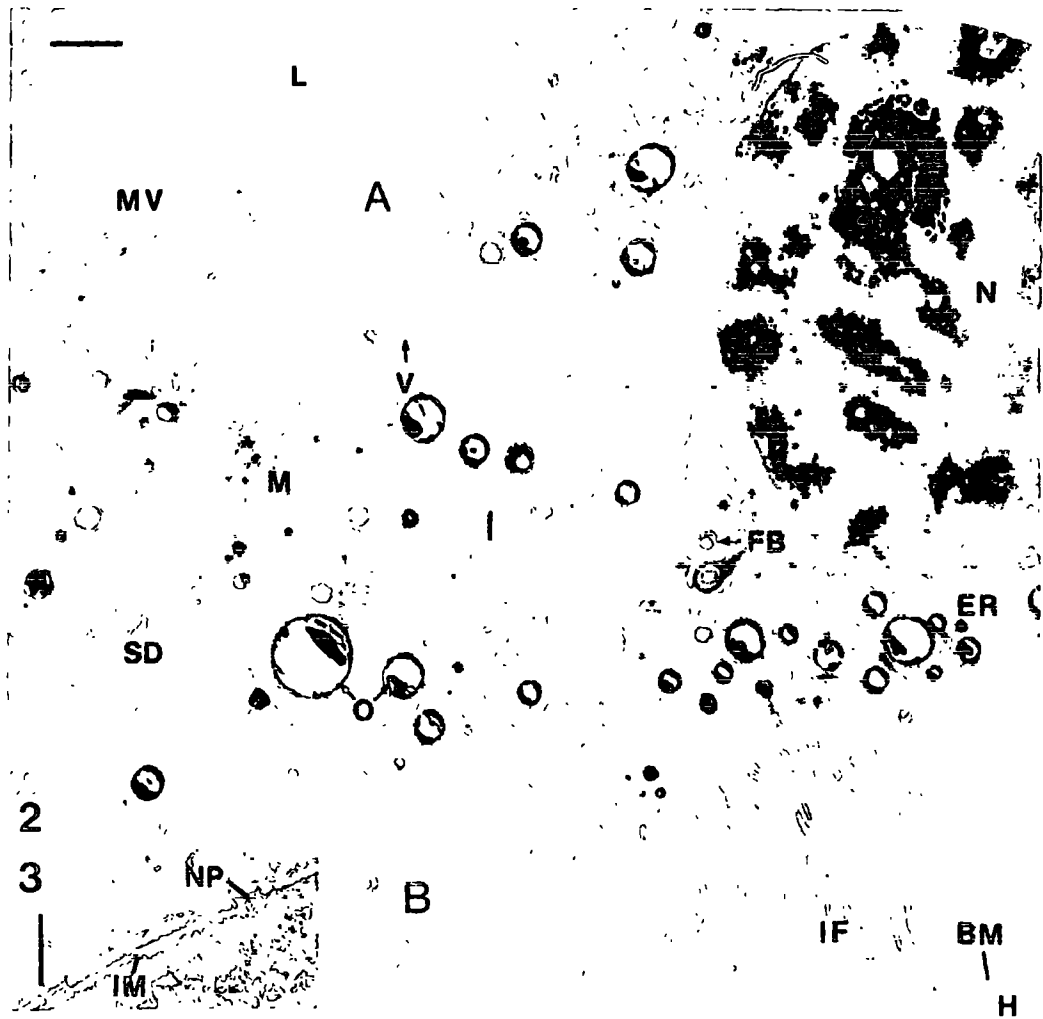
In transverse section, the extracellular channels are radially aligned, such that their long axis is in the direction of fluid flow i.e. towards the lumen of the tubule (Fig.2). The channels extend a

Fig. 2.

A low magnification electron micrograph of a section through a primary cell to show the basal (B), intermediate (I) and apical regions (A). In the basal region, the infoldings (IF) of the plasma membrane sit on a thick basement membrane (BM) in which lie bundles of longitudinal muscle fibres and tracheal elements (not shown). The space labelled (H) would in vivo correspond to the haemolymph. The intermediate region contains a large nucleus (N), mitochondria (M), formed bodies (FB), vacuoles (V), and endoplasmic reticulum (ER). The apical region possesses microvilli (MV) which project into the lumen (L). The holes (O) in the section represent areas from which the interior has been removed by the knife. The cells are joined laterally by extensive septate desmosomes (SD).  
Scale 2  $\mu\text{m}$ .

Fig. 3

Electron micrograph showing a segment of nucleus of a primary cell. Note nuclear pore (NP) and electron dense inner nuclear membrane (IM). Scale 0.25  $\mu\text{m}$ .



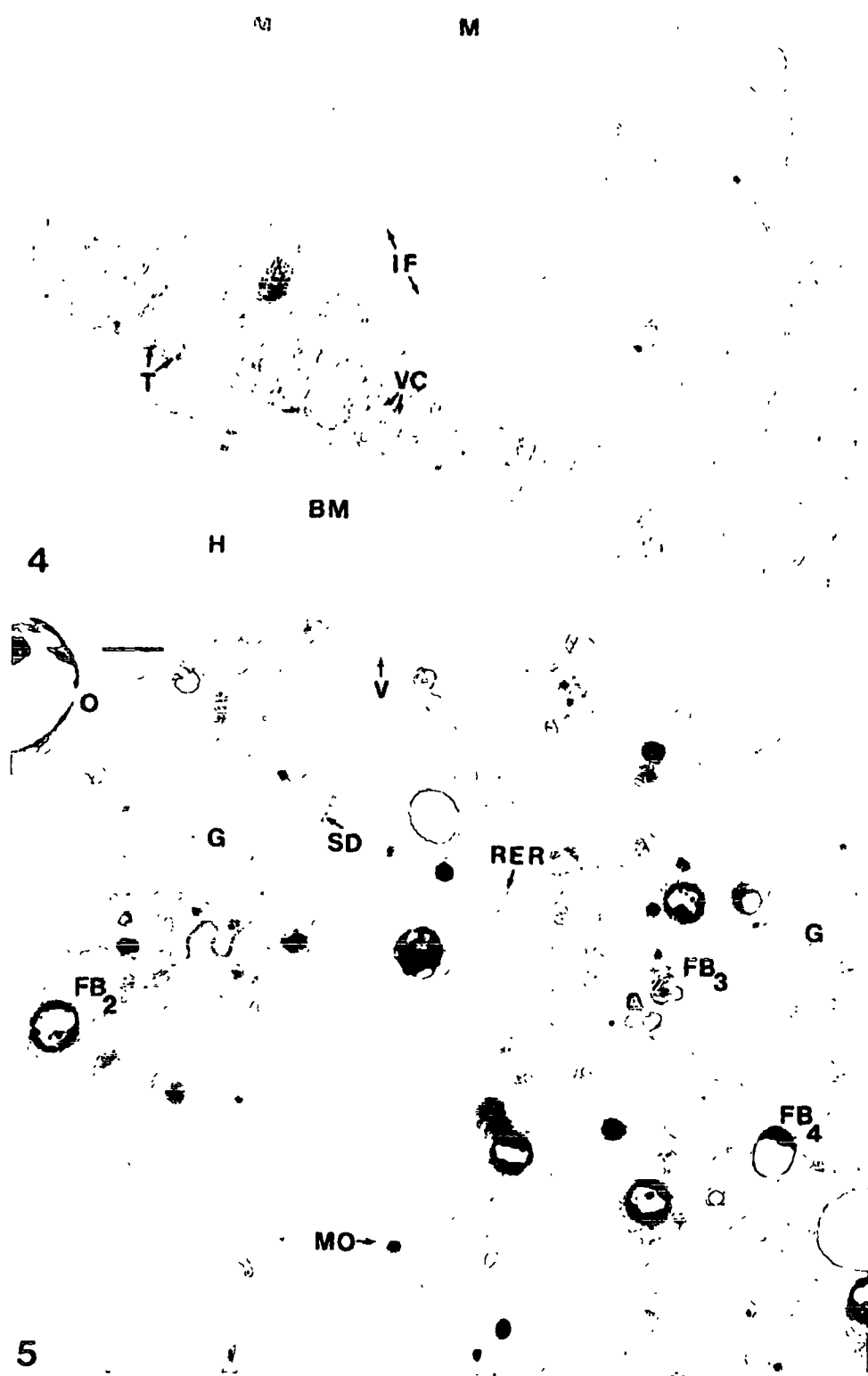
Figs. 2 and 3

Fig.4

Electron micrograph of a transverse section through the basal region of a primary cell to show the infoldings (IF) of the basal plasma membrane. Note the three layered basement membrane (BM) and the electron dense tips (T) of the infoldings. Mitochondria (M) and small vesicles (VC) are visible within the fingers of cytoplasm. H, Haemolymph. Scale 0.5  $\mu\text{m}$ .

Fig.5

Electron micrograph of the intermediate region between adjacent primary cells. G, Golgi body,  $\text{FB}_{2-4}$ , formed bodies; MO, macula occludens; O, region where the knife has torn the inclusion away from the surrounding membrane. RER, rough endoplasmic reticulum; SD, septate desmosome; V, vacuoles. Scale 1  $\mu\text{m}$ .



Figs. 4 and 5

third to a quarter of the distance from the basement membrane to the base of the microvilli (Fig.2). Occasionally apposed membranes forming the extracellular channels, fuse in the upper regions of the channels to form tight junctions of the macula occludens type (Fig.5).

#### Intermediate region

This zone contains the bulk of the cell's cytoplasm and associated organelles. The nucleus is roughly spherical in shape and has a well defined nuclear membrane, in which numerous pores are present (Figs.2,3). The inner of the two nuclear membranes is more electron dense than the outer (Fig.3). Rough endoplasmic reticulum, Golgi body, vacuoles of various sizes, rod and filamentous shaped mitochondria, as well as free ribosomes are present in the cytoplasm (Fig.5). In the majority of sections examined, there were also present, various types of inclusions that were not well preserved during preparation. As a result of this, many sections are holed where the knife has torn the interior of the inclusion away from the surrounding membrane (Fig.5.0). Some inclusions were surrounded by several layers of membrane like structures (Fig.5,FB<sub>2</sub>) whilst others appeared as a concentric series of layers when sectioned successfully (Fig.8, FB<sub>1</sub>). There are present other smaller bodies composed of concentrically arranged membranes (Fig.5, FB<sub>3</sub>) and spheres containing electron dense and vacuolated regions (Fig.5, FB<sub>4</sub>). The latter may represent stages in the degradation of mitochondria.

#### Apical region

Figure 6 shows the apical region of a primary cell at higher magnification. The apical surface is composed of tightly packed microvilli about 4.0-6.0 $\mu$ m in length and roughly 0.15 $\mu$ m in diameter. Some microvilli are cylindrical in the intermediate portion of their length, tending to be slightly bulbous at their tips and thickened

Fig. 6.

Electron micrograph of the apical region of a primary cell showing microvilli (MV) projecting into the lumen (L). Mitochondria (M) are present in the cytoplasm and occasionally in the microvilli. Pinocytotic vesicles (PV) are evident in this region. Smooth membranes (SM) are present in some of the microvilli. Scale 0.5  $\mu$ m.

Fig. 7.

Electron micrograph showing at higher magnification the junctional structures between adjacent primary cells. Section through the apical region showing the zonula adhaerens (ZA) and septate desmosome (SD). Scale 0.5  $\mu$ m.

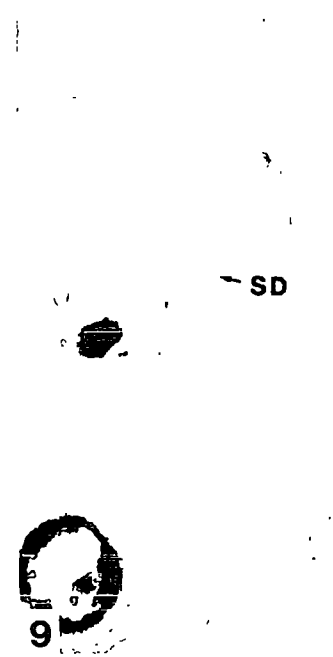
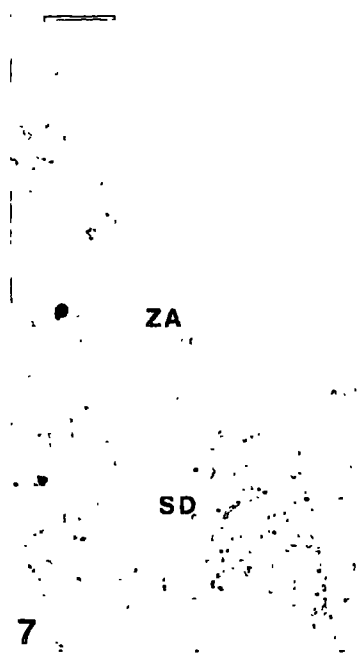
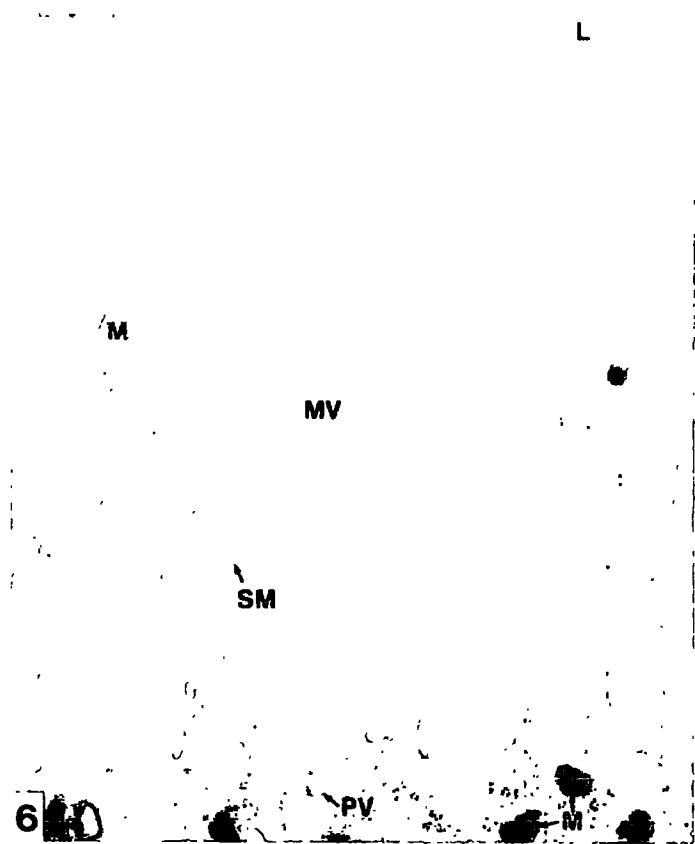
Fig. 8.

Electron micrograph showing one type of formed body (FB<sub>1</sub>) found in the cytoplasm of primary cells. Note laminated appearance. Scale 0.5  $\mu$ m.

Fig. 9.

Low power electron micrograph showing the extent of the septate desmosome junction (SD) between adjacent primary cells. Scale 0.5  $\mu$ m.





Figs. 6-9

towards their base. They arise from the apical surface, either individually or together in a clump, having a common stalk like base (Fig.6). Microvilli occasionally branch into two after leaving the apical surface of the cell (Figs.6,9), whilst some contain mitochondria (Fig.6). Small vesicles are found in the apical cytoplasm and may be either pinched off from or fusing with the apical plasmalemma (Fig.6). Similar vesicles were reported in the Malpighian tubules of Carausius morosus by Taylor (1971a). The interior of the microvilli contain two membranes running parallel with one another as well as with the sides of the filament (Fig.6).

The plasmalemmas of adjacent primary cells are joined to one another laterally by septate desmosomes (Fig.9), which, in the apical region, give way to a junctional structure of the zonula adhaerens type (Fig.7). Here, the extracellular space is slightly wider than in the septate desmosome region. A layer of electron dense material is attached to the cytoplasmic side of the membrane and there is a short zone between the zonula adhaerens and the septate desmosome where the membranes have no junctional structure (Fig.7). The mitochondria of the primary cells are associated with the apical and basal regions of the cell (Fig.2), although this is not as marked in Jamaicana flava or Locusta migratoria as it is in Carausius morosus (Taylor, 1971a).

#### Secondary cells

The electron microscopic structure of a secondary cell is shown in Figures 10,11,12. These cells are distinguishable from the primary cells, as they are smaller and also have a smaller nucleus. In addition, the overall electron density of their cytoplasm is greater than that of the primary cells (Fig.11). The basal cell membrane is invaginated to form a series of narrow channels and cytoplasmic processes (Fig.12). The

Fig. 10

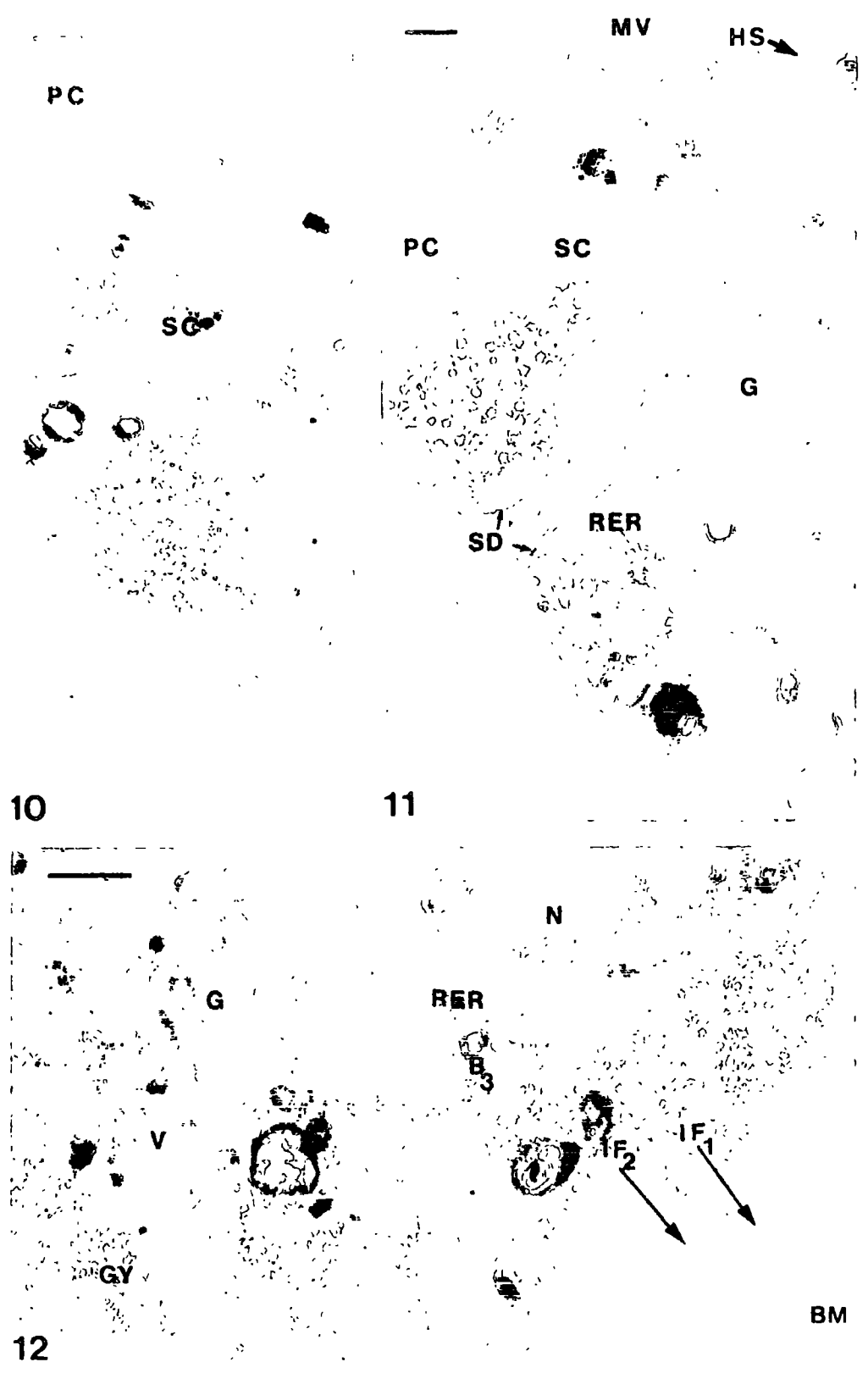
Electron micrograph showing a secondary cell (SC) lying adjacent to a primary cell (PC). Scale 1  $\mu\text{m}$ .

Fig. 11

Electron micrograph showing the apical and lateral regions of a secondary cell. G, Golgi body; HS, husk; MV, microvilli; RER, rough endoplasmic reticulum; SD, septate desmosome. Scale 0.5  $\mu\text{m}$ .

Fig. 12

Electron micrograph showing the intermediate and basal regions of a secondary cell. Basement membrane (BM); IF<sub>1</sub>, IF<sub>2</sub> basal infoldings of primary and secondary cells respectively. Note interdigitations of primary cell with the more electron dense secondary cell in this region. G, Golgi body; GY, glycogen like granules; RER, rough endoplasmic reticulum; V, vacuoles. Scale 1  $\mu\text{m}$ .



Figs. 10-12

cytoplasmic processes of both primary and secondary cells interdigitate, so that a primary cell process may lie adjacent to that of a secondary cell (Fig.12). The basal cytoplasmic processes contain mitochondria and free ribosomes. In overall length the basal infoldings are not as long as their counterparts in the primary cells and thus do not extend as far into the cell.

The apical cell surface is microvillate in which the individual filaments are shorter and thinner than those of the primary cells (Fig. 10). In addition mitochondria are not particularly associated with this region and are rarely found in the filaments. Small clear vacuoles or husks are often found among the microvilli (Fig.11).

Golgi bodies and associated vacuoles are extremely abundant in the cytoplasm (Figs.11,12) as is endoplasmic reticulum, which is present mainly in the form of flat granular cisternae (Fig.12). Glycogen like granules are numerous and occur either as discrete particles or in larger aggregations especially in the lateral regions of the cell (Fig. 11). The large concretions observed in the primary cells (Fig.8) were not present in the secondary cells, although bodies with electron dense and vacuolated regions were observed (Fig.12). Mitochondria are small, few in number and are found scattered throughout the cytoplasm. Secondary cells were only observed to join with primary cells which they did laterally, by means of extensive septate desmosomes, similar to those joining adjacent primary cells (Fig.9). Preliminary studies of the Malpighian tubules of Locusta migratoria indicate that secondary cells are also present in this species and that they are similar in structure to those of Jamaicana flava (Figs. 13,14,16).

#### Ileum

The ileum is approximately 35 mm in length and between 2 and 4 mm

## Figs. 13 and 14

Glutaraldehyde/lead preparations. Photomicrographs of whole mounts of Malpighian tubules from Locusta migratoria, showing the secondary cells as dense regions on the surface of the tubule.

## Fig. 13

Primary and secondary cells at low magnification. PC, primary cell; SC, secondary cell. Scale 50  $\mu\text{m}$ .

## Fig. 14

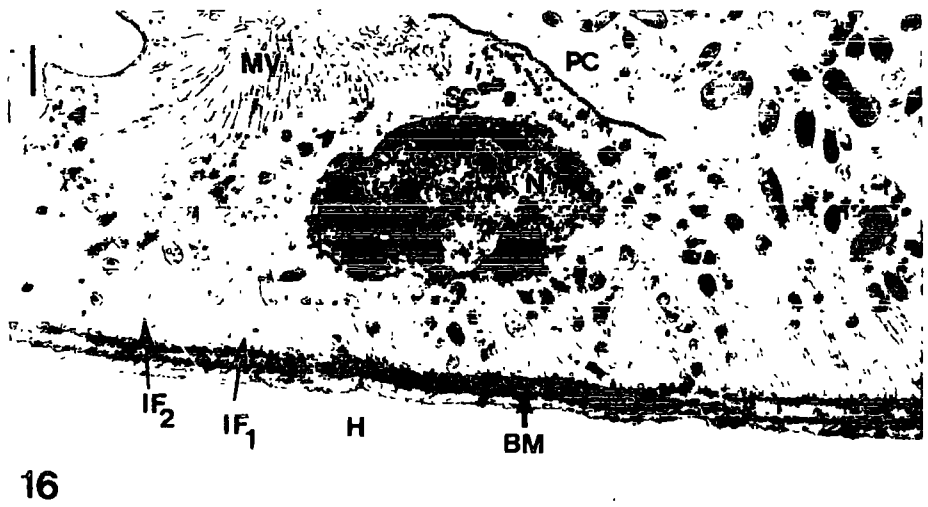
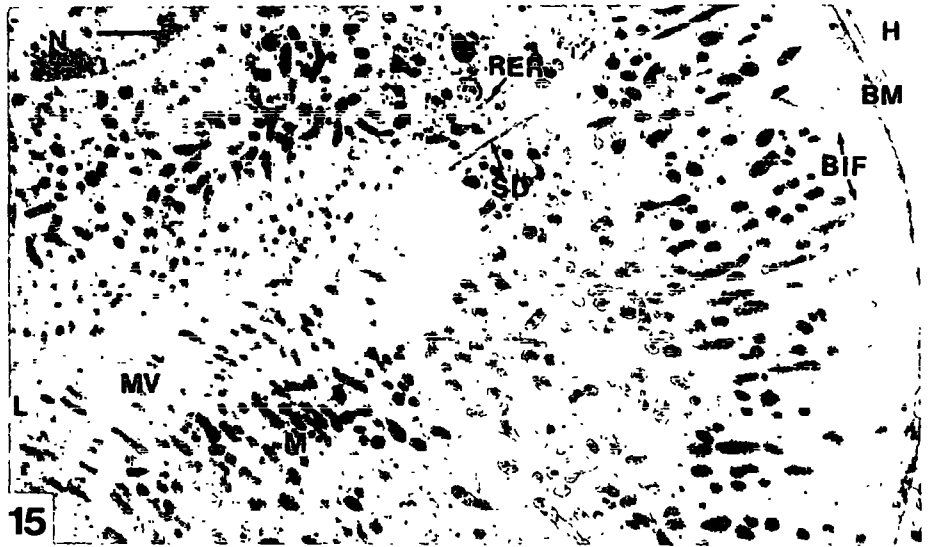
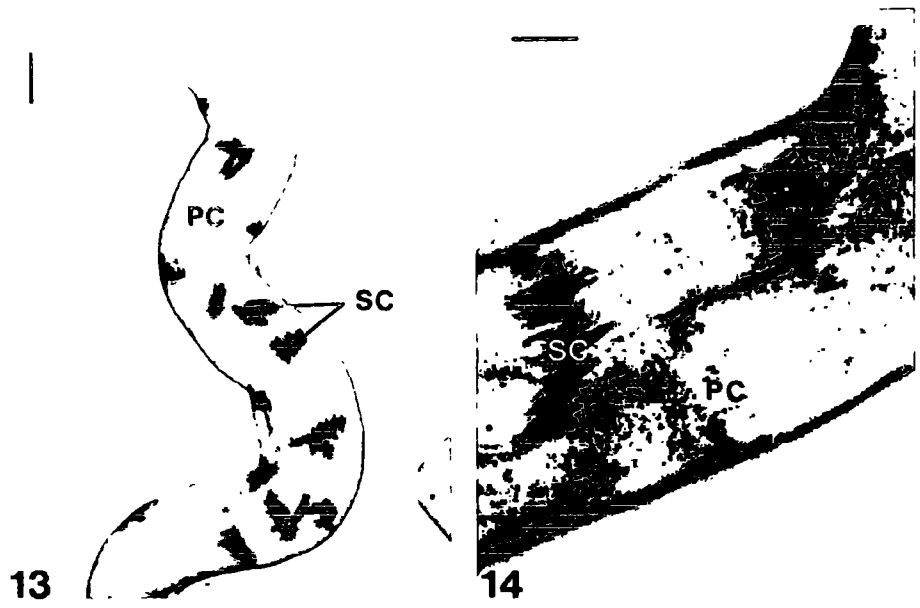
Secondary cell at higher magnification. Note interdigitations with primary cell at the periphery. PC, primary cell; SC, secondary cell. Scale 25  $\mu\text{m}$ .

## Fig. 15

Low magnification electron micrograph of a transverse section through a primary cell. These cells are very similar in structure to the primary cells of Jamaicana flava (Fig.2). BIF, infoldings of basal plasma membrane; BM, basement membrane; H, haemolymph; L, lumen; M, mitochondria; MV, microvilli; N, nucleus; RER, rough endoplasmic reticulum; SD, septate desmosome. Scale 2  $\mu\text{m}$ .

## Fig. 16

Electron micrograph showing a secondary cell. The electron dense secondary cell (SC) interdigitates with the primary cell (PC) in the basal region. IF<sub>1</sub> and IF<sub>2</sub> basal infoldings of primary and secondary cells respectively. BM, basement membrane; H, haemolymph; MV, microvilli; N, nucleus. Scale 1  $\mu\text{m}$ .



Figs. 13-16

in diameter (Fig.1,A). Figure 17 shows a transverse section through the ileum. It will be seen that the luminal surface of the epithelium is covered by a layer of cuticle, whilst the haemolymph side is surrounded by a layer of circular muscle, 2-3 fibres thick. The muscle layer is separated from the epithelium by a subepithelial sinus (Figs.17,18).

The epithelium consists of columnar cells, interrupted in six regions by a single layer of small flat cells (Figs. 17,18). Longitudinal muscle fibres are in close contact, with those of the circular muscle, in the regions of small flat cells (Figs.17,18). The epithelium is thrown into folds or hillocks, which are most prominent at the junctions of the small cell layer with the columnar cells (Figs.17,18). The characteristic features of the columnar cells, when viewed with the optical microscope, are the striated luminal surface and vacuolated regions in the basal or haemolymph region of the cell (Fig.18).

Figure 19 shows the electron microscope structure of several columnar cells. It will be seen that the striated luminal region of the cells, results from the extensive invagination of the apical cell membrane. The infoldings are packed tightly together and extend deep into the cell occupying approximately one fifth of the distance from the apical to the basal cell membrane (Fig.19). The infoldings are longer and more numerous than their counterparts in the rectum, although both possess a coat of particles on the cytoplasmic side (Fig.21). Microtubules are also characteristic of this region (Figs.20,21). Large numbers of mitochondria are found in the apical region of the columnar cells but not all are intimately associated with the infoldings of the plasma membrane (Figs.19,20). In the apical region of the cell, the lateral plasma membranes from adjacent cells, form a restricted zone of septate desmosomes (Fig.20). When in this form, the lateral cell membranes



Fig. 17

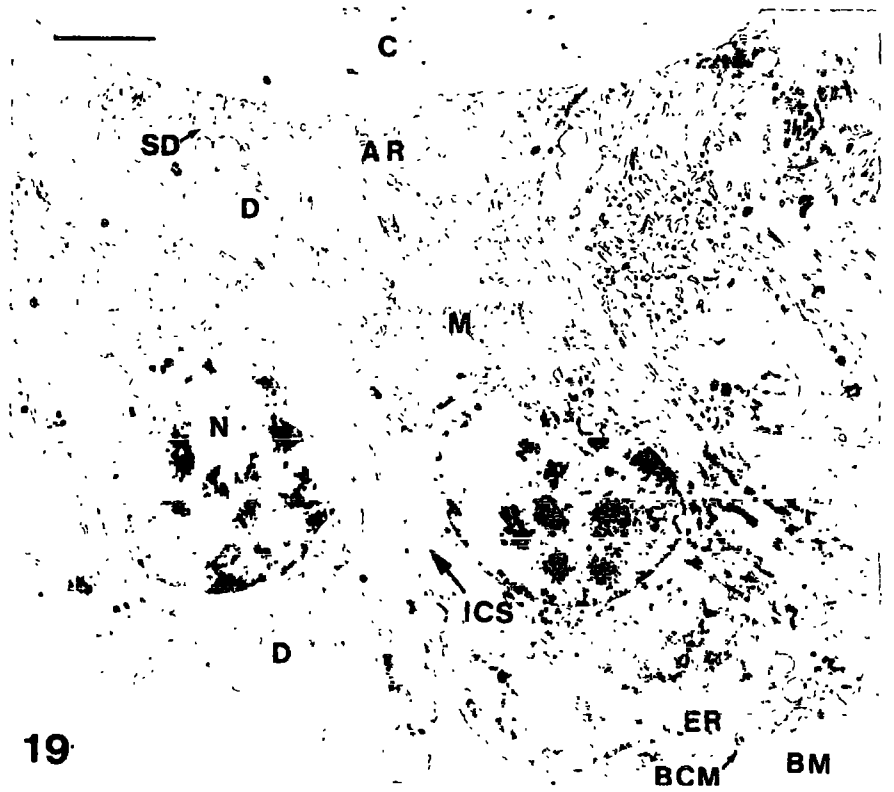
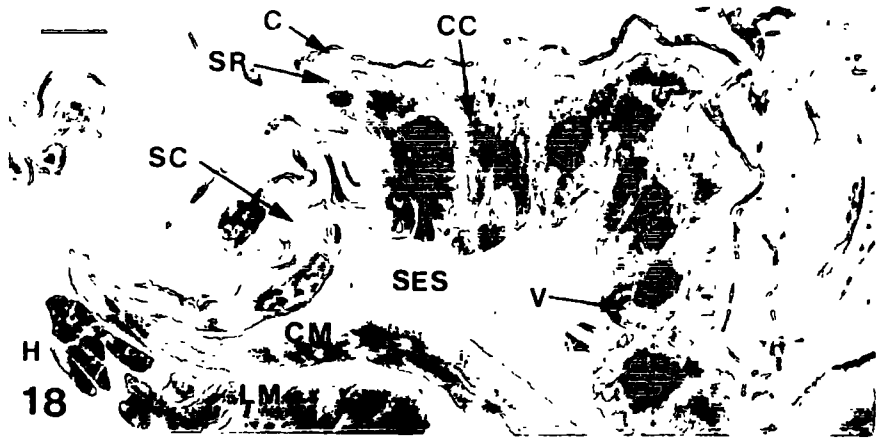
Photomicrograph showing a transverse section through the ileum. The ileal epithelium consists of columnar cells (CC), interrupted in six regions by a single layer of small, flat cells (SC). Where it joins the small cell layer, the columnar epithelium is thrown into hillocks. Circular (CM) and longitudinal (LM) muscles lie on the haemolymph side (H) of the epithelium whilst a cuticular layer (C) provides the lining of the lumen (L). Scale 500  $\mu\text{m}$ .

Fig. 18

A photomicrograph showing at high magnification the large and small cell epithelia of the ileum, (CC) and (SC) respectively. The striated apical (SR) and vacuolated basal (V) regions of the large cells are clearly visible. A layer of cuticle (C) covers the apical surface of the epithelia, whilst circular muscle (CM) separates the sub-epithelial sinus (SES) from the haemolymph (H). Longitudinal muscle (LM) is also present. Scale 50  $\mu\text{m}$ .

Fig. 19

Electron micrograph of a transverse section through the large columnar cells (CC) of the ileum. The cuticle (C) forms the lining of the lumen and lies adjacent to the invaginated apical region (AR). Mitochondria (M) are also present in this region. The nucleus (N) occupies the intermediate portion of the cells whilst "lipid like" droplets (D) are present in the basal region. In the apical region only, the lateral cell membranes of adjacent cells form septate desmosomes (SD) and it is in this region also that these membranes are highly convoluted. For most of the remainder of their length the lateral cell membranes are free from another and intercellular spaces are present (ICS). A basement membrane (BM), basal cell membrane (BCM) and endoplasmic reticulum (ER) are also visible. Scale 5  $\mu\text{m}$ .



