Some effects of insect hormones on Na($^{+}$)*, K*($^{+}$) − *ATPaseandfluidsecretionbythemalpighiantubulesoflocust*

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Some effects of Insect Hormones on Na^+, K^+-ATPase and fluid secretion by the Malpighian tubules of *Locusta migratoria* L.

by

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Abstract

A study has been made on the effects of insect hormones on fluid secretion by the Malpighian tubules of *Locusta*, and on the Na^+, K^+-ATPase activity in microsomal preparations of the tubules. A diuretic hormone present in extracts of the neurosecretory cells and corpora cardiaca accelerated rates of fluid secretion by *in vitro* preparations of the tubules but had no effect on ATPase activity. Ecdysone affected neither secretory rates nor enzyme activity whereas Juvenile Hormone had an inhibitory effect on both. Attempts have been made to explain how J.H. may inhibit Na^+, K^+-ATPase activity. It is possible that J.H. affects the membrane conformation and thus prevents the normal reaction sequence of the ATPase.

Ultrastructural studies have shown that the fine structure of the Malpighian tubules varies with development. Invaginations of the basal and apical cell membranes were found to develop with increasing age throughout the 5th stadium. At the same time the numbers of mitochondria in the tubule cells appeared to increase and the mitochondria came to lie in the cytoplasm of the basal infolds. Just prior to the larval-adult moult the invaginations of both membranes decreased and mitochondria were rarely found amongst the basal infolds. Associated with these ultrastructural changes, functional changes are also reported. Na^+, K^+-ATPase activity was low at the beginning and end of the 5th stadium, times when there was least invagination of the plasma membrane. At the same times animal relative water content was high, suggesting lower rates of secretion.

Both ouabain and ethacrynic acid were found to inhibit fluid secretion by, and Na^+, K^+-ATPase, in microsomal preparations of the Malpighian tubules. Ouabain was the more effective inhibitor of enzyme activity ($pI_{50} = 5.8$ as compared with ethacrynic acid $pI_{50} = 2.5$).

The results are discussed in terms of the relationship between insect hormones and cell structure, fluid secretion and Na^+, K^+-ATPase activity.

Glossary

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
B.S.A.	bovine serum albumen
Ci	curie
c.p.m.	counts per minute
cyclic AMP	cyclic adenosine 3',5'-monophosphate
E.D.T.A.	ethylene diamine tetra-acetic acid
g	gram
5-HT	5-hydroxytryptamine
J.H.	Juvenile Hormone
K_m	Michaelis constant
M	Molar
Mg^{2+}-ATPase	magnesium activated adenosine triphosphatase
Na^+, K^+-ATPase	magnesium dependent, sodium and potassium stimulated adenosine triphosphatase
P_i	inorganic phosphate
r.p.m.	revolutions per minute
Tris	tris (hydroxy methyl) amino methane
V_{max}	maximum reaction velocity

Contents

CHAPTER 1

INTRODUCTION

'Urine' formation by the Malpighian tubules of insects has been the subject of numerous studies in the past (Ramsay 1953, 1954, 1955, 1956; Berridge 1968; Pilcher 1970; Anstee and Bell 1975; Gee 1975a, 1976a; Maddrell 1969 and see reviews 1971, 1977). This work on a variety of insect species has shown that in most cases K^+ ions are necessary for fluid secretion by the tubules and it has been suggested that the secretion of K^+ may be the 'prime mover' in generating 'urine' flow (Ramsay 1956; Berridge 1967). This conclusion is the result of several observations: the fluid secreted by the Malpighian tubules of a variety of insects has a higher K^+ concentration than the surrounding haemolymph; measurements of transwall potential show that K^+ movements into the lumen are thermodynamically uphill i.e. K^+ entry is active; the rate of fluid secretion is dependent on the K^+ concentration of the bathing fluid. However it must also be noted that the Malpighian tubules of *Rhodnius* and *Glossina* are exceptions in that *Rhodnius* tubules will secrete Na^+ or K^+ (Maddrell 1969) whilst in *Glossina*, Na^+ is the 'prime mover' (Gee 1975a, 1976a).

Although active cation transport has been established in insect Malphigian tubules (Ramsay 1953, 1955; Berridge 1967, 1968; Pilcher 1970; Maddrell 1977; Bell 1977; Anstee *et al.* 1979), the nature of the ion 'pumps' remains uncertain. Studies on the Malpighian tubules of *Calliphora* have shown that whilst the K^+ concentration in the bathing medium was of prime importance in determining the rate of fluid secretion, the secretion of fluid was enhanced when Na^+ was present as well as K^+ (Berridge and Oschman 1969). As a result of this, Berridge and Oschman have proposed a model for cation and fluid transport across *Calliphora* Malpighian tubules. They propose that in the primary cells of the Malpighian tubules there is an apical pump transporting K^+ electrogenically into the lumen, whilst on the basal surface there is a coupled Na^+/K^+ exchange pump. It is generally accepted that an electrogenic K^+ pump is situated on the apical cell membrane (Berridge 1967; Berridge and Oschman 1969; Maddrell 1977) but objections have been raised to the presence of a Na^+/K^+ pump on the basal surface. Objections to such a pump have arisen chiefly because several workers have failed to show that fluid secretion by insect Malpighian tubules is inhibited by the cardiac glycoside ouabain, which is a specific inhibitor of Na^+, K^+-activated ATPase (Berridge 1968; Maddrell 1969; Pilcher 1970; Gee 1976b; Rafaeli-Bernstein and Mordue 1978). This enzyme has been almost universally implicated in Na^+/K^+ exchange pumps elsewhere (Skou 1965; Albers 1967; Whittam and Wheeler 1970; Bonting 1970).

Maddrell (1971) has further argued against the involvement of a Na^+/K^+ exchange pump in fluid secretion across Malpighian tubules on the basis that there would be no net transfer of solute produced. This however assumes that the $Na^+ : K^+$ exchange is on a 1 : 1 basis. This is not necessarily the case and indeed in red blood cells Na^+, K^+-activated ATPase is responsible for the exchange of $3Na^+$ in one direction for $2K^+$ in the other (Post and Sen 1967).

More recently Maddrell (1977) has suggested a model which might apply to all Malpighian tubules whether they pump K^+ or Na^+. It is proposed that K^+, Na^+ and Cl^- enter the cells passively at a rate depending on the permeability of the membrane to the ions and on the electrochemical gradients across the membrane. The apical membrane possesses an electrogenic pump which it is suggested has a higher affinity for Na^+ than K^+. Thus if the basal cell membrane is more permeable to K^+ than Na^+ (e.g. *Carausius* (Pilcher 1970) and *Rhodnius* (Maddrell 1971)), K^+ ions will enter the cell faster and be transported into the lumen whereas if Na^+ ions enter faster they would be transported since the pump has a higher affinity for Na^+. This it is argued could then explain the ability of *Glossina* Malpighian tubules to secrete a Na^+-rich fluid. However it is difficult to see how this model is consistent with some of the observed facts. It is known that the rate of fluid secretion at low K^+ concentrations is enhanced by the presence of Na^+ (Berridge 1968; Maddrell 1971), a fact which has been taken as support for the presence of a Na^+/K^+ exchange pump on the basal surface. Further, the K^+ concentration in the cytoplasm is high compared with that of the bathing medium so that it is difficult to see how adequate K^+ entry into the cell could be achieved passively.

Whilst a great deal is now known of cation movement across the Malpighian tubules, the mechanism by which water flows across the tubules remains uncertain. It is generally accepted that water movement is passive in response to an osmotic gradient set up by active ion transport (Maddrell 1971, 1977). The fluid secreted by nearly all insect Malpighian tubules is marginally hyperosmotic to the bathing fluid over a wide range of osmotic concentrations of the bathing solution (Maddrell 1971) and the rate of fluid flow is inversely proportional to the osmotic pressure of the bathing medium. Maddrell (1971) therefore suggests that solute transport is not affected by changes in osmotic pressure but water movements change so that the fluid produced is slightly hyperosmotic. Exactly how solute movements give rise to water movements is not yet clear.

A number of theories have been proposed to explain water flow across epithelia. In this regard an important step was made when Curran and Solomon (1957) suggested that intestinal water absorption in the rat ileum was a passive process achieved by active transport of salt. Curran and McIntosh (1962) proposed the double-membrane theory of osmotic coupling to explain this process. Essentially this model comprises two membranes in series with a compartment in between (Figure 1.1). The membranes (*a* and *b*) have differing permeabilities to solute and solvent molecules. Solutes are actively transported from *1* across an impermeable membrane, *a*, into an intercellular space, *2*. The osmolarity of this space increases and water flows from *1* to *2* in response to the osmotic gradient. Fluid flow across the epithelium is achieved when increases in the hydrostatic pressure within *2* cause fluid to flow through the relatively permeable second membrane into compartment *3*.

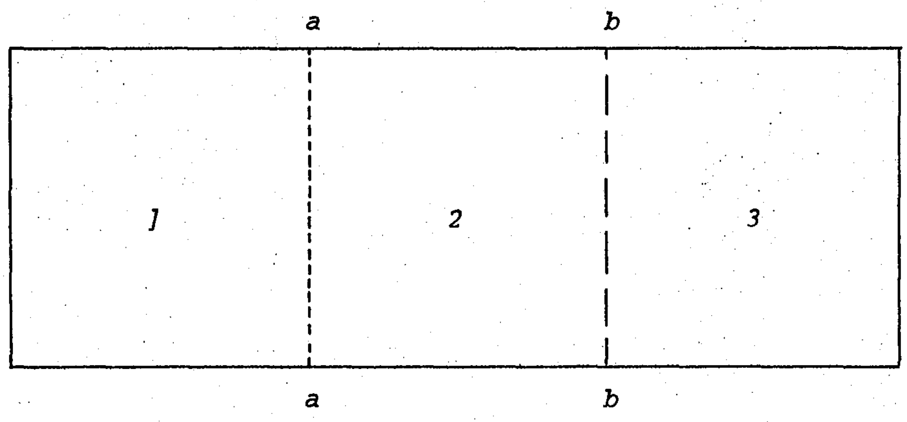
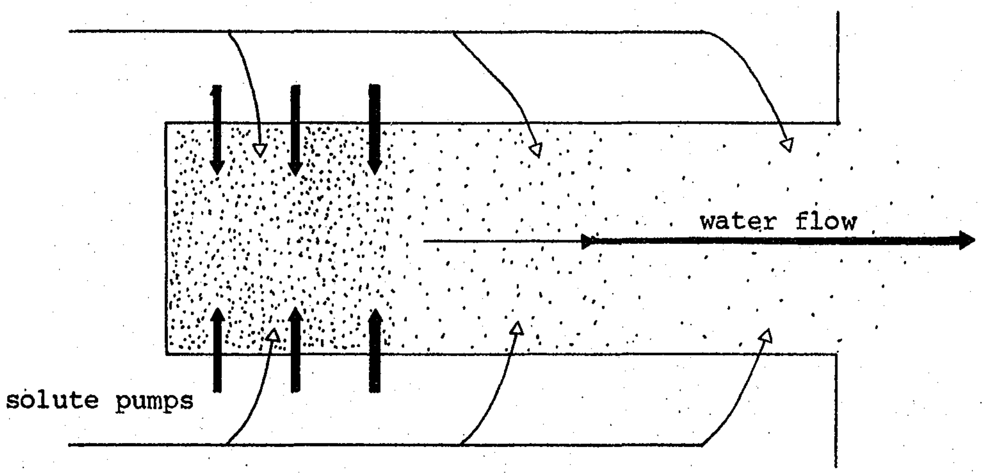


Figure 1.1 The 'double-membrane' model of Curran and McIntosh (1962)

More recently progress has been made in linking the structure of transporting cells to their function. Diamond and Bossert (1967, 1968) have proposed a model based on the 'architecture' of fluid transporting cell membranes. At the ultrastructural level the cell membranes of fluid secretory epithelia are seen to be invaginated to form a system of extracellular channels and spaces (basal infoldings, apical microvilli), which Diamond and Bossert (1968) suggest may constitute the fluid transport route. They suggest that active solute transport into the extracellular channels makes the channel contents hypertonic and permits water-solute coupling. This theory depends upon channels which are structurally or functionally closed at one end but can be applied to fluid flow in and out of, both 'forwards' and 'backwards' facing channels. Forward facing channels (Figure 1.2a) lie in the direction of fluid flow and are found in basal infoldings (salivary gland) and microvilli (gallbladder, Malpighian tubule). Backward facing channels (Figure 1.2b) face in the opposite direction to that of fluid transport and are found in the basal infoldings of avian salt gland and the basal infoldings of Malpighian tubules. Figure 1.2 shows the Diamond and Bossert (1968) model for a standing-gradient flow system. In 'forward' facing channels (Figure 1.2a), solute is actively transported into the closed end of the channel making the channel fluid hypertonic and causing water to move into the channel from the adjacent cytoplasm. As solute moves down the channel due to diffusion along its concentration gradient, more and more water will enter the channel, reducing the osmolarity until the fluid emerging at the open end would be virtually isotonic. In effect, a standing osmotic gradient would be maintained within the channel by active solute transport, the osmolarity decreasing continuously from the closed towards

a) 'forward' facing channel



b) 'backward' facing channel

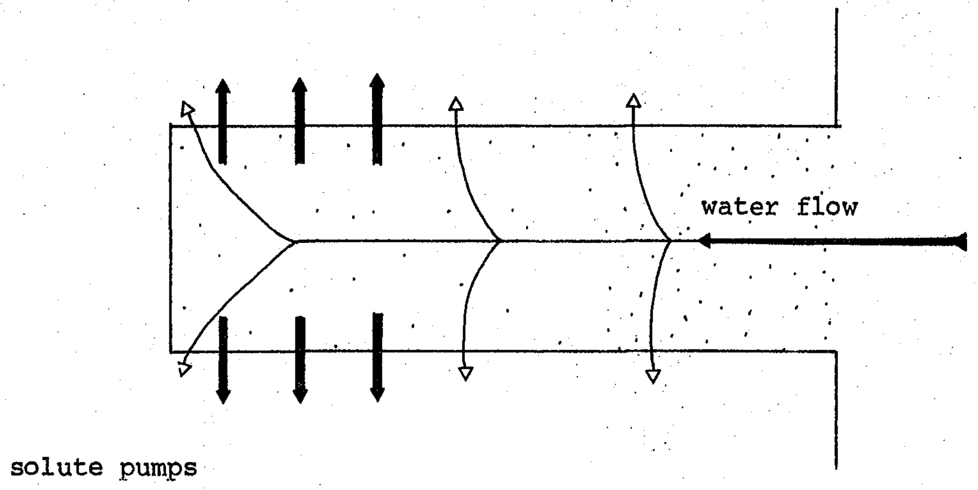


Figure 1.2 The Standing Gradient Osmotic flow system after Diamond and Bossert (1968)

the open end, and a fluid of fixed osmolarity would constantly flow from the open end.

In 'backwards' facing channels (Figure 1.2b) i.e. with the open end facing the side of the epithelium from which fluid is being taken up, solute is actively transported out of the channel making the channel fluid hypotonic. As solute diffuses down its concentration gradient towards the closed end of the channel, more and more water leaves the channel 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 will constantly enter the channel mouth and be secreted across its walls.

This model originally derived from studies on rabbit gallbladder has since been applied to insect Malpighian tubule fluid secretion (Berridge and Oschman 1969).

An alternative theory which has been proposed to explain the mechanism of ion and water transport across epithelia is based on electro-osmosis (Hill 1975b, 1977). In electro-osmosis, the transmembrane potential can move water because there is specific frictional interaction between water and one of the ions as it moves out of the cell down an electrochemical gradient drawing water with it. Maddrell (1977) has discussed how this theory could be applied to Malpighian tubules.
The action of an electrogenic cation pump would produce an electrical potential difference across the apical membrane. This gradient would draw Cl^- from the cell through the membrane. In crossing the membrane, the Cl^- would frictionally interact with water molecules and cause them also to move out of the cell. This mechanism relies on the maintenance of a potential gradient across this cell membrane so that the apical wall

must be arranged so that it is not bathed by fluids other than its own secretion. Maddrell (1977) suggests that membrane infoldings such as in the microvilli would serve this purpose and would also increase the number of pump sites by increasing the surface area.

Finally, since the 'urine' produced by several insects is hypertonic to the bathing medium (Ramsay 1953; Berridge 1968; Maddrell 1969) a simple mechanism of osmosis has been suggested to explain water and solute coupling in Malpighian tubules (Taylor 1971a). Active transport of solute across the basal surface would maintain the cytoplasm hypertonic to the haemolymph and active solute transport apically would maintain the lumen hypertonic to the cells. Water would then flow passively as a result of these small osmotic pressure differences.

The exact mechanism by which water is transported across the Malpighian tubules may remain uncertain but it has been well established that the secretion of fluid is under hormonal control. In the insects investigated to date the hormones which control excretion by the Malpighian tubules and rectum are produced by neurosecretory cells, although the source of the hormone depends on the species. The diuretic hormones of *Anisotarsus cupripennis* (Nuñez 1956), *Schistocerca* (Highnam *et al*. 1965, Mordue 1969), *Dysdercus* (Berridge 1966) and *Carausius* (Pilcher 1970) are synthesised by neurosecretory cells in the brain and released into the haemolymph via the corpora cardiaca. In *Rhodnius* (Maddrell 1963) and *Corethra* (Gersch 1967) neurosecretory cells in the mesothoracic ganglion synthesize a diuretic hormone. Diuretic hormone is also present in the thoracic ganglion of *Glossina* and is released from neurosecretory axon endings in the abdomen (Gee 1975b).

Anti-diuretic factors have also been demonstrated in several insect species. In *Schistocerca* (Mordue 1969), the glandular lobe of the corpora cardiaca produces an anti-diuretic hormone which controls the rectal glands. Anti-diuretic activity has also been attributed to the corpora cardiaca of *Apis* (Altmann 1956) and *Gryllus, Periplaneta* and *Clitumnus* (de Bessé and Cazal 1968).

It is thought that both diuretic and anti-diuretic factors are released in response to stimuli associated with feeding and with the osmotic pressure of the haemolymph. In *Rhodnius* (Maddrell 1963) and *Glossina* (Gee 1975b) diuretic hormone is released in response to the ingestion of a blood meal. In *Schistocerca* feeding results in an increased rate of fluid secretion by the Malpighian tubules (Mordue 1969) and in *Dysdercus* (Berridge 1966) and *Carausius* (Pilcher 1970) there is evidence that the titre of diuretic hormone in the haemolymph rises in response to feeding.

In the majority of insects studied it appears that water balance results from a balance between 'urine' production by the Malpighian tubules and reabsorption by the rectum (Maddrell 1966; Berridge 1966; Wall 1967; Mordue 1969; Pilcher 1970; Gee 1975a). The effect of the diuretic hormone is to increase excretion through the Malpighian tubules (as found in *Rhodnius* (Maddrell 1966), *Dysdercus* (Berridge 1966), *Carausius* (Pilcher 1970) and *Glossina* (Gee 1975b)), and perhaps as in *Locusta* (Mordue 1969) reduce reabsorption by the rectum. Less information is available about the mode of action of insect anti-diuretic hormone. Wall (1967) postulated that in *Periplaneta* the hormone exerted an anti-diuretic effect on both Malpighian tubules and rectum by reducing the passive permeability of the cells to water. It would therefore restrict water movements generated by ion transport across the Malpighian tubules and would reduce the amount of water leaking back across the rectal glands after it had been reabsorbed from the faeces (Wall 1967). In locusts the anti-diuretic hormone present in the corpora cardiaca increases rectal reabsorption (Mordue 1970, 1972; Goldsworthy and Mordue 1972).