Figs. 17-19

Fig. 20

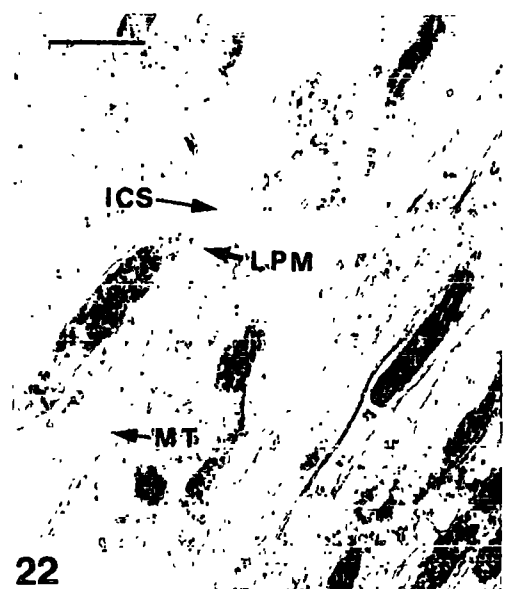
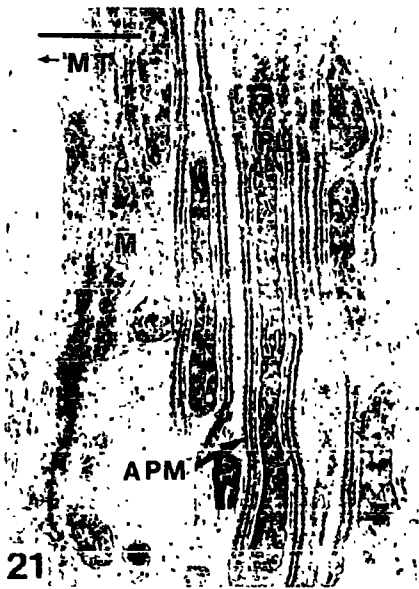
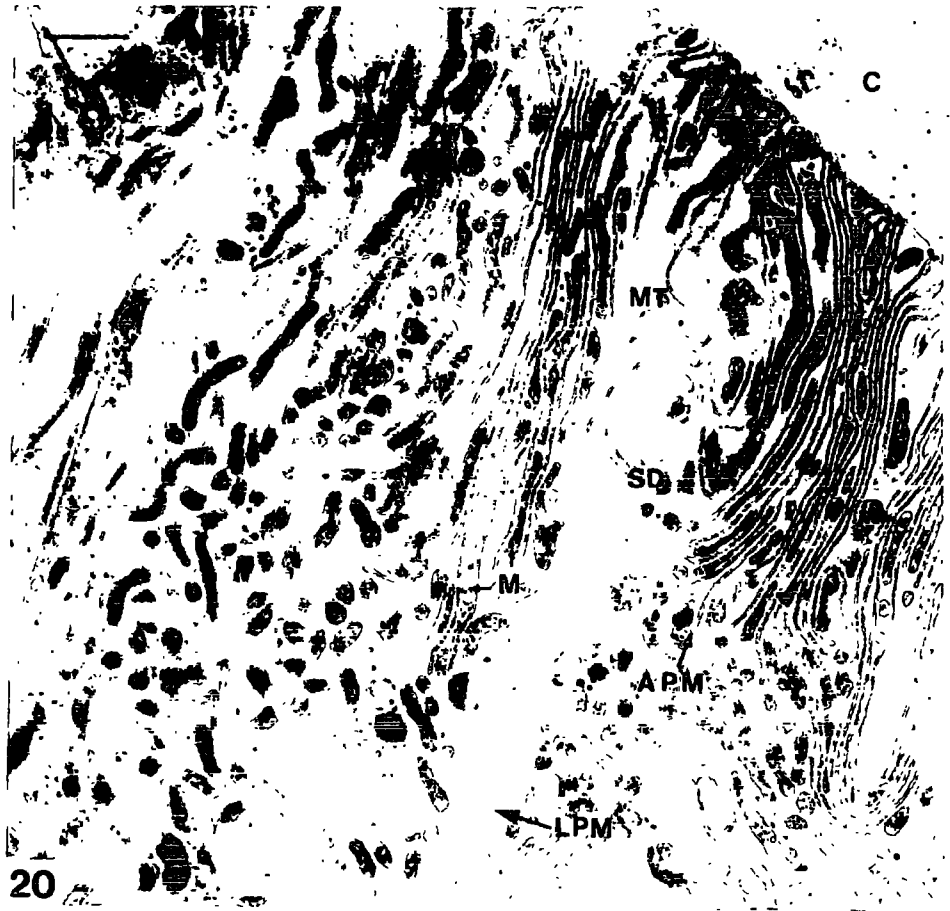
Electron micrograph showing at higher magnification the apical region of a large ileal cell. The apical plasma membrane (APM) is highly invaginated with the infoldings extending well into the cell. Mitochondria (M) associate with the infoldings and microtubules (MT) are abundant and are orientated in an apical/basal direction. The septate desmosome (SD) junction between adjacent cells, as well as the convoluted nature of the lateral cell membranes (LPM) is visible. C, cuticle. Scale 1  $\mu\text{m}$ .

Fig. 21

Electron micrograph showing the infolding of the apical plasma membrane (APM) at higher magnification. The cytoplasmic side of the infolded membrane is covered by a particulate layer (PL). Mitochondria (M) and microtubules (MT) are also present. Scale 0.5  $\mu\text{m}$ .

Fig. 22

Electron micrograph showing the lateral region between two large ileal cells in a region where mitochondria are not associated with the lateral plasma membrane (LPM). ICS, intercellular spaces; MT, microtubules. Scale 0.5  $\mu\text{m}$ .



Figs. 20-22

meander through the apical region of the cells (Fig.20). The remainder of the lateral cell membrane is moderately infolded to form extracellular spaces and channels (Fig.22). The infoldings are not as extensive as the lateral infoldings of the rectal pad cells (see below) and mitochondria do not associate with the plasma membrane to the same extent. Where mitochondria are associated with the lateral cell membrane the extracellular space forms a narrow channel as shown in (Fig.23). Bundles of microtubules are found in the lateral regions of the cells and are orientated in an apical/basal direction (Fig.24). At various points along their length the lateral cell membranes fuse together to form tight junctions of the macula occludens type (Fig.25).

The basal cell membrane is moderately invaginated and sits on a basement membrane that contains numerous trachea and tracheoles (Fig.26). The nuclei of the columnar cells are roughly spherical in shape and lie between the basally located droplets (presumably lipid) and the apical aggregations of mitochondria (Fig.19). Short strands of rough endoplasmic reticulum are found in the cytoplasm as well as Golgi bodies (Fig.27).

#### Rectum

The rectum is a bulbous sac approximately 4.0 mm in length and 2.0 mm in diameter at its widest part. Like the Malpighian tubules, and its counterpart in other insects (Wigglesworth, 1965; Hopkins, 1967; Oschman and Wall, 1969), it has an extensive tracheal supply which clearly distinguishes it from the remainder of the gut (Fig.1,D). The trachea are concentrated into six cigar shaped regions, each of which receive several tracheal trunks, whose branches pass through the outer muscle layer and ramify throughout the tissues beneath (Fig.1,D).

In transverse section the rectum is composed of three layers (Fig.28). A cuticular intima covers the luminal surface of the underlying cell

Fig. 23

Electron micrograph, similar to Figure 22, but in a region where mitochondria (M) are associated with the lateral plasma membrane (LPM). ICC, intercellular channel; N, nucleus. Scale 0.5  $\mu\text{m}$ .

Fig. 24

Electron micrograph of the adjacent region of two large ileal cells showing bundles of microtubules (MT). Scale 0.5  $\mu\text{m}$ .

Fig. 25

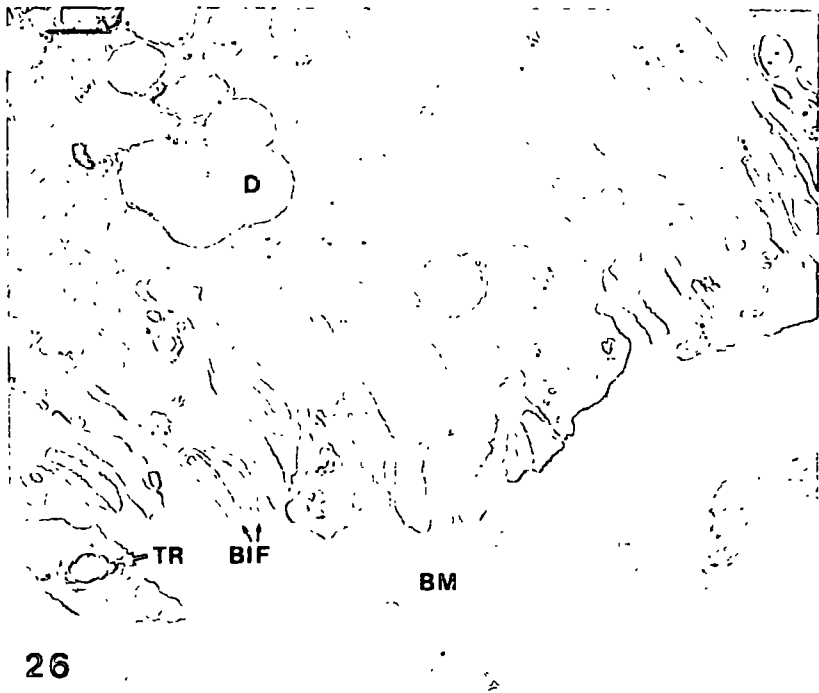
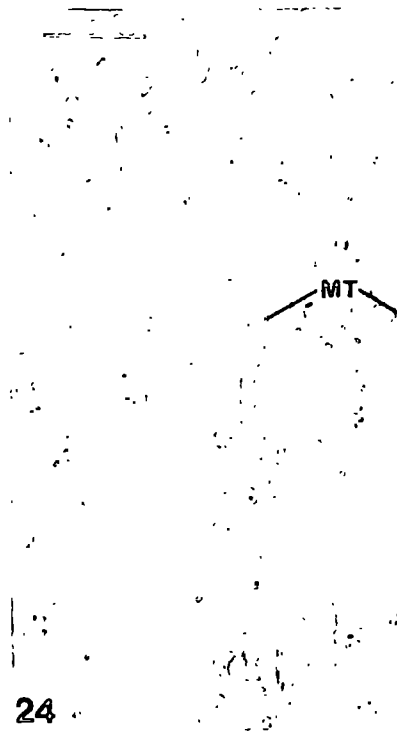
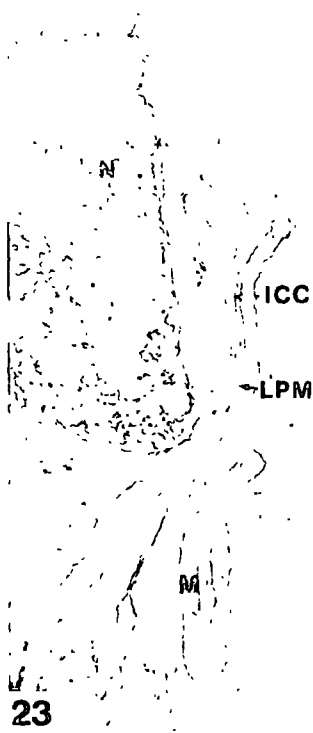
Electron micrograph showing a macula occludens junction (MO) between adjacent ileal cells. Scale 0.5  $\mu\text{m}$ .

Fig. 26

Electron micrograph showing the basal region of a large ileal cell. BIF, infoldings of basal plasma membrane. BM, basement membrane; D, droplets; TR, trachea; Scale 1  $\mu\text{m}$ .

Fig. 27

Electron micrograph of the basal region of a large ileal cell showing Golgi body (G), vacuoles (V) and droplets (D). Scale 0.5  $\mu\text{m}$ .



Figs. 23-27

layer, which is separated from the haemolymph by a layer of circular muscle. The cuticle does not have a uniform appearance throughout the rectum. In regions where it overlies the junctional cells, it is thinner and, under light microscope examination, has a different optical appearance to the cuticle of other regions (Figs. 29, 30). This suggests that the cuticle overlying the junctional cells has different properties and perhaps structure to that found elsewhere in the rectum.

The cellular layer comprises a one cell thick epithelium of small cells, thickened in six regions to form longitudinally orientated rectal pads (Fig.28). On either side of the small cell layer and adjacent to the rectal pad cells, there are tall narrow cells which correspond in position to the junctional cells described by Berridge and Gupta (1967) in Calliphora erythrocephala and by Oschman and Wall (1969) in Periplaneta americana (Figs. 29, 30). The small cell or reduced epithelium is thrown into folds and hillocks (Figs.28, 29). The folding of this epithelium is perhaps the means whereby the rectum can increase in girth. A layer of circular muscle, two-three fibres thick, lies adjacent to the rectal epithelium (Fig. 28). Bundles of longitudinal muscle fibres lie on the haemolymph side of the circular muscle and usually in the region of the reduced epithelia (Fig.28). Where longitudinal muscle fibres are present, the reduced epithelium loses its folded appearance and is flat (Fig.29). A subepithelial space lies between the circular muscle and the rectal epithelium, but is obliterated where the reduced epithelium is flat (Fig. 29).

#### Reduced epithelial cells

Figure 31 shows the electron microscopic structure of a reduced epithelial cell. It will be seen that as the cuticle approaches the apical surface of the cell it loses its lamellar appearance and a



Fig. 28

Photomicrograph showing, in transverse section, the various components of the rectum. The rectal pads (RP) are flanked on either side by a region of reduced epithelium (RE). Junctional cells (JC) are also visible. The subepithelial sinus (SES) lies between the epithelial cells and circular muscle layer (CM). The sinus is prominent in some regions and not in others. Longitudinal muscles (LM) are associated with the circular muscle in the region of reduced epithelia. C, cuticle; H, haemolymph; L, lumen. Scale 500  $\mu\text{m}$ .

Fig. 29

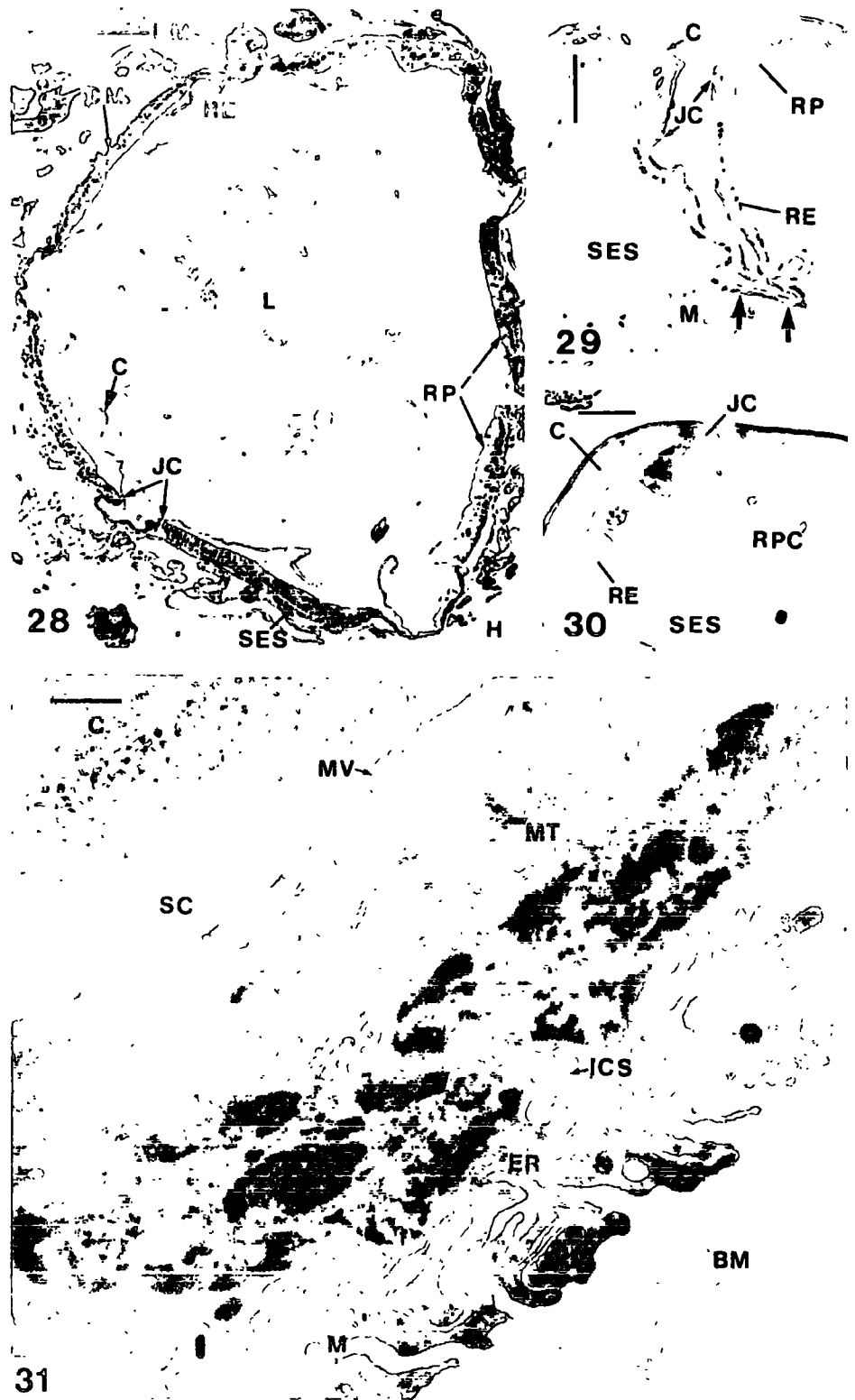
Photomicrograph illustrating the region between two rectal pads (RP). Cuticle (C); reduced epithelium (RE) junctional cells (JC); subepithelial sinus (SES) which is obliterated in the region where the muscles insert into reduced epithelium (heavy arrows). Scale 100  $\mu\text{m}$ .

Fig. 30

High magnification photomicrograph showing the three types of cell that make up the rectal epithelium. C, cuticle; JC, junctional cell; RE, reduced epithelial cell; RPC, rectal pad cell; SES, subepithelial sinus. Scale 20  $\mu\text{m}$

Fig. 31

Electron micrograph of a transverse section through two reduced epithelial cells. The cuticle (C) lies next to a subcuticle (SC). The apical plasma membrane is infolded to form short microvilli (MV). Mitochondria (M) are present in these cells but are not usually associated with any of the cell membranes. Microtubules (MT), endoplasmic reticulum (ER) are also present in the cytoplasm. Intercellular spaces (ICS) are found in the lateral region of the cell where the cell membrane is infolded. Septate desmosomes connect adjacent cells in the apical region (not shown). BM, basement membrane. Scale 1  $\mu\text{m}$ .



Figs.28-31

subcuticle is present. The subcuticle is composed of a diffuse substance which is fibrillar in nature. A subcuticle was also reported in the rectal cells of Periplaneta americana by Oschman and Wall (1969). The apical plasma membrane of the rectal cell is invaginated to form a few short microvilli (Fig. 32). Individual microtubules are found within the microvilli (Fig.32). In the apical region apposed lateral cell membranes are held together by short cross bridges to form septate desmosomes (Fig. 32). Where the membranes are connected in this way they are highly convoluted, being many times the length necessary to connect apical and basal surfaces of the cell (Fig.32). Bundles of microtubules are present in the lateral regions of the cells. They are short and orientated in a strict apical/basal direction (Figs.32,33).

In addition to the septate desmosomes the lateral cell membranes make contact with one another in the basal region of the cell, where they fuse together and form tight junctions of the macula occludens type (Fig. 33). Where apposed lateral cell membranes are free they are extensively folded and form narrow intercellular spaces (Figs.31,32). Mitochondria are not abundant in these cells and those that are present do not associate with the cell membranes. The basal cell membrane is deeply and irregularly infolded, to form meandering canaliculi. The cell rests on a basement membrane next to which lies a small subepithelial sinus (Fig.31). The ultrastructure of the junctional cells was not examined.

#### Rectal pad cells

Figure 34 shows the electron microscopic structure of a rectal pad cell at low magnification. The apical plasma membrane is moderately infolded and forms a complex series of membrane leaflets (Figs. 35, 36). In transverse section, the apical region appears as a series of short irregular cytoplasmic compartments and narrow extracellular spaces (Fig.37).

Fig. 32

High magnification electron micrograph of the apical region of a reduced epithelial cell showing the subcuticular space (SC), short microvilli (MV), septate desmosomes (SD), intercellular space (ICS) and bundles of microtubules (MT). Scale 0.5  $\mu\text{m}$ .

Fig. 33

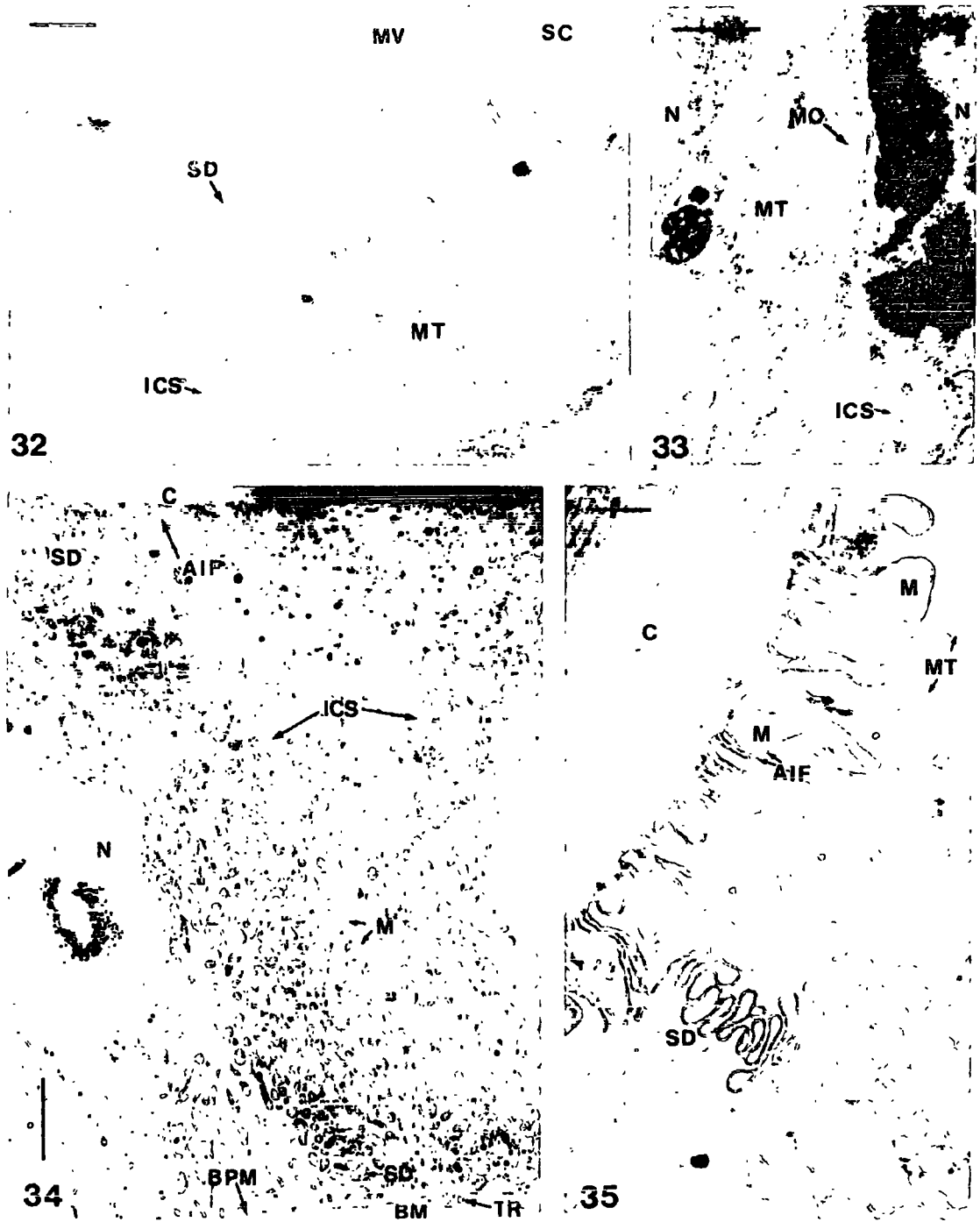
Electron micrograph showing a region between adjacent reduced epithelial cells. ICS, intercellular space; MO, macula occludens; MT, microtubules; N, nucleus; Scale 0.5  $\mu\text{m}$ .

Fig. 34

Low magnification electron micrograph showing the characteristics of rectal pad cells. A cuticle (C) covers the apical surface of the cell. The apical plasma membrane is moderately infolded (AIF). Septate desmosomes (SD) are present between adjacent rectal pad cells in restricted regions at apical and basal borders. Between these points extensive intercellular channels and spaces (ICS) are present. Most of the mitochondria (M) of the pad cells are associated with the lateral and apical membranes, with very few free in the general cytoplasm. The basal plasma membrane (BPM) is flat and sits on a basement membrane (BM) in which trachea (TR) are present. N, nucleus. Scale 5  $\mu\text{m}$ .

Fig. 35

Electron micrograph showing the apical region of a rectal pad cell. The infolded plasma membrane (AIF) is covered by cuticle (C). Mitochondria (M) are associated with the apical microvilli. MT, microtubules; SD, septate desmosome. Scale 1  $\mu\text{m}$ .



Figs. 32-35

The infolded membranes are variable in length but do not extend past a depth of 1-2  $\mu\text{m}$  into the cell (Fig.34). Apposing lengths of plasma membrane forming the infoldings, are in close proximity to one another so that the extracellular space is small (Fig. 37). A coat of small particles is located on the cytoplasmic side of the infolded membrane (Fig.37). Similar types of particles were first reported in insect recta by Gupta and Berridge (1966a) in Calliphora erythrocephala and later by Oschman and Wall (1969) in the rectal pads of Periplaneta americana. Rod and filamentous shaped mitochondria, microtubules and vacuoles of various sizes, are associated with many of the cytoplasmic compartments (Figs. 36, 37). Microtubules are randomly arranged but occasionally occupy the central region of a cytoplasmic compartment (Fig. 36).

Apposed lateral cell membranes of adjacent rectal pad cells, form septate desmosomes at restricted regions near both apical and basal borders (Figs. 35, 38). Many sections were examined and in all, extensive septate desmosomes were present in these regions of adjacent rectal pad cells. This probably means that both apical and basal desmosome junctions form a collar around the rectal pad cells in much the same way as Oschman and Wall (1969) described for these cells of Periplaneta americana. Serial sections however, through the rectal pad cells of Jamaicana flava would determine the extent of the septate desmosomes in this species. In the apical region the septate membranes are highly convoluted, often looping back on themselves (Fig.35). Between apical and basal septate junctions apposed cell membranes lose their ladder like appearance and are highly infolded, forming a maze of extracellular channels and spaces (Figs.34,40,41). The length of the channels varies as do the size of the spaces, but the channels are

Fig. 36

Similar electron micrograph to Figure 35 but of an oblique transverse section showing leaflet like arrangement of apical membrane infoldings (AIF). C, cuticle; M, mitochondria; MT, microtubules; V, vacuoles. Scale 0.5  $\mu\text{m}$ .

Fig. 37

High magnification electron micrograph of apical region showing the particulate layer (PL) on the cytoplasmic surface of the apical infoldings (AIF). Mitochondria (M) and microtubules (MT) are also present in this region. Scale 0.25  $\mu\text{m}$ .

Fig. 38

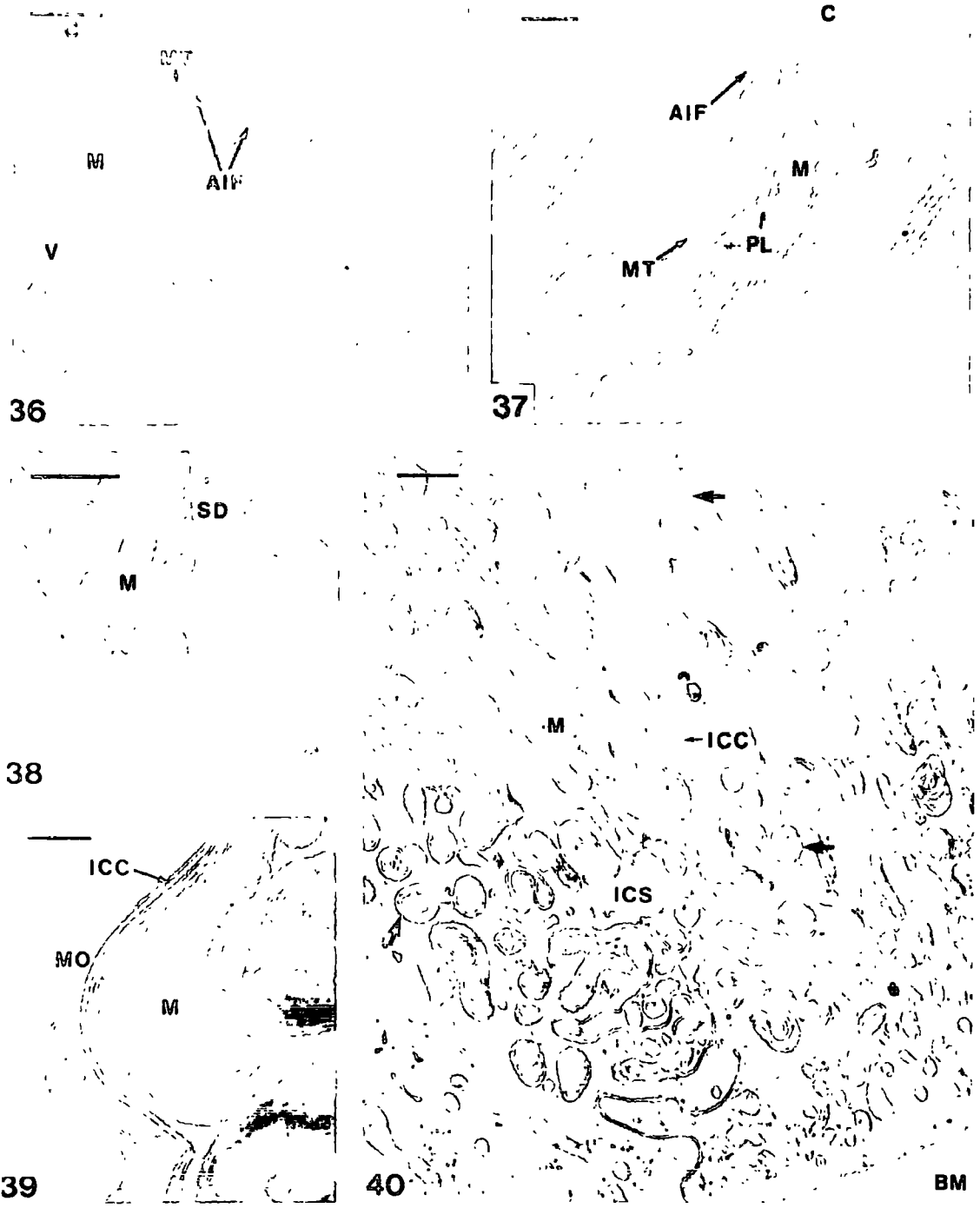
Electron micrograph showing the septate desmosome (SD) junction in the basal region between adjacent rectal pad cells. M, mitochondrion. Scale 0.5  $\mu\text{m}$ .

Fig. 39

Electron micrograph showing the five-layered structure, characteristic of macula occludens junctions (MO). Section through the lateral regions between two rectal pad cells. ICC, intercellular channel; M, mitochondrion. Scale 0.25  $\mu\text{m}$ .

Fig. 40

Electron micrograph of part of a rectal pad cell showing the intercellular spaces (ICS) and channels (ICC). BM, basement membrane; M, mitochondrion. Arrows indicate the extent to which the lateral cell membranes are invaginated. Scale 1  $\mu\text{m}$ .



Figs. 36-40

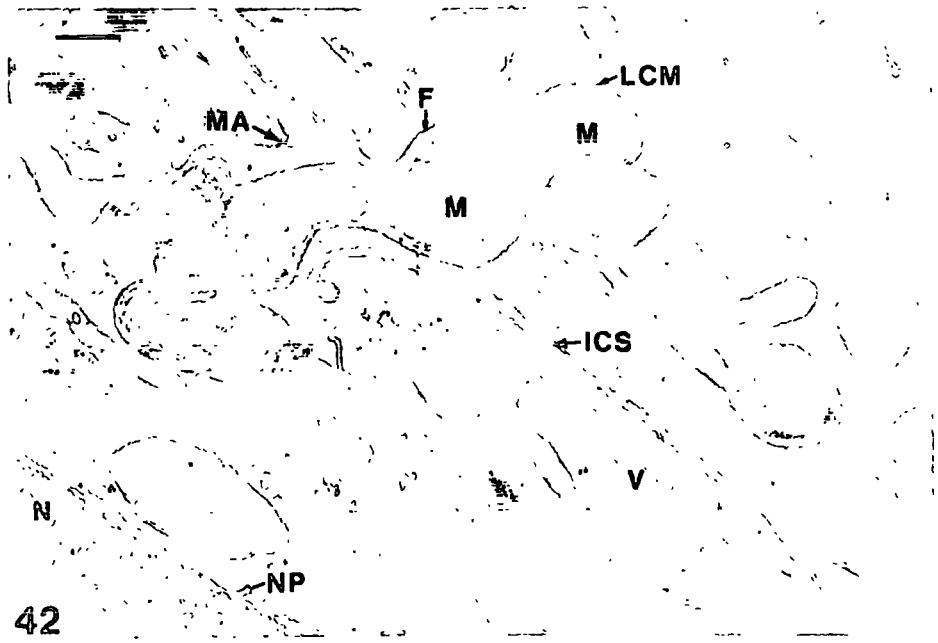
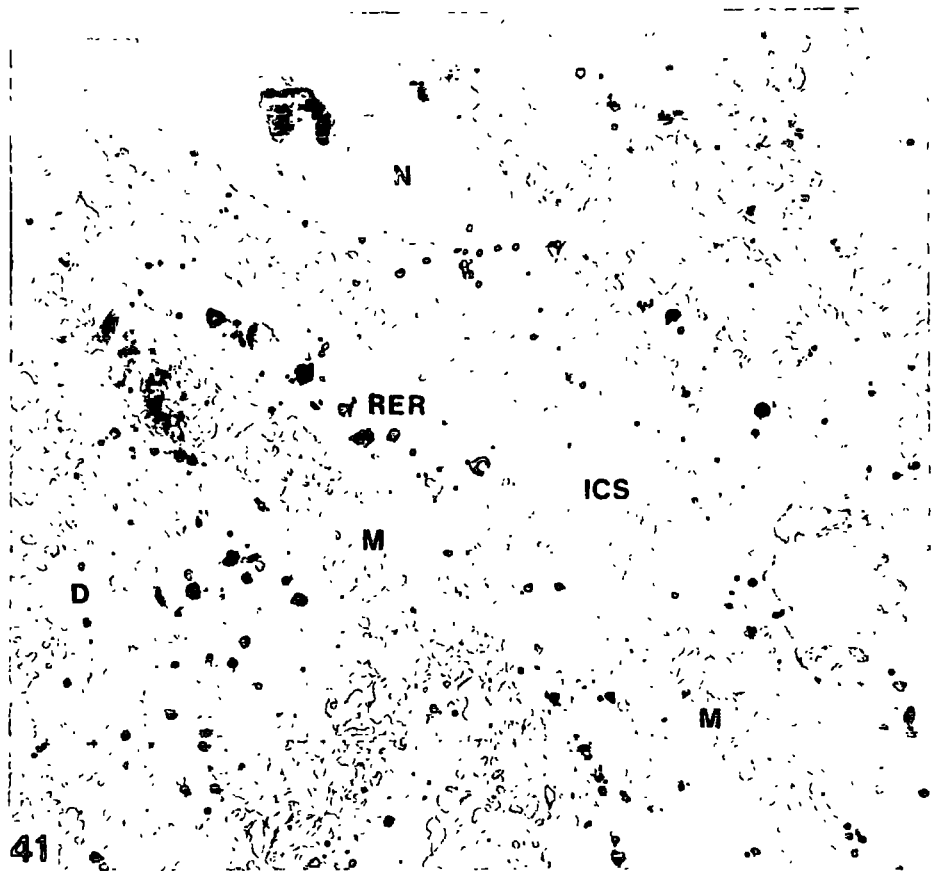


Fig. 41

Slightly oblique longitudinal section of rectal pad cell showing the association between mitochondria (M) and the lateral regions of the cell. D, droplets; ICS, intercellular space; N, nucleus; RER, rough endoplasmic reticulum. Scale 5  $\mu\text{m}$ .

Fig. 42

High magnification electron micrograph of the lateral region between adjacent rectal pad cells to show the intimate association between mitochondria (M) and the lateral cell membranes (LCM). The narrow channels between cells are joined in regions by fine filaments (F). Macula adhaerens (MA) junctions occur where channels dilate to form intercellular spaces (ICS). Part of a nucleus (N) containing nuclear pores (NP) is also shown. V, vacuole. Scale 0.5  $\mu\text{m}$ .



Figs. 41 and 42

of constant width and open into the spaces from all directions (Fig. 41). The channels are held to a constant width by a system of fine cross filaments (Fig. 42). Frequently at the junction of an intercellular channel and space, electron dense regions are present (Fig. 42) and resemble the macula adhaerens type of junction described by Farquhar and Palade (1963). Occasionally, the lateral cell membranes fuse to form tight junctions of the macula occludens type (Fig. 39). The intercellular spaces are not restricted to the apical or basal regions of the cells (Fig. 40). It is evident from Figure 42 that the mitochondria of the rectal pad cells are intimately associated with the lateral plasma membranes, especially where the cells are separated by narrow channels. The degree of intimacy can be judged by the fact that in sections, mitochondria appear to be composed of four and not two membranes. A similar mitochondrial appearance was described by Oschman and Wall (1969) in the rectal pad cells of Periplaneta americana. Numerous microtubules are present in the lateral regions of these cells and run in an apical/basal direction (Fig. 40).

The basal plasma membrane is not folded but is, however, indented in places (Fig. 44). Where indentations occur flattened vesicles are also present (Fig. 43). The presence of indentations and vesicles implies that the membrane is active in pinocytosis. Oschman and Wall (1969) found evidence of pinocytosis in a comparable region of the rectum of Periplaneta americana. In addition to the indentations, the basal cell membrane also has associated with it numerous electron dense regions which extend into the cytoplasm (Fig. 43). These regions may represent some kind of anchorage akin to the hemidesmosome described by Berridge and Gupta (1967) in the corresponding cells of

## Figs. 43 and 44

Electron micrographs showing the basal region of a rectal pad cell. The basal plasma membrane (BPM) sits on a basement membrane (BM) that contains trachea (TR); vesicles (VC); and pinocytotic vesicles (PV). Electron dense regions (EDR) are associated with the basal plasma membrane. Scale Fig. 43 0.5  $\mu\text{m}$ ; Fig. 44, 0.2  $\mu\text{m}$ .

## Figs. 45 and 47

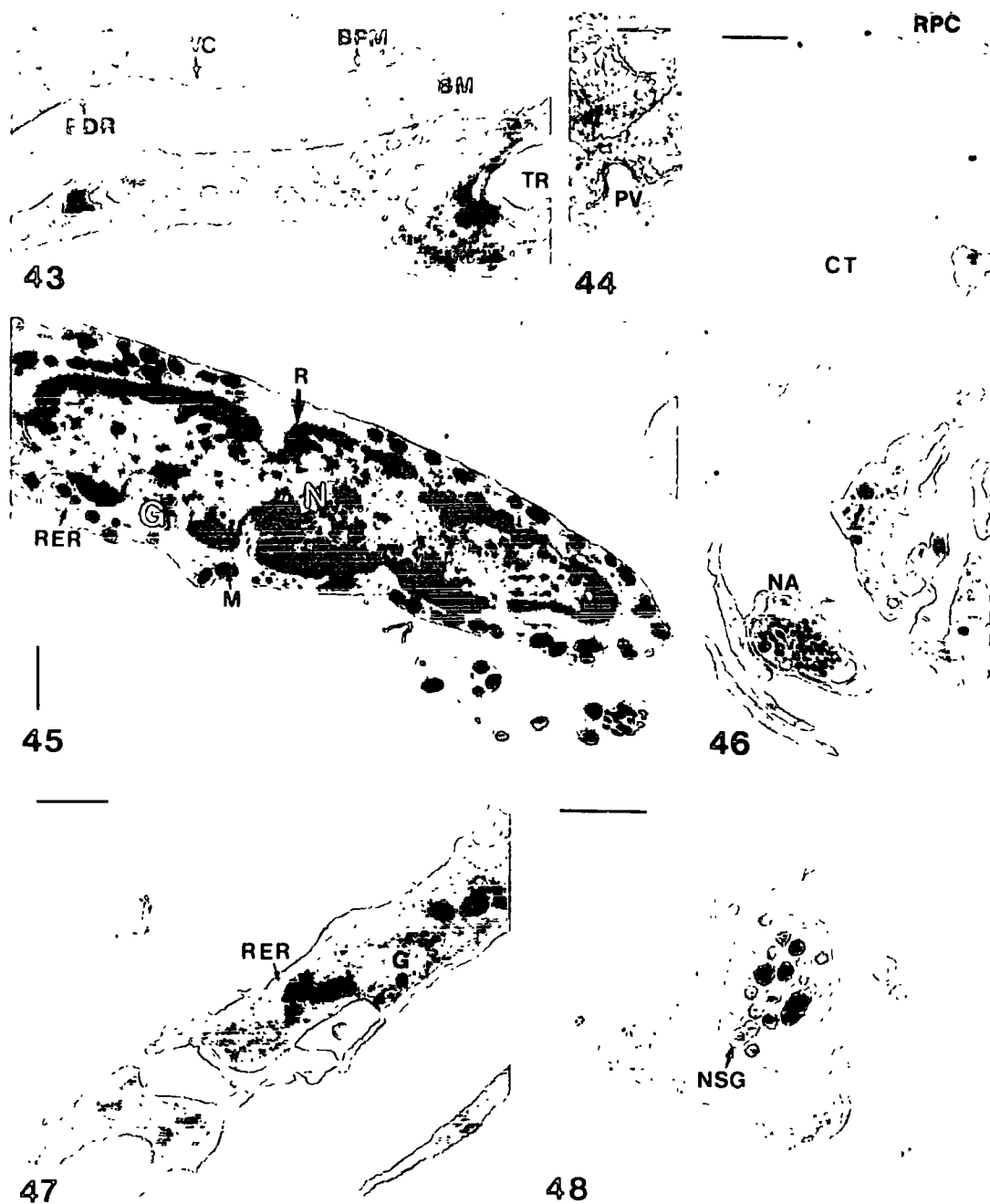
Electron micrographs showing a type of hemocyte found in the subepithelial sinus. Note, rough endoplasmic reticulum (RER); Golgi apparatus (G); mitochondrion (M); nucleus (N) and ribosomes (R) on nuclear membranes.

Scale 1  $\mu\text{m}$ .

## Figs. 46 and 48

Electron micrographs showing neurosecretory axons (NA) in the connective tissue (CT) of the subepithelial sinus. NSG, neurosecretory granule; RPL, rectal pad cell.

Scale, Fig. 46, 1  $\mu\text{m}$ ; Fig. 48, 0.5  $\mu\text{m}$ .



Figs. 43-48

Calliphora erythrocephala. The transition from basal to lateral cell membrane is marked by a short intercellular channel prior to the apposed membranes forming septate desmosomes (Fig.38). The basal cell membrane rests on a basement membrane composed of fine fibrils (Fig.43) and contains trachea and tracheoles (Fig.34).

Other organelles present in the cytoplasm of these cells include a large nucleus, rough endoplasmic reticulum, vacuoles of assorted sizes, and droplets (Figs. 34, 41, 42).

A subepithelial sinus lies between the basement membrane of the pad cells and the surrounding layer of circular muscle (Figs.28,29). It is variable in size and contains connective tissue, in which hemocytes and neurosecretory axons are present (Figs.45,46,47,48). The hemocytes are flattened cells, that have well developed endoplasmic reticulum, ribosomes and Golgi apparatus (Figs. 45,47). Oschman and Wall (1969) observed similar cells in Periplaneta americana and pointed out their similarity to vertebrate fibrocytes. These authors suggest that the flattened cells may secrete the connective tissue. Although the innervation of the rectum was not investigated, it is clear from Figures 46,48, that nerve axons are present in the connective tissue of the subepithelial sinus. The axons are filled with droplets resembling the neurosecretory granules described by Norman (1965) in the corpus cardiacum of Calliphora erythrocephala.

In general structure, the rectum of Jamaicana flava agrees with the description given by Hodge (1939) for this organ in Locusta migratoria. As in Jamaicana flava, the rectum of this species consists of an epithelium thickened in six regions to form pads. Preliminary electron microscopic examination of the large rectal pad cells of Locusta show that they are similar to their counterparts in Jamaicana

flava (this study) and other insects (Berridge and Oschman 1972).

Figures 49 - 52 show the electron microscopic structure of a rectal pad cell. These cells are characterised by: a cuticular lining to the lumen, moderately invaginated apical plasma membrane, regions of restricted septate desmosomes between adjacent cells at apical and basal borders, lateral plasma membrane extensively infolded, resulting in numerous extracellular spaces and channels, numerous mitochondria are associated with the infoldings, a subepithelial sinus is present and lies between the epithelium and the surrounding muscle layer.

## 2. Histochemical localization of $\text{Na}^+ - \text{K}^+$ ATPase in the rectum of Locusta migratoria

As a result of preliminary experiments, several additional procedures were incorporated into the method. For example, rapid freezing of the tissues was found to improve section preservation. In addition, sections were allowed to attain cryostat operating temperature ( $-25^{\circ}\text{C}$ ), prior to sectioning of the tissue immediately after freezing and carbon dioxide treatment, often produced torn and splintered sections. A short period of fixation also enhanced section integrity over that of unfixed tissue, although the effects of fixation on enzyme activity were undetermined. Time trials at a temperature of  $37^{\circ}\text{C}$ , indicated the optimum incubation period to be about twenty minutes. Longer periods caused heavy depositions of lead phosphate.

Although experimental procedures were as far as possible standardised, the results from similar experiments were variable. The variation was of two kinds. There were those incubations that did not respond in the way one would predict as necessary for the demonstration of  $\text{Na}^+ - \text{K}^+$  ATPase. Sometimes sections incubated with magnesium ions alone (Table 1, medium 2) possessed a similar quantity of reaction product to sections incubated in the presence of magnesium, sodium and potassium ions

## Figs. 49 - 52

Electron micrographs showing the characteristics of rectal pad cells from the rectum of Locusta migratoria.

## Fig. 49

Low magnification electron micrograph of a rectal pad cell. A cuticle (C) covers the apical surface of the cell. The apical plasma membrane is infolded (AIF). Septate desmosomes (SD) are present between adjacent rectal pad cells in restricted regions at apical and basal borders. Between these points extensive intercellular channels and spaces (ICS) are present. Most of the mitochondria (M) of the pad cells are associated with the lateral and apical membranes, with very few free in the general cytoplasm. Scale 3  $\mu\text{m}$ .

## Fig. 50

Electron micrograph showing the apical region at a higher magnification. AIF, apical infoldings of plasma membrane. C, cuticle; M, mitochondria; SD, septate desmosome. Scale 1  $\mu\text{m}$ .

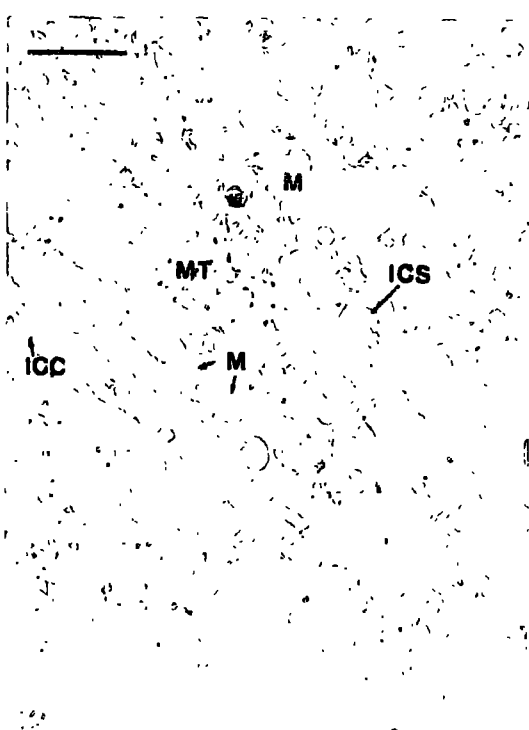
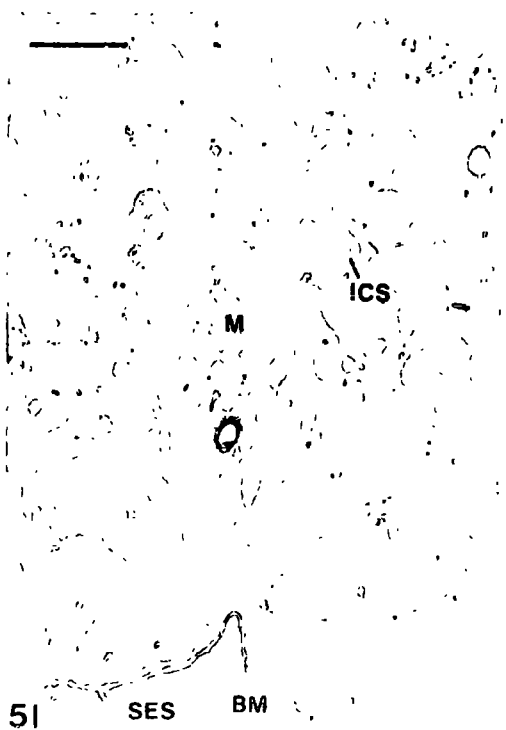
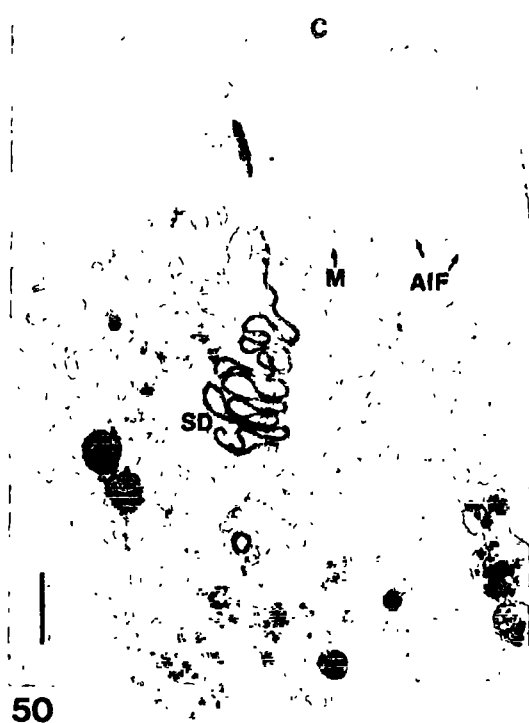
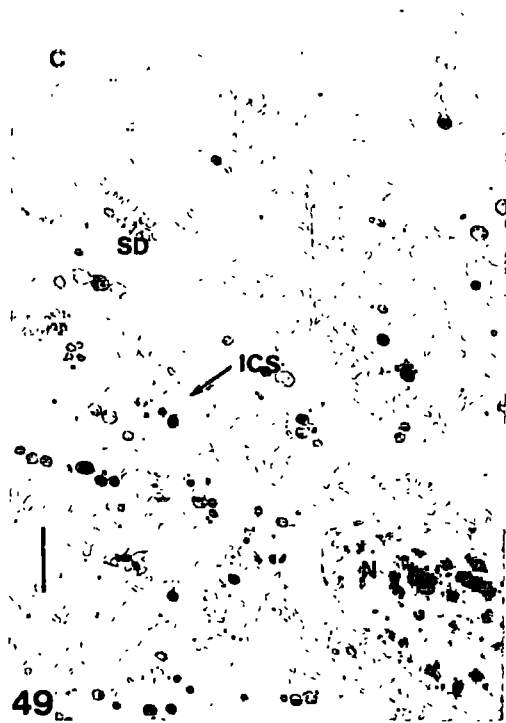
## Fig. 51

Electron micrograph showing the basal region of a rectal pad cell. BM, basement membrane; ICS, intercellular space; M, mitochondria; SES, subepithelial sinus. Scale 1  $\mu\text{m}$ .

## Fig. 52

High magnification electron micrograph of the lateral region between adjacent rectal pad cells to show the intimate association between mitochondria (M) and the lateral cell membranes. ICC, intercellular channel; ICS, intercellular space; M, mitochondria; MT, microtubules. Scale 1  $\mu\text{m}$ .





Figs. 49-52

Figs 53 and 54

Photomicrographs showing sites of ATPase activity in the rectal pad of Locusta migratoria (slightly oblique transverse section)

Fig. 53

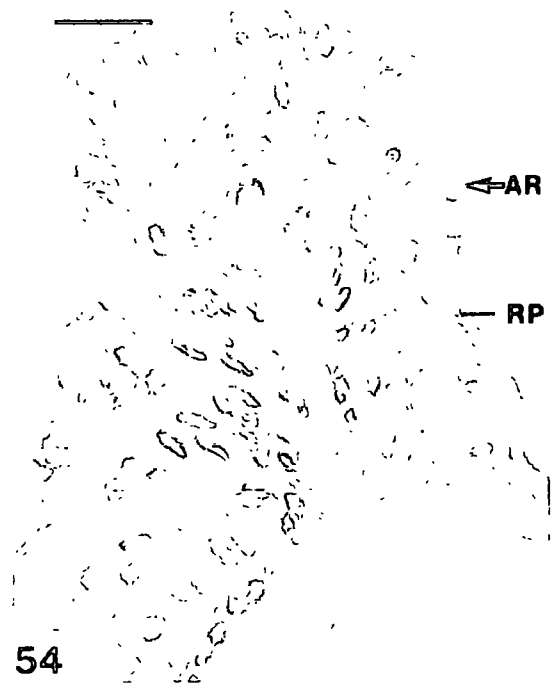
Section incubated in a medium containing 3 mM  $Mg^{2+}$ .

Scale 50  $\mu m$ .

Fig. 54

Section incubated in a medium containing 3 mM  $Mg^{2+}$ , 100 mM  $Na^+$  and 20 mM  $K^+$ . Scale 50  $\mu m$ .

In addition to the salts above, both media contained 3.3 mM ATP, 3.0 mM  $Pb(NO_3)_2$  and 24.0 mM Tris(pH 7.8). Incubation time, 20 min at 37°C. AR, apical region; RP, rectal pad.



Figs .53 and 54

(Table 1, medium 3). Secondly, sections incubated in identical media, but a different set of experiments, possessed varying amounts of reaction product. Precipitates of lead phosphate were only found in the large cells of the rectal epithelium and usually in the membrane regions (Fig.53). The deposits were not of a uniform shape or size. The cuticular edge of the cells normally had a heavy precipitate whether the cuticle was present or not. The appearance of sections incubated in a medium containing only magnesium ions is shown in Figure 53. Usually, sections incubated with magnesium, sodium and potassium ions also resembled Figure 53, but occasionally sections were obtained that appeared as in Figure 54. Phosphate deposition under these conditions ( $Mg^{2+}$ ,  $Na^+$  and  $K^+$ ) was unaffected by the presence of ouabain in the incubation medium (Table 1, medium 4). Control sections incubated without ATP (Table 1, medium 1) possessed a little precipitate which was usually less than in sections incubated with magnesium ions alone (Table 1, medium 2).

The junctional cells and cells of the reduced epithelium never contained reaction product of the type described above, but the cytoplasm did possess a very fine dark brown, deposit. Such a precipitate was found occasionally in the cells of the rectal pads but was more prominent in the junctional and epithelial cells. The quantity of this reaction product was independent of the incubation medium.

### Discussion

The uniform morphological appearance presented by the Malpighian tubules of Jamaicana flava has been reported for the tubules of Melanoplus differentialis (Beams, Tahmisian and Devine, 1955) and Dissoteria carolina (Tsubo and Brandt, 1962) but not for other species including Schistocerca gregaria (Savage, 1956) and Rhodnius prolixus

(Wigglesworth, 1931). In these insects the Malpighian tubules are morphologically differentiated into distal and proximal regions, which, in Rhodnius, have been shown to have secretory and absorptive functions respectively (Wigglesworth, 1931). Furthermore in Rhodnius, the cells from the distal region resemble the primary cells of Jamaicana flava and Locusta migratoria, whilst the cells from the proximal region are similar to those of the distal region but lack the highly microvillate apical surface (Wigglesworth and Salpeter, 1962).

The Malpighian tubules of Jamaicana flava and Locusta migratoria are composed of two types of cell, referred to in the text as primary and secondary cells. The ultrastructure of these cells in Jamaicana flava is schematically represented in Figures 55, 56. The corresponding cells from the Malpighian tubules of Locusta migratoria are structurally similar. The primary cells of Jamaicana flava are characterised by: invaginations of the basal cell membrane, long closely packed microvilli, mitochondria that tend to be associated with the apical and basal cell membranes, a thin basement membrane, cells joined to one another laterally by extensive septate desmosomes, cytoplasm that contains large concretions and vacuoles, an extensive trachea and tracheolar supply. The Malpighian tubules of a variety of insect species are composed of cells that structurally, resemble the primary cells of Jamaicana flava (reviewed by Maddrell, 1971). In addition in some species, for example Gryllus domesticus (Berkaloff, 1960), Drosophila (Wessing, 1966) and Carausius morosus (Taylor, 1971a), the invaginated basal cell membrane is reported to be the site of pinocytotic activity. Although no evidence of pinocytotic activity was observed in this region of the primary cells of Jamaicana flava, small vesicles were present within the fingers of cytoplasm. In this respect

## Figs. 55 - 57

Diagrams showing the main types of cell and their features, found in the Malpighian tubules and ileum in Jamaicana flava.

## Figs. 55

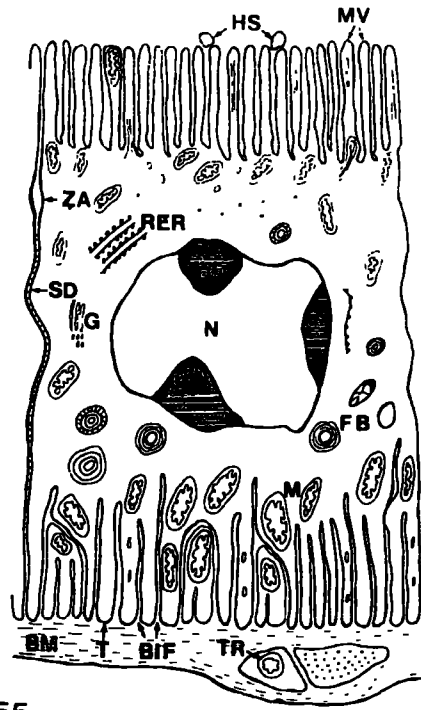
Malpighian tubule; primary cell. BIF, infoldings of basal plasma membrane, BM, basement membrane; FB, formed bodies; G, Golgi body; HS, husks; M, mitochondria; MV, microvilli; N, nucleus; RER, rough endoplasmic reticulum; SD, septate desmosome; T, electron dense tip of basal infolding; TR, trachea; ZA, zonula adhaerens.

## Fig. 56

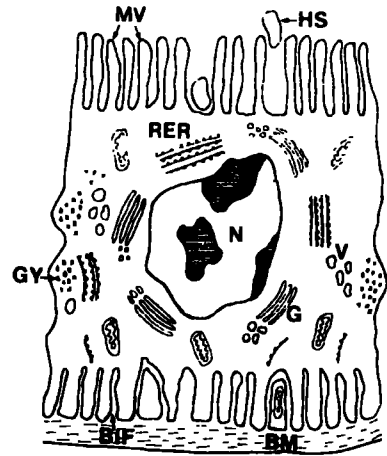
Malpighian tubule; secondary cell; BIF, infoldings of basal cell membrane; BM, basement membrane; G, Golgi body; GY, glycogen like granules; HS, husk; MV, microvilli; N, nucleus; RER, rough endoplasmic reticulum; V, vacuoles.

## Fig. 57

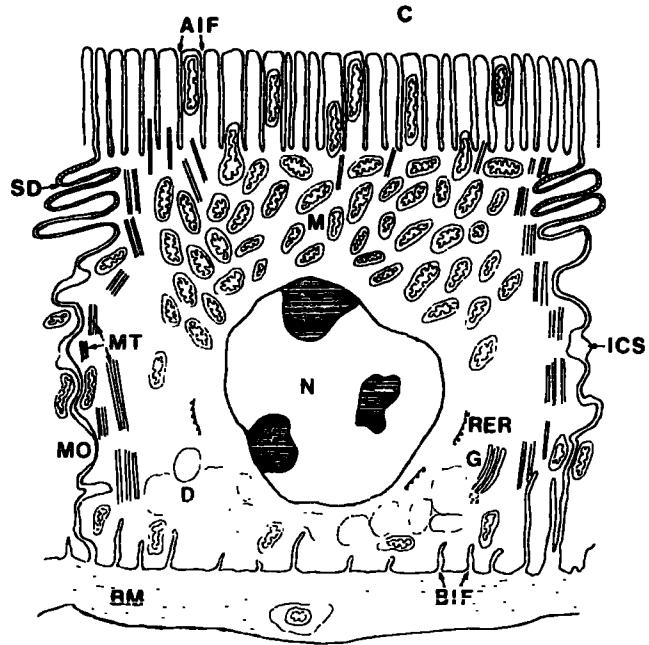
Large ileal cell. AIF and BIF, infoldings of apical and basal plasma membranes respectively; BM, basement membrane; C, cuticle; D, droplets; G, Golgi body; ICS, intercellular space; M, mitochondria; MO, macula occludens; MT, microtubules; N, nucleus; RER, rough endoplasmic reticulum; SD, septate desmosome.



55



56



57

Figs. 55 - 57

the primary cells resemble their counterparts in Calliphora erythrocephala (Berridge and Oschman, 1969) and Calpodes ethlias (Locke and Collins, 1967). Furthermore it is significant that the tubules of Gryllus, Drosophila and Carausius take up large molecules such as horse radish peroxidase, colloids and proteins, whereas the tubules of Calliphora and Calpodes do not. The ability to take up large molecules, most probably reflects differences in the permeability of the basement membrane. Further studies are required to determine whether the membrane is permeable to large molecules in these cells of Jamaicana flava.

The laminated spheres observed in the primary cells are precisely the same in character as those found in the cells of the upper segment of Rhodnius prolixus Malpighian tubules (Wigglesworth and Salpeter, 1962) and the cells of the tubules of Gryllus, Xylocopus and Bombus (Berkaloff, 1958, 1959). Wigglesworth and Salpeter (1963) point out that 'these spheres are not uric acid since they persist in osmicated preparations, whereas uratic granules are soluble in aqueous fixatives and quickly dissolve in osmium'. On the other hand Berkaloff (1958) regards the granules as being composed of uric acid, calcium urate and perhaps phosphate and suggests that these formed urates are discharged into the lumen by merocrine secretion. Berkaloff (1958) however, notes that the uratic spheres in the lumen are dissolved and appear in electron micrographs as husks, as they do in Jamaicana flava.

In addition to the laminated spheres, there are other types of body in which membranes predominate. Wigglesworth and Salpeter (1962) observed similar structures in the cells of the distal region of Rhodnius prolixus tubules and suggested they are stages in the degradation of mitochondria. The observations of the present study offer



neither support or criticism of this suggestion.

The apical and basal elaborations of the cell membranes and their association with mitochondria, are features characterising other cells noted for the transport of water and solutes. Mammalian proximal kidney tubule (Tisher, Bulger and Valtin, 1971) and insect salivary gland (Berridge and Oschman, 1969) both possess similar features. A number of functions have been assigned to the membrane elaborations including: 1. an increase in the permeability of the membrane to water (Pease, 1956) and solutes (Taylor 1971a). 2. enables mitochondria to be brought close to a large membrane surface (Taylor, 1971a), 3. provides a large surface area for the location of active transport sites (Fawcett, 1962), 4. establishes specific geometric conditions that allow osmotic coupling of water and solute transport (Diamond and Bossert, 1967, 1968).

Whilst 1-3 are attractive possibilities, physiologists now regard the geometry of the elaborations as being of particular significance in understanding the mechanism, whereby fluid moves from the haemolymph across the epithelial cells into the lumen of the tubule. Berridge and Oschman (1969) and Oschman and Berridge (1971) have discussed the application of the standing gradient hypothesis of Diamond and Bossert, (1967) to the Malpighian tubule cells of insects. These authors observe that 'fluid entering the basal (backward) channel (see Chapter One, Fig.3) is isotonic to the blood but becomes progressively hypotonic as it approaches the closed ends, due to active solute uptake into the cell. As the fluid becomes hypotonic a favourable gradient is created for passive flow of water from the channels into the cytoplasm. Since the apical cell surface is highly folded a similar mechanism may operate here, to couple solute transport to water movement from the cell into

the lumen. Solutes are pumped into the narrow channels between microvilli and establish standing gradients that would draw water out of the cell. Since these channels open in the direction of fluid flow they are analogous to forward channels such as the intercellular spaces of resorptive epithelia'.

In the present study, the dimensions of the surface elaborations of the primary cells agrees well with their counterparts in Calliphora erythrocephala (5 - 10  $\mu\text{m}$ ) (Berridge and Oschman, 1969) so that at least from a geometric point of view the apical and basal channels of the primary cells of Jamaicana flava would favour a standing osmotic gradient type of mechanism for fluid transport. Moreover Oschman and Berridge (1969) reason that the physiological parameters of Calliphoran Malpighian tubules support a mechanism invoking standing gradients along the channels.

Two kinds of cell have also been identified in the Malpighian tubules of other insects. Martoja (1959, 1961) demonstrated histochemically, mucus containing cells in the tubules of several Orthoptera, including Locusta migratoria. Such cells are interspersed with the larger epithelial cells along the length of the tubule, in much the same way as the cells, identified by the lead/glutaraldehyde procedure, are distributed along the tubules of Locusta migratoria. In addition, small isolated cells have been identified in the Malpighian tubules of Coleoptera and Lepidoptera (Wigglesworth, 1965) whilst Berridge and Oschman (1969) observed small stellate cells, intermingling with larger epithelial cells in the tubules of Calliphora erythrocephala. Taylor (1971b) has also shown two types of cell in the Malpighian tubules of Carausius morosus.

The secondary cells possess features found in both transporting

and secretory cells. Apical and basal membrane elaborations occur in cells noted for water and/or solute transport, whilst extensive systems of rough endoplasmic reticulum, Golgi bodies and vacuoles characterise secretory cells. Two main functions have been suggested for the secondary cells, the secretion of mucus into the lumen (Martoja 1956, 1961; Berkaloff, 1960; Gabe, 1962) and the resorption of ions and water from the lumen (Berridge and Oschman, 1969).

Wigglesworth (1965), Martoja (1959, 1961), Mazzi and Baccetti (1957) and Berkaloff (1960) have identified by histochemical methods, cells in the Malpighian tubules of various Orthoptera that contain acid mucopolysaccharide in addition to a high RNA content. Similarly, Gabe (1962) reports mucopolysaccharides in some cells of the Malpighian tubules of Isoptera. Whilst histochemical tests were not carried out in the present study, the abundance of rough endoplasmic reticulum and Golgi body in the secondary cells, is in keeping with a synthetic or secretory role, the end product of which may be mucus. Taylor (1971b) reaches a similar conclusion from his study of the 'Type II cells' of Carausius morosus Malpighian tubules and, in keeping with the suggestion of Gabe (1962), considers the mucus to be a lubricant, assisting the passage of faeces along the gut. Berridge and Oschman (1969) have suggested that the stellate cells of Calliphora erythrocephala are involved in the resorption of sodium ions from the lumen of the Malpighian tubule and that the resorption is responsible for the low sodium content of the tubule fluid. Taylor (1971b) has pointed out that the low sodium/potassium ratio found in the distal region of the tubules of Carausius morosus (Ramsay, 1955b) and the high ratio of these ions in the proximal region of the tubules, correlates, with an increase in the frequency of Type II cells from the proximal to the distal region of the tubule. Taylor further

suggests that the characteristics of the tubule fluid, in the distal region, may be brought about by either resorption of sodium or secretion of potassium ions. Furthermore, Berridge and Oschman (1969) noted that in Rhodnius prolixus the fluid from the distal region of the Malpighian tubules, where secondary cell types are absent, is rich in sodium ions but that fluid from the proximal region, where secondary cells are present, contains very little sodium.

In Jamaicana flava the part played by the secondary cells in very active resorption of ions is doubtful, since very few mitochondria are found in the cytoplasm of these cells. Those that are present, do not associate with the invaginations of the apical and basal plasma membranes. However, this does not preclude a passive entry of cations into the secondary cells, since it has been shown in a variety of insects that the tubule lumen is positive to the haemolymph (Maddrell, 1971).

The electron microscopic structure of the columnar cells of the ileum is schematically represented in Figure 57. These cells are characterised by; a highly invaginated apical plasma membrane, the infoldings of which penetrate deep into the cell, slightly infolded lateral and basal cell membranes, the bulk of the mitochondria are associated with the apical membrane and the region beneath it, extensive microtubule system in the lateral, and to a lesser extent in the apical region of the cell, septate desmosomes which are restricted to the apical region, tight junctions in the basal region between adjacent lateral cell membranes, a subepithelial sinus and lipid like droplets in the basal region of the cells. Physiological studies of the hindgut of several insect species (Wigglesworth, 1965) have shown that the material in the gut, becomes progressively drier as it passes along the ileum and rectum. Ramsay (1955a) showed that for Carausius morosus, fluid could be reabsorbed from the gut lumen, by the

cells of the ileum. In the ileal cells of Jamaicana flava, mitochondria do not associate with the lateral cell membranes to the same extent as they do in the rectal pad cells of this species. Instead these organelles are associated with the highly invaginated apical plasma membrane. This membrane has a particulate layer on the cytoplasmic side of the infoldings, a feature commonly found in cells engaged in solute and fluid transport (Berridge and Oschman, 1972). The distribution of mitochondria within the ileal cells suggests the apical, rather than the lateral or basal surfaces as the active regions with high energy demands. In addition, the apical infoldings are far more extensive than their counterparts in the rectum. This again suggests the apical, rather than the lateral or basal surface as the important one on these cells.

The relationship between mitochondria, membrane, particularly the lateral plasma membrane and intercellular space is considered to be important in the movement of water from the lumen of the gut to the blood (Oschman and Wall, 1969). The extent of the mitochondria lateral membrane relationship in the ileal cells, implies that the movement of water across the cells, via the lateral intercellular spaces, is of minor or secondary importance.

Recently, Wall (1970) has shown that the hyperosmotic fluid produced by the Malpighian tubules of Periplaneta americana, is hyposmotic when it enters the rectum. This suggests that solute is absorbed from the luminal contents, by the hindgut anterior to the rectum. Whether the solutes are returned immediately to the blood or stored in the cells of the hindgut, is unknown. In this respect, the 'lipid like' droplets occurring in the basal regions of the ileal cells are of interest. They may represent storage products, perhaps accumulating

from the activity at the apical surface. On the other hand the droplets, may constitute an energy reservoir for the large numbers of mitochondria found in the cells. Clearly, further physiological study of the hindgut anterior to the rectum is required.

The recta of several terrestrial species of insect Calliphora erythrocephala (Berridge and Gupta, 1967) Periplaneta americana (Oschman and Wall, 1969), Aedes aegypti (Hopkins, 1967) and Schistocerca gregaria (Phillips, 1970) are all characterised by having epithelia, thickened in parts to produce pads or papillae, separated by regions of reduced or simple rectal cells. In Jamaicana flava and Locusta migratoria the rectal epithelium is thickened in six regions to form rectal pads. The electron microscopic structure of the rectal pad cells of Jamaicana flava, is shown diagrammatically in Figure 58. The corresponding cells of Locusta migratoria possess similar features. As in the terrestrial species above, these cells are characterised by: a layer of cuticle adjacent to the apical plasma membrane, moderate infolding of the apical plasma membrane, a flat basal cell membrane, in which pinocytotic activity is evident, extensively infolded lateral cell membranes, extensive system of extracellular channels and spaces between adjacent cells, regions of septate desmosomes restricted to the apical and basal borders of adjacent cells, basement membrane containing trachea and tracheoles and a subepithelial sinus between the basement membrane and the surrounding muscle layer. Many of these features are also found in other epithelia noted for ion and water transport including vertebrate kidney (Pease, 1956) and gall bladder (Tormey and Diamond, 1967).