Several authors have proposed similar mechanisms for the control of Malpighian tubule fluid secretion (Maddrell 1964; Berridge 1966; Pilcher 1970; Mordue 1972; Gee 1975b). Essentially, diuretic hormone is released in response to feeding, leading to increased rates of fluid secretion. Once feeding stops, diuretic hormone release ceases and the haemolymph titre is reduced by degradation resulting in a reduced rate of excretion. In *Schistocerca* this theory has been extended to cover the rectum also (Mordue *et al.* 1970). During feeding, diuretic hormone from the storage lobes of the corpora cardiaca both increases secretion by the Malpighian tubules and reduces rectal water reabsorption. When water conservation is required, rectal reabsorption is increased by an anti-diuretic hormone secreted by the glandular lobes.

The mode of action of both the diuretic and anti-diuretic hormones on their target tissues is still under investigation although there is evidence that diuretic hormone may act by increasing intracellular levels of cyclic AMP (Maddrell *et al.* 1971; Gee 1976). Cyclic AMP has been shown to stimulate fluid secretion by the Malpighian tubules of *Schistocerca* (Maddrell and Klunsuwan 1973), *Carausius* and *Rhodnius* (Maddrell *et al.* 1971) and *Locusta* (Bell 1977). In addition Aston (1975) has measured increased intracellular cyclic AMP levels during stimulation of *Rhodnius* tubules by diuretic hormone.

The diuretic and anti-diuretic hormones are thought to be peptides or polypeptides (Mills 1967; Mordue and Goldsworthy 1969;

Aston and White 1974; Gee 1975) but it has also been suggested that steroids may have a role in fluid secretion (Gee *et al.* 1977). Gee *et al.* (1977) propose that ecdysone affects the rate of fluid secretion by the Malpighian tubules of *Glossina* possibly by altering the permeability of the basal cell membrane of the tubule cells. In a different context Kroeger and Lezzi (1966) have previously suggested an effect of ecdysone on the selective permeability of cell and nuclear membranes.

The control of fluid secretion in insects may then involve ecdysone and perhaps Juvenile Hormone (J.H.), hormones primarily associated with regulating growth and development. J.H. secreted by the corpora allata has, like ecdysone, been implicated in affecting the permeability of cell membranes (Wigglesworth 1957; Lezzi and Gilbert 1972; Baumann 1968, 1969). In addition, factors from the corpora allata of Locusts have been found to affect animal water content (Strong 1968; Been akkers and Van Den Broek 1974).

The present study has been carried out to investigate further the role of the Na^+, K^+-activated ATPase in ion and fluid transport across the Malpighian tubules of *Locusta*. In addition, the role of insect hormones in controlling ATPase activity and Malpighian tubule function has been examined.

CHAPTER 2

GENERAL MATERIALS AND METHODS

MAINTENANCE OF INSECTS

The insectary was maintained at a temperature of 28 ± 0.5°C and a relative humidity of 60 ± 5% with a constant photoperiod of 12 hours light and 12 hours dark. Circulation of air was effected by three electric fans and a continuous air exchange was maintained by one large ventilator. Populations of *Locusta migratoria migratorioides* R and F, phase *gregaria*, were reared in perspex fronted cages consisting of dexion angled metal framework with aluminium top and sides (43cm x 58cm x 58cm). There was a 'false floor' to each cage made of perforated aluminium. This contained four holes into which plastic cups filled with sand were placed and into which female locusts deposited their egg pods. The 'false floor' was separated from the true floor by a space 10cm high and the faeces from the locusts passed through the holes in the 'false floor' into the space beneath. Each cage was illuminated by a 40 watt bulb which resulted in temperatures within the cage varying from 30-40°C according to the proximity to the bulb. The locusts were supplied daily with fresh grass, water and Bemax.

Throughout their development, animals were reared at sufficiently high density to ensure their remaining 'gregarious' (Joly and Joly 1953).

EXPERIMENTAL ANIMALS

Some experiments required that the locusts be aged accurately. To achieve this, cages containing late 4th instar locusts were checked twice daily at 10.00 a.m. (8 ± 8 hours old) and 6.00 p.m. (4 ± 4 hours old) and any newly moulted 5th instar locusts were removed to experimental cages.

These cages were cylindrical, made from aluminium and acetate sheet ($144cm^2$ x 40cm) and provided with wooden perches.

Experimental animals were supplied daily with fresh grass and water. No special illumination was provided, resulting in a slightly lower temperature than in the stock cages, but ensuring that all animals were at constant temperature.

CHEMICALS

All chemicals used were the purest available and were generally supplied by Sigma Co., Kingston-upon-Thames, Surrey, U.K. or B.D.H., Poole, Dorset, U.K. Juvenile Hormone, synthetic, B grade, was supplied by Calbiochem. Ltd., Bishops Stortford, Herts., U.K.

GLASSWARE

Pyrex glassware was used throughout. Prior to use it was cleaned by soaking overnight in 2% 'Quadralene' laboratory detergent, followed by several rinses in hot tap water and then distilled water. The glassware was then dried in ovens except for glass/teflon homogenisers which were allowed to drain at room temperature.

STATISTICAL TECHNIQUES

Statistical comparisons of data were performed using the conventional technique described by Snedecor and Cochran (1967). Where necessary, the statistical tables of Fischer and Yates (1963) were used. Values and probabilities less than 0.05 were taken as significant.

INSECT RINGER SOLUTION

Unless otherwise stated in the text the composition of the Ringer solution used in experiments was: NaCl 100mM, KCl 8.6mM, $CaCl_2$ 2mM,

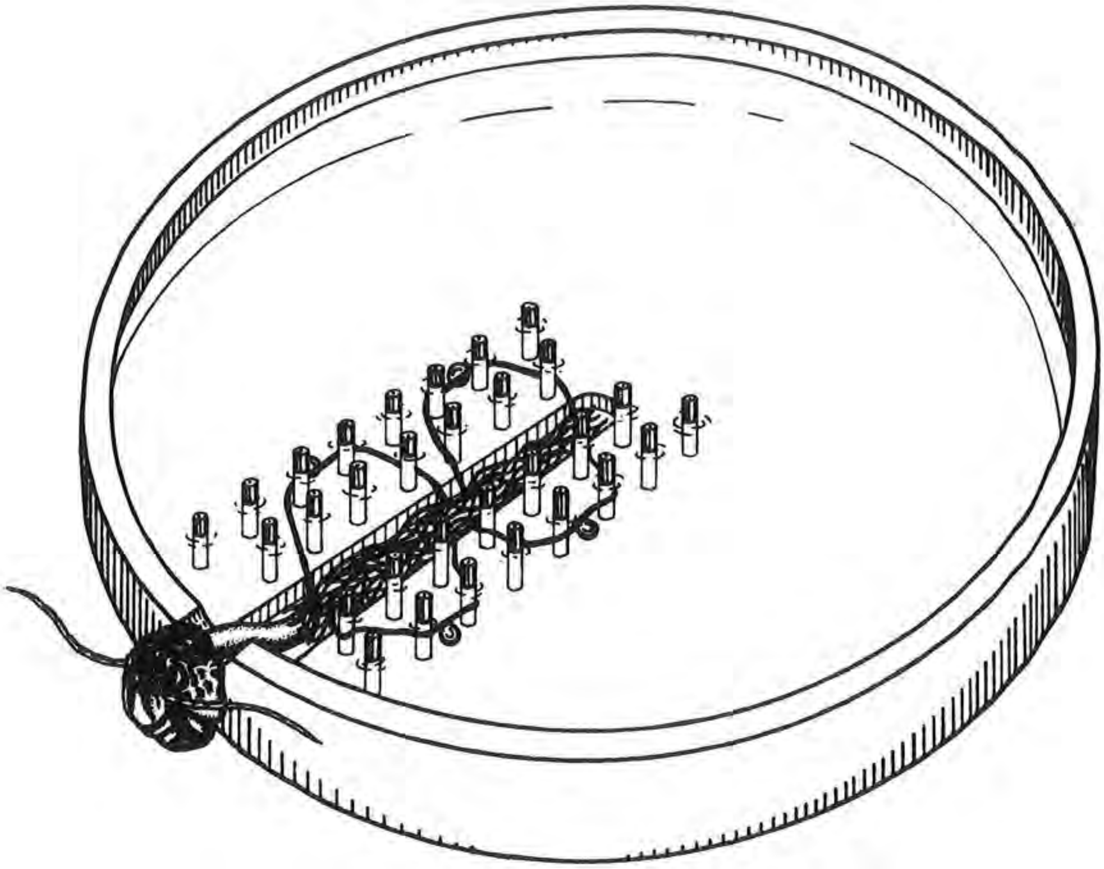


Figure 2.1

Experimental arrangement involved in setting up *in vitro* preparations of Malpighian tubules.

The trough in the perspex dish contains Ringer solution into which the whole alimentary canal is placed. The head remains outside the dish. The Malpighian tubules are looped around the stainless steel pegs, out of the Ringer solution. The entire preparation is covered with liquid paraffin.

$MgCl_2,6H_2O$ 8.5mM, NaH_2PO_4 4mM, $NaHCO_3$ 4mM, glucose 34mM, NaOH 11mM, H.E.P.E.S. 25mM, pH 7.2.

EXPERIMENTAL PROCEDURES:

1. To determine the rate of fluid secretion by the Malpighian tubules of *Locusta*

In vitro measurements of the rate of fluid secretion were carried out using essentially the same technique as that described by Maddrell and Klunsuwan (1973).

Animals were killed by twisting the head such that the 'neck' cuticle was separated from the thorax. The abdomen was then cut transversely just forward of the posterior tip and the alimentary canal drawn out through the thorax. The entire alimentary canal was immersed in Ringer solution contained in a small trough in a perspex dish. The head remained outside the dish to prevent contamination by regurgitated fluid. The whole preparation was then covered with liquid paraffin. Individual Malpighian tubules were drawn out of the Ringer solution and looped around stainless steel pegs surrounding the trough (Figure 2.1). These tubules were then partially severed at one point along their length using a fine tungsten needle. The rate of fluid secretion was determined by measuring the increase in diameter of the droplet secreted from the cut. As many as twelve tubules could be set up in this way using a single animal. The initial droplet of fluid secreted over the first 10 minutes was removed before making the experimental readings. The secretion rate for each tubule was determined by measuring the diameter of the secreted droplet every 5 minutes for 35 minutes. The 'normal' Ringer solution was then replaced with either fresh 'normal' Ringer solution or an experimental Ringer solution before redetermining the rate of secretion over a second 35 minute period.

The volume of fluid secreted was calculated, assuming the droplet to be a sphere, and expressed in nls/min. The effect of the particular treatment was determined by comparing the rates of secretion over the two 35 minute periods. In this way each tubule acts as its own control. This is necessary as the rate of secretion varies considerably from tubule to tubule.

All experiments, unless otherwise stated in the text, were carried out at 30 ± 0.1°C.

2. Microsomal preparation of a Na$^+$, K$^+$-activated ATPase (E.C.3.6.1.3. Skou 1965) from the Malpighian tubules of *Locusta*

Reagents

Homogenisation medium (pH 7.2)	:	Histidine/HCl	40mM
		Mannitol	250mM
		EDTA	5mM
		Sodium deoxycholate	0.1%
Sodium iodide extraction medium (pH 7.2)	:	$MgCl_2$	5mM
		NaI	4 M
		EDTA	10mM
Washing medium (pH 7.2)	:	NaCl	5mM
		EDTA	5mM
Ionic reaction medium for total ATPase (pH 7.2)	:	$MgCl_2$	8mM
		NaCl	200mM
		KCl	40mM
		Histidine	50mM

(gives final concentration of $MgCl_2$ 4mM, NaCl 100mM, KCl 20mM)

Ionic reaction medium for Mg^{2+}-dependent ATPase
(pH 7.2) : $MgCl_2$ 8mM
Histidine 50mM

(gives final concentration of $MgCl_2$ 4mM)

Cirrasol mixture : Mix equal volumes of 1% Cirrasol ALN-F in deionised water with 1% ammonium molybdate in 1.8M H_2SO_4

Tris ATP 12mM (final concentration 3mM)

The tris ATP used in the experiments was prepared from the disodium salt. The required amount of ATP was dissolved in a small volume of deionised water and this was then poured through 'charged' Dowex resin, in a Buchner funnel, four times. The Dowex resin was rinsed several times with deionised water which was pooled with the ATP solution to make up almost the required volume. The ATP was then in the H^+ form and it was converted to the Tris salt by the addition of a few drops of 2M Tris buffer to give a pH of 7.2. The solution was made up to final volume with deionised water and stored at -20°C.

Preparation of microsomes

Equal numbers of male and female locusts were used in all experiments, the total number used varying with the size of the experiment.

Locusts were killed by decapitation and the Malpighian tubules quickly dissected out and placed in 10mls of homogenisation medium in a glass Potter-Elvehjen homogenising tube. Homogenisation was carried out with a Teflon pestle (clearance 0.1 - 0.15mm) giving 10 passes of the plunger at 2,000 r.p.m. The homogenate was extracted with an equal volume of NaI for 30 minutes at 0°C (Nakao *et al.* 1965). The extract was diluted to 50mls with deionised water and centrifuged at 50,000g for 30 minutes at 0°C. The pellet was discarded and the supernatant centrifuged at 100,000g for 60 minutes. The resulting pellet was suspended in washing medium and centrifuged at 100,000g for 45 minutes. This washing procedure was then repeated twice, centrifuging at 100,000g for 30 minutes each time. The final pellet was suspended in deionised water by homogenisation.

ATPase assay

Unless otherwise stated in the text, all experiments were run for 30 minutes at 30°C.

Pairs of tubes containing 1.0ml of appropriate reaction media and 0.5ml ATP were set up and equilibrated at 30°C for five minutes. The reaction was started by the addition of 0.5ml of the microsomal preparation and stopped by the addition of 4.0ml freshly prepared Cirrasol solution (Atkinson *et al.* 1973). The tubes were then left for 10 minutes at room temperature for the yellow colour to develop. The optical density of the final solutions was measured at 390nm in a Pye Unicam Sp 1800 dual beam spectrophotometer. Inorganic phosphate was measured by reference to a calibration graph prepared by assay of standard phosphate solutions. A reagent blank measuring non-enzymatic hydrolysis of ATP was prepared by addition of Cirrasol solution before the enzyme was introduced.

Calculation of Na^+, K^+-activated ATPase activity

Enzyme activity was measured by determining the amount of inorganic phosphate released. Na^+, K^+-activated ATPase activity was obtained as the difference in inorganic phosphate liberated in reaction media containing Na^+, K^+ and Mg^{2+} and that in media containing Mg^{2+} alone. The Mg^{2+}-dependent ATPase activity was obtained as the difference in inorganic phosphate liberated in reaction media containing Mg^{2+} and the control tubes (reagent blank).

Analysis of inorganic phosphate

Standard phosphate solutions were prepared from a stock solution containing 20g phosphorus (as KH_2PO_4/ml). Serial dilution of this stock solution gave samples of 20, 15, 10, 5, 2, 1, 0g Pi/ml. To 2mls of each sample 4mls of cirrasol solution were added. The tubes were allowed to stand at room temperature for 10 minutes before measuring the optical density at 390nm. A calibration graph was then prepared by plotting the concentration of inorganic phosphate in nmoles against absorbancy. A typical example of this can be seen in Figure 2.2.

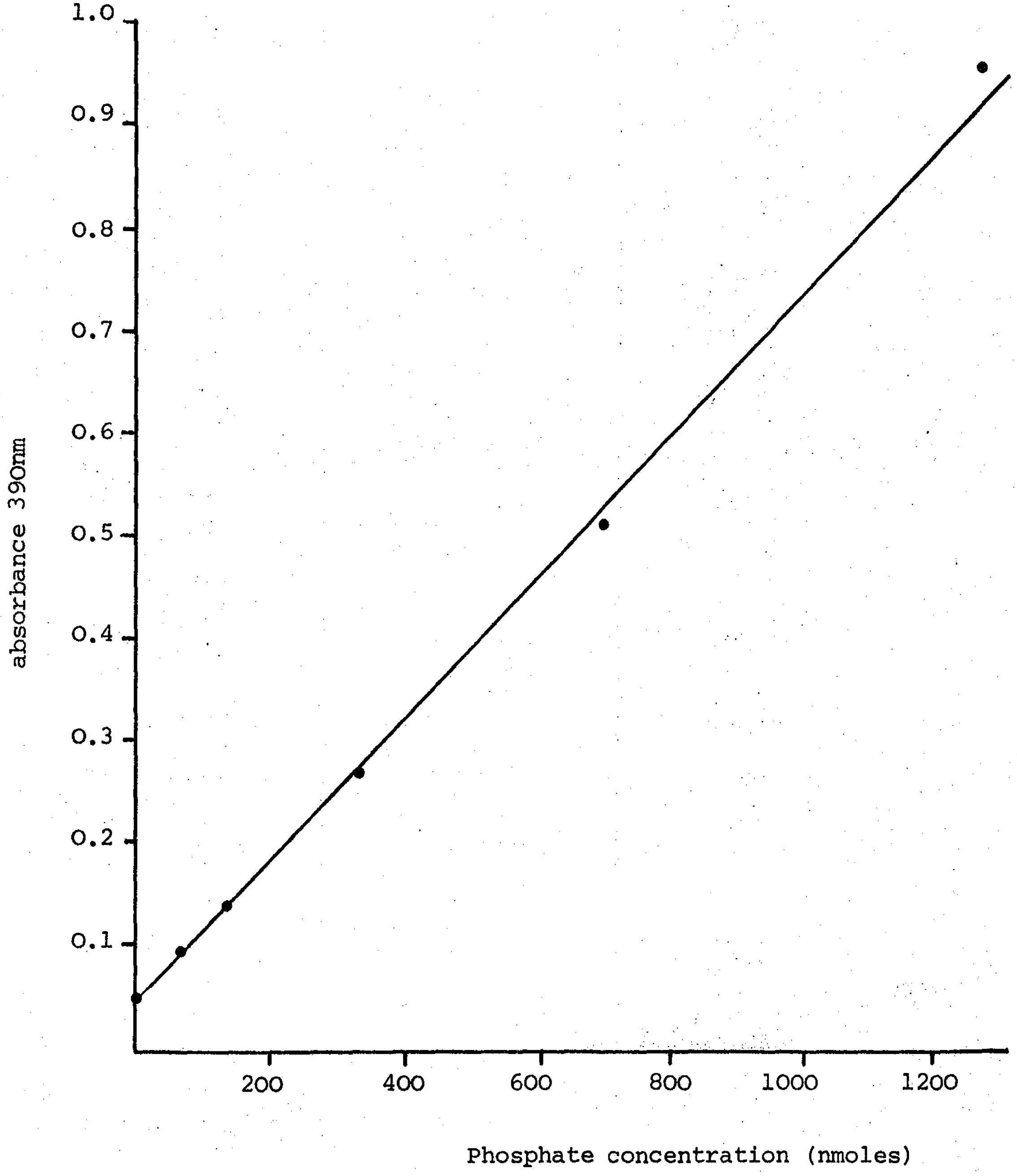
3. Estimation of protein

The method used was that of Lowry *et al.* (1951) using Bovine Serum Albumen (B.S.A.) Fraction V as standard.

Reagents:

(i) 2% Na_2CO_3
(ii) 0.5% $CuSO_4$
(iii) 1% KNa Tartrate

Figure 2.2 Standard calibration curve for determination of inorganic phosphate



Folins Solution A:

Prepared by mixing equal volumes of (ii) and (iii) and to each volume of this adding 50 volumes of (i).

Folins Solution B:

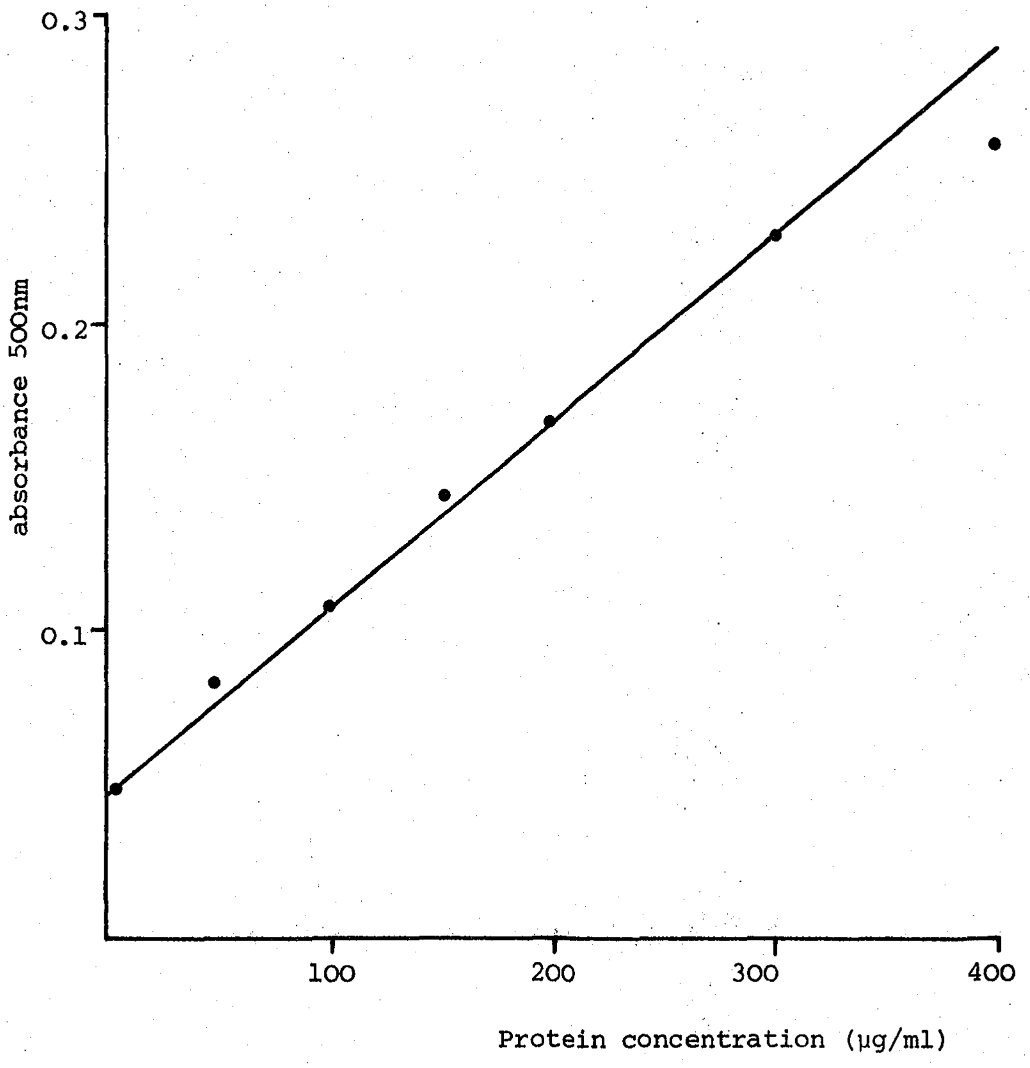
Prepared by diluting 4 volumes of Folin Ciocalteaus phenol reagent with 6 volumes of distilled water.

Method

3mls of Folins Solution A were added to 0.2mls of protein solution and this was allowed to stand for 30 minutes at room temperature. 0.3ml of Folins Solution B was then added and the resulting solution allowed to stand for a further 60 minutes at room temperature. The optical density was then measured at 500nm.

Protein standards of 400, 300, 200, 150, 100, 50, 0g/ml were prepared. A calibration graph of protein concentration vs. absorbancy could then be constructed from which unknowns could be determined. A new calibration graph was constructed each time an assay was carried out. A typical calibration graph can be seen in Figure 2.3.

Figure 2.3 Standard calibration curve for determination of Protein



CHAPTER 3

ULTRASTRUCTURAL STUDIES ON THE MALPIGHIAN TUBULES OF *LOCUSTA MIGRATORIA*

Numerous studies have been carried out to determine the fine structure of the Malpighian tubules of a wide variety of insect species. These include *Melanoplus differentialis* (Beams *et al*. 1955), *Dissosteira carolina* (Tsubo and Brandt 1962), *Calliphora erythrocephala* (Berridge and Oschman 1969), *Carausius morosus* (Taylor 1971a and b), *Periplaneta americana* (Wall *et al*. 1975), *Jamaicana flava* (Peacock and Anstee 1977), and *Locusta migratoria* (Bell and Anstee 1977). Several researchers have reported the presence of two distinct cell types (Berridge and Oschman 1969; Taylor 1971a and b; Wall *et al*. 1975; Peacock 1975; Charnley 1975; Bell 1977). These authors report that the majority of the tubule is composed of 'primary' (Berridge and Oschman 1969) or 'Type I' (Taylor 1971a) cells which are structurally different from the so-called 'stellate' (Berridge and Oschman 1969), 'Type II' (Taylor 1971b) or 'secondary' (Peacock 1975) cells which are less frequently encountered. The primary cells are characterised by the following features: They exhibit extensive invaginations of the basal cell surface forming a system of extracellular channels and spaces whilst the luminal surface forms a microvillar border. Closely associated with both the apical microvilli and the basal cell membrane are large numbers of mitochondria. These features are characteristic of many other transporting epithelial cells e.g. mammalian kidney (Rhodin 1958), mammalian gall bladder (Kaye *et al*. 1966) and insect salivary gland (Oschman and Berridge 1970). As mentioned earlier (see Chapter 1), several models, proposed to explain fluid movement across epithelia, have attempted to relate these fine structural features to function (Diamond and Bossert 1967, 1968; Berridge and Oschman 1969;

Taylor 1971a). Perhaps the model which has attracted most attention in recent years is the Standing Gradient Hypothesis of Diamond and Bossert (1967, 1968) which has been used to explain fluid movement across the Malpighian tubules of *Calliphora* by Berridge and Oschman (1969).

The secondary cells are smaller than the primary cells and have reduced basal infoldings and a less dense apical microvillar border. Furthermore, the cytoplasm of the secondary cells is very rich in endoplasmic reticulum, secretory vesicles, Golgi bodies and lysosomes (Berridge and Oschman 1969; Taylor 1971b; Peacock 1975). Whilst the precise function of these cells remains uncertain it has been suggested that they may secrete mucopolysaccharides (Martoja 1956, 1959, 1961; Berkaloff 1960). In contrast, Berridge and Oschman (1969) suggest that they may be involved in the absorption of ions and water from the lumen.

Previous studies on the ultrastructure of insect Malpighian tubules have been carried out on mature adult insects (Berridge and Oschman 1969; Taylor 1971a and b; Bell and Anstee 1977; Peacock and Anstee 1977). With the exception of a study by Ryerse (1977) relatively little is known of how Malpighian tubule structure varies with development. Ryerse (1977) found that the Malpighian tubules of *Calpodes ethlius* were extensively remodelled at the larval-pupal metamorphosis. In particular, mitochondria were retracted from the apical microvilli and the latter and the infolds of the basal cell membrane were reduced. Associated with these fine structural changes Ryerse (1978) found marked changes in Malpighian tubule fluid secretion. Fluid transport ceased when the mitochondria were retracted and resumed during the pupal stage when the mitochondria were reinserted into the microvilli. Similarly, changes in cell ultrastructure have been correlated with functional variation in other secretory epithelia. Diehl *et al.* (1977) report that the salivary gland cells of the tick *Amblyomma hebraeum* undergo radical ultrastructural changes during the feeding period. They noted that there was increased development of the plasma membrane invaginations and an associated increase in mitochondrial numbers. At the same time the rate of fluid secretion by the salivary glands increased (Kaufman *et al.* 1976).

The purpose of the present study was to examine the ultrastructure of the Malpighian tubules of mature adult *Locusta*. In addition this study has been extended to determine what fine structural changes occur throughout the 5th stadium and into early adult life to provide a basis for subsequent physiological and biochemical studies. The observations made will be presented in two sections: I. The ultrastructure of the Malpighian tubules from mature adult insects. II. Changes in the fine structure of the Malpighian tubules throughout the 5th stadium into early adult life.

Materials and Methods

Sexually mature locusts of both sexes were used for ultrastructural studies. The animals were killed by decapitation and the Malpighian tubules, together with an adjoining 'collar' of gut, were quickly dissected out in ice-cold Ringer solution. The tubules were fixed in 5% glutaraldehyde buffered with 0.1M sodium cacodylate (pH 7.3) overnight. The tissue was then washed for 2hrs in 0.1M buffer containing 0.2M sucrose prior to post-fixation with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.3) for 2hrs. The material was then dehydrated through a graded series of alcohols; 10 minutes in each of 50%, 70% and 95% alcohol, followed by two 30 minute periods in absolute alcohol. After two 10 minute rinses in propylene oxide the material was left in a 50:50 mixture of [1]Epon epoxy resin and propylene oxide overnight. Following infiltration in Epon for 8hrs, the material was embedded in fresh Epon and polymerisation was effected at 60°C for 48hrs.

Silver/silver-gold sections were cut on a Reichert NK ultratome, expanded with diethyl ether vapour and mounted on uncoated copper grids. Sections were stained with uranyl acetate followed by lead citrate (Reynolds 1963) prior to their examination in an AEI 801 electron microscope.

In the study on fine structural changes associated with development, the tubules from 5th instar and early adult locusts were subjected to a somewhat different method of fixation and embedding. Aged animals were killed as described above and the tubules fixed in [2]Karnovsky's fixative for 1-1½hrs at 4°C. The tissue was post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1hr before dehydration through a graded series of alcohols: 15 mins in each of 70%, 95% and absolute alcohol with 3 changes at 5 min intervals in each; 1:1 absolute alcohol: acetone, 3 changes in 30 mins; acetone, 3 changes in 30 mins. The material was then placed in a 1:1 mixture of propylene oxide: Araldite at 45°C for 30 mins prior to embedding in [3]Araldite. Polymerisation was effected at 45°C for 12hrs followed by 48hrs at 60°C.

1. Epon 812 epoxy resin: equal parts of <u>A</u>

Epon 812	(62 vols)
D.D.S.A.	(100 vols)

and <u>B</u>

Epon 812	(100 vols)
M.N.A.	(89 vols)

2. Karnovsky's fixative:

paraformaldehyde	2g
distilled water	40mls
1N NaOH	2-6 drops

+

25% glutaraldehyde	10ml
0.2M sodium cacodylate	10ml pH 7.3

3. Araldite:

Araldite	10ml
D.D.S.A.	10ml
dibutyl phthalate	2ml
DMP 30	1ml

Observations and Discussion

Section I. The Malpighian tubules of mature adult *Locusta migratoria*

Mature adult *Locusta migratoria* possess approximately 200 Malpighian tubules. These are blind-ending tubules, approximately 15-20mm. long and 50-80μm in diameter, which open into the alimentary canal by way of 12 ampullae at the junction of the mid-gut and the hind-gut. The Malpighian tubules lie free in the haemocoel extending forward to the mid-gut caeca and backward to the rectum. They are well supplied with tracheae which give off numerous tracheoles which are in immediate contact with individual tubules.

The Malpighian tubules have a uniform morphological appearance along their length. They are composed of two types of cell, primary and secondary (Plates 2,11); the primary cells making up the majority of the tubule (Plate 1). The outer surface (i.e. the basal surface) of each tubule is completely ensheathed by a thick (0.4μm) basement membrane (Plate 3). A transverse section through a primary cell is shown in Plate 2. Each primary cell can be divided morphologically into 3 distinct regions: basal, intermediate and apical.

Basal region

The basal cell membrane is extensively infolded forming a complex system of long narrow extracellular channels running perpendicular to the basement membrane and extending for variable distances into the cell (Plate 3). These extracellular channels measure 4-8μm in length and are ca. 0.03μm across. The cytoplasmic compartments between the extracellular channels are ca. 0.075μmwide. Where the basal cell membrane meets the basement membrane, the cytoplasmic processes exhibit electron dense tips (Plate 3) which are similar to the hemi-desmosome junctions described by Berridge and Oschman (1969) in the Malpighian tubules of *Calliphora*, by Taylor (1971a) in *Carausius morosus* and by Bell and Anstee (1977) in *Locusta migratoria*. The cytoplasmic compartments between the basal infolds contain numerous mitochondria and small vesicles measuring 0.05-0.25μm in diameter (Plate 3).

Intermediate region

This region contains the nucleus and numerous other cellular inclusions. The nucleus is a large roughly spherical body surrounded by a well-defined nuclear membrane (Plate 4). The cytoplasm contains many vacuoles of different sizes (0.4-1.0μm in diameter). Some of these appear empty whilst others contain granules of varying electron density (Plate 5). Indeed, many inclusions contain concentric layers of electron dense material (Plate 6). Several multi-vesicular bodies (Berridge and Oschman 1969; Taylor 1971a) were also observed. The cytoplasm in this region also contains numerous free ribosomes, fragments of rough endoplasmic reticulum, occasional Golgi bodies and mitochondria (Plate 7).

Apical region

The apical surface of the primary cells is composed of numerous closely-packed microvilli which project into the lumen of the tubule (Plate 8). The microvilli are 3-5μm long, club-shaped and approximately 0.15-0.4μm in diameter at their widest point. A number of the microvilli contain processes of the apical mitochondria. In general, those microvilli containing mitochondria appeared to have larger diameters than those from which mitochondria were absent. Small vesicles were infrequently observed at the bases of the microvilli and in their 'swollen' tips (Plate 9).

The plasma membranes of adjacent cells are joined together laterally by septate desmosome junctions (Plate 10). This sort of junction was first described by Locke (1965) and designated comb or septate desmosome by Danilova *et al.* (1969). Towards the luminal border, the desmosomes widen out to form the so-called *Macula adhaerens* junctions (Plate 10) in which a layer of electron dense material is attached to the cytoplasmic side of the adjacent membranes.

Throughout the present study numerous, small, coated vesicles were observed in the cytoplasm of the primary cells of *Locusta* (Plate 2). Similar vesicles have been described in the tubules of *Gryllus* (Berkaloff 1960), *Drosophila* (Wessing 1965; Eichelberg and Wessing 1975), *Carausius* (Taylor 1971a) and *Jamaicana* (Peacock and Anstee 1977). Experiments on *Drosophila* demonstrated that the vesicles arise from the basal cellular infoldings and pass through the cell cytoplasm to the lumen where they empty their contents (Eichelberg and Wessing 1975). In this way these structures could perhaps be involved in the entry of urinary constituents into the cells and their exit into the lumen (Eichelberg and Wessing 1975). It has been suggested that water and solutes could traverse the cell inside such vesicles by the mechanism known as cytopempsis (Wigglesworth and Salpeter 1962; Wessing 1964, 1965). The presence of similar vesicles throughout the cytoplasm of *Locusta* primary cells suggests that some substances may be transported across the tubules in this manner. However, it has been pointed out by Taylor (1971a) that it is unlikely that this process contributes significantly to normal 'urine' production as the vesicles would have to form and disappear very rapidly to account for the volume of 'urine' produced.

There are a variety of other cytoplasmic inclusions found in the intermediate region of *Locusta* primary cells. These range from multivesicular bodies, residual bodies and vacuoles to various stages of lamellated concretions. Most structural investigations of Malpighian tubules have noted the existence of granular structures variously termed 'dense bodies', mineralised spheres and concretions (Berkaloff 1958; Wigglesworth and Salpeter 1962; Wessing and Eichelberg 1969; Sohal *et al.* 1976). The chemical nature and the physiological significance of these concretions is still poorly understood. Most of the current knowledge of their chemical composition comes from histochemical studies. Uric acid, calcium urate and possibly phosphate have been reported in concretions from *Gryllus* (Berkaloff 1958). Similar concretions in *Rhodnius* however were considered to consist of minerals such as Ca^{2+}, Mg^{2+} and Fe^{3+} carbonates rather than urate (Wigglesworth and Salpeter 1962). Gouranton (1968) has identified similar minerals in the concretions of homopterans and Wall *et al.* (1975) suggest that these spheres have a role in calcium phosphate storage in *Periplaneta*. Mucopolysaccharides, sodium and potassium have been localised in the concretions of *Drosophila* larvae (Wessing and Eichelberg 1975) and it has been suggested that the concretions may be involved in the transepithelial movement of substances. Using X-ray microanalysis Sohal *et al.* (1976) have identified phosphate, chloride, sulphur, potassium, calcium and iron in the concretions from *Musca* tubules. The specific role of these mineralised spheres in the excretory process remains controversial. It has been suggested that they may concentrate substances removed from the haemolymph and extrude them into the tubule lumen (Berkaloff 1960; Wessing and Eichelberg 1975). However, there is no evidence to suggest the extrusion of intracytoplasmic concretions into the tubule lumen. Sohal *et al.* (1976) suggest that sequestration of metal ions within the concretions may provide a means for the effective excretion of these elements.

The close association of large numbers of mitochondria with the basal and apical cell surfaces, referred to above, suggests that energy requiring processes take place on or near the basal and apical cell membranes in *Locusta*. Similarly, mitochondria have been found associated with the basal and/or apical surfaces in the Malpighian tubules of other insects (Berridge and Oschman 1969; Taylor 1971a; Wigglesworth and Salpeter 1962; Peacock and Anstee 1977; Ryerse 1977). Studies on a variety of insect species have established that in the majority active transport of K^+ across the tubule into the lumen is necessary to generate 'urine' flow (Ramsay 1956; Berridge 1968; Pilcher 1970; Anstee *et al.* 1979). Such active transport is an energy requiring process and the concentration of mitochondria near to the apical and basal surfaces of *Locusta* tubules suggests that energy demanding processes are likely to be taking place at both surfaces. This is consistent with previous suggestions (Berridge and Oschman 1969) that there are two separate components of K^+ transport in insect Malpighian tubules; a Na^+/K^+ exchange pump at the basal surface and an electrogenic K^+ pump at the apical surface.