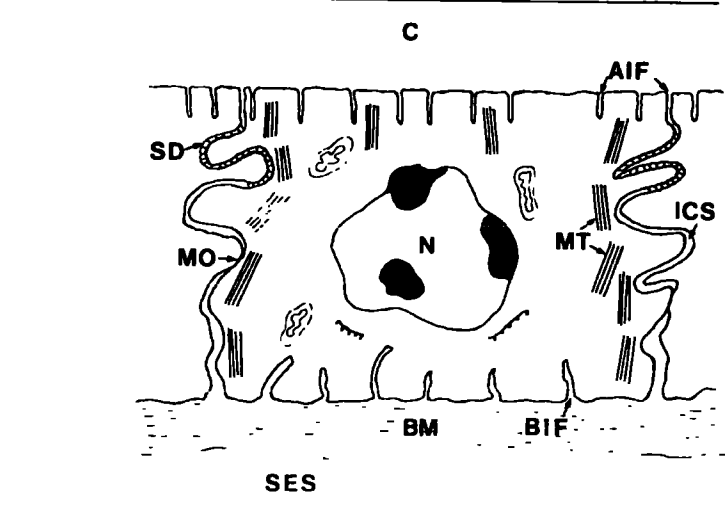
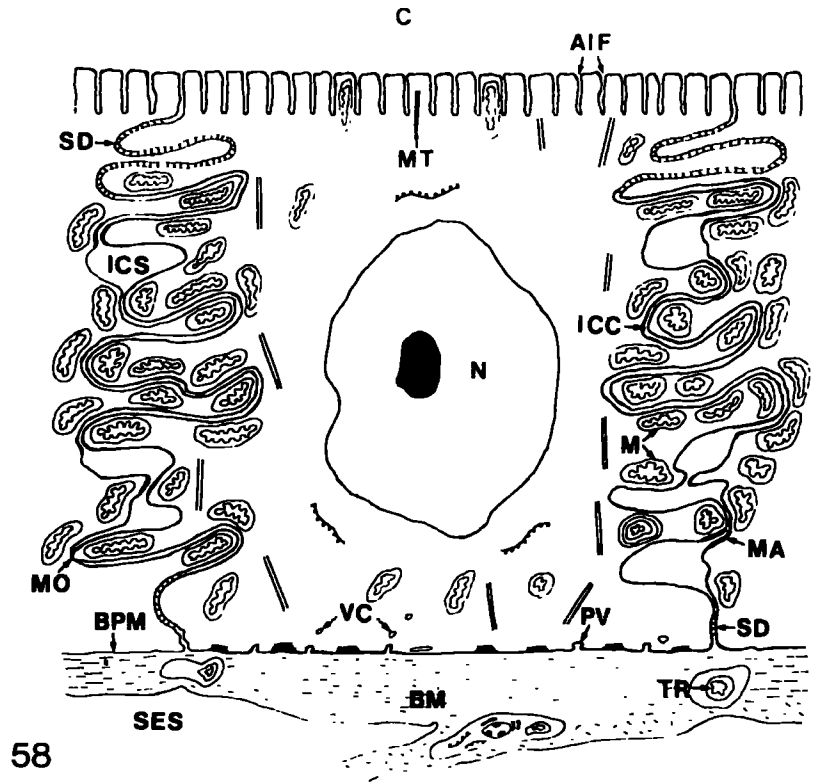
Recently much progress has been made in linking the structure of transporting cells to their function (reviewed by Berridge and Oschman, 1972). In this respect the features listed above, are considered to be important in the absorptive role of the rectum. Phillips and Dockrill (1968)

Fig. 58

Rectal pad cell. AIF, infoldings of apical plasma membrane; BM, basement membrane; BPM, basal plasma membrane; C, cuticle; ICC, intercellular channel; ICS, intercellular space; M, mitochondria; MA, macula adhaerens; MO, macula occludens; MT, microtubules; N, nucleus; PN, pinocytotic vesicles; SD, septate desmosome; SES, subepithelial sinus; TR, trachea; VC, vesicles

Fig. 59

Reduced epithelial cell of rectum. AIF and BIF, infoldings of apical and basal plasma membranes respectively; BM, basement membrane; C, cuticle; ICS, intercellular space, MO, macula occludens; MT, microtubules; SD, septate desmosome; SES, subepithelial sinus.



Figs. 58 - 59



have shown that the rectal cuticle in Schistocerca gregaria acts as a passive molecular sieve, allowing small molecules, water and ions to move between the rectal lumen and the rectal pad cells, but preventing large molecules from doing so. The latter pass out in the faeces. Similar studies on the rectal cuticle of Jamaicana flava would determine whether the cuticle plays a similar role in this insect. The distribution of mitochondria within the rectal pad cells of both Jamaicana flava and Locusta migratoria, indicates that the lateral, and to a lesser extent, the apical plasma membranes, are regions of high energy demands. The infoldings of the apical plasma membrane may, as suggested by Pease (1956) serve to increase the surface area of the cells. On the other hand Diamond and Bossert (1967, 1968) have proposed that the geometry of such infoldings is important in the transport of ions and water from the lumen into the cells. From their studies of the rectum of Periplaneta americana Oschman and Wall (1969) suggested that standing gradients could account for the coupling of solutes and water in the apical region of the rectal pad cells in a manner similar to that described above for the basal infoldings of Malpighian tubule cells. The mitochondria would provide the energy for the coupling and the particulate layer of the membranes may be involved in some as yet unexplained way (Oschman and Wall, 1969). The lateral regions of the rectal pad cells vary in complexity between species. Figure 60 shows the types of association that have so far been found between mitochondria and lateral cell membranes of insect rectal cells. In the termite Cephalotermes rectangularis (Noirot-Timothee and Noirot, 1967) (Fig. 60,A) the lateral cell membranes are not extensively infolded and the mitochondria lie opposite one another in the cytoplasm of adjacent cell membranes. In Periplaneta americana (Oschman and Wall, 1969) the lateral cell membranes are infolded and engulf mitochondria as illustrated in

Figure 60(B), whilst in the mosquito Aedes aegypti (Hopkins, 1967) the infolding of the lateral membranes is more extensive, so that several infolded membranes and associated channels are in close proximity to a mitochondrion (Fig.60(C)). This condition is taken a step further in Calliphora erythrocephala (Berridge and Gupta, 1967), where the lateral cell membranes are infolded to form membrane stacks with which mitochondria are associated (Fig.60(D)). It will be seen that the lateral regions of the rectal pad cells of Jamaicana flava and Locusta migratoria, closely resemble those of Periplaneta americana. The association between the lateral cell membrane and the mitochondria has been referred to by Oschman and Wall (1969) as a 'plasmalemma mitochondrial complex' or 'PMC'.

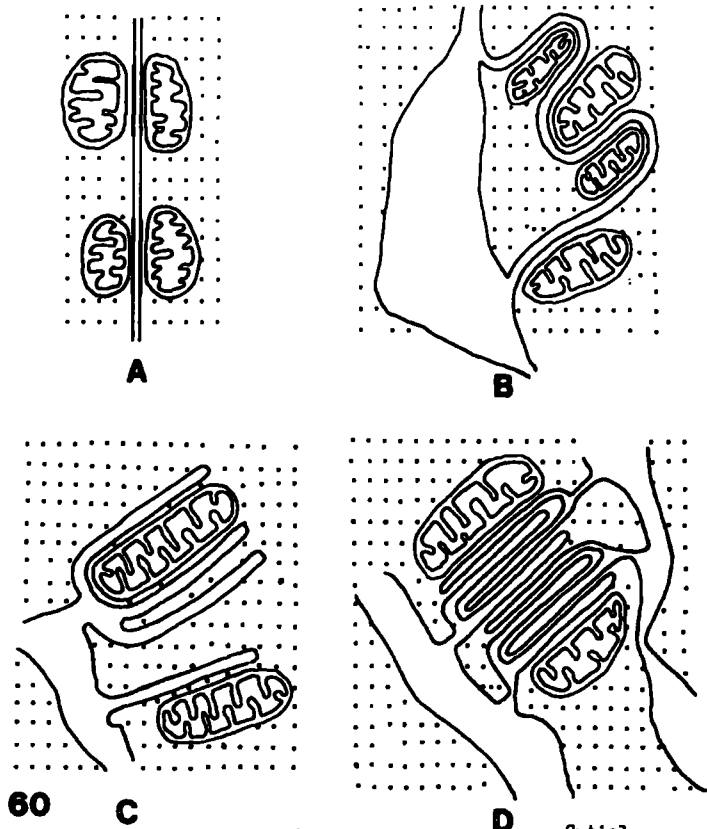
Berridge and Gupta (1967) and Oschman and Wall (1969) consider the relationship between the lateral cell membrane and the mitochondria to be of crucial importance in the function of the rectum. These authors have discussed rectal structure, particularly the role of the intercellular channels and spaces, in relation to the hypotheses advanced by Curran and MacIntosh (1962) and Diamond and Bossert (1967, 1968) (see Chapter One for details of the hypotheses). They conclude that the rectal papillae of Calliphora erythrocephala and the rectal pads of Periplaneta americana are structurally similar to the double membrane model of Curran and MacIntosh (1962), and as proposed by Diamond and Bossert (1968), suggest that water flows passively out of the cell into the intercellular spaces, in response to local osmotic gradients, created by the active transport of solute into them. Figure 61 shows the model of fluid transport in the rectal pads of Periplaneta americana proposed by Oschman and Wall (1969) (Figure redrawn from Maddrell, 1971). The replenishment of ions can come either from the rectal lumen i.e. across

Fig. 60

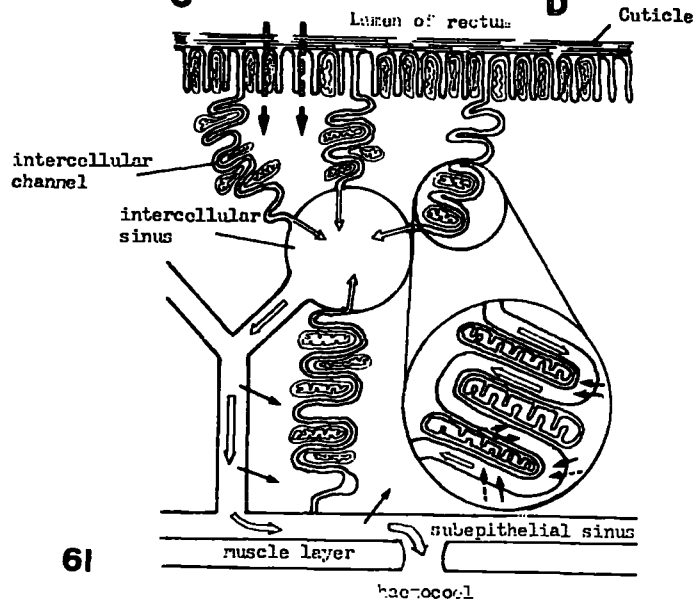
Plasmalemma-mitochondrial complexes of the lateral membranes of transporting epithelia in insects. (A) the termite, Cephalotermes rectangularis (Noirot-Timothee and Noirot, 1967). (B) the cockroach, Periplaneta americana (Oschman and Wall, 1969). (C) the mosquito, Aedes aegypti (Hopkins, 1967). (D) the blowfly, Calliphora erythrocephala (Berridge and Gupta, 1967). (redrawn from Oschman and Wall, 1969).

Fig. 61

Hypothetical scheme to explain water absorption from the rectum. The drawing is based on that of Oschman and Wall (1969) of Periplaneta americana but the general scheme would apply to Calliphora erythrocephala, Locusta migratoria and Jamaicana flava. The solid continuous arrows refer to active movements of solutes, the solid broken arrows to passive movements of water and the open continuous arrows to fluid movements in the extracellular spaces (redrawn from Maddrell, 1971).



60



61

Figs. 60 and 61

the apical region of the cells, or, in those species that transport water when no ions are present in the lumen e.g. Schistocerca gregaria (Phillips, 1964a), from the subepithelial sinus. The model proposed by Oschman and Wall (1969) for the rectal pads of Periplaneta americana can be directly applied to the rectal pads of Jamaicana flava. There are sufficient similarities in structure for this to be so. It is suggested that the narrow channels in the lateral region of the cells contain fluid that is hyperosmotic to the cell. Water would then flow out of the cell into the channels. The fluid of the channels would drain into the large intercellular spaces with replenishment of ions and water coming via one of the routes described above. As in Periplaneta the fluid in the intercellular spaces and channels of the rectal pad cells of Jamaicana flava is probably prevented from entering the rectal lumen and the subepithelial space, by the presence of septate desmosomes at the apical and basal borders of the cell. The studies of Lowenstein and Kanno (1964) have shown this type of junction to act as a barrier to the passage of fluid out of the intercellular space. Thus the question arises how the fluid in the intercellular space is returned to the haemolymph? Oschman and Wall (1969) have pointed out that, in Periplaneta americana, access to the haemolymph from the subepithelial space, is limited to points of penetration of trachea and even here, a one way muscle valve operates favouring passage of fluid into the blood. A similar arrangement was described for Calliphora erythrocephala (Berridge and Gupta, 1967) although in this species a single tracheal trunk penetrates the surrounding muscle layer. Although further work is required to clarify the relationship between the trachea, rectal pad cells subepithelial sinus and haemolymph in Jamaicana flava, the return of fluid to the blood, via the intercellular spaces round the trachea, is an

attractive possibility.

The distribution of solute pumps along the extracellular channels would seem to be important in the formation of local osmotic gradients. In this respect the histochemical localization of possible pumping mechanisms is of interest. The Wachstein and Meisel (1957) and McClurkin (1964) methods of demonstrating  $\text{Na}^+ - \text{K}^+$  ATPase, rely on the principle that inorganic phosphate released from the hydrolysis of ATP, is captured by a lead salt, resulting in the precipitation of insoluble lead phosphate. The location of lead phosphate is assumed to be the active centre of the enzyme. Recently Tormey (1966), Moses, Rosenthal, Beaver and Schuffman (1966) and Ernst (1972) have questioned the validity of results obtained using lead salts as capture agents. These workers confirmed the earlier findings of Bonting, Caravaggio and Hawkins (1962) that lead ions, as well as the fixation process, effectively inactivates the  $\text{Na}^+ - \text{K}^+$  ATPase in renal preparations. Furthermore, lead ions were found to facilitate non-enzymatic hydrolysis of ATP. These factors together with undetermined effects of fixation most probably are responsible for the variation in the amount of lead phosphate deposited.

Sections incubated with magnesium, sodium and potassium ions rarely showed an increase in the quantity of reaction product over preparations incubated with magnesium ions alone. Ouabain was however, without effect on such preparations. Therefore the deposits are interpreted as being sites of  $\text{Mg}^{2+}$  ATPase activity. Tormey (1966) observed that  $\text{Mg}^{2+}$  ATPases have been demonstrated in membranes thought to be intimately involved in active transcellular transport. However the relationship of this ATPase to the 'pump'  $\text{Na}^+ - \text{K}^+$  ATPase is unclear, since the studies of Nakao, Tashima, Nagano and Nakao (1965), Bowler and Duncan (1968) and Charnock, Cook, Almeida and To (1973) suggest that  $\text{Mg}^{2+}$  ATPase and  $\text{Na}^+ - \text{K}^+$  ATPase may well be different enzymes.

## CHAPTER SIX

BIOCHEMICAL STUDIES ON THE EXCRETORY SYSTEM OF SEVERAL  
ORTHOPTERAN INSECTSIntroduction

The transport of sodium and potassium ions in many secretory and absorptive tissues is known to involve a sodium/potassium exchange pump (see Whittam and Wheeler, 1970 for references). Furthermore, the activity of the cation transport mechanisms in a particular tissue, appears to be correlated with the activity of a particular ATPase enzyme system (Whittam, 1958; Caldwell, Hodgkin, Keynes and Shaw, 1960). This ATPase system has two components. One is dependent only on magnesium ions and is not inhibited by the cardiac glycoside ouabain ( $Mg^{2+}$  ATPase), and which has been suggested by Duncan (1967) and Bowler and Duncan (1967) to be responsible for the control of passive permeability of cells and by Wins and Schoffeniels (1966) and Schatzmann and Vincenzi (1969) to be involved in transport of calcium ions. The second enzyme is sodium, potassium dependent and inhibited by ouabain ( $Na^+ - K^+$  ATPase). It is the latter enzyme which has been implicated in the active transport of the monovalent cations, sodium and potassium (see reviews by Skou, 1965; Whittam and Wheeler, 1970).

$Na^+ - K^+$  ATPase activity has been demonstrated in homogenates of a wide variety of tissues which transport those ions. Nervous tissue is a very rich source of this enzyme (Nakao, Tashima, Nagano and Nakao, 1965) as is vertebrate kidney (Chignell and Titus, 1966; Skou, 1962). The plasma membrane of erythrocytes (Whittam and Ager, 1964; Rega, Garrahan and Pouchan, 1970), skeletal muscle (Peter, 1970; Parkin, 1975) and many other vertebrate tissues have been shown to possess this enzyme in a membrane particulate fraction. It has been isolated from a variety of

other organisms too, for example fish gill (Epstein, Katz and Pickford, 1967; Kamiya and Utida, 1968), crustacean gill (Philippot, Thuet and Thuet, 1972), crustacean nerve (Skou, 1957) and muscle (Bowler and Duncan, 1967; Bowler, Gladwell and Duncan, 1973).

The only works on  $\text{Na}^+ - \text{K}^+$ ATPase from insect tissues are those of Grasso, (1967) on cockroach nerve cord, Cheng and Cutkomp (1972) on honeybee C.N.S. preparation and Koch, Cutkomp and Do (1969) on cockroach muscle. Wareham, Duncam and Bowler (1968) however, report using sucrose extracted microsomes, the lack of a synergistic  $\text{Na}^+ - \text{K}^+$ ATPase in cockroach muscle microsomes. This contradiction is resolved by the fact that the preparation used by Koch, Cutkomp and Do (1969) was not microsomal (see below for definition of microsome), sedimenting at 13,000xg and described by them as containing mitochondria and nerve endings. Moreover, further work has demonstrated the presence of this enzyme in insect muscle, using mannitol homogenised and sodium iodide extracted microsomes (Bowler personal communication). Although a monovalent cation transport ATPase would be predicted for Malpighian tubules and rectum of insects when argued these structures are analogous to vertebrate kidney, no  $\text{Na}^+ - \text{K}^+$ ATPase could be demonstrated by Berridge and Gupta (1968) although they reported a  $\text{Mg}^{2+}$ ATPase to be present.

Many workers have used techniques essentially developed for mammalian tissues, that is homogenization in buffered sucrose (usually 0.25 M) containing a chelating agent such as ethylenediaminetetra-acetic acid (EDTA) from the resulting homogenate was then obtained, either microsomal preparations (Grasso, 1967; Berridge and Gupta, 1968) or mitochondrial fraction, containing nerve endings (Koch, Cutkomp and Do, 1969).

Perhaps the paucity of literature and the failure of some researchers



to demonstrate  $\text{Na}^+ - \text{K}^+$ ATPase activity in certain insect tissues, has resulted from inadequacies of their preparative technique. Indeed, buffered sucrose media have been replaced, or modified, in search for purified membrane preparations rich in  $\text{Na}^+ - \text{K}^+$ ATPase, from a wide variety of tissues. Skou (1962) showed that the addition of sodium deoxycholate to the homogenization medium raised the ratio  $\frac{\text{total } \text{Na}^+ - \text{K}^+\text{ATPase}}{\text{Mg}^{2+}\text{ATPase}}$  activity, from less than two, to between four and six times. Other work by Nakao, Nagano, Adachi and Nakao (1963) has illustrated the effectiveness of high concentrations of sodium iodide in producing preparations which have strong  $\text{Na}^+ - \text{K}^+$ ATPase activity. Using both deoxycholate and sodium iodide, Robinson (1967) has shown that such a combination increases the  $\frac{\text{Na}^+ - \text{K}^+\text{ATPase}}{\text{Mg}^{2+}\text{ATPase}}$  ratio considerably more than when deoxycholate alone was used. Ahmed and Judah (1964) observed that mannitol instead of sucrose in the homogenization medium was important in obtaining  $\text{Na}^+ - \text{K}^+$ ATPase activity from a liver preparation. Parkin (1975) isolated this enzyme from rat diaphragm muscle, following unsuccessful attempts to demonstrate it using buffered sucrose and sucrose deoxycholate media, by homogenization in a mannitol, deoxycholate medium, after which the preparation underwent a short period of extraction in 2 M sodium iodide.

The source of the microsomes, together with the extraction method employed in obtaining them, do not appear to markedly affect the properties of  $\text{Na}^+ - \text{K}^+$ ATPase. Moreover, it seems that the enzyme systems retain very similar properties to the extent that Skou (1965) lists several criteria which an enzyme must obey before being assigned to this type. These are:- 1.  $\text{Na}^+ - \text{K}^+$ ATPase activity must be shown in the microsomal fraction of the tissue. 2. It utilizes ATP almost exclusively as a substrate, which it hydrolyses to ADP and inorganic

phosphate:  $\text{ATP}^{4-} + \text{H}_2\text{O} = \text{ADP}^{3-} + \text{HPO}_3^{2-} + \text{H}^+$ . 3. The enzyme system requires magnesium ions and it has been suggested that the substrate is an magnesium/ATP complex. 4. For maximal activity the enzyme requires the simultaneous presence of sodium and potassium ions. 5. The response due to sodium and potassium ions is abolished by the presence of the cardiac glycoside, ouabain. 6. There must be close correlation between the effect of cardiac glycosides on the cation transport in the intact cell and their effect on this enzyme system. 7. The hydrolysis of ATP proceeds at a rate dependent on the concentration of sodium ions inside the cell and also on the concentration of potassium ions outside the cell. Usually these enzymes have a sodium ion site on the internal face of the membrane and a potassium ion site on the outer face. Although the mechanism by which this enzyme functions in sodium and potassium transport is unclear, the hydrolysis of ATP somehow causes translocation of the cations across the membrane. Charnock and Post (1963) and Post, Sen and Rosenthal (1965) have shown that the hydrolysis of the substrate complex is accomplished in two steps. The first is sodium dependent and involves the transfer of the terminal phosphate of ATP to the protein of the  $\text{Na}^+ - \text{K}^+$ ATPase and a second, potassium dependent and involves dephosphorylation of the protein. Other studies by Nagano, Kanazawa, Nizuno, Tashima, Nakao and Nakao (1965) and Badger, Sen and Post (1966), indicate that the phosphorylated intermediate is an acylphosphate.

The biochemical studies on  $\text{Na}^+ - \text{K}^+$ ATPases have, in the main, been carried out on subcellular fractions obtained by differential centrifugation of cell homogenates. These fractions are usually referred to as microsomal preparations. They are more precisely defined by Siekevitz (1965) as the "high speed pellet (100,000-250,000 xg for

60-120 min) resulting when the supernatant fluid from the mitochondrial fraction is sedimented". The reports in the literature indicate that the microsomal pellets do not originate from one subcellular organelle but are mainly derived from the cell's membranous components. From their work on rat liver microsomes Manitius, Bensch and Epstein (1968), consider the fraction to be derived from the plasma membranes, whilst Landon and Norris (1963) assumed from their ultrastructural studies that  $\text{Na}^+ - \text{K}^+$ ATPase activity was located in the endoplasmic reticulum. On the other hand Katz and Epstein (1967), who also worked with kidney microsomes, were uncertain about the location of  $\text{Na}^+ - \text{K}^+$ ATPase at the subcellular level. Thus it can only be inferred that the  $\text{Na}^+ - \text{K}^+$ ATPase activity resides in the cell's membranous components. While the reports of microsomal structure for vertebrates are relatively numerous there are only two for insect preparations (Brindley, 1966; Cassidy, Smith and Hodgson, 1969). Cassidy, Smith and Hodgson (1969) worked with the abdomen, gut and fat body microsomal preparations, from two adult species of insect and concluded that the buffering system, centrifugation speed, centrifugation time and species influenced the composition of the microsomal fraction.

The recent work of Peacock, Bowler and Anstee (1972) has demonstrated a ouabain sensitive  $\text{Na}^+ - \text{K}^+$ ATPase in preparations from the Malpighian tubules and hindgut of several insect species. Furthermore, Anstee and Bell (1975) in contrast to Berridge (1968) and Pilcher (1970b) have shown that ouabain, when present in the medium (at pharmacological concentrations) bathing the tubules of Locusta migratoria reduces urine production to a very low level. In addition, Mordue (1969) has shown that water flow across the Malpighian tubules of locusts is also reduced by interference with the stomatogastric system, whilst Clarke and Anstee (1971) observed alterations in the activity of certain enzymes following frontal

ganglionectomy. Thus it was of interest to determine the effect of interference with the stomatogastric nervous system on the ouabain sensitive  $\text{Na}^+ - \text{K}^+$ ATPase of Malpighian tubules as well as the ileum and rectum.

In the present study four species of insect were used. These were Homorocoryphus nitidulus vicinus, Jamaicana flava, Locusta migratoria migratorioides and Schistocerca gregaria. The initial extraction procedures were performed with the classical buffered sucrose technique, developed for nervous tissue by Skou (1957). However, for reasons given above, all subsequent preparations were isolated using a modified version of the mannitol, deoxycholate and sodium iodide technique.

The aim of the present work was four-fold: 1. to extract and examine the distribution of  $\text{Na}^+ - \text{K}^+$ ATPase in the Malpighian tubules and hindguts of several insect species. 2. to determine the properties of the extracted  $\text{Na}^+ - \text{K}^+$ ATPase. 3. to carry out an electron microscopic examination of the membrane fractions. 4. to determine the effect of surgical interference with the stomatogastric nervous system on  $\text{Na}^+ - \text{K}^+$ ATPase activity of microsomal preparations from the Malpighian tubules, ileum and rectum.

#### Materials and Methods

##### 1. Dissection and isolation of excretory system.

Approximately equal numbers of sexually mature male and female individuals, numbering eight-twelve in total, were used for each experiment. The Malpighian tubules and hindgut were dissected from the severed abdomen under the appropriate ice cold homogenization medium in a glass petri dish, surrounded by ice. The hindgut was washed

free of gut contents and transferred to a known volume of homogenization medium in a homogenization tube surrounded by ice. The medium in the petri dish was replaced afresh after every fourth dissection. The latter step was a precaution against contamination of the preparation by gut contents.

In a second set of experiments the excretory system was separated into Malpighian tubules, ileum and rectum. This operation was performed in ice cold homogenization medium, after dissecting out the excretory system from the abdomen. The midgut was removed anterior to the junction of the Malpighian tubules with the alimentary tract. The tubules were separated at the posterior margin of this junction. The ileum was removed from the rectum at the anterior tips of the rectal pads. After removal, each part of the excretory system was placed in a separate volume of homogenization medium in a homogenization tube surrounded by ice.

## 2. Homogenization and centrifugation procedures

i. Homogenization was performed by twelve passes of the plunger at 1000 rpm, in a Potter - Elvehjem homogenizer with a teflon pestle, clearance, 0.1 - 0.15 mm. The homogenizer was surrounded by ice throughout this procedure.

ii. Programme for sucrose extraction of microsomes.

(a) Homogenization medium

250 mM Sucrose

1 mM Ethylenediaminetetra-acetic acid (sodium salt)

50 mM Histidine (pH 7.25)

Homogenization was performed in 15 ml of homogenization medium and then diluted to 25 ml with the same buffered sucrose.

## (b) Centrifugation

The homogenate was transferred to thin walled polypropylene centrifuge tubes and centrifuged at 2000 xg for 10 min at 0°C in a MSE 2L. The pellet was discarded and the supernatant was then centrifuged at 12,500 xg for 20 min at 0°C in a MSE HS 18, head number 69182 and again the pellet was discarded. The supernatant was respun at 12,500 xg and the pellet discarded. The resulting supernatant was then spun at 107,000 xg for 60 min at 0°C in a MSE Automatic Superspeed 40, head number 2409, and the final pellet resuspended in an appropriate volume of resuspension medium (see below). The steps involved in centrifugation are shown diagrammatically in Figure 1 (A).

(iii) Programme for mannitol, deoxycholate and sodium iodide extraction of microsomes.

## (a) Homogenization medium

250 mM Mannitol

0.5 mM Ethylenediaminetetra - acetic acid (sodium salt)

30 mM Histidine

0.1 % Disodium deoxycholate.

## (b) Extraction medium

4 M NaI

5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O

10 mM Ethylenediaminetetra-acetic acid (sodium salt)

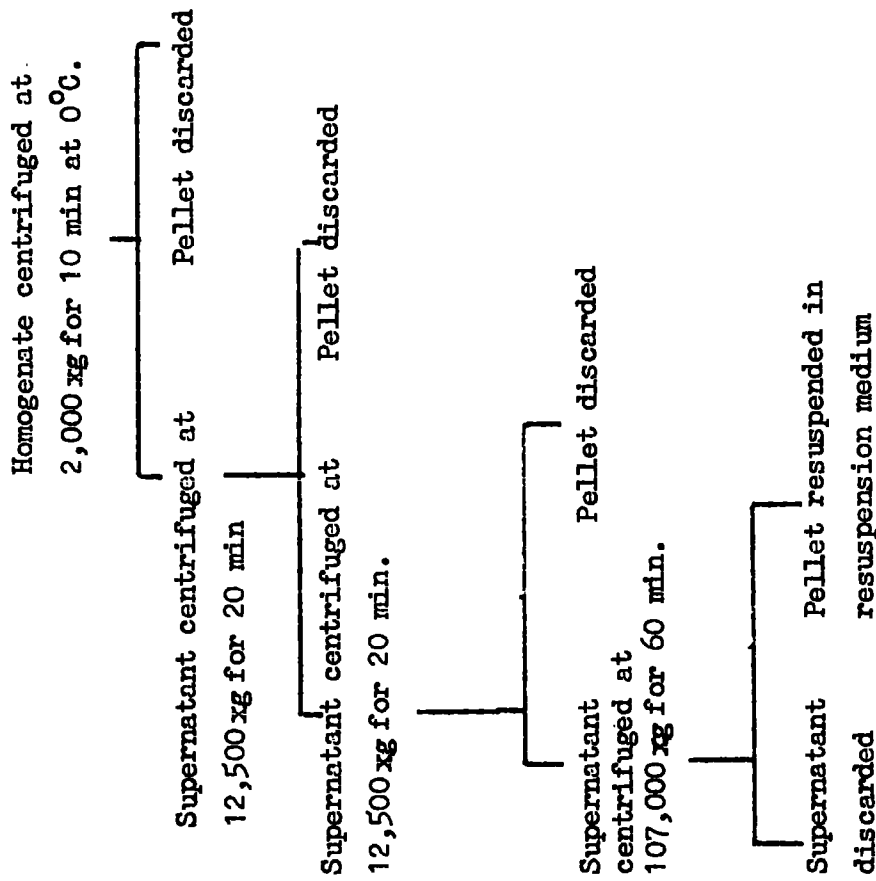
## (c) Washing medium

5 mM NaCl

5 mM Ethylenediaminetetra-acetic acid (sodium salt)

All solutions were brought to pH 7.25 and stored at 0 - 4°C. During an experiment they were kept on ice. The collected Malpighian tubules and hindguts were homogenized in 3 ml of homogenization medium. The

A



B

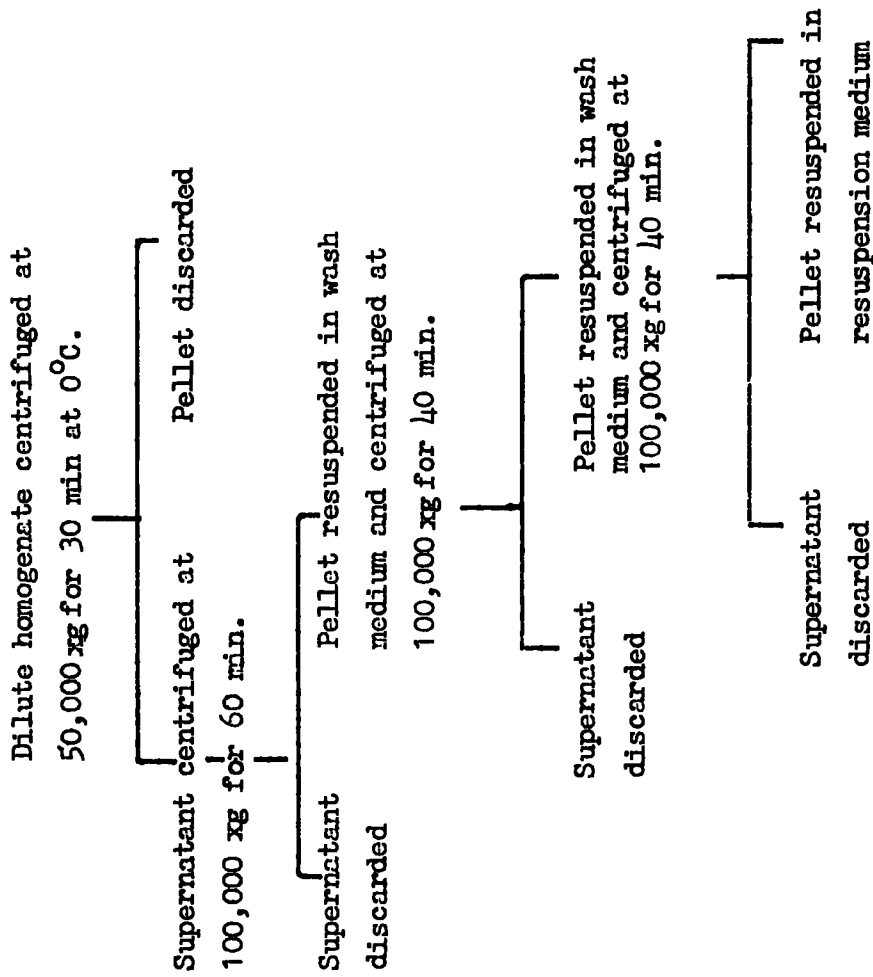


Fig.1. Diagram outlining the centrifugation steps in: A) sucrose method of extracting microsomes

B) mannitol, deoxycholate and sodium iodide method of extracting microsomes.

homogenate was transferred to a boiling tube (22mm diameter) containing an equal volume of the sodium iodide extraction medium. The suspension was mixed by vortex stirring for 2 min , after which it was left to stand on ice for a further 30 min. On completion of this extraction period, the sodium iodide concentration was diluted to 0.8 M (two and a half times) by the addition of 9 ml of resuspension medium (see below) and again thoroughly mixed.

(d) Centrifugation

The diluted suspension was transferred to thin walled polypropylene centrifuge tubes and centrifuged at 50,000 xg (28,000 rpm) for 30 min at 0°C in a MSE Automatic Superspeed 40, head number 2409. The pellet from this spin was discarded and the supernatant retained and centrifuged at 100,000 xg (38,000 rpm) for 60 min at 0°C. The supernatant was discarded and the pellet resuspended in washing medium by gentle uptake and extrusion from a Pasteur pipette. The preparation was then centrifuged at 100,000 xg for 40 min. This washing procedure was repeated once more. The final pellet was resuspended in cold resuspension medium (see below) and kept on ice until required. The steps involved in centrifugation are shown diagrammatically in Figure 1 (B).

iv. Resuspension of microsomes

(a) Resuspension medium

Deionized water neutralised to pH 7.0 by the addition of a small volume of 30 mM histidine and HCL.

Great care was taken to ensure that an even suspension of microsomes was obtained. This was done by gentle hand homogenization. The volume of the final suspension was determined by the size of the experiment and number of animals used but the protein content of the solution was maintained between 80 - 200 ug protein per 2 ml.