The basal and apical invaginations of the cell membrane are features characteristic of cells from other transporting epithelia (Pease 1956; Rhodin 1958; Fawcett 1962; Kaye *et al.* 1966; Berridge and Oschman 1970). It has been variously suggested that the membrane elaborations may (a) provide a large surface area for location of active transport sites (Fawcett 1962), (b) increase the membrane permeability to water (Pease 1956; Taylor 1971a), (c) enable the mitochondria to be brought close to the membrane surface (Taylor 1971a), and (d) provide geometric conditions for the formation of osmotic gradients (Diamond and Bossert 1967, 1968). In recent years much attention has been paid to the 'architecture' of the basal and apical unfoldings and their significance in the mechanism of fluid transport. Berridge and Oschman (1969) have discussed the application of the Standing Gradient Hypothesis of Diamond and Bossert (1967) to the Malpighian tubules of *Calliphora* (see Chapter 1). Basically it is suggested that the membrane infoldings enable the formation of standing solute gradients within the extracellular channels and that these gradients provide the osmotic force necessary for fluid flow. However, several objections have been raised against the application of the Standing Gradient Hypothesis to insect Malpighian tubules. Maddrell (1971, 1977) has pointed out that the depth of the basal infoldings of insect Malpighian tubules (5-10μm) is very much shorter than the model systems of up to 100μm long analysed by Diamond and Bossert (1967). Taylor (1971a) has calculated that the standing osmotic gradients in the channels of *Carausius* Malpighian tubules would therefore be very small. Hill (1975a, 1977) also argues against the Standing Gradient theory since the osmotic permeabilities of the cell membranes required to ensure isotonic flow would be so high as to be virtually impossible. Furthermore, Gupta *et al.* (1977) have determined the ionic concentrations in the basal cytoplasm of *Calliphora* Malpighian tubules using electron probe X-ray microanalysis. They found that a significant gradient of K^+ exists in this region although the direction of this gradient is the reverse of that postulated by Berridge and Oschman (1969) when applying the Standing Gradient Hypothesis. Similarly, they found that the concentrations of Na^+, K^+ and Cl^- measured along the channels between microvilli in *Rhodnius* are directionally opposite to that needed to support the Standing Gradient theory.

The primary cells of the Malpighian tubules of *Locusta* are very similar in ultrastructure to those of *Calliphora*, as described by Berridge and Oschman (1969). The dimensions of the basal infolds and the apical microvilli agree well with those same regions in *Calliphora*. On this basis the Berridge and Oschman model for *Calliphora* could also be applied to explain fluid transport across *Locusta* tubules. However, in view of the objections mentioned above, it seems unlikely that this is in fact the mechanism in operation. Several other theories for fluid transport across epithelia have been described in Chapter 1, and of these the theory of Taylor (1971a) seems most plausible for Malpighian tubules. Taylor (1971a) suggests a very simple mechanism, which is essentially as envisaged by Ramsay (1953), to explain water and solute coupling. Active transport of solute (mainly K^+ and accompanying anions) across the basal surface would maintain the cytoplasm hypertonic to the haemolymph. At the same time, active solute transport across the apical surface would maintain the lumen hypertonic to the cells. Water would then flow as a result of these small osmotic pressure differences, their magnitude being determined by the rate of solute transport and the osmotic permeability of the membrane. This being so, the invaginations of the basal and apical cell membranes would be developments to increase the surface area for the presentation of active transport sites as well as increasing the overall permeability of the cells to water.

As mentioned above, the Malpighian tubules of *Locusta* are completely ensheathed in a thick basement membrane. It is thought that in some Malpighian tubules the basement membrane denies large molecules access to the tubule cells. Locke and Collins (1967, 1968) showed that the basement membrane did not allow blood proteins and injected peroxidase access to the Malpighian tubules of *Calpodes*. Berridge and Oschman (1969) also suggest that the basement membrane operates as a filter, preventing high molecular weight proteins from accumulating in the basal channels. Evidence from electron micrographs showed that the basal infolds did not accumulate protein, and there was no evidence of micropinocytosis from the channels into the cells (Berridge and Oschman 1969). However, this feature is not common to all insects and the basement membranes of other Malpighian tubules seem to be more permeable. Uptake of proteins including horseradish peroxidase (m.w. 40,000) from the haemolymph has been demonstrated for the Malpighian tubules of *Gryllus* (Berkaloff 1960), *Drosophila* (Wessing 1965) and the dragonfly *Libellula* (Kessel 1970). It may be significant that in these tubules micropinocytosis does take place at the basal surface. In *Carausius* too there is evidence of extensive micropinocytosis, and Taylor (1971a) suggests that the main function of the basement membrane in this insect is to protect the tubules against distortion by intraluminal pressures created by muscular activity and trans-tubular transport.

There was no evidence of pinocytic activity at the basal surface of *Locusta* tubules in the present study but further studies are clearly required to determine whether the basement membrane is permeable to large molecules in this insect.

Secondary Cells

These cells were found infrequently in electron micrographs of the Malpighian tubules. They are immediately distinguishable from the primary cells which are larger and more electron dense (Plate 2). The structure of a typical secondary cell is shown in Plate 11. The appearance is very characteristic of secretory cells; Golgi bodies associated with vacuoles are abundant in the cytoplasm as is well-developed endoplasmic reticulum consisting of parallel arrays of rough-surfaced membranes (Plate 12). The basal cell membrane is invaginated to form a series of narrow channels (ca. 1-1.5μm in length) and cytoplasmic processes. However, these are much less extensive than in the primary cells and the infolds do not extend as far into the cell (Plate 11). The basal cytoplasmic processes contain mitochondria but these mitochondria are somewhat smaller than those found in primary cells.

The apical cell membrane gives rise to microvilli but these are sparse and shorter (ca. 1-2μm) than those of the primary cells (Plate 11). Mitochondria are rarely associated with this region and do not extend into the microvilli. In several sections numerous vacuoles were found at or near the apical border (Plate 11, 13). The contents of some of these vacuoles appeared to be being discharged into the tubule lumen by the process of exocytosis (Plate 13). This process of fusion between the plasma membrane and the membrane surrounding an intracellular vesicle or vacuole has been noted in many cell types.

These secondary cells in the Malpighian tubules of *Locusta* are similar in structure to those reported in tubules from other species. Berridge and Oschman (1969) have described 'stellate cells' in the Malpighian tubules of *Calliphora* which also have extensive endoplasmic reticulum, well-developed Golgi complexes, numerous vacuoles and reduced infoldings of the basal and apical cell surfaces. Similarly, Taylor (1971b), Wall *et al.* (1975) and Peacock (1975) have described such cells in the tubules of *Carausius morosus, Periplaneta americana* and *Jamaicana flava* respectively. Secondary cells have been described previously in the Malpighian tubules of *Locusta* by Martoja (1959, 1961), Peacock (1975) and Charnley (1975).

Two main functions have been proposed for these secondary cells, the secretion of mucopolysaccharides (Martoja 1956, 1961; Berkaloff 1960), and the absorption of ions and water from the tubule lumen (Berridge and Oschman 1969). Martoja (1956, 1961) and Berkaloff (1960) have demonstrated histochemically cells in the Malpighian tubules of various Orthoptera that contained acid mucopolysaccharides. Berridge and Oschman (1969) have suggested that the stellate cells of *Calliphora* are involved in the reabsorption of Na^+ from the tubule lumen and that reabsorption is responsible for the low Na^+ content of the tubule fluid. They point out that the high Na^+ concentration of the fluid secreted by the distal segments of *Rhodnius* tubules (Ramsay 1952) might arise because this region appears to lack stellate cells (Wigglesworth and Salpeter 1962), whilst in the proximal region the 'urine' becomes hypertonic as Na^+ is returned to the blood. Taylor (1971b) has also noted that the low Na^+/K^+ ratio found in the distal region of *Carausius* tubules by Ramsay (1955) 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.

The observations of the present study offer neither criticism or support of these proposals. The secondary cells of *Locusta* possess features characteristic of both transporting and secretory epithelia. Whilst histochemical tests were not carried out in the present study the abundance of rough endoplasmic reticulum, Golgi bodies and vacuoles is suggestive of a secretory role, the product of which may be mucus. Taylor (1971b) also suggests a secretory role for the Type II cells of *Carausius morosus* and considers the mucus produced to be a lubricant assisting the passage of faeces along the gut. Whether or not the secondary cells of *Locusta* are involved in Na^+ reabsorption remains to be established. It is doubtful that they could play a part in active reabsorption of Na^+ since very few mitochondria are found in the secondary cells and those that are present do not associate with the apical cell surface. However, this would not preclude a passive entry of Na^+ into the secondary cells. In addition, Berridge and Oschman (1969) have also suggested that Na^+ reabsorption may be by way of septate desmosomes directly from the primary cells. The septate desmosomes are thought to offer little resistance to such transcellular movements whilst being impermeable to transepithelial movements (Loewenstein 1966).

Plate 1

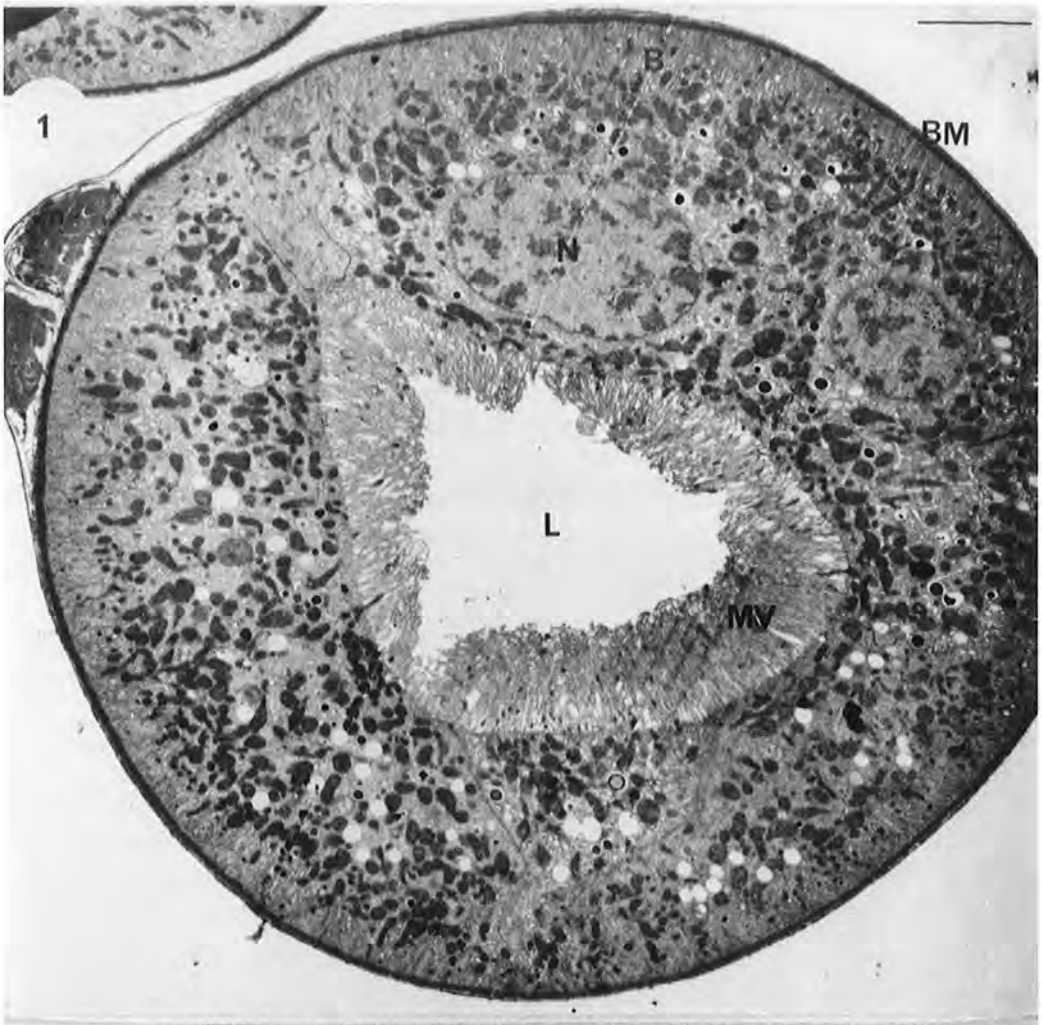
Low power transmission electronmicrograph showing a tranverse section through a Malpighian tubule. The tubule is completely surrounded by a basement membrane (BM) which envelops the muscles (m) which run along the length of the tubule. The majority of the tubule is made up of primary cells which are characterised by invaginations of the basal cell membrane (B) and numerous microvilli (mv) which project into the lumen (L).

Scale = 10μm

Plate 2

Transverse section electronmicrograph showing a primary cell. The infoldings of the basal cell membrane (Bl) extend for up to one-third of the length of the cell. The apical cell membrane forms a microvillar border (mv). Note the presence of numerous mitochondria (M) and concretions (C).

Scale = 1μm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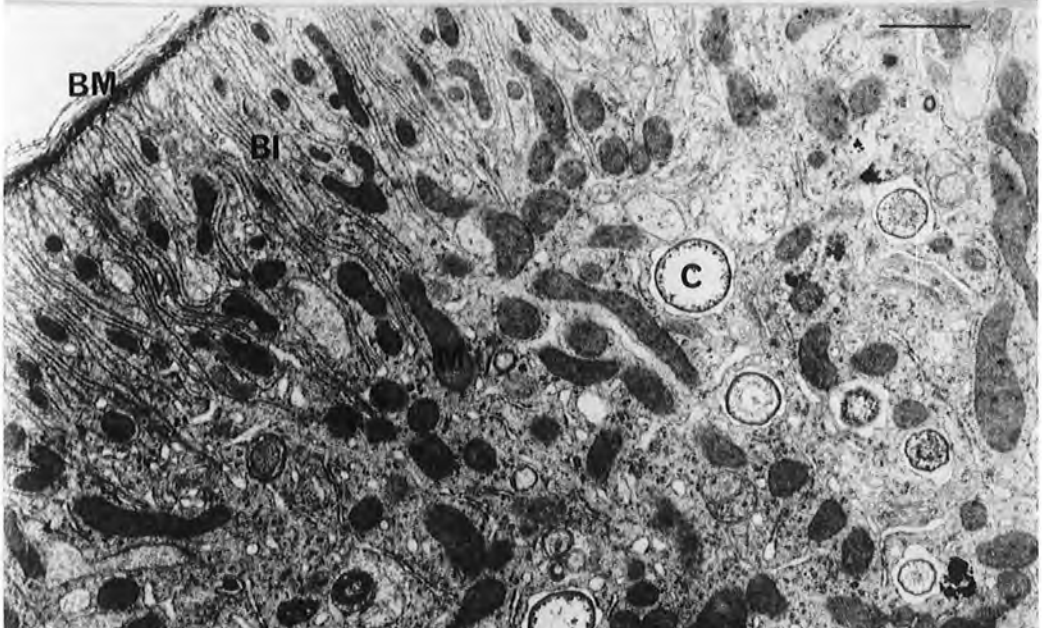
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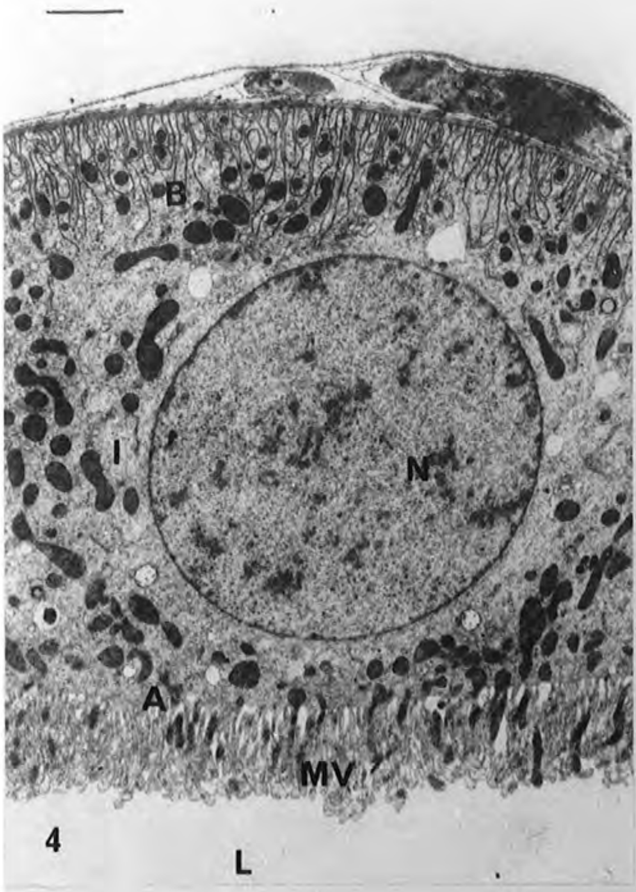

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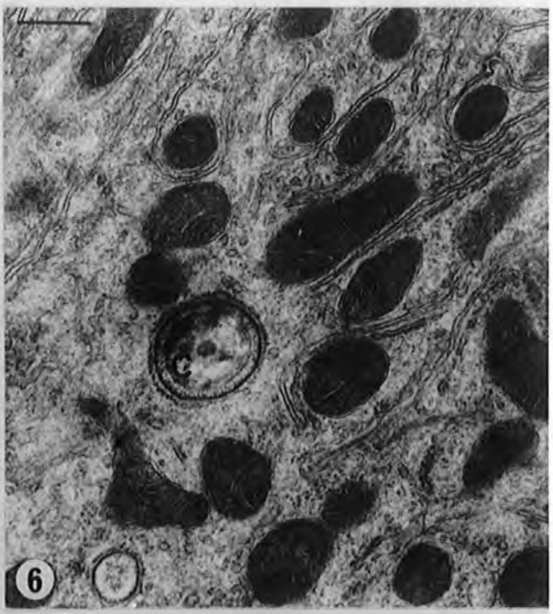

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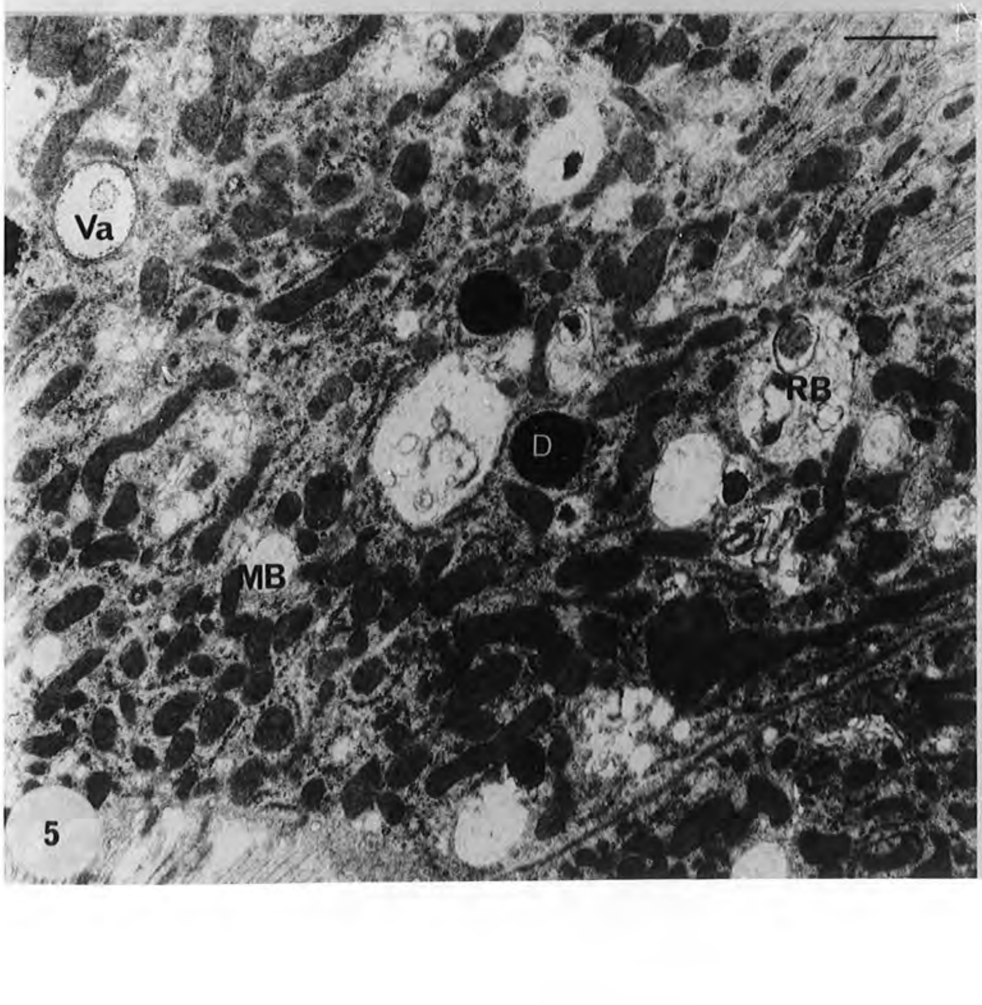

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Plate 3

Higher power transmission electronmicrograph showing a T.S. of a primary cell. The basement membrane (BM) can be seen to be a layered structure. The infoldings of the basal cell membrane (Bl) give rise to extracellular channels (EC). Where the basal cell membrane meets the basement membrane there is an increase in electron density on the cytoplasmic side forming hemidesmosome junctions (H). The primary cells are joined together laterally by septate desmosome junctions (SD). In the apical region the desmosome widens out to form a zonula adhaerens junction (ZA). Numerous mitochondria (M) are present in the cytoplasmic compartments formed by the infoldings of the basal plasma membrane. At the apical surface too there is a large number of mitochondria some of which are found in the microvilli(MV). Note also the presence of numerous coated vesicles (V), occasional Golgi bodies (G), and vacuoles (Va), multi-vesicular bodies (MB) and residual bodies (RB).

Scale = 1μm

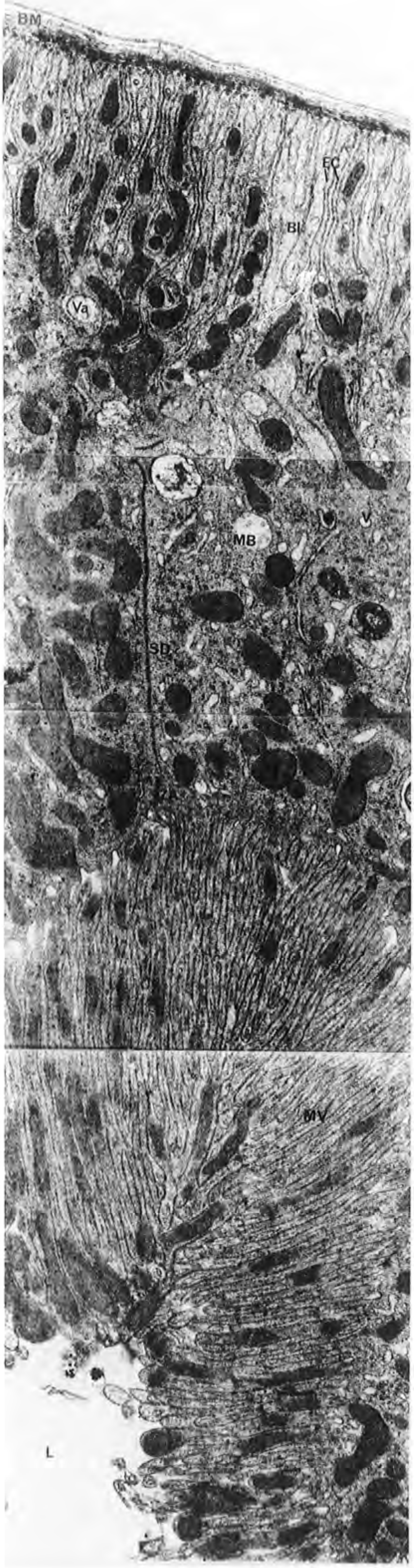

BM
EC
Bl
Va
MB
V
SD
MV
L

Plate 4

Low power T.S. through a primary cell showing the three main regions : Basal (B) characterised by infoldings of the basal plasma membrane; intermediate (I) containing the nucleus (N), vacuoles and concretions; Apical (A) which possesses numerous microvilli (MV) which project into the lumen (L) of the tubule.

Scale = 4μm

Plate 5

The cytoplasm of the intermediate region contains many vacuoles (Va) of different sizes as well as multivesicular bodies (MB), residual bodies (RB) and dense bodies (D).

Scale = 1μm

Plate 6

Note the presence of concretions (C) which appear to be made up of concentric layers of electron dense material.

Scale = 0.5μm

Plate 7

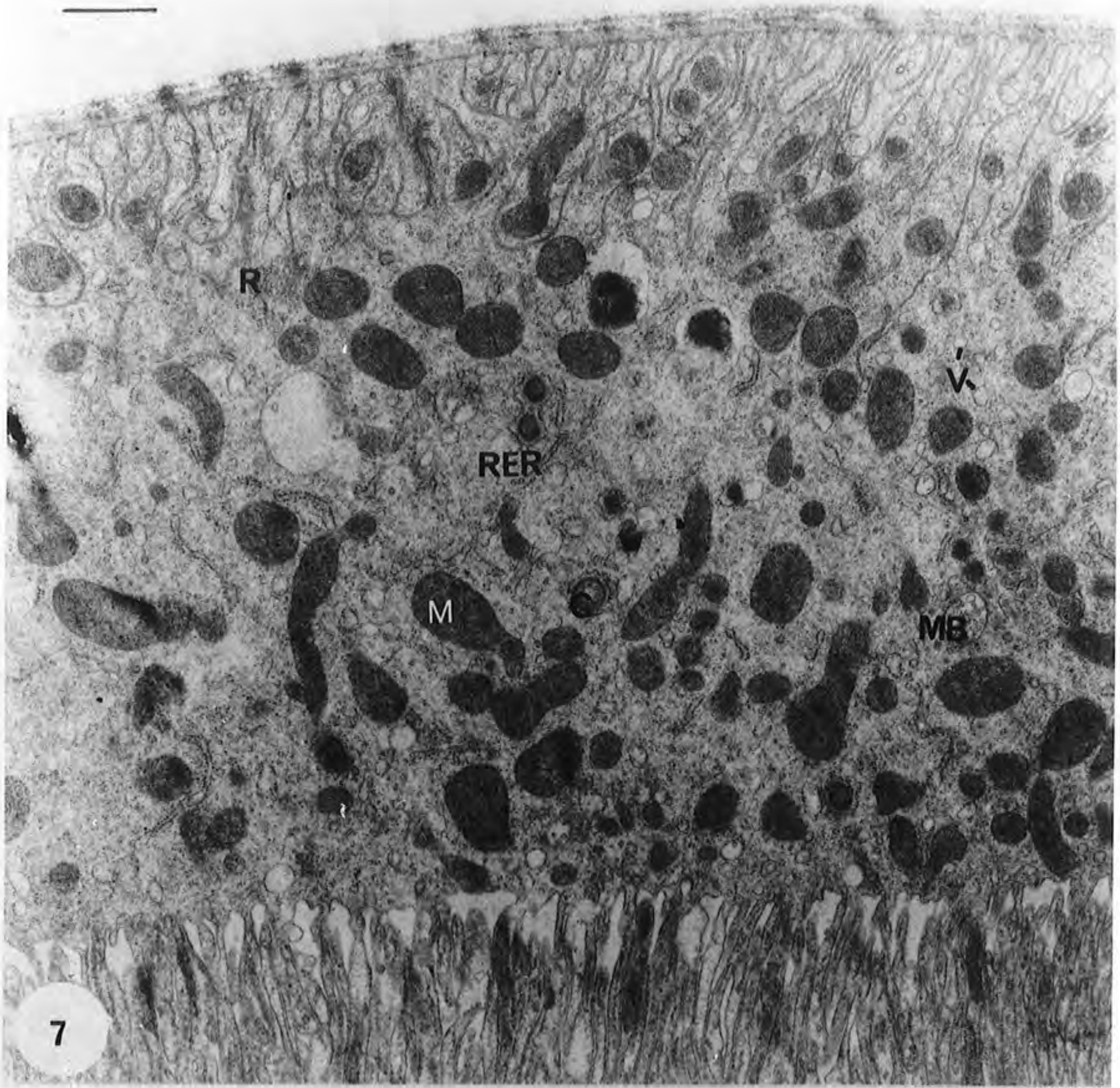
Note the presence in the intermediate region of the cell of numerous mitochondria (M), rough endoplasmic reticulum (RER), free ribosomes (R), vesicles (V) and multi-vesicular bodies (MB).

Scale = 1µm

Plate 8

Transverse section electronmicrograph of the intermediate region showing a Golgi body (G)

Scale = 1µm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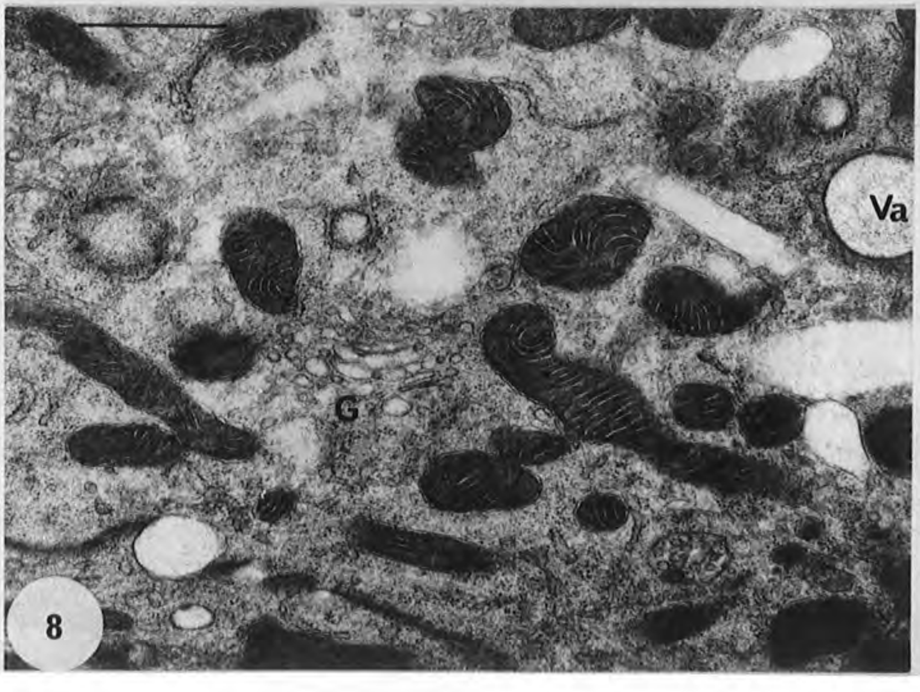

8

Plate 9

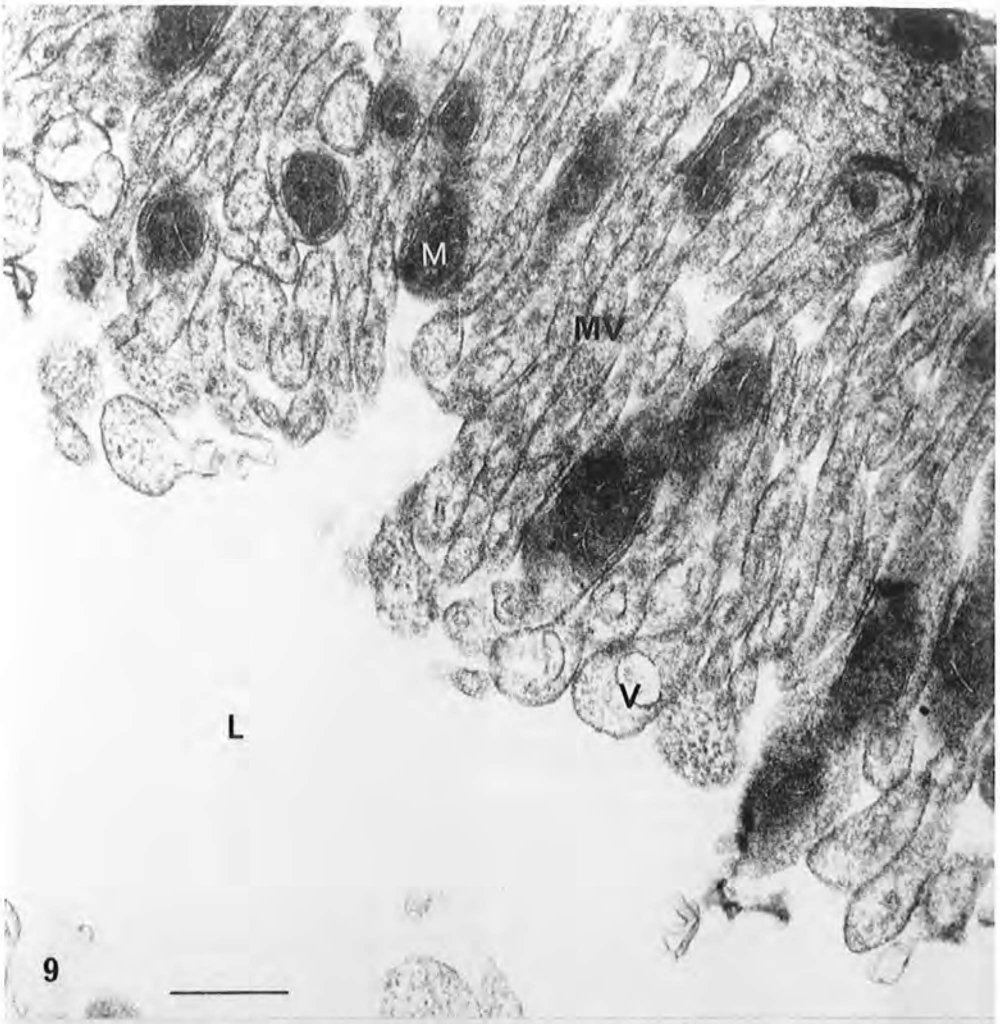
High power transmission electronmicrograph showing a transverse section through the apical region of a primary cell. The apical surface is composed of tightly packed microvilli (MV) which project into the lumen (L) of the tubule. The microvilli are club-shaped and a number contain processes of the apical mitochondria (M). Small vesicles (V) are occasionally found in the tips of the microvilli.

Scale = 0.5µm

Plate 10

High power transverse section showing a septate desmosome junction (SD). In the apical region the desmosome widens out to form the macula adhaerens junction (ZA) in which a layer of electron dense material is attached to the cytoplasmic side of the adjacent membranes.

Scale = 0.25µm



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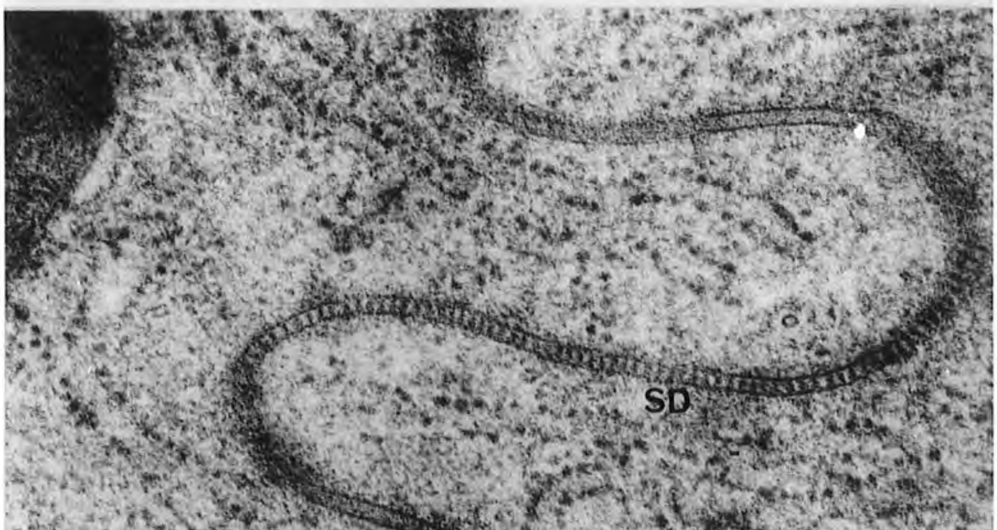

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Plate 11

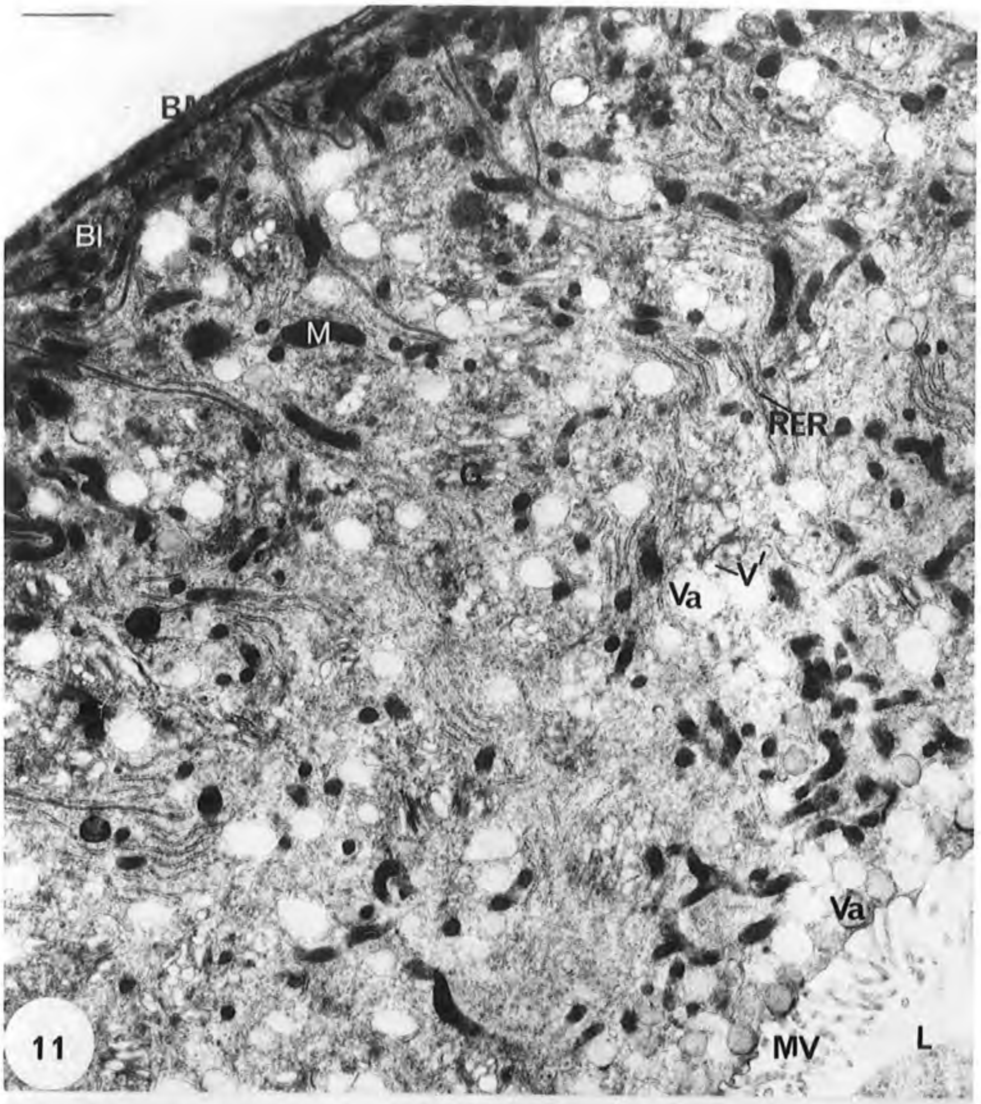
Transmission electronmicrograph showing a transverse section through a secondary cell. Note the reduced infoldings of the basal cell membrane (Bl) and the sparse microvilli (MV). The intermediate region contains numerous vacuoles (Va), vesicles (V) associated with Golgi bodies (G) and there is a well-developed system of endoplasmic reticulum (RER). Numerous vacuoles (Va) are also found at the apical cell surface.