### 3. Reaction media and incubation conditions

Five different incubation media, having the following composition were used:

- i. 4 mM  $Mg^{2+}$
- ii. 4 mM  $Mg^{2+}$ , 100 mM  $Na^+$
- iii. 4 mM  $Mg^{2+}$ , 20 mM  $K^+$ ,
- iv. 4 mM  $Mg^{2+}$ , 100 mM  $Na^+$ , 20 mM  $K^+$ ,
- v. 4 mM  $Mg^{2+}$ , 100 mM  $Na^+$ , 20 mM  $K^+$ ,  $10^{-3}$  M ouabain

Other conditions of incubation were 2 mM ATP (Tris salt) and 50 mM imidazole buffer pH 7.25 (unless otherwise stated) to a final volume of 2 ml. Incubation was carried out in boiling tubes (22 mm diameter) in a water bath at  $30^{\circ} \pm 0.1^{\circ}C$ . All tubes were thermoequilibrated for 10 min prior to starting an experiment. The reaction was usually started by the addition of 0.5 ml of enzyme suspension. An incubation time of 45 min was used, over which period  $Na^+ - K^+$ ATPase activity was linear and that of  $Mg^{2+}$  ATPase, variable or curvilinear (Fig.2).

The reaction was terminated by the addition of 4 ml of a mixture of 1.0 % lubrol and 1.0 % ammonium molybdate in 1.8 N sulphuric acid. The tubes were removed from the water bath and allowed to stand at room temperature for 10 min, in order to allow the development of the yellow colour. They were then transferred to precooled glass centrifuge tubes on ice. Any protein that precipitated was removed by centrifugation at 1000 xg in a MSE Mistral 2L refrigerated centrifuge for 10 min and the supernatant returned to fresh pre-cooled glass centrifuge tubes on ice. The determination of inorganic phosphate and protein were as described below. Control tubes assaying non enzymatic hydrolysis of ATP were used at each incubation. Such tubes were treated in a similar manner to the experimental tubes and contained either medium (ii) or (iii) above. The

Fig. 2. Na<sup>+</sup> - K<sup>+</sup> (● - ●) and Mg<sup>2+</sup> (▲ - ▲) ATPase activities as a function of time

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. Mg<sup>2+</sup> ATPase activity being the Pi released in the presence of 4 mM Mg<sup>2+</sup> only. All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation temperature 30°C. Ordinate: ATPase activity expressed as nmoles Pi liberated/mg protein. Abscissa: time in minutes. Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

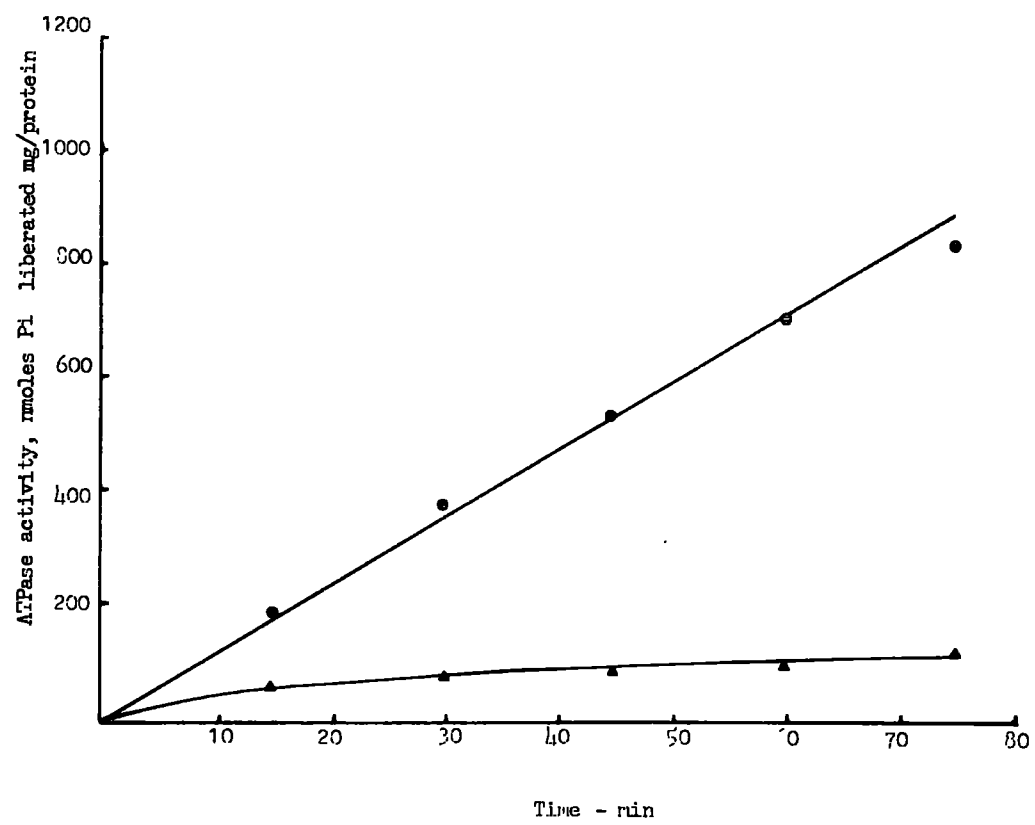


Fig. 2

salts medium and ATP were incubated in the same way as in experimental tubes at the end of the incubation period they had 4 ml of lubrol, ammonium molybdate, sulphuric acid mixture added, followed by the enzyme suspension.

$\text{Na}^+$  -  $\text{K}^+$ ATPase activity was obtained as the difference in Pi liberated in media containing  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$  and in  $\text{Mg}^{2+}$  alone.  $\text{Mg}^{2+}$  ATPase activity was obtained as the difference in Pi liberated in media containing  $\text{Mg}^{2+}$  and the control tubes (see above).

#### 4. Assay of inorganic phosphate

The method adopted for the assay of inorganic phosphate was that developed by Atkinson (1970). A stock solution of 20 ug of phosphorus per ml was used to construct a calibration curve (see Fig.3).

##### Method

The following standard inorganic phosphate solutions were obtained by serial dilution of a stock solution (20 ug Pi/ml) 2.5, 5.0, 10.0, 15.0, 20.0 ug Pi/ml. Duplicate 2 ml samples of standards and unknowns were made up in boiling tubes. Equal volumes of lubrol and ammonium molybdate in sulphuric acid were mixed and thoroughly stirred. 4 ml of this mixture were added to the 2 ml samples of standards and unknowns and left 10 min at room temperature. The contents of the boiling tubes were then poured into glass cuvettes, 10 mm light path and the optical density read at 390 nm using a Hilger Watt spectrophotometer. The boiling tubes and their contents could be stored on ice for periods of up to one hour without any appreciable variation in the concentration of phosphate being recorded.

#### 5. Assay of protein

The method used to determine the concentration of microsomal protein was the Folin - phenol method of Lowry, Rosebrough, Farr and Randall (1951)

Fig. 3. Calibration curve for inorganic phosphate

Ordinate: Absorbance at 390 nm

Abscissa: Phosphate concentration (nmoles/ml)

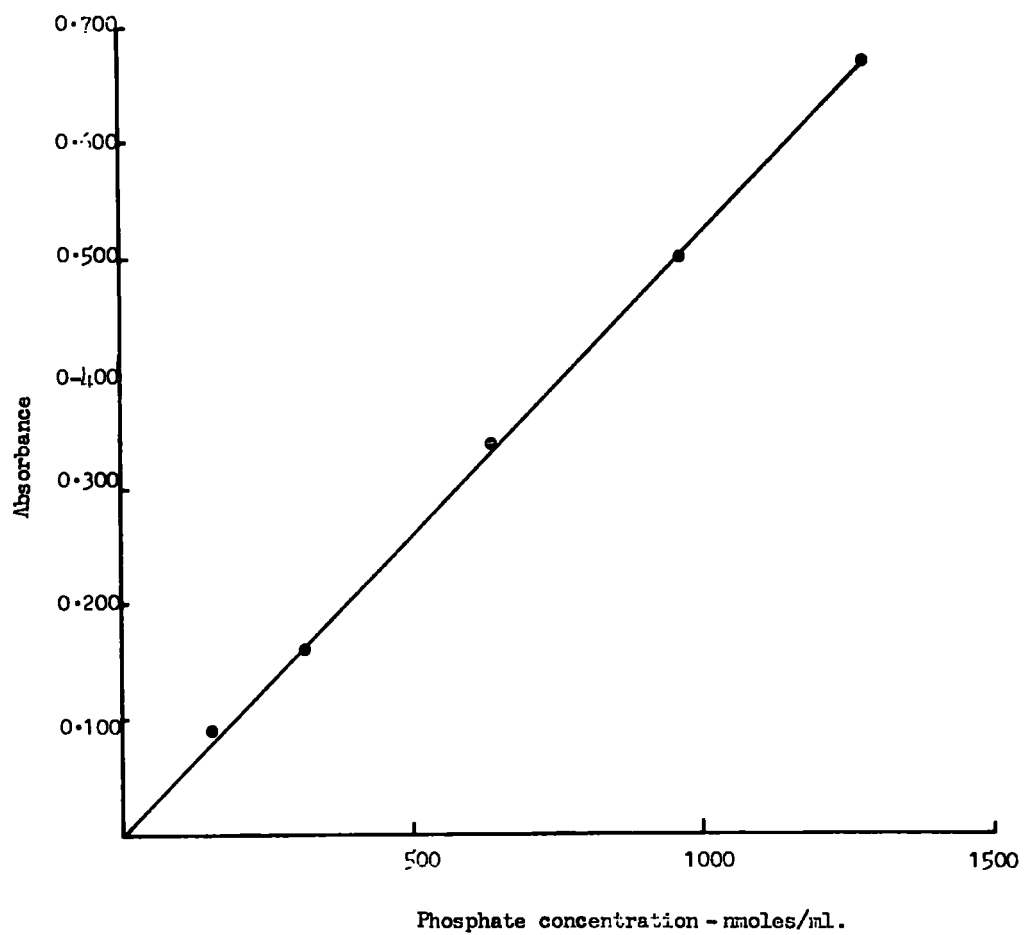


Fig. 3

using bovine serum albumen, Fraction V, as standard.

#### Reagents

- i. 2.0% ( $W/v$ ) Sodium carbonate in 0.1 N NaOH
- ii. 0.5% ( $W/v$ ) Copper sulphate
- iii. 1.0% ( $W/v$ ) Sodium potassium tartrate

Folin mixture A is prepared by mixing equal volumes of solutions (ii) and (iii) and one volume of this mixture was added to fifty volumes of solution (i). Folin mixture B was prepared by diluting four volumes of Folin Ciocalteau phenol reagent with six volumes of distilled water.

#### Method

Standard protein solutions were prepared containing 0, 50, 100, 150, 200, 250, 300, 350 and 400  $\mu$ g BSA per ml. Unknown protein samples were centrifuged, the supernatant removed and the protein pellet was then re-suspended in deionised water. (The volume of deionised water equalled the volume of supernatant removed). Duplicates of standards and unknowns were prepared. 0.2 ml of protein was added to 3 ml of folin mixture A in a boiling tube and allowed to stand at room temperature for 30 min 0.3 ml. of folin mixture B was then added and the resulting mixture allowed to stand at room temperature for a further 60 min. The solutions were poured into glass cuvettes, 10 mm light path and the optical density measured at 500 nm in a Hilger Watt spectrophotometer. A blank was run in which distilled water replaced the protein solution. From the standard protein solutions a calibration curve of absorbance against protein concentration was plotted from which unknowns could be determined. For each batch of unknown solutions to be assayed a fresh calibration curve was determined, one such curve is shown in Figure 4.

#### 6. Preparation of the Tris salt of adenosine triphosphate (ATP)

Tris ATP was prepared from the disodium salt of the nucleotide

Fig. 4. Calibration curve for protein

Ordinate: Absorbance at 500 nm

Abscissa: Protein concentration ( $\mu\text{g/ml}$ )



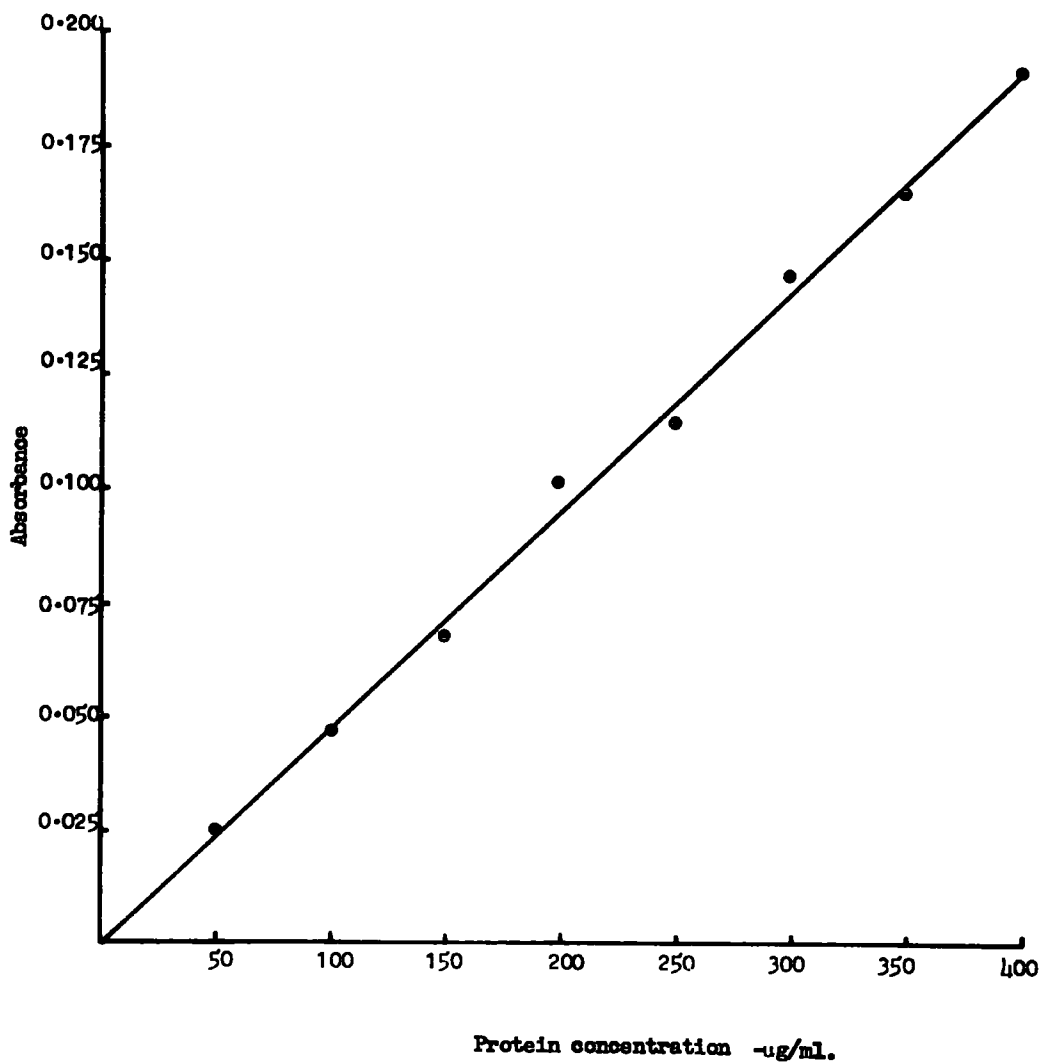


Fig. 4

using an ion exchange Dowex resin. A quantity of the resin was rinsed with distilled water using a Buchner funnel. The wet weight was found and noted. The resin was then washed in 3N HCl (AnalaR), 150 ml acid per 25 g wet weight of resin. This was followed by washing in distilled water until the effluent had a pH between 3 - 4. This step removed residual acid from the resin which was now in the  $H^+$  form. It was then resuspended in its own volume of distilled water, and could be kept in this form at  $0^\circ - 4^\circ C$  until required. A known quantity of disodium ATP was dissolved in a known, small volume of distilled water. To this was added a small quantity of resin ( $H^+$  form) and both were thoroughly mixed using a vortex mixer. The resin was allowed to sediment and the supernatant was removed and retained. The resin was then washed three times with a little distilled water, both were thoroughly mixed on each occasion. The fluid resulting from each wash was pooled. The ATP was now in the  $H^+$  form. It was converted to the Tris salt by the drop wise addition of 2 M Tris, until the pH was 7.25. It was then made up to the required volume and stored in boiling tubes at  $-20^\circ C$ .

## 7. Electron microscopic study of membrane fractions

### i. Preparation of membrane fractions

Preparations were obtained from the combined Malpighian tubules and hindguts of Homorocoryphus nitidulus vicinus as described under Materials and Methods 1, 2 above. Both the 50,000 xg and 100,000 xg fractions were examined.

### ii. Fixation, embedding and staining of membrane fractions.

All steps were performed at  $0^\circ - 4^\circ C$  unless otherwise stated.

Membrane fractions were:-

1. gently resuspended in resuspension medium by alternate withdrawal into and release from a Pasteur pipette,

2. recentrifuged at 100,000 xg for 60 min and the supernatant discarded,
  3. resuspended in 2.5 % gluteraldehyde and 0.05 M cacodylate as in 1 above and left 3 h,
  4. recentrifuged as in step 2 above,
  5. resuspended as in 3 above and left 14 h,
  6. washed in cacodylate buffer, 10 min by gentle agitation followed by a 15 min spin at 100,000 xg to compress pellet,
  7. post fixed in 1.0 % osmic acid and left for 3 h,
  8. rinsed 5 times in distilled water and thereafter treated as described in Chapter Five Materials and Methods.
8. Surgical interference with the anterior stomatogastric system; its effect on  $Mg^{2+}$  and  $Na^+ - K^+$  ATPase activities of Malpighian tubule, ileal and rectal preparations

Fifth instar male Locusta migratoria were used throughout this part of the work. Only one type of operation was carried out and involved severing both frontal connectives close to the frontal ganglion. This operation was chosen since Allum (1973) has shown in Locusta migratoria, given certain precautions, it has similar effects on growth and metabolism, to the surgically more drastic frontal ganglionectomy. Operations were carried out with  $48 \pm 12$  h of emergence from the fourth instar. All instruments used in the operations were sterilised by soaking in absolute ethanol for 30 min, followed by rapid flaming in a bunsen flame. Insects were anaesthetised singularly in a small screw top jar, containing a cotton wool pad soaked in anaesthetic ether. A few filter papers were placed on top of the cotton wool and prevented the insect from coming into direct contact with the liquid anaesthetic. Insects were anaesthetised for 2 min after which time they were usually immobile. Anaesthetised individuals were placed in a perspex operating jig and secured

with plasticene (Fig.5, A). The jig was transferred to the stage of a binocular microscope and secured with plasticene. Illumination was provided by a high intensity lamp fitted with a polaroid heat filter.

Three cuts were made in the frons (Fig.5, B,1-3) and the rectangular flap of cuticle and hypodermis turned ventral, to reveal the three large air sacs at the front of the head. The air sacs were parted to reveal the frontal ganglion and associated nerves on the dorsal surface of the pharynx (Fig.5, C). After operations, insects were placed in clean perspex containers at room temperature, to recover. Insects were unfed on the day of the operation (day one) but received fresh grass on day two. On day three they were returned to the insectary. The time taken to carry out the operation was never more than 2 min. Mortality, among the operated insects was approximately 50 % after 7 days. Control operations were carried out in which insects were treated in an identical manner to the operated group, except that the frontal connectives were merely touched and not cut. Deaths among control insects were rare. Seven days after the operation the  $Mg^{2+}$  and  $Na^+ - K^+$  ATPase activity of the Malpighian tubules, ileum and rectum were assayed for both operated and control groups of insects. ATPase activity was assayed as described above, using the mannitol, deoxycholate and sodium iodide extraction procedure. Both operated and control groups of insects were weighed on a torsion balance at the time of the operation and at 24 h intervals thereafter upto the time of sacrifice. Although the number of insects at the beginning of an experiment varied from one experiment to another, the number of insects in the operated and control groups at the time of ATPase assay was never less than eight. Heads were examined at the time of ATPase assay to ensure that the nerves had been severed.

**Fig. 5**

- A. Diagram to show the position of an anaesthetised insect, in the perspex jig during operations.
- B. Anterior view of a locust's head showing the position of the three cuts, made in the cuticle of the frons.
- C. Similar to B, but with the rectangular flap of cuticle removed to show the frontal ganglion and frontal connectives on the dorsal surface of the foregut.

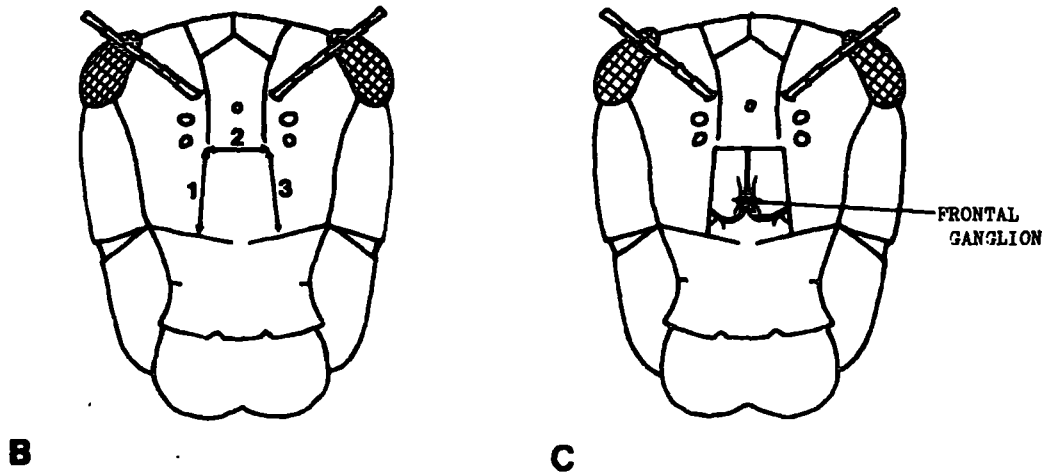
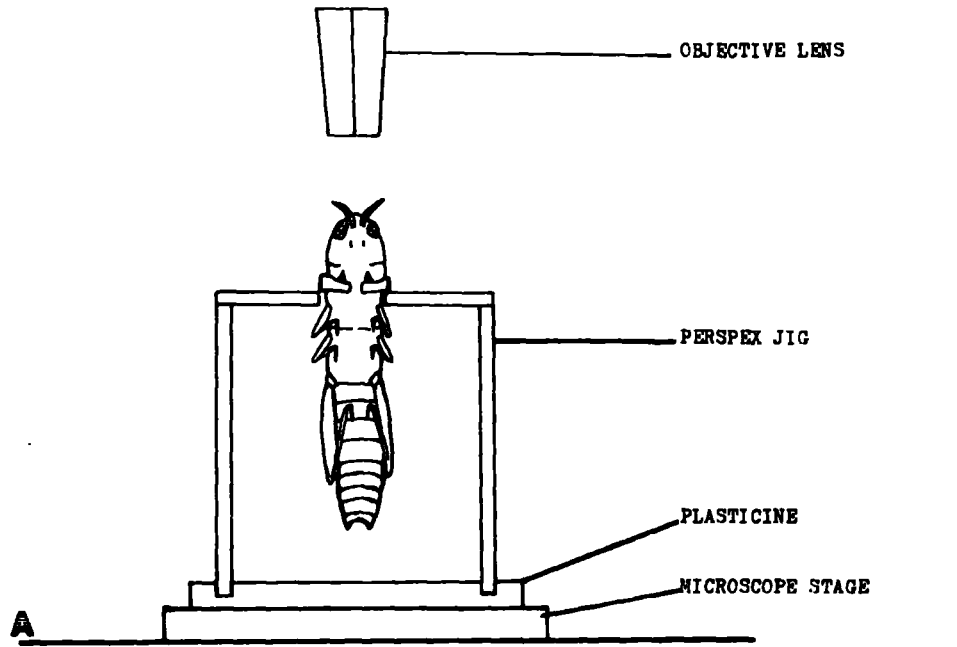


Fig.5.

## Results

### 1. Extraction and distribution of $\text{Na}^+$ - $\text{K}^+$ - and $\text{Mg}^{2+}$ ATPases.

The results obtained from the combined Malpighian tubule and hindgut preparations extracted in a buffered sucrose medium are shown in Table 1. Experiment (a) shows the ATPase activity of the preparation in the presence of magnesium ions was not stimulated by the addition of potassium and was slightly inhibited by the addition of sodium ions. The basal  $\text{Mg}^{2+}$  ATPase activity is also reduced when both sodium potassium ions are present. The addition of ouabain further inhibits the enzymatic splitting of ATP when both sodium and potassium ions are present and with potassium alone, although it did not affect enzyme activity when only sodium was present. If  $\text{Na}^+$  -  $\text{K}^+$  ATPase activity is expressed as the difference in enzyme activity in the presence of sodium, potassium and magnesium ions and sodium, potassium and magnesium ions plus ouabain, then the preparation has a low  $\text{Na}^+$  -  $\text{K}^+$  ATPase activity of 5.3 nmoles Pi/mg protein/min.

In experiments (b-j), where the mannitol, deoxycholate and sodium iodide extraction method was used, a very different picture emerges. Preparations normally gave very low basal  $\text{Mg}^{2+}$  ATPase levels (5.1, 8.3 nmoles Pi/mg protein/min for Schistocerca gregaria experiments b, c respectively). Although as can be seen this varied from experiment to experiment. This activity is only slightly stimulated by sodium or potassium ions alone. The greatest stimulation giving a two fold increase in activity (see experiment j), more usually however the stimulation was less than 30% higher. This depended somewhat on the source of the preparation. For example, in Jamaicana flava preparations it was below 10%. However when sodium and potassium ions are present together they have a dramatic stimulatory effect. The ratio of  $\text{Na}^+$  -  $\text{K}^+$  ATPase/ $\text{Mg}^{2+}$  ATPase

Table 1.

The  $Mg^{2+}$  and  $Na^+ - K^+$  ATPase activities of microsomal preparations extracted from the combined Malpighian tubules and hindguts of four insect species after homogenization in buffered sucrose medium (expt.a) or mannitol, deoxycholate medium and extraction in sodium iodide (expts. b - j).

Salts present in various combinations, at the following concentrations:  $4Mg^{2+}$ , 20 mM  $K^+$ , 100 mM  $Na^+$ .

All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Ouabain concentration  $10^{-3}M$ . Incubation 45 min at  $30^{\circ}C$ .



ATPase activity nmoles Pi liberated/mg protein/min		Activity in the presence of:									
Insect species	Expt	Mg <sup>2+</sup>		Mg <sup>2+</sup> + Na <sup>+</sup>		Mg <sup>2+</sup> + K <sup>+</sup>		Mg <sup>2+</sup> + Na <sup>+</sup> + K <sup>+</sup>		Mg <sup>2+</sup> + Na <sup>+</sup> + K <sup>+</sup> minus Mg <sup>2+</sup>	Mg <sup>2+</sup> + Na <sup>+</sup> + K <sup>+</sup> minus ouabain
		-	+	-	+	-	+	-	+		
<u>Schistocerca gregaria</u>	a	29.3	29.0	21.3	21.7	29.2	27.9	25.7	20.4	-3.6	+5.3
	b	5.1						71.9	4.6	+66.8	+67.3
	c	8.3		6.2		8.3		71.5	7.6	+63.2	+63.9
<u>Jamaicana flava</u>	d	11.2		13.6		11.5		26.4	11.1	+15.2	+15.3
	e	15.3		13.3		15.6		33.7	15.2	+18.5	+18.5
<u>Homocoryphus nitidulus vicinus</u>	f	16.4		17.0		20.1		290.3	17.2	+273.9	+273.1
	g	3.0		3.4		1.5		97.7	7.7	+94.7	+90.0
	h	15.4		19.9		20.0		191.6	16.5	+176.2	+175.1
	i	21.0		23.0		24.0		269.7	12.1	+248.7	+247.2
<u>Locusta migratoria migratorioides</u>	j	10.3		21.1		23.4		118.2	12.1	+107.9	+106.1

Table 1

activities varying from one-two (experiments d,e) to thirty one (experiment g) but was more usually about twelve. This activity in the presence of sodium and potassium ions was abolished by ouabain. When considering the activities of the enzymes for the various insect species it will be seen that the activity in the presence of both sodium and potassium ions is lowest in Jamaican flava (experiments d, e) and largest in Homorocoryphus nitidulus vicinus (experiments f, i). The other two species have intermediate values with Schistocerca gregaria slightly lower than that for Locusta migratoria.

Table 2 shows the results of eight experiments in which the excretory system was separated into Malpighian tubules, ileum and rectum, before the  $Mg^{2+}$  and  $Na^+ - K^+$  ATPase activities were determined. A considerable degree of variation exists between experiments in both levels of  $Mg^{2+}$  and  $Na^+ - K^+$  ATPase activity. From the table it can be seen that in the species examined, all parts of the excretory system possess a  $Na^+ - K^+$  ATPase which is ouabain sensitive.

Taking the  $Mg^{2+}$  ATPase activity for each species in turn, it can be seen that for Jamaicana flava the largest activity is found in the Malpighian tubule preparation, values varying between 20.4 - 37.0 nmoles Pi/mg protein/min. Activity in the ileal and rectal preparations varied between 4.3 - 17.5 and 4.9 - 31.4 nmoles/Pi/mg protein/min, respectively. In the one experiment using Homorocoryphus nitidulus vicinus the  $Mg^{2+}$  ATPase was most active in the ileum at 55.9 nmoles Pi/mg protein/min. In the Malpighian tubule preparation  $Mg^{2+}$  ATPase activity was 23.9 nmoles Pi/mg protein/min, and the preparation from the rectum was least active at 10.9 nmoles Pi/mg protein/min. The preparations from Locusta migratoria were all low in  $Mg^{2+}$  ATPase activity, varying between 1.5 - 11.8 nmoles Pi/mg protein/min in all three preparations from each portion of the excretory system.  $Na^+ - K^+$  ATPase activity was not uniformly

Table 2

The  $Mg^{2+}$  and  $Na^+ - K^+$ ATPase activities of microsomal preparations extracted from the Malpighian tubules, ilea and recta of four insect species after homogenization in mannitol, deoxycholate medium and extraction with sodium iodide.

Salts present in various combinations, at the following concentrations: 4 mM  $Mg^{2+}$ , 20 mM  $K^+$ , 100 mM  $Na^+$ . All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25) Ouabain concentration  $10^{-3}$ M. Incubation 45 min at  $30^\circ C$ .

Column		ATPase activity nmoles Pi liberated/mg protein/min															
		Malpighian tubules				Ileum				Rectum							
		Activity in the presence of:				Activity in the presence of				Activity in the presence of:							
Insect species	Expt	Mg <sup>2+</sup> (A)	Mg <sup>2+</sup> (B)	Mg <sup>2+</sup> (B-A)	(C)	Mg <sup>2+</sup> (A)	Mg <sup>2+</sup> (B)	Mg <sup>2+</sup> (B-A)	(C)	Mg <sup>2+</sup> (A)	Mg <sup>2+</sup> (B)	Mg <sup>2+</sup> (B-A)	(C)	Mg <sup>2+</sup> (A)	Mg <sup>2+</sup> (B)	Mg <sup>2+</sup> (B-A)	(C)
		Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	minus Mg <sup>2+</sup> ouabain	ouabain (B-C)	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	minus Mg <sup>2+</sup> ouabain	ouabain (B-C)	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	minus Mg <sup>2+</sup> ouabain	ouabain (B-C)	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	Mg <sup>2+</sup> Na <sup>+</sup> K <sup>+</sup>	minus Mg <sup>2+</sup> ouabain	ouabain (B-C)
<u>Jamaicana</u>	a	32.6	39.9	7.3	24.7	15.2	4.3	13.3	9.0	4.7	8.6	11.4	62.4	51.0	9.5	52.9	
<u>flava</u>	b	37.0	81.9	44.9	30.9	51.0	17.5	70.2	52.7	17.5	52.7	31.4	183.0	151.6	34.4	148.6	
	c	20.4	73.7	53.3	17.1	56.6	10.2	62.4	52.2	7.3	55.1	4.9	55.3	50.4	2.1	53.2	
	d	29.0	75.7	46.7	25.0	50.7	13.2	37.3	24.1	6.5	30.8	12.7	103.4	90.7	7.6	95.8	
<u>Homoro-</u> <u>coryphus</u> <u>nitidulus</u> <u>vicinus</u>	e	23.9	75.4	51.5	22.5	52.9	55.9	58.7	2.8	56.3	2.4	10.9	36.2	25.3			
<u>Locusta</u> <u>migratoria</u> <u>migratori-</u> <u>oides</u>	f	3.0	64.8	61.8	3.5	61.3	3.4	36.4	33.0	4.1	32.3	5.4	135.6	130.2	2.6	133.0	
	g	2.0	12.7	10.7	2.3	10.4	3.9	46.3	42.4			1.5	213.8	212.3	5.5	208.3	
	h	11.8	55.9	44.1			2.0	12.9	10.9			6.8	99.0	92.2			

Table 2

distributed between the different parts of the excretory system in any of the three species. The activity of this enzyme can be assessed either by the difference in phosphate liberated in solutions containing magnesium ions only from that containing magnesium, sodium and potassium ions (Table 2, (B-A)) or by the ouabain sensitive activity that is (B-C). High levels of  $\text{Na}^+ - \text{K}^+$ ATPase exist in preparations from all three parts of the excretory system of Jamaicana flava. In three of the four experiments (a,b,d) more activity was found in the rectal preparations, where it varied between 50.4 and 151.6 nmoles Pi/mg protein/min. Rather similar levels of activity were found in the preparations from ileum and Malpighian tubules, which varied between 7.3 and 53.3 nmoles Pi/mg protein/min. In Homorocoryphus nitidulus vicinus the preparation from ileum had very low activity and that from Malpighian tubules the highest at 51.5 nmoles Pi/mg protein/min. In Locusta migratoria as with Jamaicana flava the highest activity was found in the rectal preparations 92.2 - 212.3 nmoles Pi/mg protein/min. The  $\text{Na}^+ - \text{K}^+$ ATPase activities of ileum and Malpighian tubules were variable but similar and ranged between 10.9 - 42.4 nmoles Pi/mg protein/min for ileum and 10.7-61.8 nmoles Pi/mg protein/min for the Malpighian tubules.

A convenient way of comparing the  $\text{Na}^+ - \text{K}^+$ ATPase activity between preparations is by the  $\frac{\text{Na}^+ - \text{K}^+ \text{ATPase}}{\text{Mg}^{2+} \text{ATPase}}$  activity ratio (Table 3). It is evident that for Jamaicana flava the largest ratios occur in the rectal preparations varying between 4.47 and 10.29 and the lowest in the Malpighian tubule fraction where it varies between 0.22 and 2.61. The values for the ileal fraction lie between those of the rectal and Malpighian tubule preparations. In Locusta migratoria the picture is essentially similar. Very high ratios are to be found in the rectal preparations 24.11, 141.56, whilst the lowest ratios usually occur in the

Table 3.

Ratios of  $\frac{\text{Na}^+ - \text{K}^+ \text{ATPase}}{\text{Mg}^{2+} \text{ATPase}}$  activity for the microsomal preparations extracted from the Malpighian tubules, ilea and recta of three insect species. In column 1, the  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  activity was calculated by subtracting the activity in the presence of  $\text{Mg}^{2+}$  from that in the presence of  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$ . In column 2,  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  activity was calculated by subtracting the activity in the presence of  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$  plus ouabain, from that in the presence of  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$ . Data taken from Table 2.

Insect species	Expt	Ratio of $\frac{\text{Na}^+ - \text{K}^+ \text{ATPase}}{\text{Mg}^{2+} \text{ATPase}}$ activity					
		Malpighian tubules		Ileum		Rectum	
		1	2	1	2	1	2
<u>Jamaicana flava</u>	a	0.22	0.47	2.00	2.00	4.47	4.64
	b	1.21	1.38	3.01	3.01	4.82	4.74
	c	2.61	2.77	5.12	5.40	10.29	10.86
	d	1.61	1.75	1.83	2.33	7.14	7.54
<u>Homocoryphus nitidulus vicinus</u>	e	2.15	2.21	0.05	0.40	2.32	
<u>Locusta migratoria</u>	f	20.60	20.43	9.71	9.50	24.11	24.63
<u>migratorioides</u>	g	5.35	5.20	10.87		141.53	138.88
	h	3.74		5.45		13.56	

Table 3.

Malpighian tubule fraction. The ratios for the activities in the ileal preparations are intermediate. Although on the basis of one experiment only, calculation of the ratio for Homorocoryphus nitidulus vicinus reveals that similar values of 2.32 and 2.15 exist for the rectal and Malpighian tubule preparations respectively. The value of the ratio for the ileal fraction is exceedingly low at 0.05.