Scale = 1μm

Plate 12

Note the well-developed system of endosplasmic reticulum consisting of parallel arrays of rough surfaced membranes (RER) and the numerous Golgi complexes (G).

Scale = 1μm


BM
BI
M
RER
G
V
Va
Va
MV
L
11

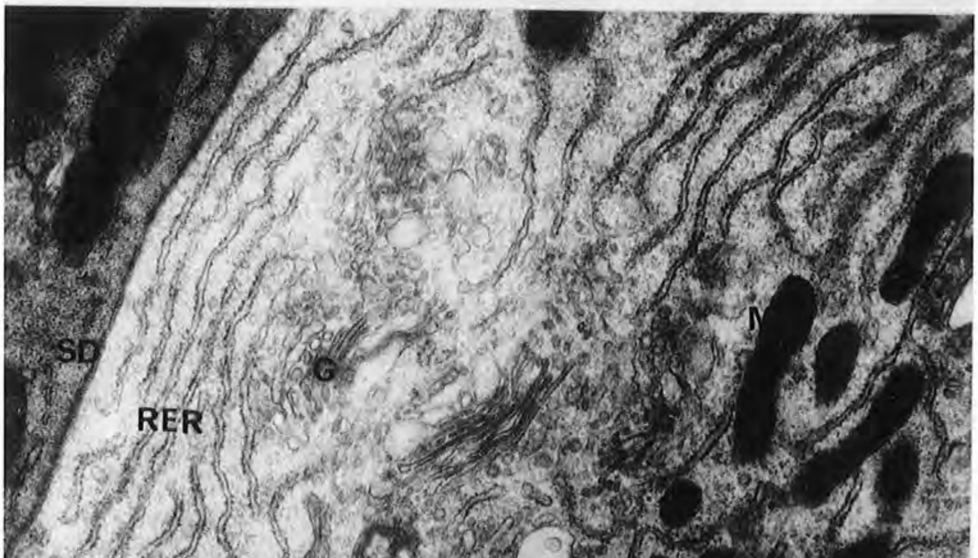
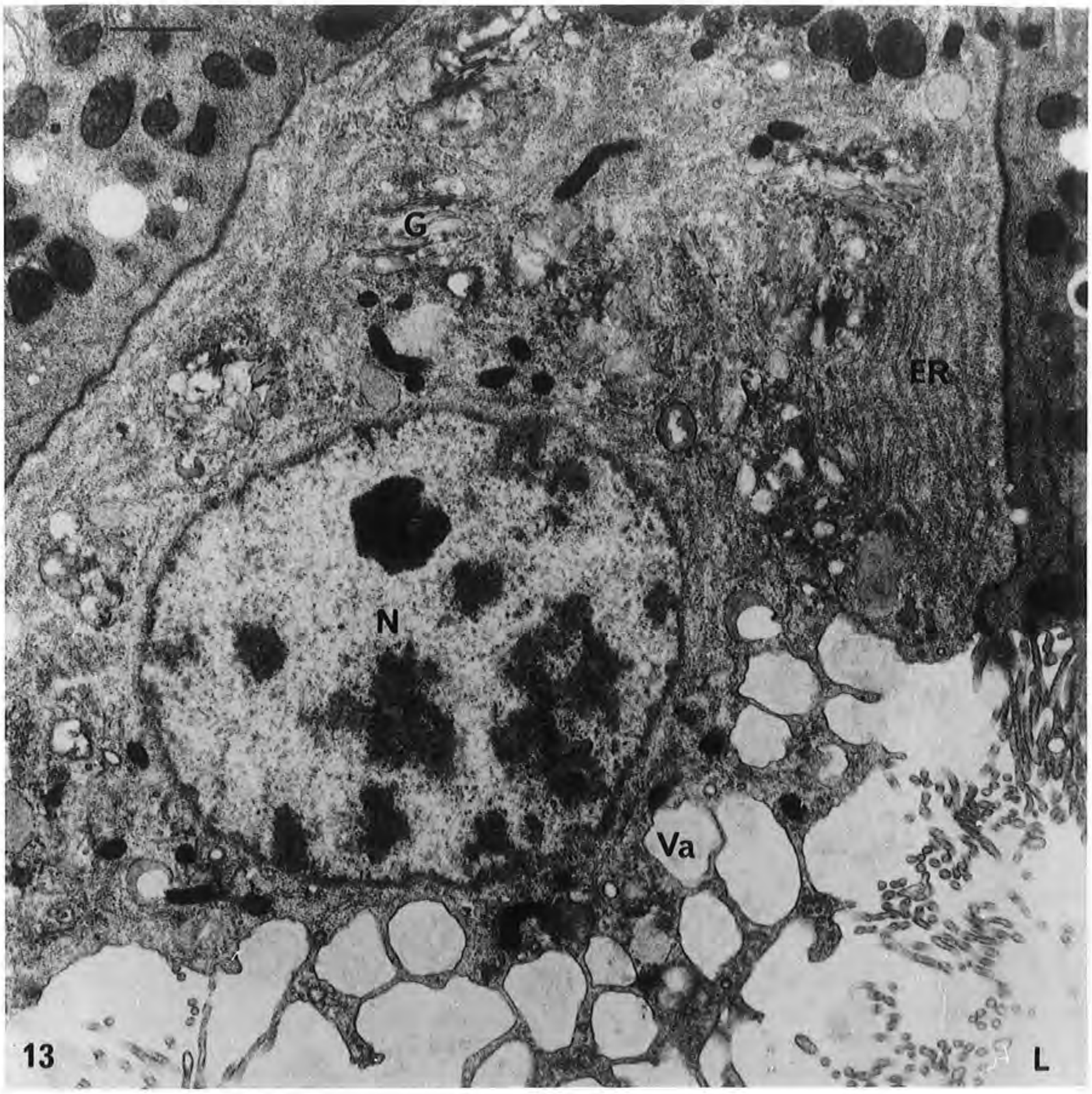

M
SD
G
RER
ZA
12

Plate 13

Transverse section through a secondary cell showing the numerous vacuoles (Va) at the apical surface. The contents of these vacuoles appear to be discharged into the tubule lumen by the process of exocytosis.

Scale = 1μm

13



Section II Changes in the fine structure of the Malpighian tubules throughout the 5th instar and in early adult *Locusta*

The general ultrastructural appearance of the Malpighian tubules from 5th instar *Locusta* was essentially the same as that described in Section I. The tubules consisted of the two types of cell already referred to. The ultrastructure of the secondary cells appeared not to change with development but several features of the primary cells varied with the age of the insect. It is convenient to describe these age-dependent changes under the following headings.

Basal Membrane

Invaginations of the basal cell membrane were observed throughout the 5th instar (Plates 14 - 17). However, the levels to which the infolds extend into the cytoplasm varied with age (Plates 14-17). Using transverse section electronmicrographs the mean extracellular channel length was determined by measuring the distance from the basement membrane into the cytoplasm of each of 20 adjacent channels for each micrograph. Examination of Table 3.1 shows that the trend was for the extracellular channels to increase in length with increased age throughout the stadium until just before the larval-adult moult when the channels became somewhat shorter. There was a significant increase in channel length between 1 day old 5th instar locusts (1.45 ± 0.1μm) and 7 day old insects (4.26 ± 0.1μm) and a significant decrease in the length of the extracellular channels between 9 day old insects (4.8 ± 0.5μm) and newly moulted adults (2.2 ± 0.2μm).

Despite this variation in the length of the extracellular channels their width remained fairly constant at approximately 0.03μm. However, the width of the cytoplasmic compartments between the channels did vary with age. The results in Table 3.2 show that the cytoplasmic compartments become narrower with increasing age, varying from 0.11 ± 0.009µm in 1 day old 5th instar locusts to 0.06 ± 0.001 on Day 7 (Plates 18,19). Just before the larval-adult moult the width of the cytoplasmic compartments increased to the size observed on day 1.

The conclusion one must draw from these measurements is that the degree of membrane invagination varies throughout the 5th instar. Furthermore, the fact that extracellular channel width remained more or less constant whilst there was a decrease in the width of the cytoplasmic compartments between channels suggests that the number of channels increased over the first 7 days of the 5th stadium.

Table 3.1 Variation in the length of the extracellular channels throughout the 5th stadium

Age (days)	n	mean extracellular channel length µm ± S.E.	P	
2hrs	60	1.93 ± 0.1		
1 day	60	1.45 ± 0.1	2hrs :1	<0.01
3	60	1.5 ± 0.1	1:3	not sig
5	60	2.15 ± 0.4	3:5	not sig
7	60	4.26 ± 0.1	5:7	<0.001
9	60	4.8 ± 0.5	7:9	not sig
11	60	2.0 ± 0.2	9:11	<0.001
1 day adult	60	2.2 ± 0.2	9:1 11:1	0.001 not sig

P values are based on the application of a Students 't' test.

Table 3.2 Variation in width of the cytoplasmic compartments throughout the 5th stadium

Age (days)	n	mean width of cytoplasmic compartments µm ± S.E.	P
2hrs	60	0.083 ± 0.062	
1	60	0.11 ± 0.009	2hrs:1 not sig
3	60	0.09 ± 0.001	1:3 <0.001
5	60	0.06 ± 0.004	3:5 <0.001
7	60	0.06 ± 0.001	5:7 not sig
9	60	0.07 ± 0.004	7:9 <0.05
11	60	0.1 ± 0.009	9:11 <0.01
1 day adult	60	0.08 ± 0.01	11:1 not sig

P values are based on the application of a Students 't' test

Apical Microvilli

As in the case of the infoldings of the basal cell surface, changes were noted at the apical surface. Using transverse section electronmicrographs mean microvillar length was determined by measuring 20 microvilli for each micograph. Difficulty was encountered in measuring microvillar length as the microvilli frequently curve in and out of the plane of the section. Consequently only those microvilli which could be traced from the apical cell surface to their tips were measured. The results are shown in Table 3.3. It can be seen (Plates 20-23) that the microvillar length increased significantly over the first 7 days of the instar (2.8 ± 0.1µm - 3.3 ± 0.12µm) and then decreased before the larval-adult moult (Day 11, 1.75 ± 0.15µm).

Table 3.3 Variation in microvillar length throughout the 5th stadium

Age (days)	n	mean length of microvilli m ± S.E.	P	
2hrs	60	2.37 ± 0.063		
1	60	2.8 ± 0.1	2hrs:1	<0.01
3	60	2.37 ± 0.14	1:3	<0.02
5	60	2.44 ± 0.1	3:5	not sig
7	60	3.3 ± 0.12	5:7	<0.001
9	60	1.57 ± 0.04	7:9	<0.001
11	60	1.75 ± 0.15	9:11	not sig
1 day adult	60	3.36 ± 0.19	11:1	<0.001

P values are based on the application of a Students 't' test

The mean diameter of the microvilli (measured 1μm from the apical surface) was also determined. Examination of Table 3.4 shows that mean microvillar diameter decreased from 0.14 ± 0.002μm in 1 day old 5th instar locusts to 0.07 ± 0.004μm in 7 day old insects (Plates ^{24,25}). Just before the larval-adult moult the microvillar diameter began to increase (0.1μm, Day 11).

Table 3.4 Variation in microvillar diameter throughout the 5th stadium

Age (days)	n	mean diameter apical microvilli $\mu m \pm$ S.E.	P	
2hrs	60	0.08 ± 0.005		
1	60	0.14 ± 0.002	2hrs:1	<0.001
3	60	0.12 ± 0.007	1:3	<0.02
5	60	0.1 ± 0.001	3:5	<0.02
7	60	0.07 ± 0.004	5:7	<0.001
9	60	0.07 ± 0.001	7:9	not sig
11	60	0.1 ± 0.001	9:11	<0.001
1 day adult	60	0.13 ± 0.007	11:1	not sig

P values are based on the application of a Students 't' test

Mitochondria

The number of mitochondria in the Malpighian tubule primary cells appears to vary throughout the 5th stadium. Examination of Plates 26 and 27 suggests that there is an enormous increase in mitochondrial number between Day 1 and Day 9 of the 5th stadium. And, comparing Plates 27 and 28 there is an apparent decrease in mitochondrial number between Day 9 and the first day of adult life.

The distribution of the mitochondria throughout the cytoplasm also appears to vary according to age. Throughout the stadium the majority of mitochondria are found in 2 main zones associated with the basal and apical cell surfaces. In newly moulted 5th instar locusts the basal zone of mitochondria is very narrow and is located a short distance above the basement membrane (Plate 29). An apical zone of similar width is situated immediately below the bases of the microvilli (Plate 30). As the age of the insect increases, the width of the basal zone of mitochondria increases until on Day 9 the mitochondria are found in the cytoplasm immediately above the basement membrane (Plate 31). Thus, with increasing age, the mitochondria come to lie in the cytoplasmic compartments between the extracellular channels formed by infoldings of the basal plasma membrane (Plates 32 - 34). Just before the larval-adult moult (Day 11) and in newly moulted adult insects, mitochondria were rarely found in the cytoplasm alongside the extracellular channels.

At the apical surface the mitochondrial zone varied only slightly in width and the percentage of microvilli containing mitochondria remained roughly constant at ca. 15-20% throughout the 5th stadium.

Similar ultrastructural changes to those described above (i.e. variation in degree of basal and apical cell membrane infolding, and position of mitochondria) have been described previously in the Malpighian tubules of *Calpodes ethlius* (Ryerse 1977). Ryerse (1977) observed that the apical microvilli of *Calpodes* tubule cells normally contain numerous mitochondria. However, at the larval-pupal metamorphosis the mitochondria were withdrawn into the apical cell cytoplasm and degraded whilst the microvilli remained intact. At the same time the intracellular and extracellular channels were reduced as the basal infolds and the microvilli shortened. Coincident with these ultrastructural changes Ryerse (1978) found a cessation of fluid secretion by the Malpighian tubules. Renewal of fluid transport at the mid-pupal stage was associated with the reinsertion of mitochondria into the apical microvilli and the reformation of the basal and apical channels.

Changes in cell ultrastructure have also been found to correspond with changes in fluid secretory rates in other transporting epithelia

(Kaufman *et al.* 1976). Studies on the salivary gland cells of the tick *Amblyomma hebraeum* have shown that radical ultrastructural changes occur in these cells during the feeding period (Diehl *et al.* 1977). Diehl *et al.* (1977) found an enormous development in plasma membrane invagination along with an increase in the numbers of mitochondria. In these arthropods the elimination of excess fluid taken in with a blood meal is achieved by the salivary glands. Associated with the ultrastructural changes described above, Kaufman *et al.* (1976) observed vastly increased rates of fluid secretion during the feeding period.

Vacuoles and Concretions

The cytoplasm of the tubule cells contains numerous vesicles, vacuoles and concretions. These range from small vesicles and clear vacuoles (Plate 35), through lamellated concretions (Plate 36), to 'dense bodies' (Plate 36) as well as 'residual bodies' (Plate 37); multivesicular bodies (Plate 37) and double-membrane bound vacuoles (Plate 38).

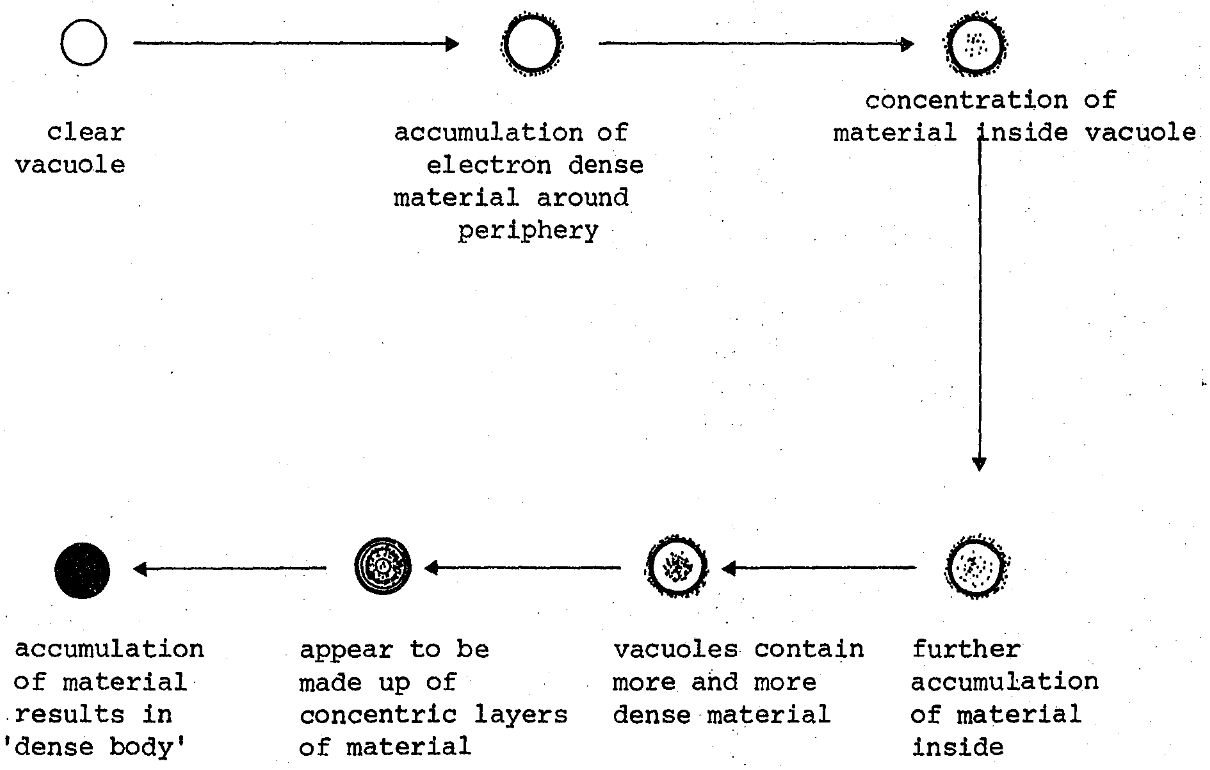
Coated vesicles were found in the cytoplasmic compartments between the basal infoldings, in the intermediate region and at the apical surface (Plates 35, 39). These vesicles were found to be particularly numerous in newly moulted 5th instar locusts (Plate 40). Comparing Plates 40 and 41 it can be seen that there is a dramatic reduction in the number of these vesicles over the first 24hrs of the stadium. The possible significance of these coated vesicles has already been discussed in Section I. Wigglesworth and Saltpeter (1962) and Wessing (1965) suggest that water and solutes may cross the cell inside such vesicles by the mechanism of cytopempis. If this is so, it may be that such a mechanism has a greater significance during the first 24 hours after moulting, perhaps to quickly rid the body of waste products.