The largest ratio for any of the species examined, occurs in the rectal preparations of Locusta migratoria where values of 141.2 and 24.1 were recorded. Disregarding the single result from Homorocoryphus nitidulus vicinus, the lowest values occur in the Malpighian tubule preparations of Jamaicana flava where values of 0.22 and 1.21 were obtained. In Locusta migratoria each component of the excretory system has a larger ratio than its counterpart in other species. Table 3 also includes the ratio  $\frac{\text{Na}^+ - \text{K}^+ \text{ATPase}}{\text{Mg}^{2+} \text{ATPase}}$  expressed as  $\frac{\text{ouabain sensitive ATPase}}{\text{Mg}^{2+} \text{ATPase}}$  (column 2). It will be seen that these ratios are in agreement with those already described.

## 2. Properties of $\text{Na}^+ - \text{K}^+ \text{ATPase}$ .

The mannitol, deoxycholate and sodium iodide method has been shown to produce microsomes with a relatively high level of  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  activity (Chapter Six Results 1.). For this reason this method only was employed in this part of the work. Active preparations were obtained from the combined Malpighian tubule and hindgut tissues of Homorocoryphus nitidulus vicinus and occasionally Locusta migratoria. In all experiments two tubes were incubated simultaneously and under identical conditions. One tube contained 4 mM magnesium and the other 4 mM magnesium as well as the stated concentrations of sodium and potassium ions. Other incubation conditions and calculation of  $\text{Na}^+ - \text{K}^+ \text{ATPase}$  activity were as described above under Materials and Methods, 3. Any deviation from these conditions are stated in the text.



The effect of preincubation of enzyme, in the absence of ATP, on its subsequent activity.

The possibility that at an incubation temperature of 30°C some denaturation of the enzyme may occur was investigated. Experimental tubes containing media were thermoequilibrated for 10 min at 30°C. The enzyme preparation was then added and after the required preincubation period the reaction was started by the addition of 0.5 ml 2 mM ATP. Preincubation times ranged from 0 - 120 min. As shown in Figure 6 preincubation of Na<sup>+</sup> - K<sup>+</sup>ATPase at 30°C reduces the activity by approximately 4.0% for each 15 min preincubation period, so that after some 60 min the activity has fallen by about 15.0%. It will also be seen that Mg<sup>2+</sup> ATPase activity is relatively unaffected by this treatment.

The effect of pH on Na<sup>+</sup> - K<sup>+</sup> ATPase activity.

A 50 mM Bis Tris propane buffer system was used to produce an uninterrupted, stable range of pH from 5 - 9. Adjustment to the required pH was made with HCl at the incubation temperature of 30°C. Figure 7 shows Na<sup>+</sup> - K<sup>+</sup>ATPase activity as a function of pH. The pH optimum of the different Na<sup>+</sup> - K<sup>+</sup>ATPase preparations lay between pH 7.25 - 8.00. The Na<sup>+</sup> - K<sup>+</sup>ATPase, extracted from the combined Malpighian tubule and hindgut of Homorocoryphus nitidulus vicinus (Fig.7(A)), gave maximum activity in the region of pH 7.25 - 7.50 whilst those from the Malpighian tubule, ileal and rectal preparations of Locusta migratoria (Fig.7(B)) each had a pH optimum of about 8.00. Mg<sup>2+</sup> ATPase activity was variable over the whole range of pH and for this reason is not included in Figure 7.

The effect of temperature on ATPase activity

Pairs of tubes, one containing 4 mM magnesium and the other 4 mM magnesium, 100 mM sodium and 20 mM potassium were equilibrated at

Fig. 6

Effect of preincubation at 30°C, without ATP on  
Na<sup>+</sup> - K<sup>+</sup> (● - ●) and Mg<sup>2+</sup> (▲ - ▲) ATPases.

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup> 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. Mg<sup>2+</sup> ATPase activity being the Pi released in the presence of 4 mM Mg<sup>2+</sup> only. All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation 30 min at 30°C. Ordinate: ATPase activity expressed as nmoles Pi liberated/ mg protein/min. Abscissa: Preincubation time in minutes. Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

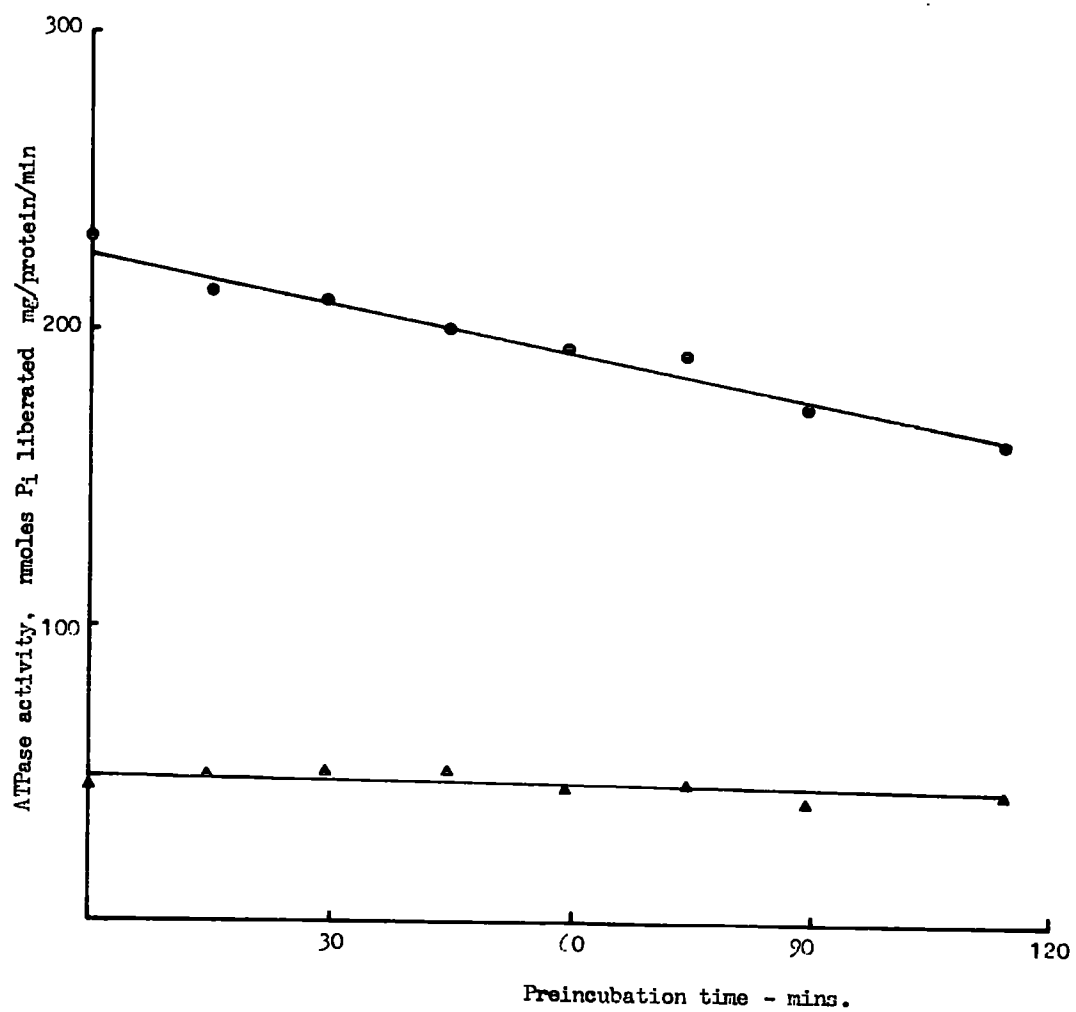


Fig. 6

Fig. 7

Effect of pH on Na<sup>+</sup> - K<sup>+</sup> ATPase

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. Both reaction media contained 2 mM ATP, 50 mM Bis Tris propane. Incubation 30 min at 30°C.

Ordinate, Na<sup>+</sup> - K<sup>+</sup>ATPase activity expressed as nmoles Pi/liberated/mg protein/min.

Abscissa: pH units.

A Preparation obtained from the combined Malpighian tubule and hindgut of Homorocoryphus nitidulus vicinus.

B ● preparation obtained from the rectum of Locusta migratoria

▼ preparation obtained from the Malpighian tubules of Locusta migratoria

■ preparation obtained from the hindgut of Locusta migratoria.

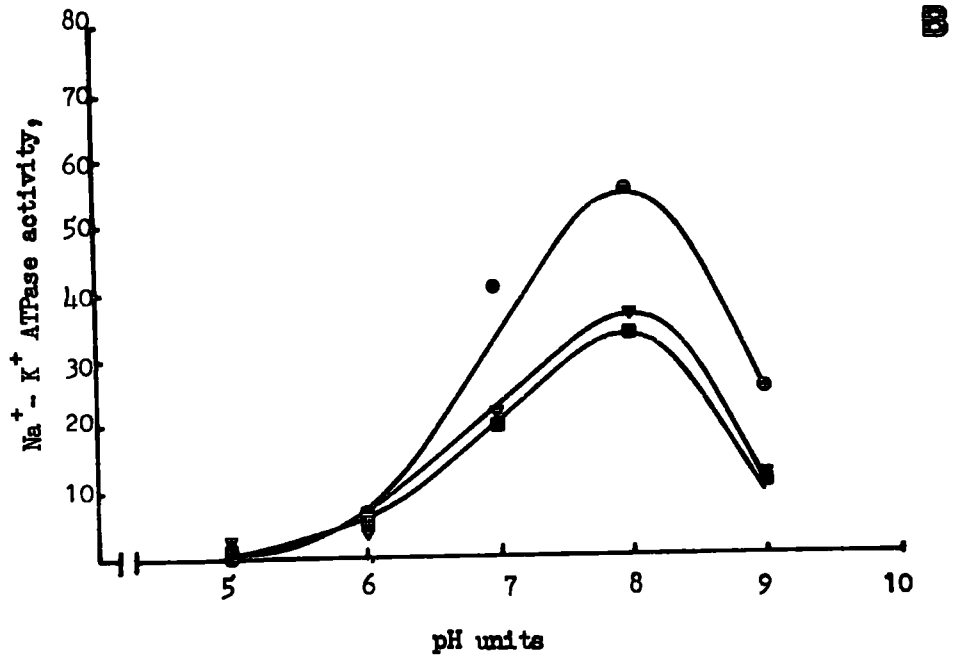
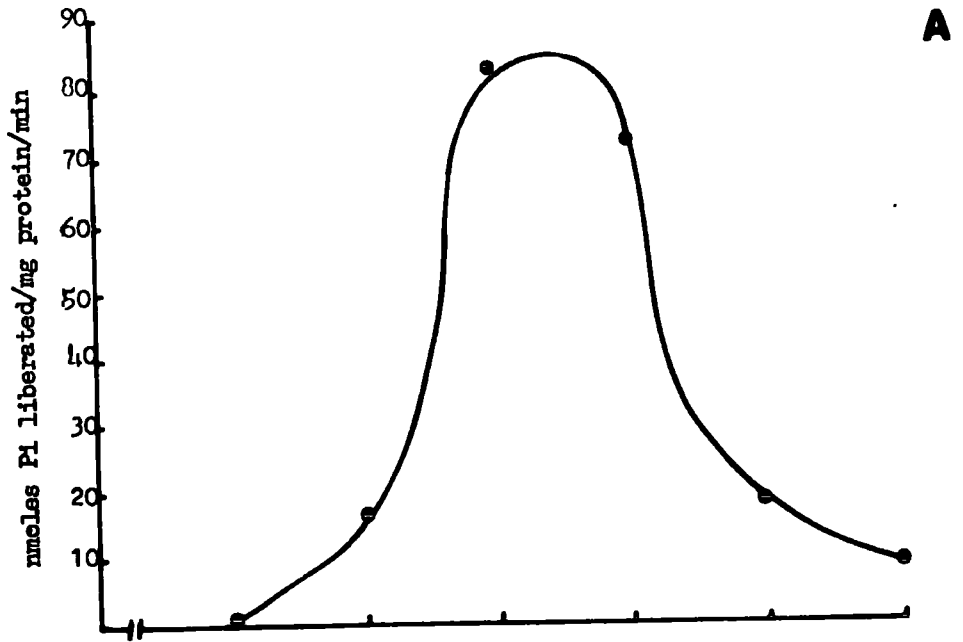


Fig. 7

temperatures in the range  $10^{\circ}$  -  $55^{\circ}\text{C}$  for 10 min. The reaction was started by adding 0.5 ml of enzyme suspension and was terminated by adding 4 ml of lubrol -molybdic acid mixture. The reaction was allowed to proceed for 60 min at temperatures of  $15^{\circ}\text{C}$  and below, 40 min at  $20^{\circ}$  and  $25^{\circ}\text{C}$ , 20 min at  $30^{\circ}$  and  $35^{\circ}\text{C}$  and 10 min at  $40^{\circ}\text{C}$  and  $55^{\circ}\text{C}$ . The effect of incubation temperature on  $\text{Na}^{+}$ - $\text{K}^{+}$ - and  $\text{Mg}^{2+}$ ATPases is shown in Figure 8, where the results are plotted as enzyme activity against incubation temperature. It can be seen that maximum activation of  $\text{Na}^{+}$ - $\text{K}^{+}$ ATPase occurred at about  $42^{\circ}\text{C}$  and for  $\text{Mg}^{2+}$ ATPase at approximately  $45^{\circ}\text{C}$ , Figure 9 shows the effect of temperature on enzyme activity in the form of Arrhenius  $\mu$  plots. From such plots, values for the activation energy of  $\text{Na}^{+}$ - $\text{K}^{+}$ ATPase were determined. However, the shapes of the plots in Figure 9 are curvilinear making absolute values of  $\mu$  difficult to calculate. Nevertheless over the temperature range  $15^{\circ}$  -  $24^{\circ}\text{C}$  approximate activation energies lay between 17.66 - 24.38 Kcals mole $^{-1}$ .

The effect of temperature on potassium stimulation of  $\text{Na}^{+}$ - $\text{K}^{+}$ ATPase.

$\text{Na}^{+}$ - $\text{K}^{+}$ ATPase activity was assayed over a 0-40 mM range of potassium concentration at temperatures of  $10^{\circ}$ ,  $20^{\circ}$  and  $30^{\circ}\text{C}$ . The results are shown in Figure 10. At 100 mM sodium, low potassium concentrations were limiting at all three temperatures. At  $10^{\circ}\text{C}$  maximum activity occurred at 10 mM potassium, whereas at temperatures of  $20^{\circ}$  and  $30^{\circ}\text{C}$ , higher concentrations of potassium were required of 20 mM, before maximal activity was achieved.

The effect of temperature on inhibition of  $\text{Na}^{+}$ - $\text{K}^{+}$ ATPase by ouabain

$\text{Na}^{+}$ - $\text{K}^{+}$ ATPase activity was assayed over the temperature range  $10^{\circ}$  -  $30^{\circ}\text{C}$ , both in the presence and absence of ouabain. Three tubes were incubated at each temperature. One contained 4 mM magnesium, a second 4 mM magnesium 100 mM sodium and 20 mM potassium and a third similar to the second but contained in addition to the salts,  $10^{-7}$  M

Fig. 8. Effect of temperature on  $\text{Na}^+ - \text{K}^+$  ( $\bullet - \bullet$ ) and  $\text{Mg}^{2+}$  ( $\blacktriangle - \blacktriangle$ ) ATPase

$\text{Na}^+ - \text{K}^+$  ATPase activity obtained as the difference between the activity in the presence of 100 mM  $\text{Na}^+$ , 20 mM  $\text{K}^+$ , 4 mM  $\text{Mg}^{2+}$  and in the presence of 4 mM  $\text{Mg}^{2+}$  alone.  $\text{Mg}^{2+}$  ATPase activity being the Pi released in the presence of 4 mM  $\text{Mg}^{2+}$  only. All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation 60 min at 15°C and below, 40 min at 20° and 25°C, 20 min at 30° and 35°C and 10 min at 40° and 55°C. Ordinate: ATPase activity expressed as nmoles Pi liberated/mg protein/min. Abscissa: temperature °C. Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

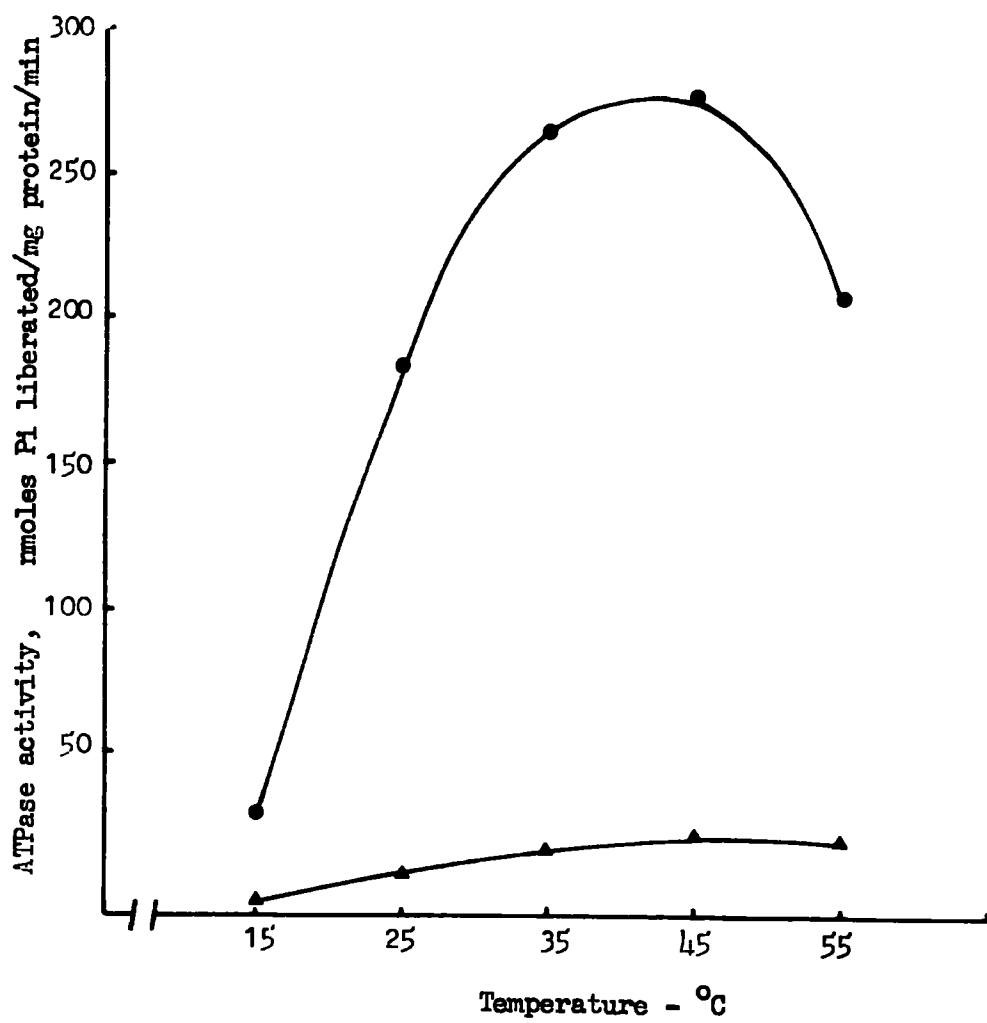


Fig. 8



Fig. 9 Effect of temperature on Na<sup>+</sup> - K<sup>+</sup>ATPase

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation times varied between 10 min at 50°C and 60 min at 15°C.

Ordinate: Log<sub>10</sub> Na<sup>+</sup> - K<sup>+</sup>ATPase activity expressed as nmoles Pi liberated/mg protein/min.

Abscissa: Reciprocal of temperature in degrees Absolute.

Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

The curves represent 5 different experiments from which Arrhenius  $\mu$  values were calculated.

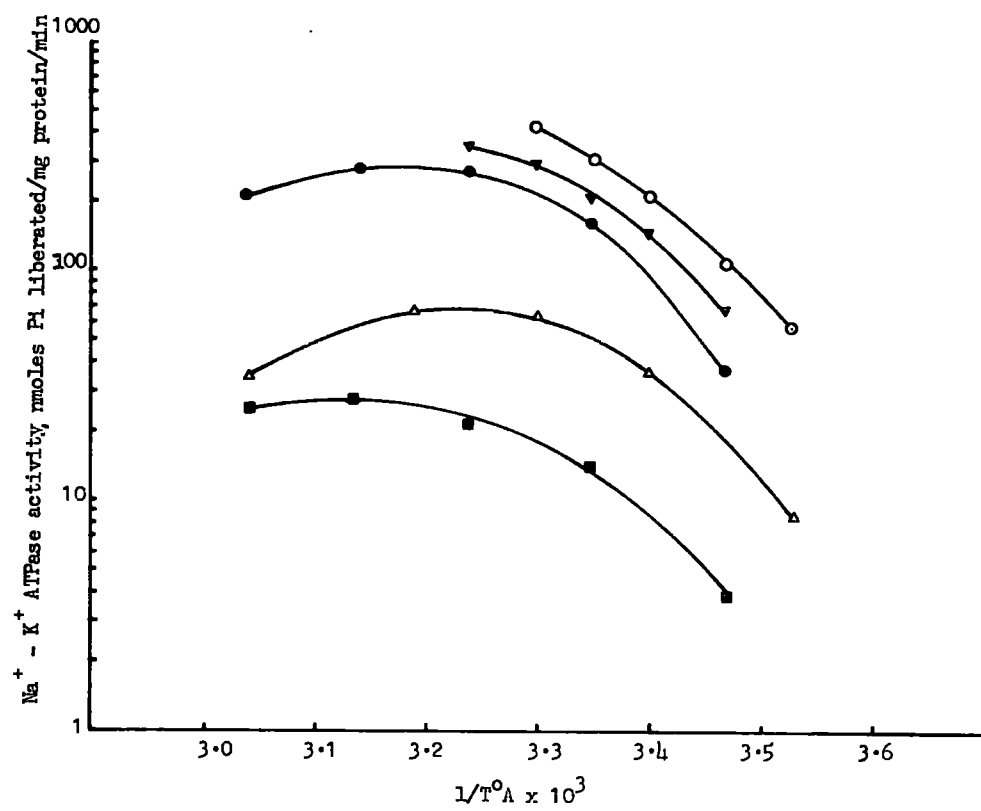


Fig. 9

Fig.10 Na<sup>+</sup> - K<sup>+</sup>ATPase activity as a function of K<sup>+</sup> concentration at 3 different temperatures

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 4 mM Mg<sup>2+</sup>, and amounts of K<sup>+</sup> indicated on the abscissa and in the presence of 4 mM Mg<sup>2+</sup> alone. Both reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation 75 min at 10°C, 30 min at 20°C and 30°C. Ordinate: Na<sup>+</sup> - K<sup>+</sup> ATPase activity expressed as nmoles Pi liberated/mg protein/min. Abscissa: K<sup>+</sup> concentration (mM). Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

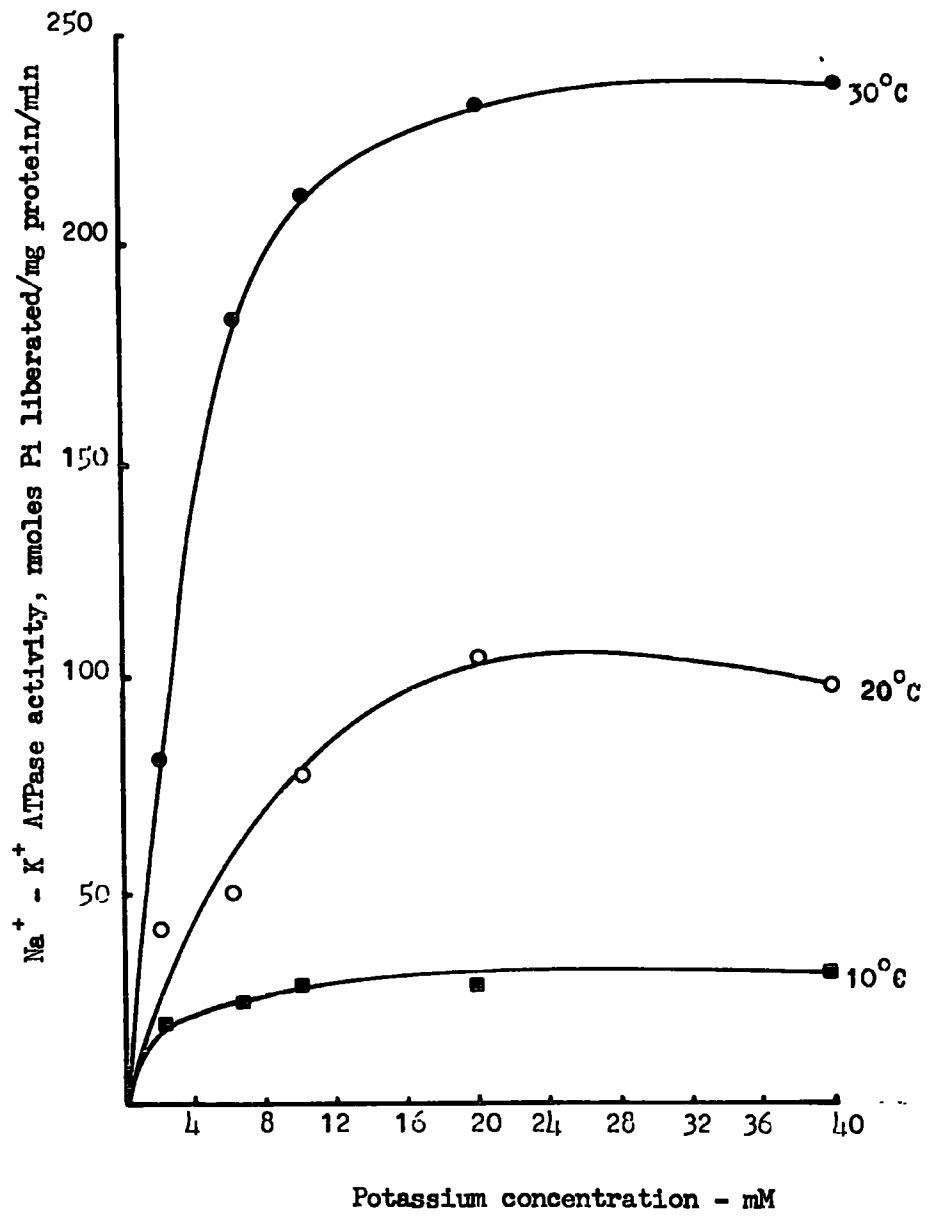


Fig. 10

ouabain. The latter concentration of inhibitor was chosen since it inhibited enzyme activity by about 65% at 30°C (see Fig.19). Na<sup>+</sup> - K<sup>+</sup>ATPase activity in the absence of ouabain was calculated as described above under Materials and Methods. Na<sup>+</sup> - K<sup>+</sup>ATPase activity in the presence of ouabain was obtained in a similar way except that activity in the presence of 4 mM magnesium was subtracted from the activity in the presence of all three cations and ouabain. Figure 11 (A),(B) shows the effect of temperature on ouabain inhibition of Na<sup>+</sup> - K<sup>+</sup>ATPase. It will be seen that increasing the incubation temperature produces an increase in the ouabain inhibition of Na<sup>+</sup> - K<sup>+</sup>ATPase. There was about 10% inhibition at 10°C which rose to approximately 40% at 30°C. (It should be noted that whilst the inhibition was always less at low than at high temperatures, there was however some variation in the activity, especially at the lower temperatures). Figure 11 (B) also shows that ouabain inhibition at low temperatures can be made comparable with that at higher temperatures, by raising the concentration of ouabain. It will be seen that approximately 40% inhibition is produced at 30°C by 10<sup>-7</sup> M ouabain, whilst a concentration of 10<sup>-6</sup> M is required for a similar effect at 15°C.

The effect of storage at -20°C and freezing and thawing on ATPase activity

The enzyme suspension was prepared as described above under Materials and Methods, 1,2. It was then divided into two portions. One part was used immediately to determine the Na<sup>+</sup> - K<sup>+</sup>ATPase activity at zero time, whilst the second was placed at -20°C. At 24 h intervals from time 0, determinations of Na<sup>+</sup> - K<sup>+</sup>ATPase activity of this stored preparation were made. On removal from storage the preparation was allowed a period of 40 min at room temperature in which to thaw. The same stored preparation was used for each determination of Na<sup>+</sup> - K<sup>+</sup>ATPase activity. Thus,

Fig. 11. Ouabain inhibition of  $\text{Na}^+ - \text{K}^+$ ATPase as a function of temperature.

- A.  $\text{Na}^+ - \text{K}^+$ ATPase activity obtained as the difference between the activity in the presence of 100 mM  $\text{Na}^+$ , 20 mM  $\text{K}^+$ , 4 mM  $\text{Mg}^{2+}$  and in the presence of 4 mM  $\text{Mg}^{2+}$  alone. Both reaction media contained 2 mM ATP, 50 imidazole (pH 7.25). Incubation time 75 min at 10°C, 30 min at 20° and 30°C, Ordinate:  $\text{Na}^+ - \text{K}^+$ ATPase activity expressed as nmoles Pi liberated/mg protein/min. Abscissa: temperature °C. Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.
- $\text{Na}^+ - \text{K}^+$ ATPase activity in the absence of ouabain
  - ▲ " " activity when  $10^{-7}$ M ouabain is present in the reaction medium
  - " " activity when  $10^{-6}$ M ouabain is present in the reaction medium
- B. Total inhibition corresponds to the activity in the presence of 4mM  $\text{Mg}^{2+}$  alone. Zero inhibition equal to the difference between the activity in the presence of 4 mM  $\text{Mg}^{2+}$ , 20 mM  $\text{K}^+$ , 100 mM  $\text{Na}^+$  and in the presence of 4 mM  $\text{Mg}^{2+}$  only. Ordinate: percentage inhibition of  $\text{Na}^+ - \text{K}^+$ ATPase activity. Abscissa: temperature °C. (● - ●) inhibition produced by  $10^{-6}$ M ouabain in the reaction medium, (▲ - ▲) inhibition produced by  $10^{-7}$  ouabain in the reaction medium.

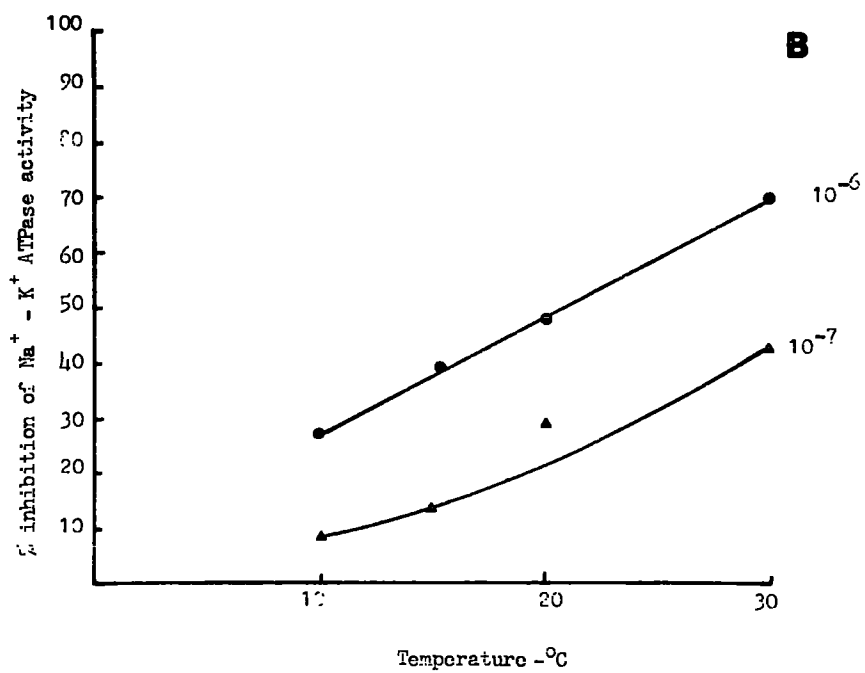
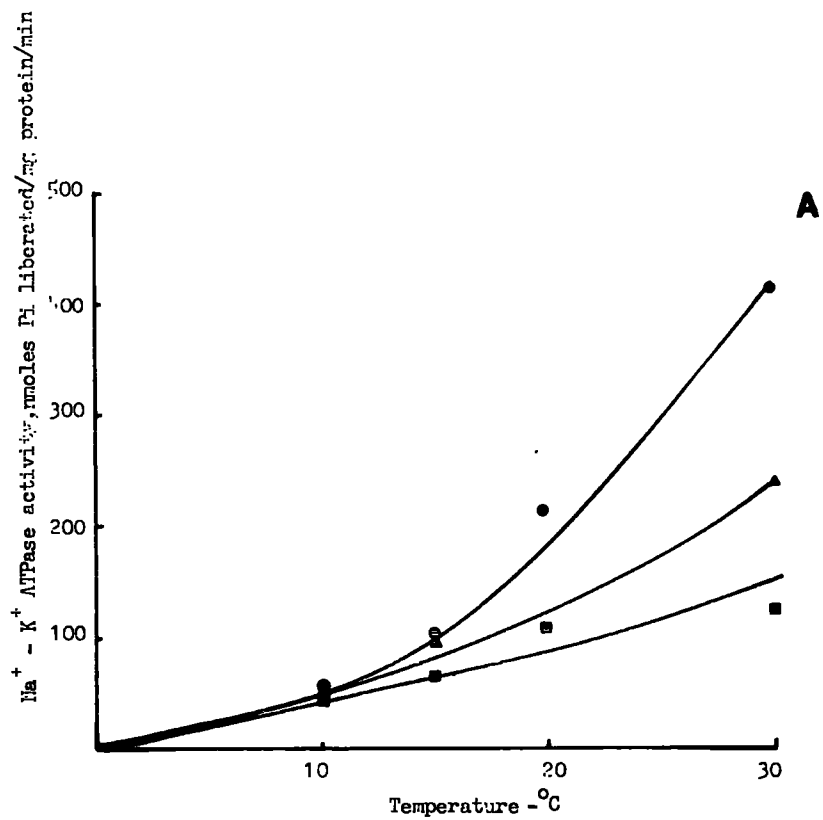


Fig. 11

not only storage but repeated freezing and thawing is to be considered. The effect of this treatment on both  $\text{Na}^+ - \text{K}^+$  and  $\text{Mg}^{2+}$  ATPases is shown in Figure 12. Both enzymes are extremely sensitive, losing approximately 50% of their activity after 24 h and roughly 88% after 48 h. In a second set of experiments only the effect of freezing and thawing on ATPase activity was determined. After isolation the enzyme preparation was divided into two parts. One was placed on ice and was used as a control preparation, its ATPase activity was assayed at zero time and also at the times when the "frozen" preparation was also assayed. The second or frozen portion, underwent the freeze - thaw procedure. This consisted of first freezing the preparation at  $-20^\circ\text{C}$  for 45 min, followed by thawing at room temperature for a further 45 min. The volume of enzyme suspension required to determine ATPase activity was removed from the thawed stock preparation, the remainder was refrozen at  $-20^\circ\text{C}$  for a further 45 min. This was rethawed a second time and the ATPase activity assayed and compared with that of the preparation standing on ice. It is evident from Figure 13 that freeze - thaw treatment affects  $\text{Na}^+ - \text{K}^+$  ATPase activity. Although only two freeze-thaw treatments were carried out, the decline in  $\text{Na}^+ - \text{K}^+$  ATPase activity appears to be linear. It will also be seen that there is a decline in  $\text{Na}^+ - \text{K}^+$  ATPase activity when the preparation is allowed to remain on ice for any length of time.  $\text{Mg}^{2+}$  ATPase activity is unaffected by maintaining the preparation at  $0^\circ\text{C}$  but is slightly enhanced by the freeze/thaw treatment.

#### The dependence of ATPase activity on magnesium ion concentration

The relationship between ATPase activity and magnesium ion concentration is shown in Figure 14. The concentration of magnesium ions was varied from 0-8 mM whilst maintaining the ATP concentration at 2 mM. In the absence of magnesium ions there was no  $\text{Na}^+ - \text{K}^+$  ATPase



Fig. 12 Effect of storage at  $-20^{\circ}\text{C}$  and freezing and thawing on  $\text{Na}^+ - \text{K}^+$  ( $\bullet - \bullet$ ) and  $\text{Mg}^{2+}$  ( $\blacktriangle - \blacktriangle$ ) ATPases

$\text{Na}^+ - \text{K}^+$  ATPase activity obtained as the difference between the activity in  $100 \text{ mM Na}^+$ ,  $20 \text{ mM K}^+$ ,  $4 \text{ mM Mg}^{2+}$  and in the presence of  $4 \text{ mM Mg}^{2+}$ .  $\text{Mg}^{2+}$  ATPase activity being the  $\text{Pi}$  released in the presence of  $4 \text{ mM Mg}^{2+}$  only. All reaction media contained  $2 \text{ mM ATP}$ ,  $50 \text{ mM imidazole (pH } 7.25)$ . Incubation  $30 \text{ min at } 30^{\circ}\text{C}$ . Preparation used on Day 2 was that refrozen from Day 1. Ordinate: ATPase activity expressed as  $\text{nmoles Pi liberated/mg protein/min}$ . Abscissa: Time at  $-20^{\circ}\text{C}$  in days.

Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

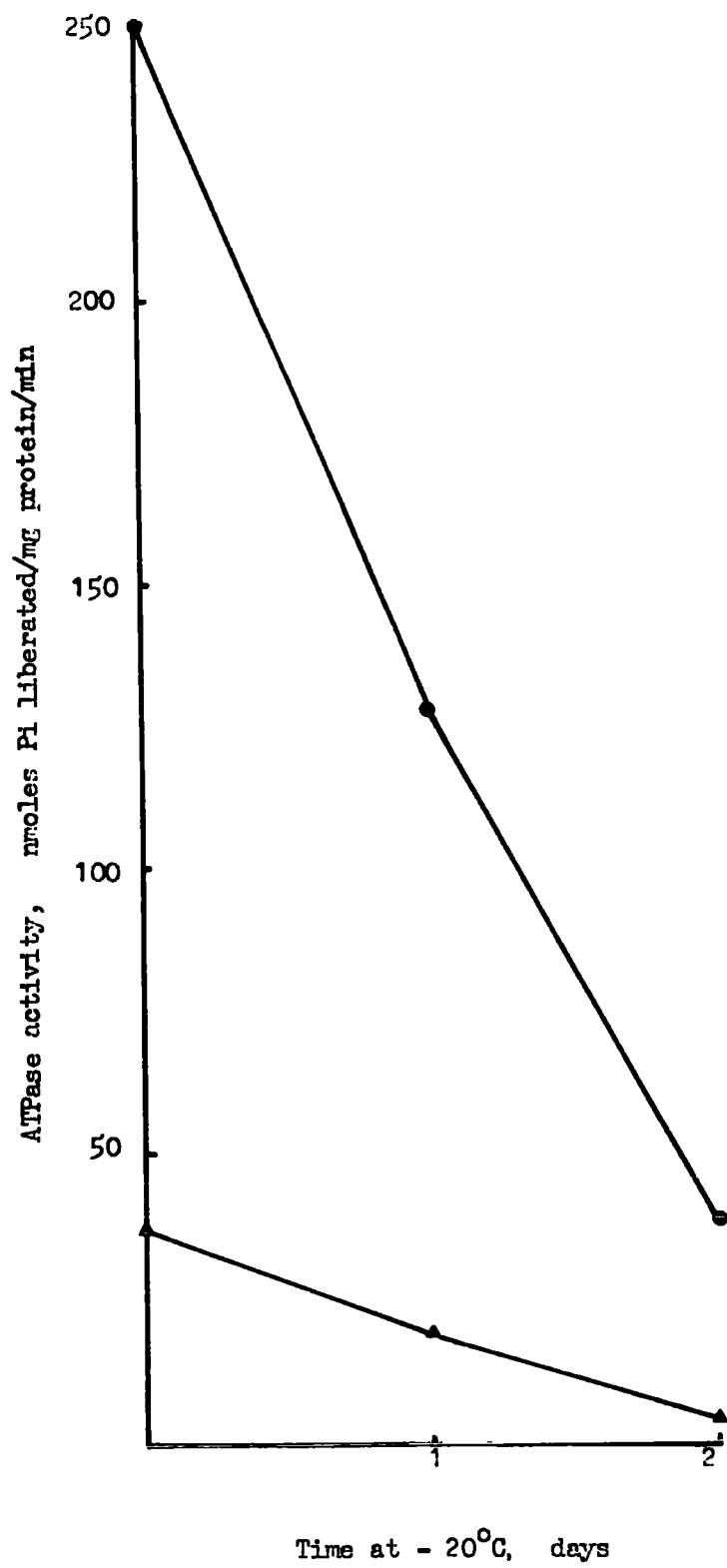


Fig. 12

Fig. 13 Effect of freezing and thawing on Na<sup>+</sup> - K<sup>+</sup> and Mg<sup>2+</sup> ATPases

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. Mg<sup>2+</sup> ATPase activity being the Pi released in the presence of 4 mM Mg<sup>2+</sup> only. All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation 30 min at 30°C. Ordinate: ATPase activity expressed as nmoles Pi liberated/mg protein/min.

Abscissa: number of times frozen and thawed.

(● - ●) Na<sup>+</sup> - K<sup>+</sup> ATPase activity of preparation which has been maintained at 0°C; (○ - ○) Na<sup>+</sup> - K<sup>+</sup> ATPase activity of preparation which has undergone one or two cycles of freezing and thawing; (▲ - ▲) Mg<sup>2+</sup> ATPase activity of preparation which has been maintained at 0°C; (△ - △) Mg<sup>2+</sup> ATPase activity of preparation which has undergone one or two cycles of freezing and thawing.

Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

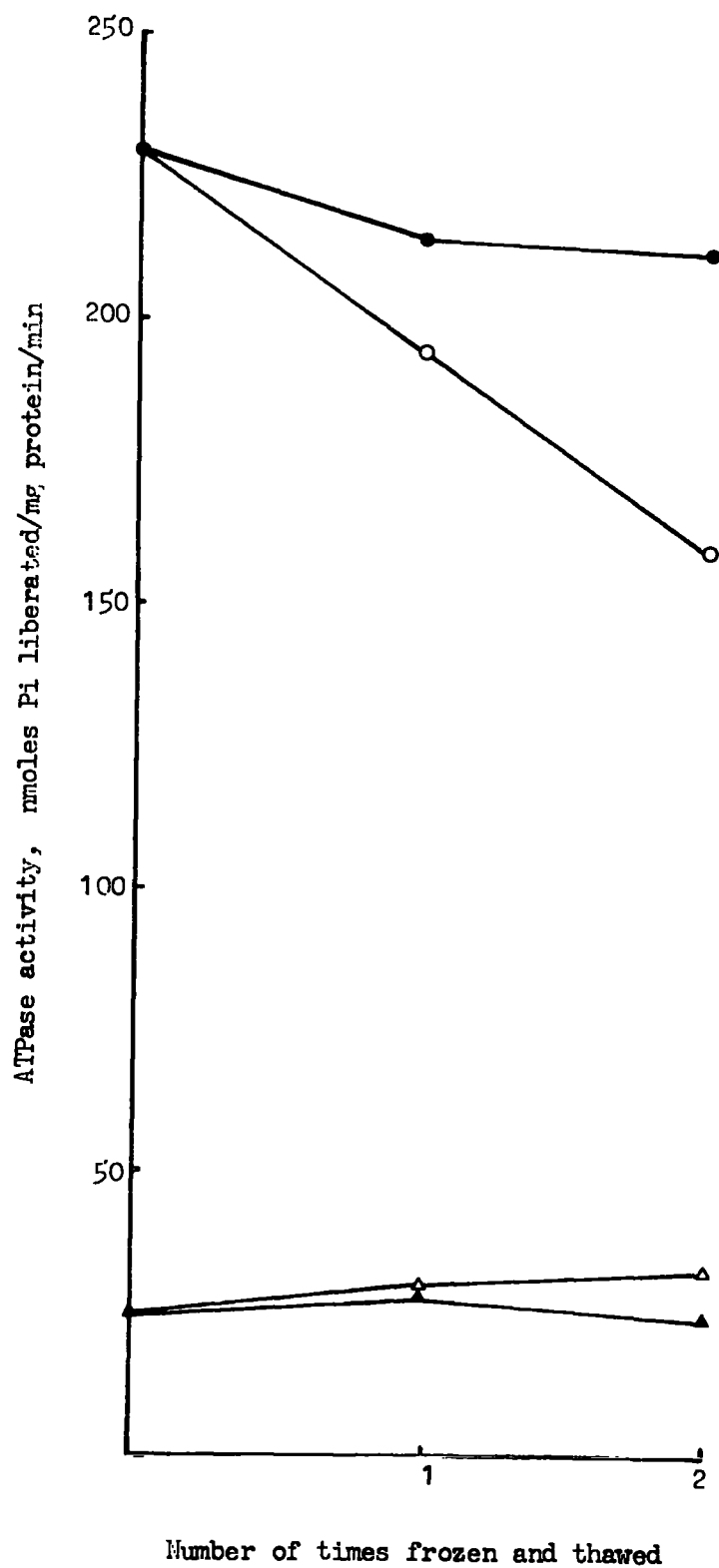


Fig. 13

Fig. 14 Effect of  $Mg^{2+}$  concentration on  $Na^+ - K^+$  ( $\bullet - \bullet$ ) and  $Mg^{2+}$  ( $\blacktriangle - \blacktriangle$ ) ATPases

$Na^+ - K^+$  ATPase activity obtained as the difference between the activity in the presence of 100 mM Na, 20 mM  $K^+$  and concentrations of  $Mg^{2+}$  indicated on the abscissa and in the presence of  $Mg^{2+}$  alone.

$Mg^{2+}$  ATPase activity being the Pi released in the presence of  $Mg^{2+}$  only. All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25).

Incubation 30 min at 30°C. Ordinate: ATPase activity expressed as nmoles Pi liberated/mg protein/min. Abscissa:  $Mg^{2+}$  concentration (mM)  
Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

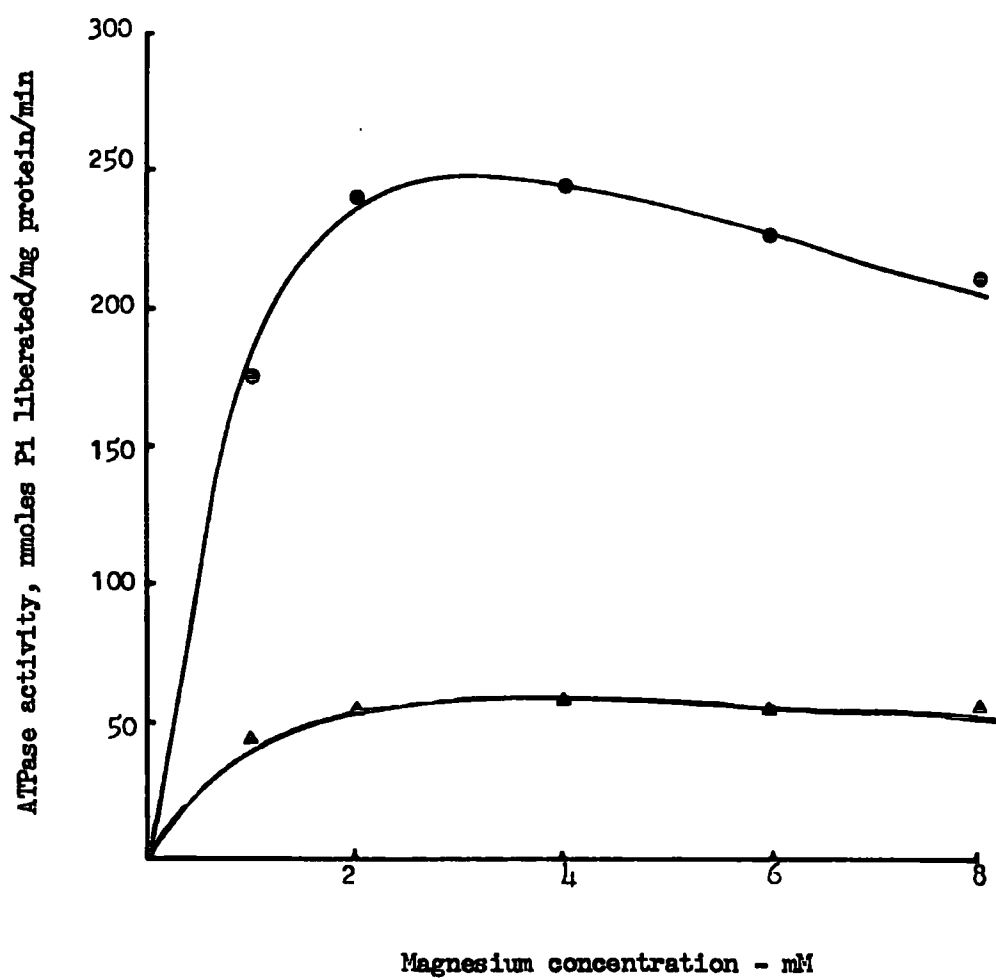


Fig. 14

activity. It will be seen that activation of this enzyme was maximal in the region of 2 - 4 mM magnesium. Further increase in magnesium concentration caused a decline in  $\text{Na}^+ - \text{K}^+$ ATPase activity.  $\text{Mg}^{2+}$ ATPase was relatively unaffected by the range of magnesium concentrations used in the experiments.

The effect of sodium and potassium concentration on  $\text{Na}^+ - \text{K}^+$  ATPase activity.

Figure 15 shows the variation in  $\text{Na}^+ - \text{K}^+$ ATPase activity as a function of potassium concentration for five different sodium concentrations 10, 50, 75, 100 and 200 mM and for three species of insect. The basal  $\text{Mg}^{2+}$ ATPase activity was only slightly stimulated by the presence of sodium ions. However as Figure 15 illustrates, the addition of small quantities of potassium ions to the medium containing magnesium and sodium ions has a pronounced effect upon enzyme activity. As can be seen a similar pattern of stimulation occurred for each preparation. In general an increase in potassium ions results in an increase in ATPase activity upto a certain potassium concentration, above which there is a reduction of enzyme activity. It is also evident that the concentration of potassium ions giving maximal stimulation is dependent on sodium, the higher the sodium ion concentration the higher the potassium required to yield maximal enzyme activity. This is perhaps clearest shown in Figure 15 (B) where at 10 mM sodium, maximal activation occurs at about 10 mM potassium whereas at 50 mM sodium the point of maximal activation occurs at 20 mM potassium. Increasing the sodium concentration to 100 mM requires a potassium concentration of 30 mM to give maximal activity. It is also clear that the higher overall activity levels occurred at 50 and 100 mM sodium. In one preparation

Fig. 15. Effect of Na<sup>+</sup> and K<sup>+</sup> concentration on Na<sup>+</sup> - K<sup>+</sup> ATPase

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 4 mM Mg<sup>2+</sup>, 10, 50, 75, 100 or 200 mM Na<sup>+</sup> and amounts of K<sup>+</sup> as indicated on the abscissa and in the presence of 4 mM Mg<sup>2+</sup> alone. Both reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation 30 min at 30°C. Ordinate: Na<sup>+</sup> - K<sup>+</sup> ATPase activity expressed as nmoles Pi liberated/mg protein/min. Abscissa: K<sup>+</sup> concentration (mM).

- A. preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus
- B. preparation obtained from the combined Malpighian tubules and hindgut of Jamaicana flava.
- C. preparation obtained from the rectum of Locusta migratoria.



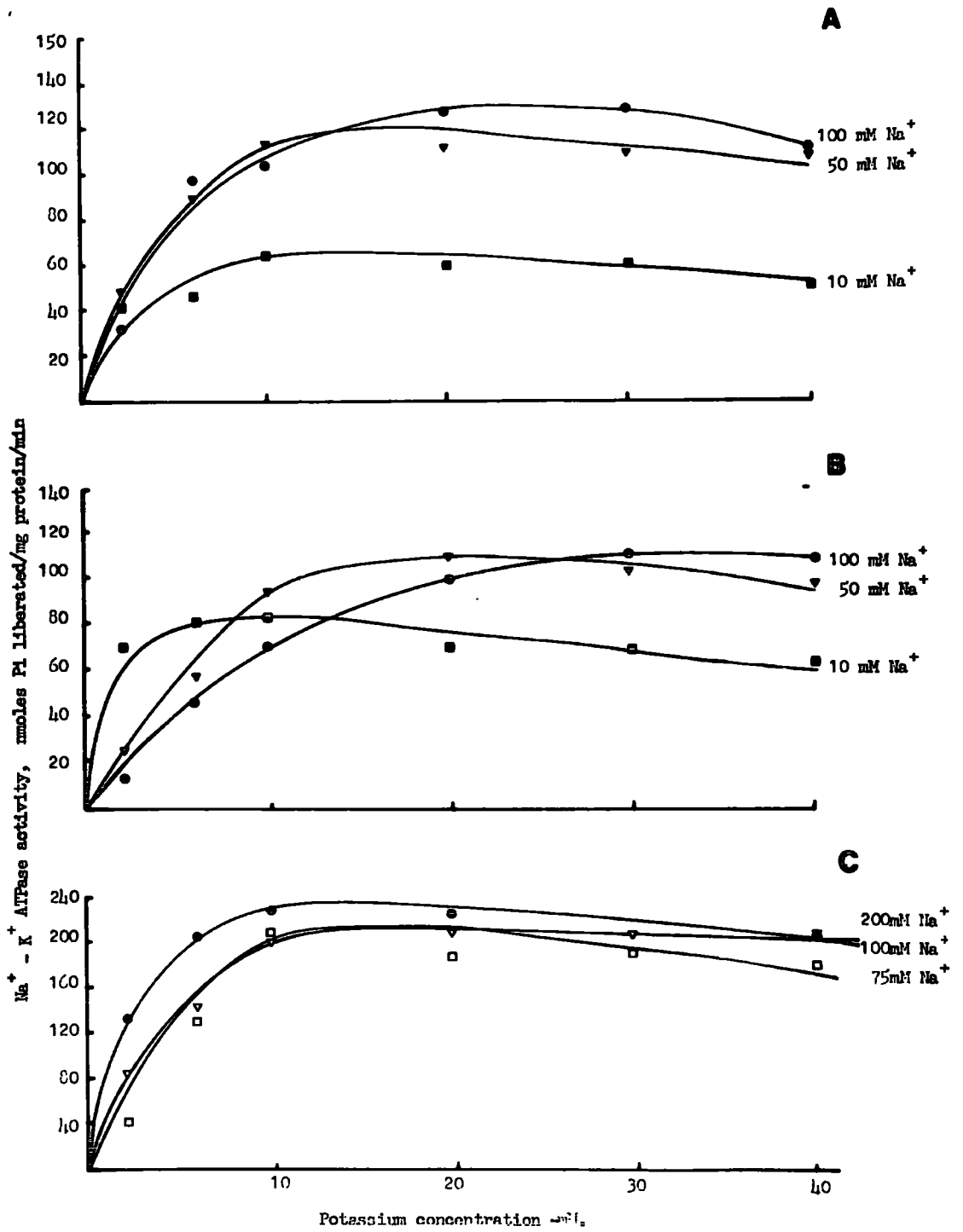


Fig. 15

(Fig.15(C)) a sodium concentration of 200 mM was used which was slightly inhibitory as compared with 100 mM sodium, at all concentrations of potassium used. The concentration of monovalent cations that produced maximal stimulation of  $\text{Na}^+ - \text{K}^+$  ATPase at  $30^\circ\text{C}$  were obtained for each preparation and are as follows: Homorocoryphus nitidulus vicinus, 100 mM sodium and 20-25 mM potassium; Jamaicana flava 100 mM sodium and 30 mM potassium; Locusta migratoria, 100 mM sodium and 10-20 mM potassium. In addition, at 100 mM sodium, the concentrations of potassium ions required for half maximal activation were 3.0, 7.5 and 1.5 mM for each species respectively.

#### The effect of ATP concentration on ATPase activity

Earlier experiments (Fig.2) showed that at 2 mM ATP and  $30^\circ\text{C}$ ,  $\text{Na}^+ - \text{K}^+$  ATPase activity was linear for at least 45 min. The question arises whether or not the reaction proceeds linearly at ATP concentrations below 2 mM. The results of some experiments in which ATP concentrations of 1.0, 0.5 and 0.25 mM were used are shown in Figure 16. It will be seen that activity was linear over 30 min for all three ATP concentrations used. Consequently an incubation time of 20 min was adopted for subsequent experiments in which the effect of substrate concentration on enzyme activity was followed. From earlier work, a magnesium : ATP ratio of approximately one gave maximal activity of  $\text{Na}^+ - \text{K}^+$  ATPase. Confirmation of this value was sort by performing complementary experiments in which the concentration of magnesium was maintained at 4 mM whilst the ATP concentration was varied between 0 - 4.0 mM. The results are given in Figure 17. It can be seen that optimal  $\text{Na}^+ - \text{K}^+$  ATPase activity occurs in the presence of 2 mM ATP i.e. at a magnesium : ATP ratio of two. It will also be seen that ATP concentrations of 3 - 4 mM very slightly depressed  $\text{Na}^+ - \text{K}^+$  ATPase activity.  $\text{Mg}^{2+}$  ATPase activity was unaffected

Fig.16 Na<sup>+</sup> - K<sup>+</sup> ATPase activity as a function of time  
at various substrate concentrations

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. All reaction media contained 50 mM imidazole (pH 7.25) as well as the stated concentrations of ATP. Incubation temperature 30°C.

Ordinate: Na<sup>+</sup> - K<sup>+</sup> ATPase activity expressed as nmoles Pi liberated/mg protein.

Abscissa: Time (minutes).

Preparation from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus

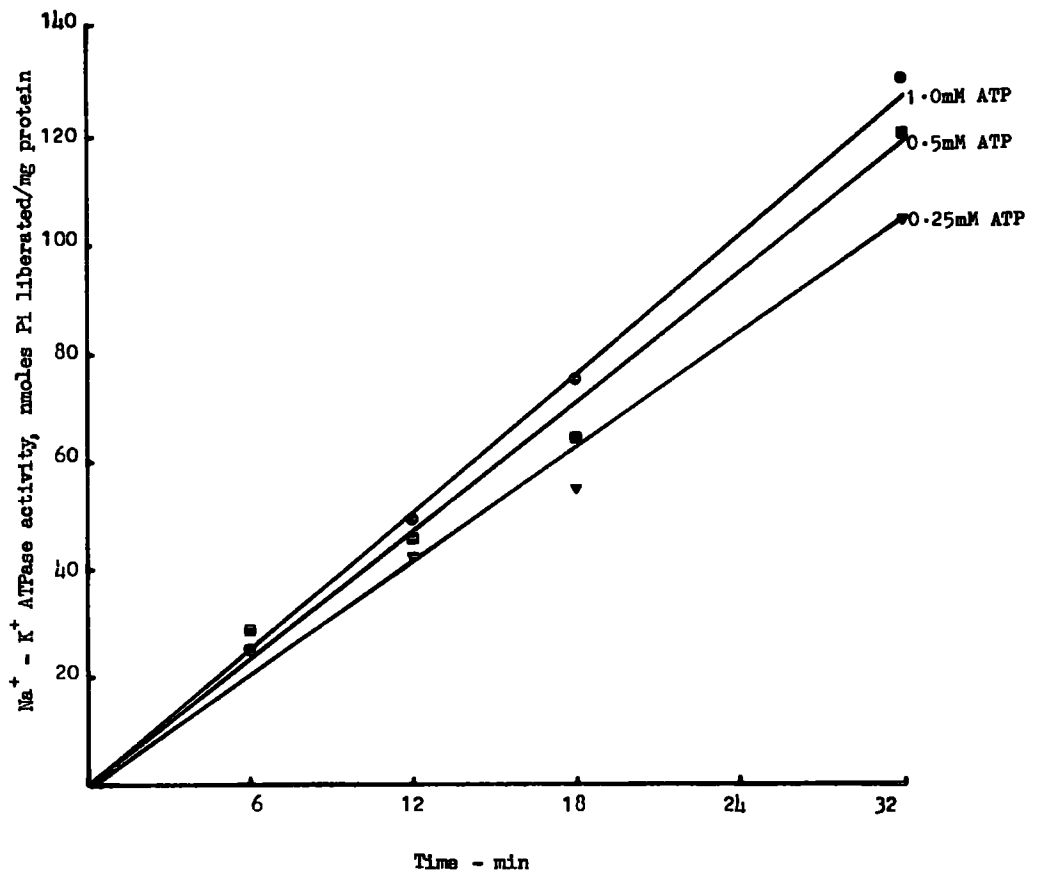


Fig.16

Fig. 17 Effect of ATP concentration on Na<sup>+</sup> - K<sup>+</sup> (● - ●) and Mg<sup>2+</sup> (▲ - ▲) ATPases

Na<sup>+</sup> - K<sup>+</sup> ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4 mM Mg<sup>2+</sup> alone. Mg<sup>2+</sup> ATPase activity being the Pi released in the presence of 4 mM Mg<sup>2+</sup> only. All reaction media contained ATP at one of the concentrations indicated on the abscissa, 50 mM imidazole (pH7.25)

Ordinate: ATPase activity expressed as nmoles Pi liberated/mg protein/min. Abscissa: ATP concentration (mM). Incubation 20 min at 30°C.

Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

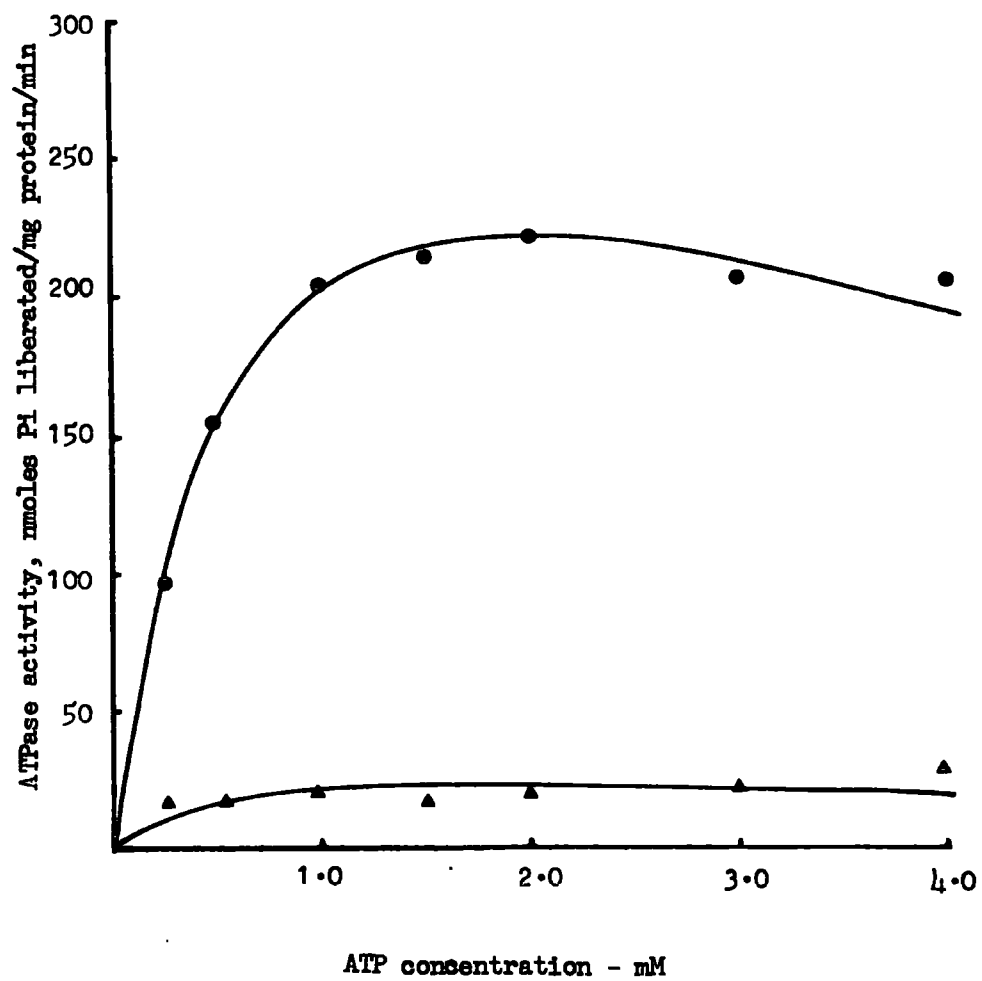


Fig. 17

by the concentrations of ATP used in these experiments. Figure 18 shows a Lineweaver - Burke plot of the data in which a double reciprocal plot of ATP concentration and reaction velocity was plotted. The  $K_m$  of  $\text{Na}^+ - \text{K}^+$  ATPase was found to lie between 0.12 and 0.19  $\mu\text{moles l}^{-1}$  ATP.  $V_{\text{max}}$  was approximately 250  $\mu\text{moles Pi liberated/mg protein/min}$ .

#### The effect of inhibitors on $\text{Na}^+ - \text{K}^+$ ATPase

$\text{Na}^+ - \text{K}^+$  ATPase activity was assayed in the presence of varying concentrations of ouabain, ethacrynic acid and alcohol. The results are shown in Figure 19. All three substances were found to inhibit enzyme activity to different degrees. Whilst it is difficult to compare all three directly, it appears that ouabain is a far more potent inhibitor of  $\text{Na}^+ - \text{K}^+$  ATPase activity than ethacrynic acid. A concentration of  $10^{-5}\text{M}$  ouabain is sufficient to reduce the sodium and potassium ion stimulated hydrolysis of ATP to almost zero, whilst at  $5 \times 10^{-3}\text{M}$  ethacrynic acid there was still substantial enzyme activity. Concentrations of between  $10^{-6} - 10^{-7}\text{M}$  ouabain and 2 - 3 mM ethacrynic acid produced 50% inhibition of enzyme activity. Israel and Salazar (1967) have shown that ethanol markedly inhibits the  $\text{Na}^+ - \text{K}^+$  ATPase from beef brain. In the present work, small quantities of ethanol in the incubation medium also reduced  $\text{Na}^+ - \text{K}^+$  ATPase activity. The shape of the curve for ethanol inhibition resembles that for ouabain inhibition of the enzyme. Low "concentrations" of ethanol (0.5 - 1.0%) have very little effect whilst between 1.0 - 2.5 % there was a rapid decline in enzyme activity. A "concentration" of 2.5 % ethanol was found to reduce  $\text{Na}^+ - \text{K}^+$  ATPase activity by 50%.

### 3. Electron microscopic examination of membrane fractions

The fraction sedimenting at 50,000  $\text{xg}$  was heterogenous in composition and contained elements resembling trachea and cuticle. Membrane fragments, vesicles and electron dense particles were also present. A small

Fig. 18 Lineweaver-Burke plot of Na<sup>+</sup> - K<sup>+</sup> ATPase activity against ATP concentration

Na<sup>+</sup> - K<sup>+</sup>ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and in the presence of 4mM Mg<sup>2+</sup> alone. Both reaction media contained ATP, 50 mM imidazole (pH 7.25) Incubation 20 min at 30°C.

Data from one typical experiment. Preparation obtained from the combined Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus.

Ordinate: Reciprocal of Na<sup>+</sup> - K<sup>+</sup>ATPase activity (nmoles Pi liberated/mg protein/min) x 10<sup>3</sup>,

Abscissa: reciprocal of ATP concentration (mM)

(line drawn to points by the method of least squares).



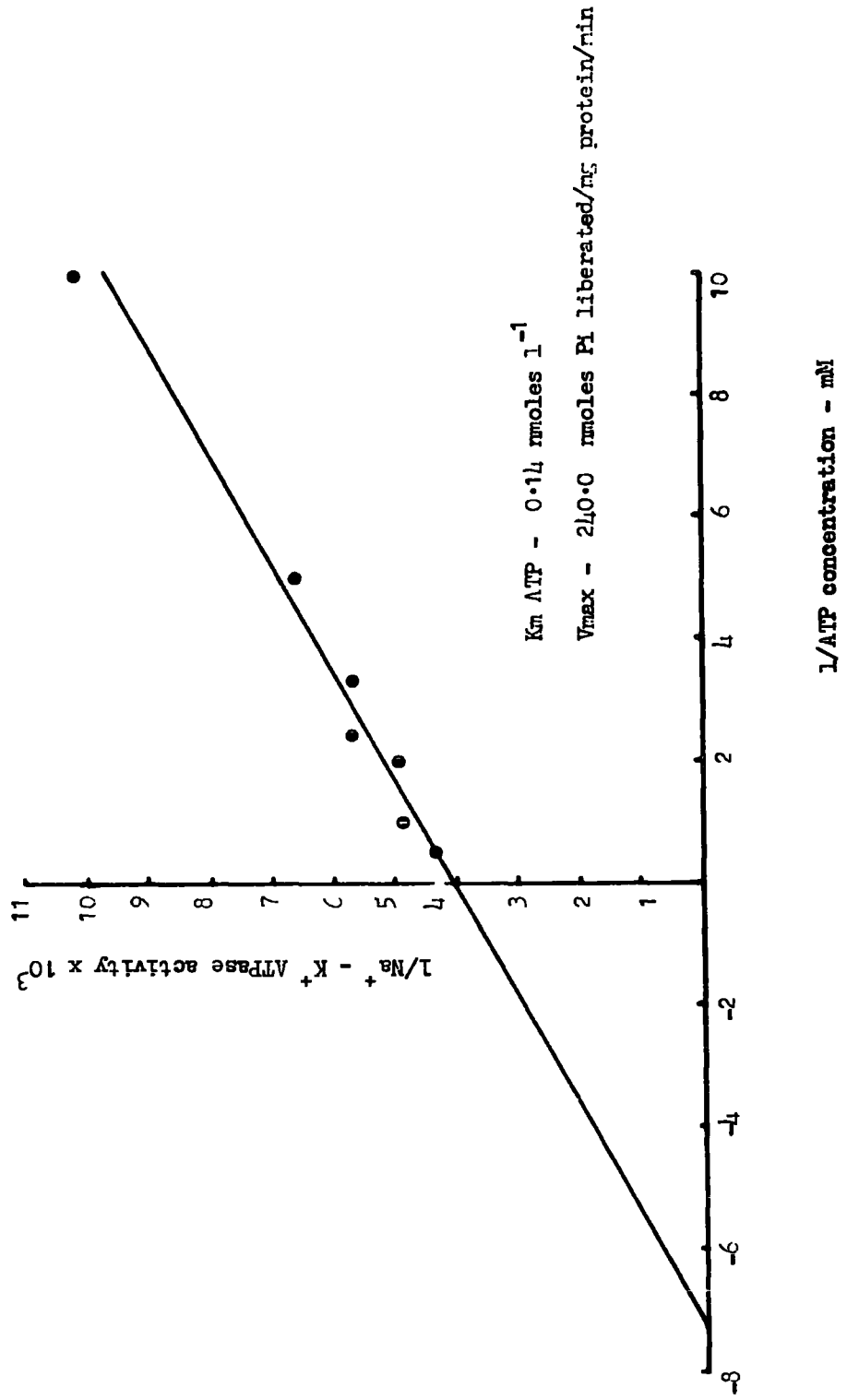


Fig. 18

Fig. 19 Effect of inhibitors on Na<sup>+</sup> - K<sup>+</sup>ATPase

Na<sup>+</sup> - K<sup>+</sup>ATPase activity obtained as the difference between the activity in the presence of 100 mM Na<sup>+</sup>, 20 mM K<sup>+</sup>, 4 mM Mg<sup>2+</sup> and the activity in the presence of 4 mM Mg<sup>2+</sup> alone. Both media contained 2 mM ATP, 50 mM imidazole (pH 7.25).

Ordinate: Na<sup>+</sup> - K<sup>+</sup>ATPase activity expressed as nmoles Pi liberated/mg protein/min.