Multi-vesicular bodies, usually associated with Golgi complexes, were found in the cytoplasm of the intermediate region throughout the 5th stadium. Similarly 'residual bodies' were also found at all the stages observed.

A variety of vacuolar structures containing variable amounts of dense material were also found in the cytoplasm of the intermediate region. These ranged from clear vacuoles to lamellated concretions. The concretions found in the Malpighian tubules of *Locusta* are similar to those described in the Malpighian tubules of *Rhodnius* (Wigglesworth and Salpeter 1962) and *Gryllus* (Berkaloff 1958). The nature of these concretions has been discussed in Section I. Although similar concretions have been described previously, their origin remains obscure. Observation of several micrographs at different ages throughout the 5th instar of *Locusta* has provided evi dence for a speculative sequence of formation of concentric concretions. In newly moulted 5th instar locusts several vacuoles were observed. Some of these appeared empty, others had a slight granular inclusion. These vacuoles appeared to increase in number with age and to contain more and more dense material. Towards the end of the instar most of the inclusions appeared to be made up of concentric layers of material. This variation in the density of the vacuoles suggests a progressive increase in the concentration of material (Fig. 3.1).

Sohal *et al.* (1976) also suggest possible concretion formation from the accumulation of dense material inside membrane-bound vacuoles. *Musca domestica* tubules were found to contain three different types of concretion (Sohal *et al.* 1976). Apart from accumulation of material inside vacuoles, Sohal *et al.* (1976) suggest that concretions may originate from multi-vesicular bodies and from lysosomes. Some of the inclusions in *Locusta* Malpighian tubules resemble the 'residual bodies' described by Sohal *et al.* (1976). Sohal *et al.* (1976) suggest that these 'residual bodies'

Figure 3.1 Speculative sequence of formation of the concretions found in *Locusta* Malpighian tubules



may derive from the multi-vesicular bodies as well as cytolysomes. There was no evidence for this in the present study although it must be stated that it is very difficult to determine any sequence of transformation from static electronmicrographs.

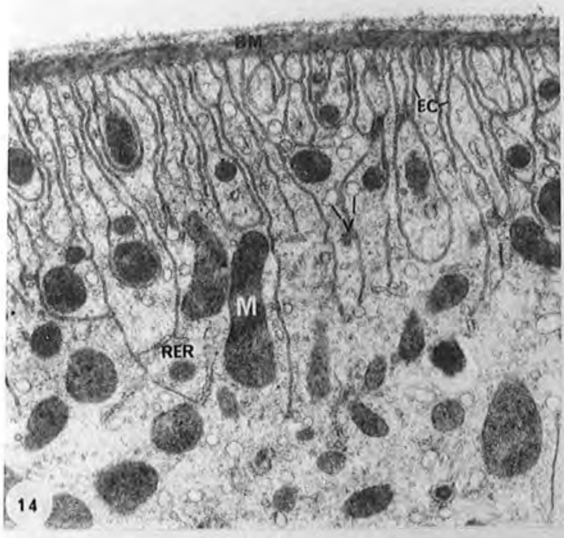
Conclusion

The ultrastructure of the Malpighian tubules of *Locusta* closely resembles that of several other species studied previously (Beams *et al.* 1955; Tsubo and Brandt 1962; Berridge and Oschman 1969; Taylor 1971a,b; Wall *et al.* 1975; Peacock and Anstee 1977). They have a similar morphological appearance along their length as do the Malpighian tubules of *Melanoplus differentialis* (Beams *et al.* 1955), *Dissosteira carolina* (Tsubo and Brandt 1962) and *Jamaicana flava* (Peacock and Anstee 1977). The Malpighian tubules of *Locusta* are composed of two distinct types of cell referred to above as primary and secondary. The primary cells are predominant and it is these cells which are thought by most researchers to be responsible for 'urine secretion' (Berridge and Oschman 1969; Taylor 1971a; Peacock and Anstee 1977). The primary cells from *Locusta* tubules exhibit several features characteristic of cells from transporting epithelia. The basal cell membrane is extensively infolded whilst the apical membrane is produced into closely packed microvilli. Large numbers of mitochondria are found associated with the basal infolds and the apical microvilli, suggesting a high energy requirement at both cell surfaces. These features of *Locusta* primary cells are found to vary with development throughout the 5th stadium and into early adult life. The degree of basal and apical membrane invagination, as well as the number of mitochondria, are found to increase with age until just before the larval-adult moult. During this period the mitochondria increasingly come to lie in the cytoplasm between the basal channels. At the larval-adult moult the basal infolds and apical microvilli shorten and mitochondria are rarely found closely associated with the basal infolds. In previous studies (Ryerse 1977, 1978; Kaufman *et al*. 1976; Diehl *et al*. 1977) changes such as those described above have been correlated with changes in fluid secretory activity. In *Locusta* too it may be expected that functional changes will accompany structural changes since mitochondria provide the energy for active ion transport resulting in fluid secretion. Also, the degree of membrane invagination would be expected to affect rates of fluid secretion. Physiological and biochemical changes associated with development in 5th instar *Locusta* will be described in Chapter 5.

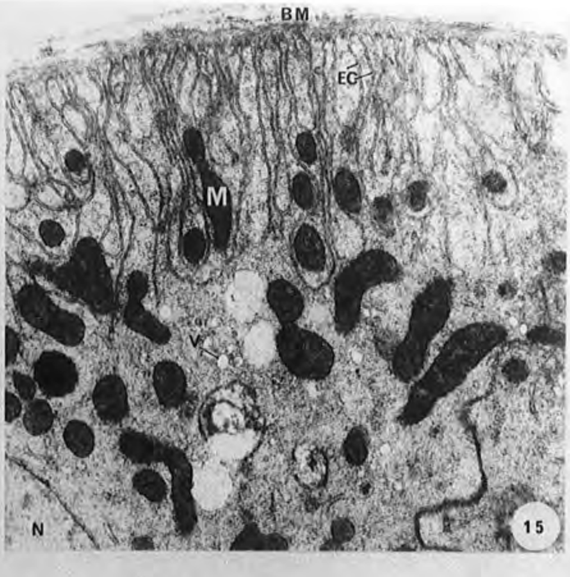
Plates 14-17

Transmission electronmicrographs showing transverse sections through the basal region of the primary cells at different stages throughout the 5th instar. Whilst infoldings of the basal cell membrane are found throughout, the extent to which the extracellular channels (EC) project into the cytoplasm varies with age. The extracellular channels increase in length over the first 9 days of the instar but then begin to decrease (Day 11) before the larval-adult moult.

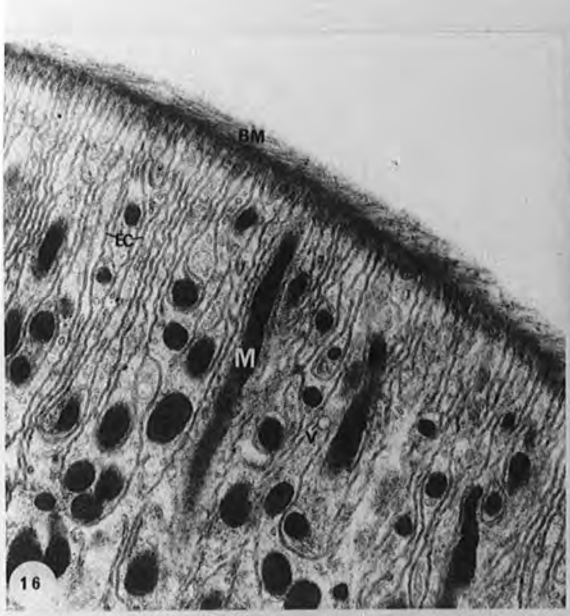
Scale = 1µm



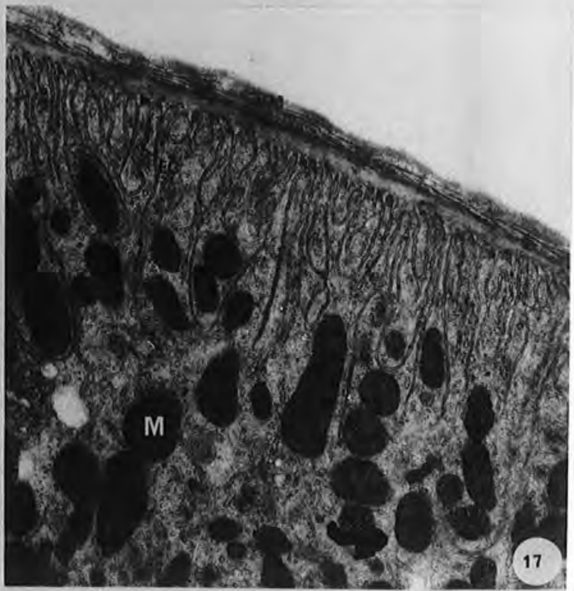
Day 1



Day 5



Day 9

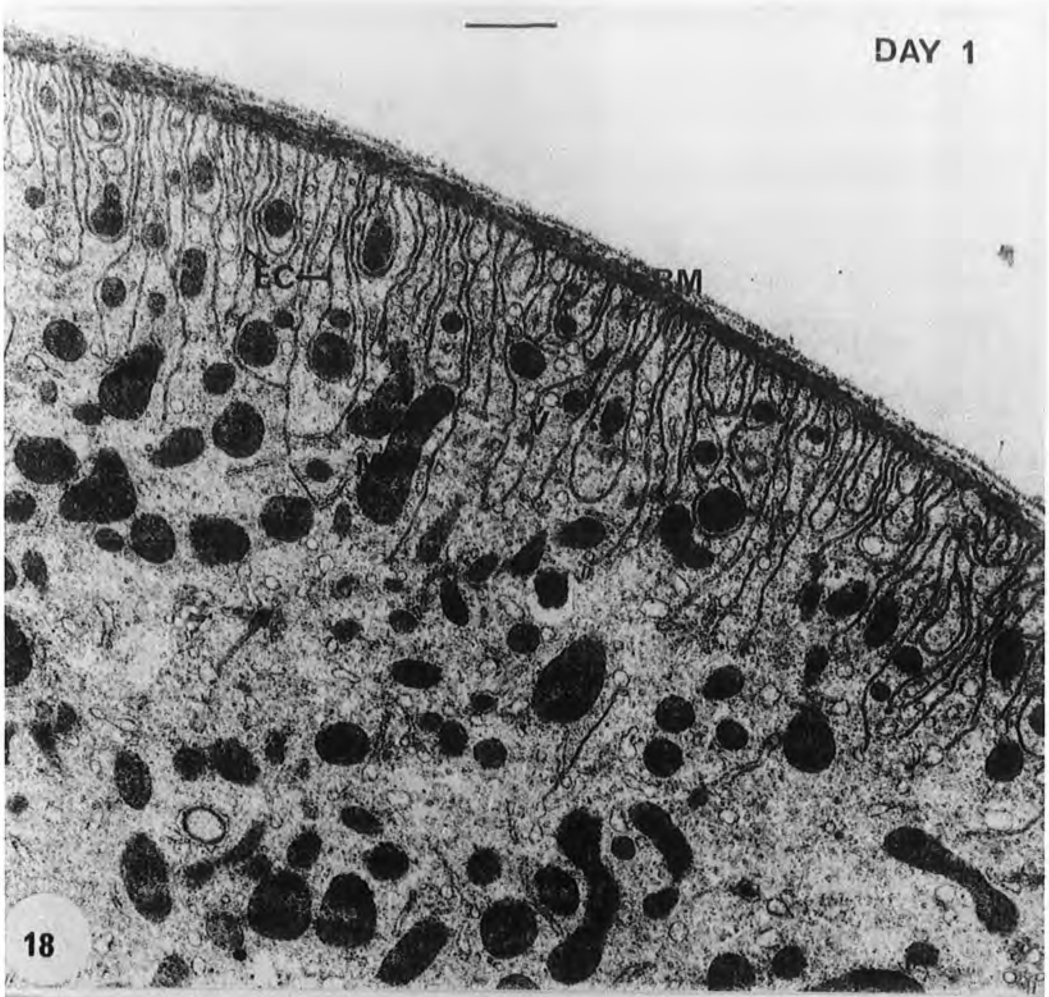


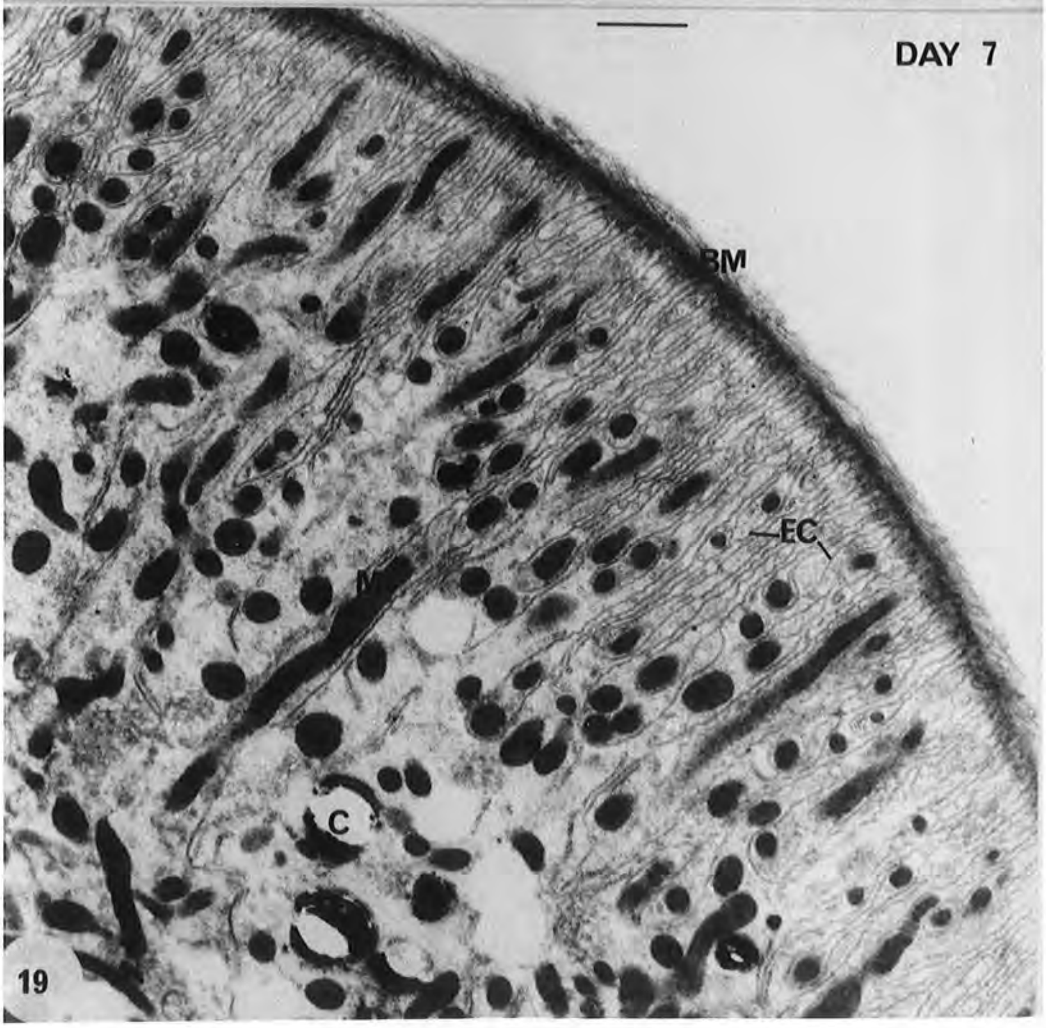
Day 11

Plates 18, 19

Transverse sections through the basal region of the primary cells to compare the width of the cytoplasmic compartments at different ages in the 5th instar. Comparing Day 1 with Day 7 it can be seen that the width of the cytoplasmic compartments between the extracellular channels (EC) decreases with increasing age. It is also apparent that the degree of membrane invagination has greatly increased over the first 7 days of the instar.

Scale = 1µm

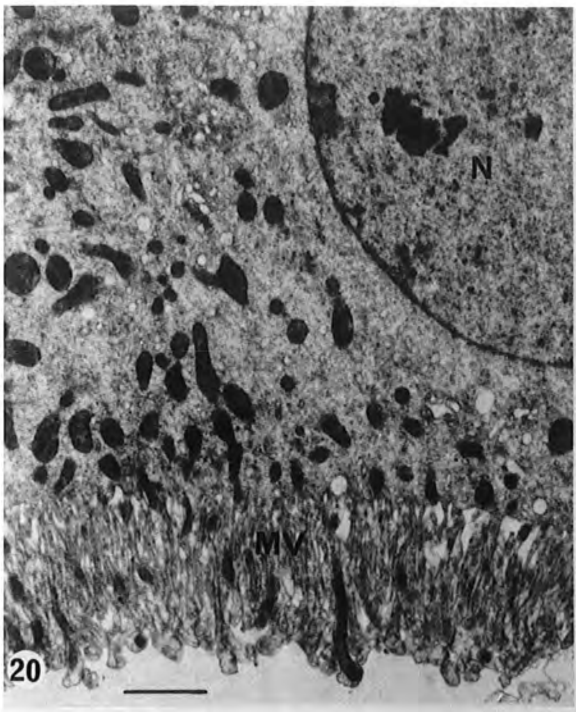

DAY 1
EC
BM
V
M
18


DAY 7
BM
EC
M
C
19

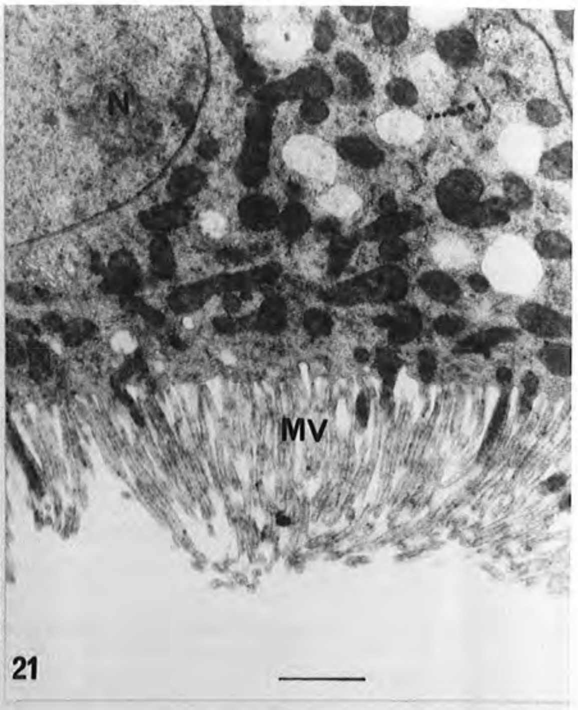
Plates 20-23

Transmission electronmicrographs showing transverse sections through the apical region of primary cells at different stages throughout the 5th instar. It can be seen that the microvilli (MV) increase in length over the first 7 days of the instar but then decrease again (Day 11) just prior to the larval-adult moult.