Abscissa: A Ouabain concentration (M)  
 B Ethacrynic acid (mM)  
 C % Ethanol

Preparations obtained from the Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus

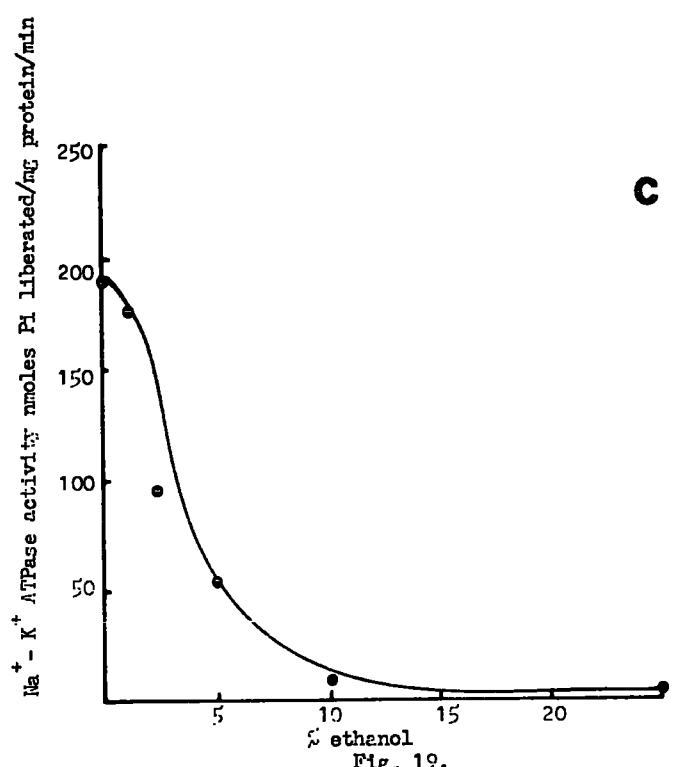
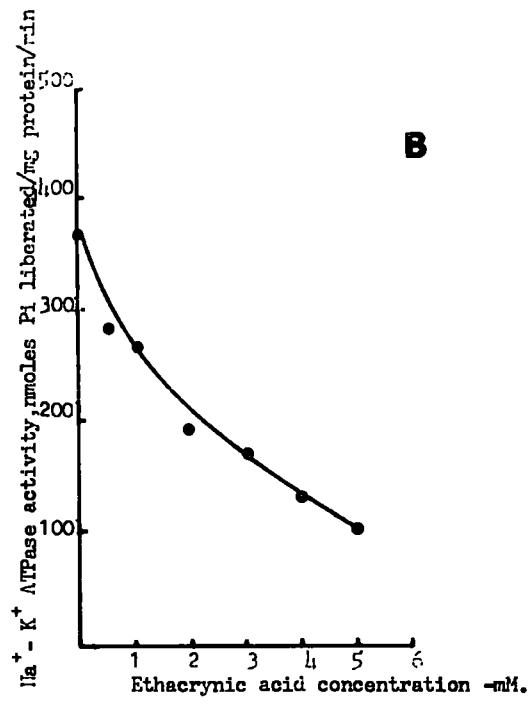
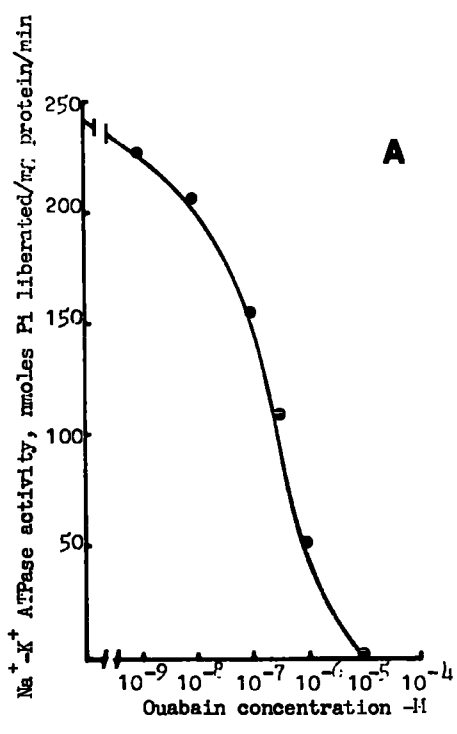


Fig. 19.

portion of a pellet is shown in Figures 20,21. It will be seen that the membrane fragments sometimes occurred as sheets in layered stacks or appeared to be embedded in material resembling the ground cytoplasm or nucleoplasm of intact cells. Both the membrane fragments and vesicles were devoid of any particulate attachment such as ribosomes. The vesicles were of various sizes, some were intact (closed vesicles) whilst others were open. The vesicles possessed no electron dense inclusions.

Figures 22, 23 shows the composition of the 100,000 xg or microsomal fraction. It will be seen that it presents an uniform appearance of compact membranous vesicles of similar size and shape, although larger types occur at the bottom of the pellet. The vesicles were a mixture of open and closed elements, only rarely containing any type of inclusion. Particulate attachments were not observed on the membranes.

#### 4. The effect of frontal connective severance on $\text{Na}^+ - \text{K}^+$ and $\text{Mg}^{2+}$ ATPase activity of Malpighian tubule, ileal and rectal preparations

Figure 24 shows the effect of the operation on growth as indicated by the weight changes of individuals. It will be seen that both operated and control insects fail to grow immediately after the operation and lose weight. When fresh grass was present, control insects grew and showed a 30 - 40% increase in weight over a six day period. Operated individuals however showed one of two trends. There were those that showed some growth, increasing in weight by roughly 11% over six days and others that failed to grow and did not regain the initial weight loss.

Table 4, shows the results of four experiments in which the effect of frontal connective severance on  $\text{Na}^+ - \text{K}^+$  and  $\text{Mg}^{2+}$  ATPase activity of Malpighian tubules, ileal and rectal preparations was examined. It is evident that a degree of variation exists within the same and different groups of insects. In all parts of the gut, of both operated and control

Fig. 20

An electron micrograph of a transverse section through the pellet of the 50,000 $\times$ g fraction, showing open vesicles (OV), membrane layers (ML) and dense ground substance (GS). Scale 0.25  $\mu$ m.

Fig. 21

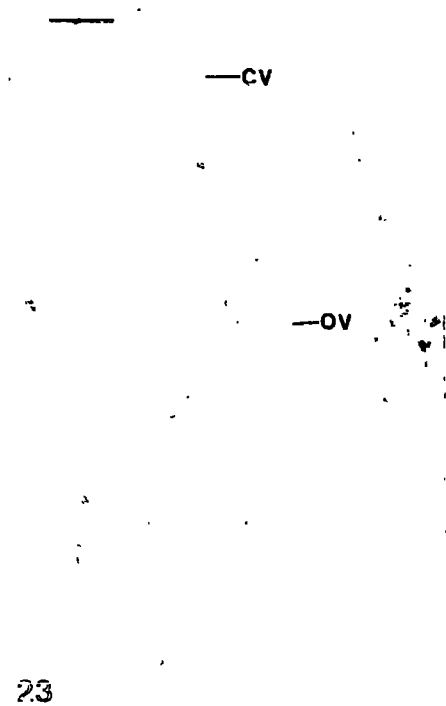
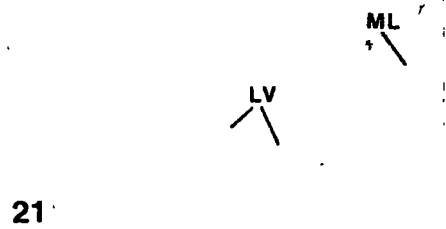
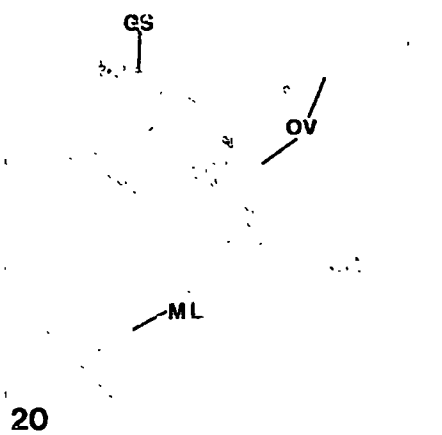
An electron micrograph of a section through the pellet of the 50,000 $\times$ g fraction showing layers of membranes (ML) and large vesicles (LV). Scale 0.2  $\mu$ m.

Fig. 22.

An electron micrograph of a section through the pellet of the 100,000 $\times$ g fraction, to show its vesicular nature. Some of the vesicles are open (OV) and others closed (CV). Scale 0.5  $\mu$ m.

Fig. 23.

An electron micrograph similar to Figure 22 but at a higher magnification. CV, closed vesicles; OV, open vesicles; Scale 0.1  $\mu$ m.



Figs.20-23

Fig. 24 Effect of frontal connective severance on growth,  
as indicated by the weight changes of 5th instar  
male Locusta migratoria

(●-●) control insect; (▲-▲), (△-△) operated insects. Each point represents the data from one insect. Measurements were made on the same insect during seven days.

Ordinate: weight expressed as percentage of the initial weight. Abscissa: Time in days after operation.

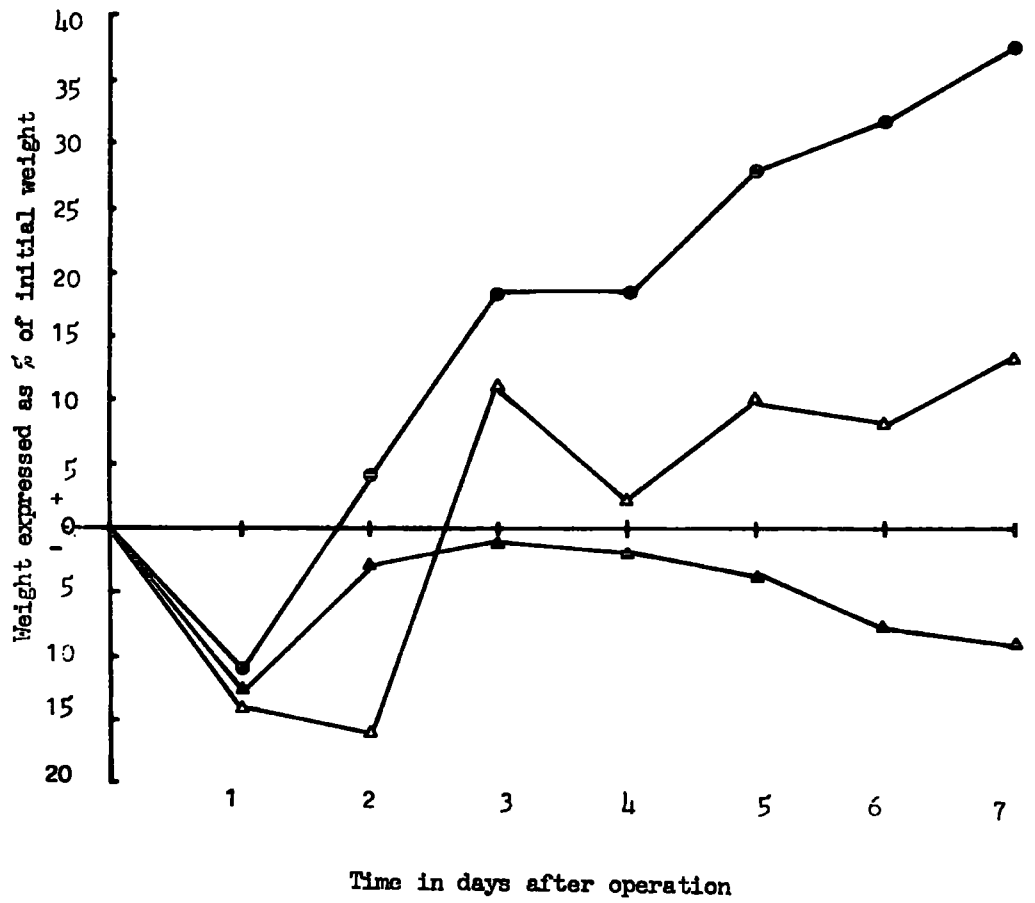


Fig. 24



Table 4

$Mg^{2+}$  and  $Na^+ - K^+$  ATPase activity of microsomal preparations isolated from the Malpighian tubules, ilea and recta of control and operated fifth instar male Locusta migratoria. The Malpighian tubules and hindguts of eight insects were used in each determination of enzyme activity. Microsomal preparations obtained after homogenization in mannitol and deoxycholate medium followed by extraction with sodium iodide. Operation: severance of both frontal connectives.

Incubation conditions:

Salts present in various combinations, at the following concentrations: 4 mM  $Mg^{2+}$ , 20 mM  $K^+$ , 100 mM  $Na^+$ . All reaction media contained 2 mM ATP, 50 mM imidazole (pH 7.25). Incubation 45 min at 30°C.

ATPase activity nmoles Pi liberated/mg protein/min			
Experiment	Malpighian tubule		Rectum
	Activity in the presence of: Mg <sup>2+</sup> Mg <sup>2+</sup> , Na <sup>+</sup> , K <sup>+</sup> (A) (B)	Activity in the presence of: Mg <sup>2+</sup> Mg <sup>2+</sup> , K <sup>+</sup> , Na <sup>+</sup> (A) (B)	Activity in the presence of: Mg <sup>2+</sup> Mg <sup>2+</sup> , Na <sup>+</sup> , K <sup>+</sup> (A) (B)
1 (Operated)	80.0 42.9	43.0 5.9	62.9 36.6
2 (Control)	120.7 114.7	10.2 9.2	75.9 74.0
3 (Operated)	58.1 49.7	51.4 31.7	80.9 72.6
4 (Control)	118.2 112.8	26.5 22.4	156.3 134.6

Table 4

insects,  $\text{Na}^+ - \text{K}^+$ ATPase activity was usually stronger than  $\text{Mg}^{2+}$  ATPase activity, with values of between 114.7 - 42.9 and 16.0 - 5.4 nmoles Pi/mg protein/min respectively, being recorded for the Malpighian tubule preparations. Furthermore, except in one instance (ileum),  $\text{Na}^+ - \text{K}^+$  ATPase activity was higher in control than in operated groups of insects. For example, in experiments 1 and 2,  $\text{Na}^+ - \text{K}^+$  - ATPase activities of 74.0 and 36.6 nmoles Pi/mg protein/min were recorded for the rectal preparations of control and operated groups respectively whilst in experiments 3 and 4 112.8 and 49.7 nmoles Pi/mg protein/min were recorded for the Malpighian tubule preparations of control and operated insects respectively.

### Discussion

Two methods of extracting microsomes with  $\text{Na}^+ - \text{K}^+$ ATPase activity were used in the present study. The buffered sucrose technique developed for nervous tissue, did little more than indicate the presence of a ouabain sensitive component. Results similar to those reported above were obtained from studies on insect muscle (Wareham, Duncan and Bowler, 1968) and mammalian muscle (Radcliffe, 1973; Parkin, 1975) and can be largely explained by sodium inhibition of the basic  $\text{Mg}^{2+}$ ATPase activity. Such inhibition would mask the coupled stimulation when both sodium and potassium ions are present, if the  $\text{Na}^+ - \text{K}^+$ ATPase proportion of the total ATPase activity is relatively small, and the  $\text{Mg}^{2+}$  ATPase activity large. The second extraction method in which mannitol, deoxycholate and sodium iodide were used, support the findings of Skou (1962), Nakao, Nagano, Adachi and Nakao (1963), Ahmed and Judah (1964) and Robinson (1967). The use of these substances in the extraction media resulted in an apparent increase in  $\text{Na}^+ - \text{K}^+$  ATPase activity. The latter may be attributable to a reduction in the basic level of  $\text{Mg}^{2+}$  ATPase activity, which may, as Skou (1962) suggests, be caused by a cleaner separation of these enzymes

during centrifugation. Indeed the  $Mg^{2+}$  ATPase activity of the preparations made in this study was usually between 3 - 20% of the total ATPase activity and for this reason the properties of this enzyme were not examined further.

The variation encountered in the present experiments may be attributable to a number of factors. The extraction procedure and estimation of inorganic phosphate and protein were standardised as far as possible. However, it is possible that small differences in extraction technique contributed to the variation seen from one experiment to another.

The results in section (a) are amongst the first biochemical demonstrations of  $Na^+ - K^+$  ATPase in the Malpighian tubules and hindguts of insects and at least for the Malpighian tubules, provide experimental support for the earlier postulations of Berridge and Gupta (1968). The properties of  $Na^+ - K^+$  ATPase reported in section (b) of Results are in good agreement with those published for other preparations from different sources (Skou, 1957; Bakkeren and Bonting, 1968; Philippot, Thuet and Thuet, 1972). However several features were found to be slightly different, for example affinities for sodium and potassium ions and the concentration of ouabain required to abolish  $Na^+ - K^+$  ATPase activity. Furthermore Skou (1965) has noted that these same features differ quantitatively throughout the literature.

$Na^+ - K^+$  ATPase activity was linear over 20-30 min and was largely unaffected by preincubation in the absence of ATP at 30°C. Similar results were reported by Baskin and Leslie (1968) for a beef brain preparation. However these workers reported an increase in ATPase activity immediately after preincubation which they attributed to some kind of advantageous alteration in the enzyme's stereochemistry. This effect was not observed in the present work. Curvilinear plots were obtained, when the effect of temperature on  $Na^+ - K^+$  ATPase activity, was

plotted as log rate against reciprocal of absolute temperature. Approximate activation energies of between 17.66 - 24.38 Kcals mole<sup>-1</sup> were obtained for the temperature range 15<sup>o</sup> - 25<sup>o</sup>C. Results of this nature were reported by Bowler and Duncan (1968) from whose data an Arrhenius  $\mu$  value of 27.5 Kcals. mole<sup>-1</sup> was calculated over the temperature range 15<sup>o</sup> - 25<sup>o</sup>C. However other workers Kohonen, Tirri and Lagerspetz (1973) report linear plots for earthworm nerve cord Na<sup>+</sup> - K<sup>+</sup>ATPase with an activation energy of 26.0 Kcals. mole<sup>-1</sup>. On the other hand Charnock, Cook and Casey (1971) and Grisham and Barnett (1973) described a sharp change in Na<sup>+</sup> - K<sup>+</sup>ATPase activity at 20<sup>o</sup>C for a mammalian kidney preparation with activation energies of 15.2 and 13.5 Kcals. mole<sup>-1</sup> above 20<sup>o</sup>C and 32.6 and 28.5 Kcals. mole<sup>-1</sup> below 20<sup>o</sup>C respectively. Thus the effect of temperature appears to depend on the source and extraction method used to obtain the enzyme preparation.

Experiments in which microsomal preparations were stored at - 20<sup>o</sup>C illustrate the acute temperature sensitivity of both Mg<sup>2+</sup> and Na<sup>+</sup> - K<sup>+</sup>ATPases. These results indicate that it would be unwise to store the preparation at - 20<sup>o</sup>C and hence each experiment was carried out with freshly obtained material. In similar studies Schwartz (1962) reported that Mg<sup>2+</sup>ATPase activity decreased faster than Na<sup>+</sup> - K<sup>+</sup>ATPase. This was not the case in the present work since very low Mg<sup>2+</sup>ATPase activities were obtained in fresh preparations. Freezing and thawing also affected Na<sup>+</sup> - K<sup>+</sup>ATPase activity but contributed little to the general reduction in activity after storage at - 20<sup>o</sup>C. The pH optima of the various Na<sup>+</sup> - K<sup>+</sup>ATPase preparations lay between 7.25 - 8.00. Skou (1957), Grasso (1967) and Philippot, Thuet and Thuet (1972) reported pH optima of between 7.2 - 7.6 for crab nerve, insect nerve and crustacean gill respectively. On the other hand Canessa - Fischer, Zambrano and Riveros - Moreno (1967)

showed a wide pH optimum for  $\text{Na}^+ - \text{K}^+$ ATPase from squid axon of between 6.0 - 8.0. Thus the enzyme from insect excretory system is fairly typical in its pH optimum.  $\text{Na}^+ - \text{K}^+$ ATPase activity was maximum at an ATP concentration of 2 mM and 4 mM magnesium. High concentrations of ATP depressed enzyme activity thus agreeing with the results of Skou (1960) and Dunham and Glynn (1961). The  $K_m$  for the enzyme was between  $1.2 - 1.9 \times 10^{-4}$  moles  $l^{-1}$  ATP which is higher than the  $1.0 - 2.7 \times 10^{-5}$  moles  $l^{-1}$  ATP reported by Bourgoignie, Klahr, Yates, Guerra and Bricker (1970). However comparison of  $K_m$  values should be performed with caution since Robinson (1967) has demonstrated that the concentration of sodium and potassium ions, affects the enzyme's affinity for its substrate.

The magnesium : ATP ratio of two was also reported for  $\text{Na}^+ - \text{K}^+$ ATPases extracted from insect nerve (Grasso, 1967), giant axon of squid (Canessa Fischer, Zambrano and Riveros - Moreno, 1967) and crustacean gill (Philippot, Thuet and Thuet, 1972). However it is common in vertebrate brain preparations for maximum activation to occur at an magnesium : ATP ratio of unity (Bakkeren and Bonting, 1968; Bourgoignie, Klahr, Yates Guerra and Bricker, 1970).

The majority of  $\text{Na}^+ - \text{K}^+$ ATPases are reported to require 100 mM sodium and 10 mM potassium for maximum activity (see Whittam and Wheeler, 1970 for references). The present work indicates that only the preparation from the rectum of Locusta migratoria conforms to these requirements. The preparations from Homorocoryphus nitidulus vicinus and Jamaicana flava both require 100 mM sodium for maximum activity but between 20 - 30 mM potassium for maximum activity.

$\text{Na}^+ - \text{K}^+$ ATPase activity was completely abolished by  $10^{-5}$  M ouabain and agrees with the observation of Glynn (1964). Other studies on ouabain inhibition relevant to the present discussion are those of Ahmed and Judah (1965) who showed that decreases in temperature produced a parallel

decrease in the effectiveness of ouabain as an inhibitor which they attributed to an increase in the affinity of  $\text{Na}^+ - \text{K}^+$ ATPase for potassium ions. The results of the present study support this view.

Electron microscopic examination of both the 50,000 and 100,000 xg fractions showed that the only recognizable elements in either were the cell membranes whilst the more resistant tissues, trachea and cuticle were distinguishable in the 50,000xg fraction. Cassidy, Smith and Hodgson (1969) used a buffered sucrose method of extracting microsomes and showed that intact mitochondria were easily distinguishable in preliminary centrifugation pellets, obtained from the gut and fat body tissues of two insect species. Furthermore these workers also report that preliminary centrifugation of between 20,000 - 30,000 xg was sufficient to remove all traces of mitochondria from the final microsomal pellet obtained from the gut of Prodenia eridania. In view of the preliminary centrifugation of 50,000xg used in the present study, the microsomal pellet was considered to be free from any mitochondrial contamination. The content of the microsomal fraction was very similar to that reported by Manitius Bensch and Epstein (1968) and Sperelakis (1972), all of whom found the fraction to consist of membranes organised as vesicles without ribosomes or other distinguishing features. These may correspond to the smooth microsomes of mammalian liver preparations reported by Dallner (1963) and Gram, Rogers and Fouts (1967). The origin of the membranes constituting the microsomal fraction is unclear. Some authors consider them to be derived from the endoplasmic reticulum and others the plasma membranes (see Introduction for references).

Considering first the effect of frontal connective severance on growth. The results of the present study agree with the findings of Roome (1968), who showed that some third and fourth instar Locusta migratoria increased in weight and produced new cuticles, following

severance of both frontal connectives, whilst others did not. Similar results were also observed by this author in Periplaneta americana. Moreover Allum (1973) reported growth in some and not in other fifth instar Locusta migratoria, following frontal connective severance and suggested that sensory information was passing to the protocerebrum by routes other than the frontal connectives. This author suggests branches of the frontal connectives and the hypocerebral, NCC I pathway of Strong (1966b) as alternative sensory routes. In the biochemical experiments individuals were not selected from the operated group of insects on the basis of their weight changes i.e. those that gained in weight and those that did not. Instead all insects in an operated group were used in the assay of  $\text{Na}^+ - \text{K}^+$  ATPase activity. This means, therefore, that two physiologically different kinds of insect are contributing to the assay of enzyme activity. Indeed Clarke and Gillot (1967a,b) have shown that the cessation of growth, following interference with the stomatogastric nervous system, is associated with a very low 'maintenance level' of protein metabolism.

In view of this, and the small number of experiments carried out, too much emphasis cannot be placed on the differences observed between the  $\text{Na}^+ - \text{K}^+$  and  $\text{Mg}^{2+}$  ATPases of control and operated groups of insects.



## CHAPTER SEVEN

## DISCUSSION

A variety of methods have been used in the search for microsomal preparations with high  $\text{Na}^+ - \text{K}^+$ ATPase activity (Skou, 1965). The present study shows that the use of a detergent, (sodium deoxycholate) and sodium iodide, instead of a buffered sucrose medium, produces preparations with high specific activity. Although the effect of these substances on tissue homogenates is well established (Skou, 1965), the events in the action of sodium deoxycholate and iodide remain unclear. Landon and Norris (1963) and Jarnefelt (1964) suggested that the effect of deoxycholate could be explained by the conversion of  $\text{Mg}^{2+}$  ATPase to  $\text{Na}^+ - \text{K}^+$ ATPase enzyme. From their studies on mammalian kidney, Jørgenson and Skou (1971) do not consider this to be the case, since they observed that deoxycholate increased the activity of  $\text{Na}^+ - \text{K}^+$ ATPase several fold, without noticeably changing the  $\text{Mg}^{2+}$ ATPase activity. Moreover, these workers suggest that deoxycholate removes lipid and protein from the membranes as well as unfolding membrane vesicles, and in doing so, exposes latent  $\text{Na}^+ - \text{K}^+$ ATPase sites in the membranes. A somewhat similar mode of action has been proposed for sodium iodide (Kawai, Nakao, Nakao and Fujita, 1973).

As an extraction procedure, the mannitol, deoxycholate and sodium iodide method, employed in the present study, was still at an early stage of development. Several features of the method require further investigation and refinement. For example, the concentrations of mannitol, deoxycholate and sodium iodide, the duration of the sodium iodide extraction period as well as homogenization require further examination. The homogenization process in particular would seem to be important since the differences observed in the concentrations of enzyme protein from one preparation

to another could be due to variations in this process. Indeed Epstein and Silva (1974) have noted that the level of  $\text{Na}^+ - \text{K}^+$ ATPase activity changes considerably with the degree of fragmentation of the plasma membrane accomplished by homogenization. Perhaps with the introduction of these modifications, some of the variation encountered in enzyme activity from one preparation to another, would be removed.

Homogenization in mannitol and deoxycholate medium followed by treatment with sodium iodide did not appear to markedly affect the functioning of  $\text{Na}^+ - \text{K}^+$ ATPase in the membrane, since the properties of the enzyme obtained from the excretory system of Homorocoryphus nitidulus vicinus are very similar to those of other  $\text{Na}^+ - \text{K}^+$ ATPases isolated from different sources (Skou, 1965).

In the Orthopteran species examined,  $\text{Mg}^{2+}$  and  $\text{Na}^+ - \text{K}^+$ ATPase activity was demonstrated in all parts of the excretory system. It would be of interest to determine the ATPase activity of all parts of the excretory system in relation to that of other tissues, e.g. fat body and muscle. The distribution of  $\text{Na}^+ - \text{K}^+$ ATPase might then throw some light on its functional importance in the excretory system, in much the same way as the high concentrations of this enzyme in the mammalian kidney, compared with liver and skeletal muscle, implicated its role in renal function (Epstein and Silva 1974).

The question arises as to the function of  $\text{Na}^+ - \text{K}^+$ ATPase in the excretory tissues. Is this enzyme primarily concerned with maintaining intracellular levels of sodium and potassium, or is it involved in a specialised, secondary function?

Phillips (1964b) has shown that the rectal pad cells of Schistocerca gregaria have a high concentration of potassium and a low concentration of sodium ions. Similar information is unavailable for the Malpighian tubule cells, although high potassium and low sodium concentrations

appear to be universal in living cells (Tosterson, 1964; Hope, 1971). Thus it is not unreasonable to suggest that  $\text{Na}^+ - \text{K}^+$ ATPase is involved in maintaining the intracellular levels of sodium and potassium in the cells of the excretory tissues. In addition to this role, sodium linked transport is known to be involved in the generation of membrane potentials (Kerkut and York, 1971), absorption of hexose sugars and amino acids in the vertebrate intestine (Grane, 1965) and the transfer of water in a wide variety of epithelia (Berridge and Oschman, 1972). With regard to the insect excretory system there is evidence to suggest a role for the sodium pump in the movement of fluid across these epithelia.

The present work, as well as the studies of Weber-Von Grothaus, Hevert, Atzabacher and Wessing (1974), Atzabacher, Hevert, Weber-Von Grothaus and Wessing (1974) and Anstee and Bell (1975) support the suggestion that a sodium/potassium exchange pump is involved in the transfer of potassium and hence fluid into the Malpighian tubule cells (Berridge, 1968; Pilcher, 1970b). Anstee and Bell (1957) have shown that fluid secretion by the Malpighian tubules of Locusta migratoria is ouabain sensitive. These workers monitored urine production by the tubules in the presence and absence of ouabain in the bathing medium. Ouabain is thought to compete with potassium ions for the potassium site of the  $\text{Na}^+ - \text{K}^+$ ATPase (Glynn 1964). The potassium site is envisaged as being located on the outer surface of the cell membrane (Skou, 1965). This suggests that  $\text{Na}^+ - \text{K}^+$ ATPase is probably located on the basal plasma membrane of Malpighian tubule cells, where it moves potassium into the cell to supply an apical potassium pump (Berridge, 1968). Ouabain, by inhibiting the basal entry of potassium, reduces the supply of this ion to the proposed apical potassium pump (Berridge 1968; Pilcher, 1970b) the overall effect of which is to decrease urine production

by the Malpighian tubules. Thus in the presence of sodium ions, a ouabain sensitive  $\text{Na}^+ - \text{K}^+$  ATPase may be actively transporting potassium ions into the Malpighian tubule cells, whereas when sodium ions are absent, a ouabain insensitive mechanism, possibly similar to the hydrogen ion potassium ion exchange proposed by Kafatos (1968), operates. Indeed, from their work on the midgut of Hyalophora cecropia, Anderson and Harvey (1966) have suggested two such mechanisms to explain the active transport of potassium in this tissue.

Maddrell (1971) has pointed out that one of the objections to a sodium/potassium exchange pump in Malpighian tubule cells is that no net movement of solute would be accomplished by this mechanism (see Chapter One for the importance of net solute movement in achieving fluid flow). Although further work is required on the proportions in which potassium ions enter and sodium ions leave the Malpighian tubule cells via the ATPase, evidence from other tissues indicates that these ions are not always transported across cell membranes in a one : one ratio. In the red blood cell, three sodium ions are pumped out of the cell in exchange for two potassium ions, whilst in the kidney the influx of potassium is only one tenth of the efflux of sodium, which suggests something more than a one : one transfer of these cations (Epstein and Silva 1974).

The case for considering potassium and chloride as the important ions in water resorption, at least in the rectum of Calliphora erythrocephala (Berridge and Gupta, 1967) is strong, particularly when it is remembered that potassium is the dominant cation in the fluid secreted by the Malpighian tubules (Maddrell, 1971) and is resorbed more rapidly than other monovalent cations from the rectal lumen (Phillips, 1964b). Recently Hopkins and Srivastava (1972) have shown, in the rectum of Leucophaea maderae that lumen potassium stimulates water uptake by the rectal epithelium and that this stimulated uptake is abolished by lumen

ouabain. (These authors point out that total water absorption is also significantly reduced by lumen ouabain). Hopkins and Srivastava (1972) suggest that the stimulated uptake may result from potassium ion transport through the apical membranes of the rectum or by supplying ions to a pumping mechanism within the rectal pads.

More recently Oschman and Wall (1969) and Phillips (1969) have pointed out the necessity of modifying the Berridge and Gupta (1967) hypothesis to take account of resorption of solutes in the intercellular sinus (see Chapter Five, Figure 61), since fluid resorption occurs in the absence of net solute transfer across the rectal epithelium. Wall and Oschman (1970) consider the recycling of ions involves sodium instead of potassium (see Chapter Five, Figure 61). From consideration of the electro-potential profiles, Phillips (1969) has presented evidence supporting the role of sodium in 'local osmosis' and the recycling process. This author suggests that the electropotential gradient would permit passive flow of sodium at the apical surface, but that sodium would have to be actively removed at the haemocoel-facing membrane, which from ultrastructural evidence, are the membranes bounding the lateral intercellular spaces which are in continuity with the haemocoel.

Evidence implicating the lateral cell membranes of the rectal pad cells in rectal function, comes from the ultrastructural studies of these cells (see Chapter Five, Introduction for references). In these cells the bulk of the mitochondria are associated with the lateral cell membranes. This implies that the lateral plasma membranes are regions where the demand for energy (ATP?) is very high. If a mechanism, which utilizes ATP as a substrate in pumping solute into the intercellular spaces were located in the lateral plasma membranes, then ATP would only have a short distance to diffuse to the reaction site. It is not unreasonable to suggest that  $\text{Na}^+ - \text{K}^+$ ATPase is the pumping mechanism involved in

solute recycling and fluid re-absorption by the rectum. Objections to a  $\text{Na}^+ - \text{K}^+$ ATPase being involved in rectal fluid absorption come from the studies of Irvine and Phillips (1971). These workers observed that fluid resorption by the rectum is insensitive to pharmacological concentrations of ouabain when the latter is present in the haemolymph. If, as has been suggested above,  $\text{Na}^+ - \text{K}^+$ ATPase is located in the lateral cell membranes of the rectal pad cells, the ultrastructural evidence suggests that the enzyme would be inaccessible to substances placed in the haemolymph. A muscle layer, and under conditions of rectal resorption, the return flow of fluid to the haemocoel, would hinder the passage of substances from the haemolymph to the lumen. It is worth noting that Irvine and Phillips (1971) did obtain a reduction in fluid resorption when the concentration of ouabain was high ( $10^{-2}\text{M}$ ), and suggest that under these conditions, the concentration of ouabain may have been sufficient to overcome a long diffusion path.

More direct support for the role of the lateral regions of the rectal pad cells in fluid transport comes from the studies of Berridge and Gupta (1967) and Wall, Oschman and Schmidt-Nielsen (1970). The latter workers observed rectal pads of Periplaneta americana in vivo and noted that the intercellular spaces distend during water absorption. Furthermore, the intercellular fluid was consistently hyperosmotic to the rectal contents. The analysis of the intercellular fluid, as well as that of other compartments within the rectal epithelium would throw some light on the nature of the solute transported.

Recently Stobbart (1968) has shown in the rectum of Schistocerca gregaria that there is a rapid turnover of sodium and potassium ions between the haemolymph and the rectal wall which does not differ significantly in recta absorbing and those not absorbing water. Stobbart suggests that water resorption is occurring continually via the double

membrane model (see Chapter One), but that in individuals not absorbing water, e.g. diuresis, the resultant resorbed water is degraded by diffusion back into the rectum through the regions of the reduced epithelium. In individuals absorbing water, e.g. antidiuresis, the back diffusion is reduced in some way, possibly by hormonal action.

The presence of neurosecretory axons within the subepithelial sinus of the rectum of a variety of insects (see Phillips, 1970 for references) including Jamaicana flava suggests a hormonal control of rectal function. Gupta and Berridge (1966b) suggested that in Calliphora erythrocephala the axons release a hormone that regulates the passive permeability of the apical plasma membrane. Nerve endings, similar to those described for Calliphora (Gupta and Berridge, 1966b) have also been reported in the rectal pads of Periplaneta americana (Oschman and Wall, 1969). In Periplaneta, these nerves originate in the last abdominal ganglion, extracts of which increase the uptake of water by in vivo recta (Wall, 1967).

There is now evidence relating neurosecretory material to protein metabolism (Clarke and Langley, 1963a-d, Gillot, 1964). Under optimum temperature conditions (30°C) growth and hence protein metabolism of certain larval Jamaicana flava was stimulated and resulted in a release of neurosecretory material from the neurosecretory system, hence the almost empty corpora cardiaca found. On the other hand under sub optimal conditions, growth and hence protein synthesis is less rapid and under these conditions neurosecretory material is not released as rapidly, hence the presence of abundant neurosecretory material in the corpora cardiaca of insects maintained at 35°C and 25°C.

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