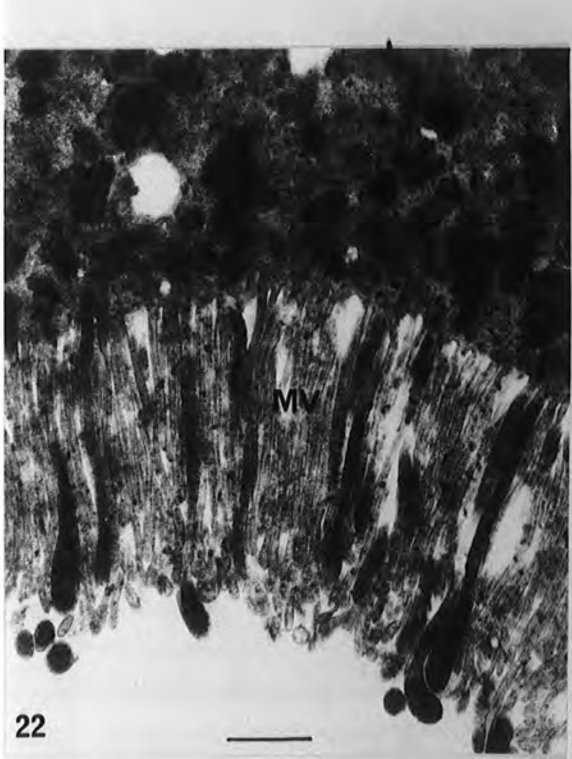
Scale = 1μm



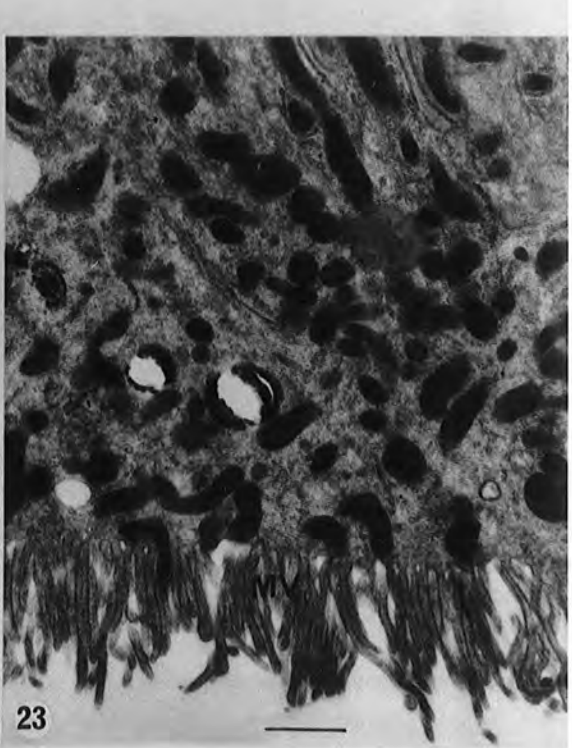
Day 1



Day 3



Day 7

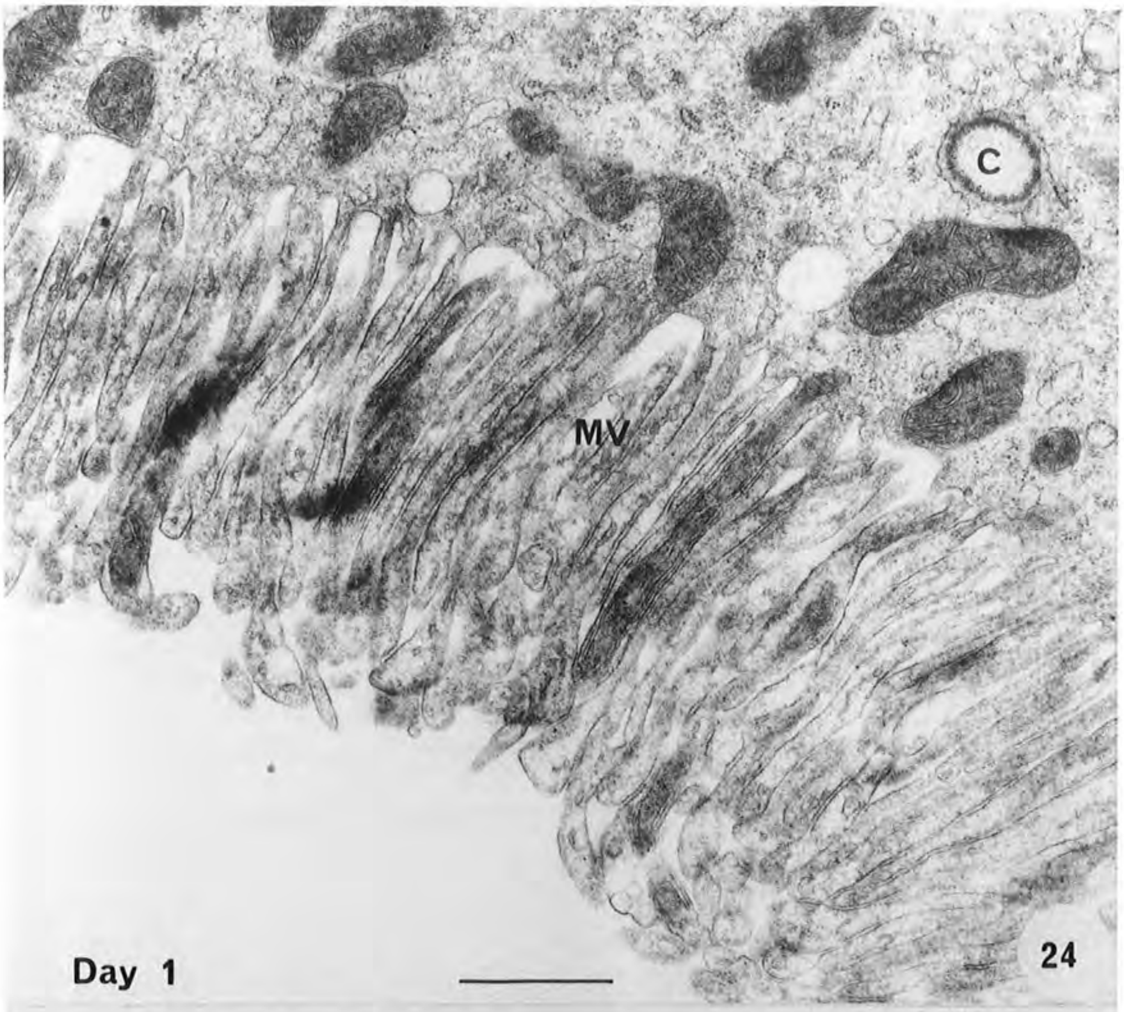


Day 11

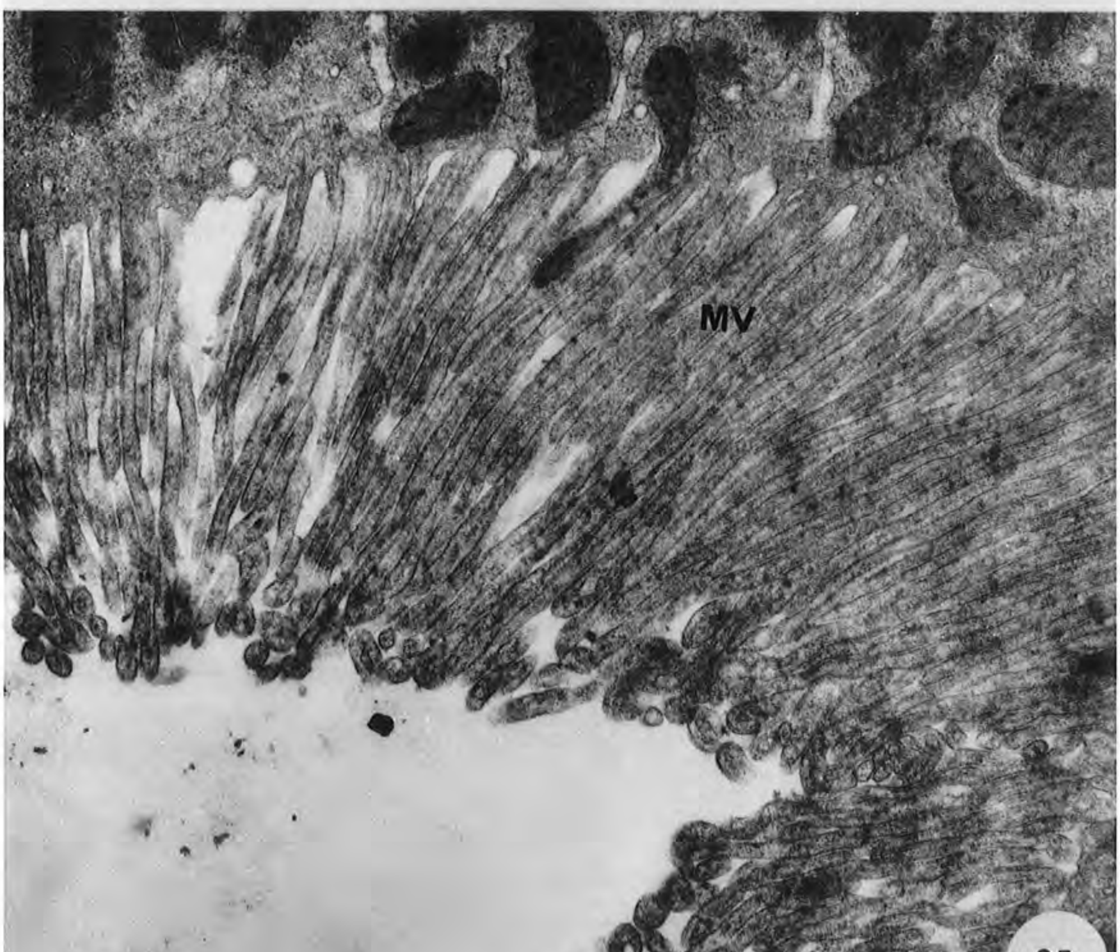
Plates 24,25

Higher power transverse sections through the apical region showing that the width of the microvilli decreases with increasing age over the first 7 days of the 5th instar.

Scale = 1µm



24

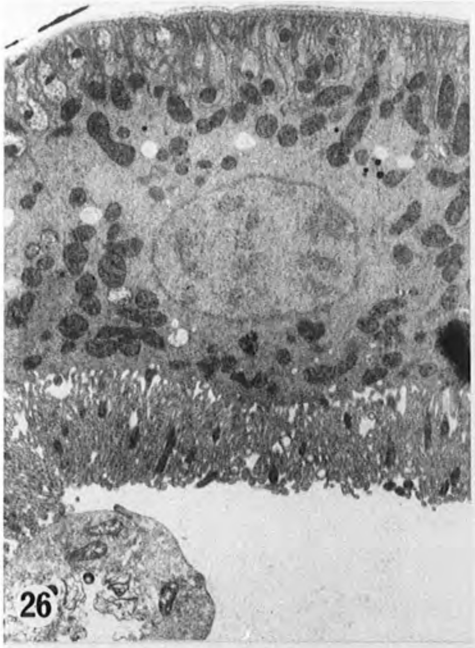


25

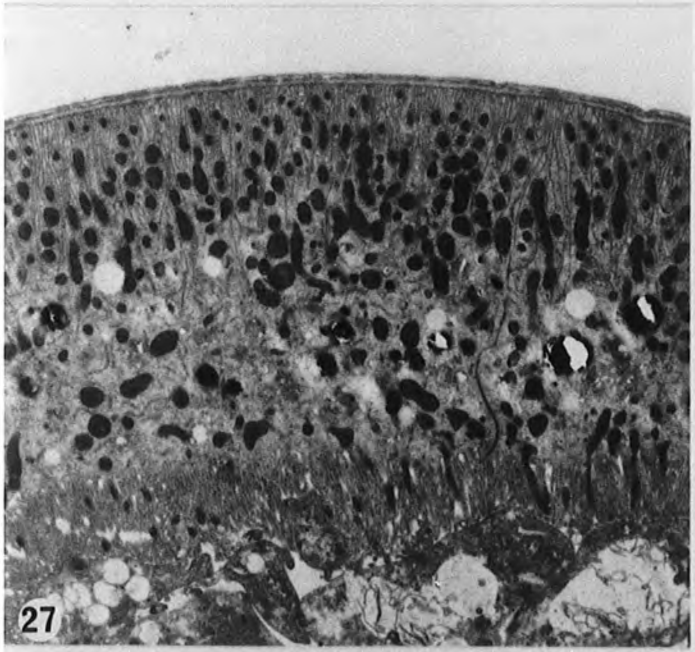
Plates 26-28

Low power transmission electronmicrographs showing transverse sections through the primary cells at different stages of development. There would appear to be an enormous increase in the number of mitochondria between Day 1 and Day 7 of the 5th instar and a subsequent decrease on the first day of adult life.

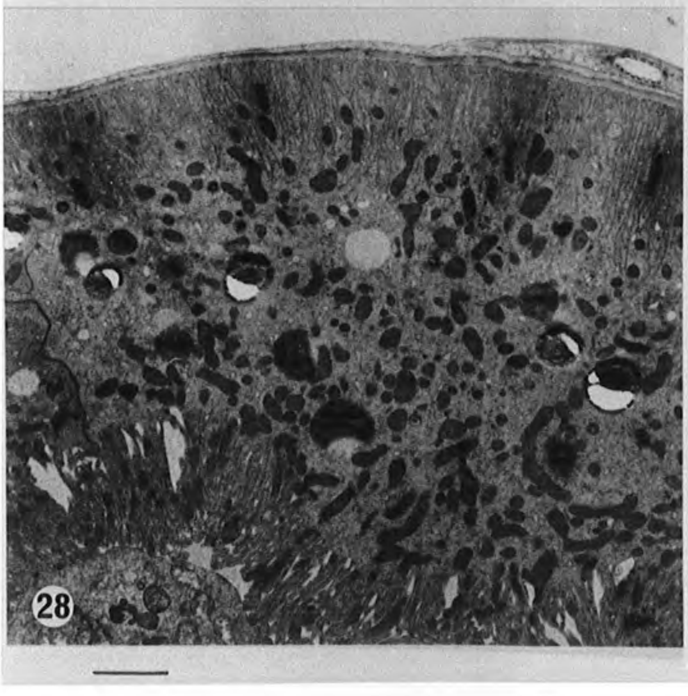
Scale = 2µm



Day 1



Day 9



Day 1 Adult

Plate 29

Transverse section through the basal region of a primary cell from a 1 day old 5th instar locust. This shows the basal zone of mitochondria which is very narrow and is located a short distance below the basement membrane.

Scale = 1μm

Plate 30

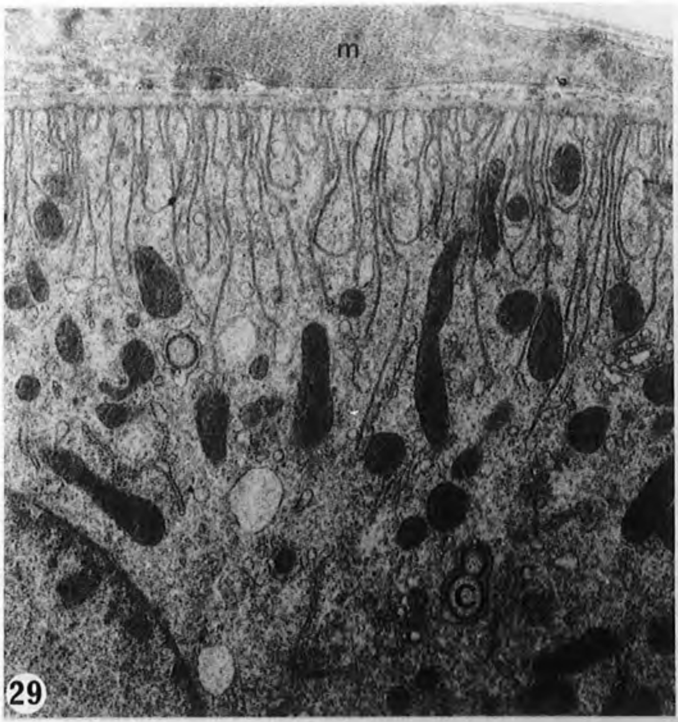
Transverse section through the apical region of a primary cell from a 1 day old 5th instar locust. This shows the apical zone of mitochondria which is situated just above the bases of the microvilli with some mitochondria extending down into the microvilli.

Scale = 1μm

Plate 31

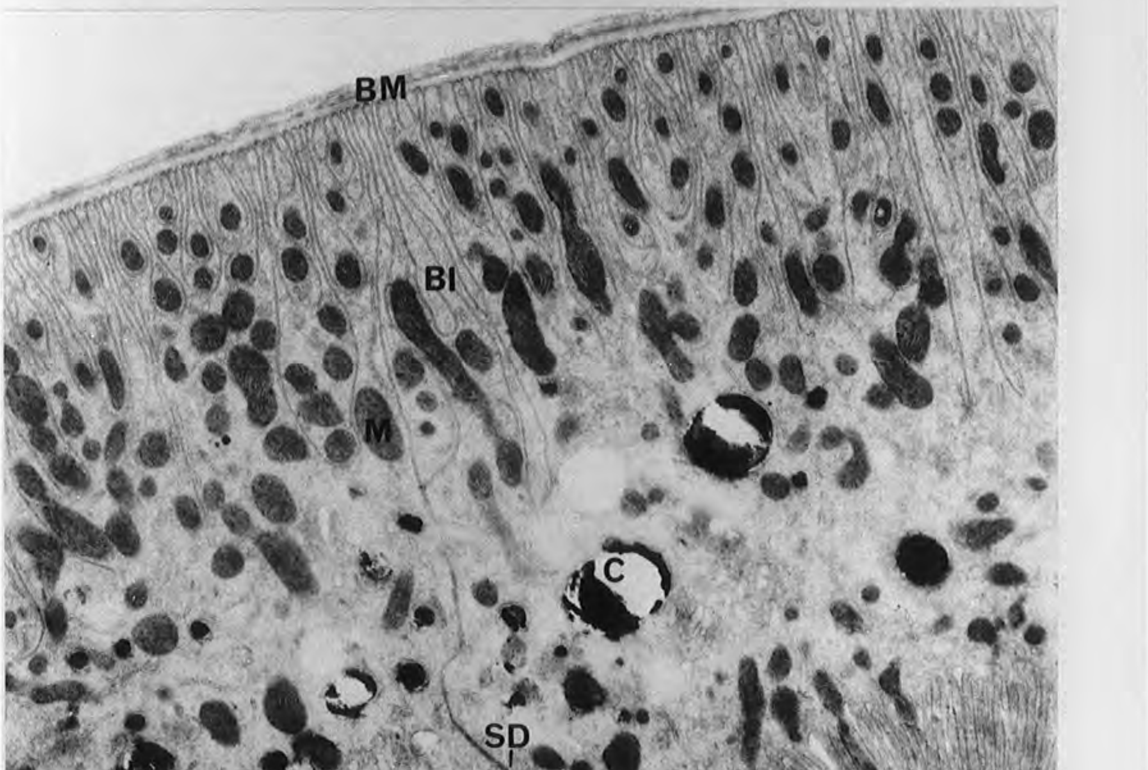
Transverse section through a primary cell from a 9 day old 5th instar locust. Note that the width of the basal zone of mitochondria has increased with mitochondria being found alongside the extracellular channels formed by the infoldings of the basal cell membrane. These mitochondria extend along the length of the cytoplasmic compartments and are found immediately below the basement membrane. There is no change in the apical zone of mitochondria.

Scale a 1μm



29

30

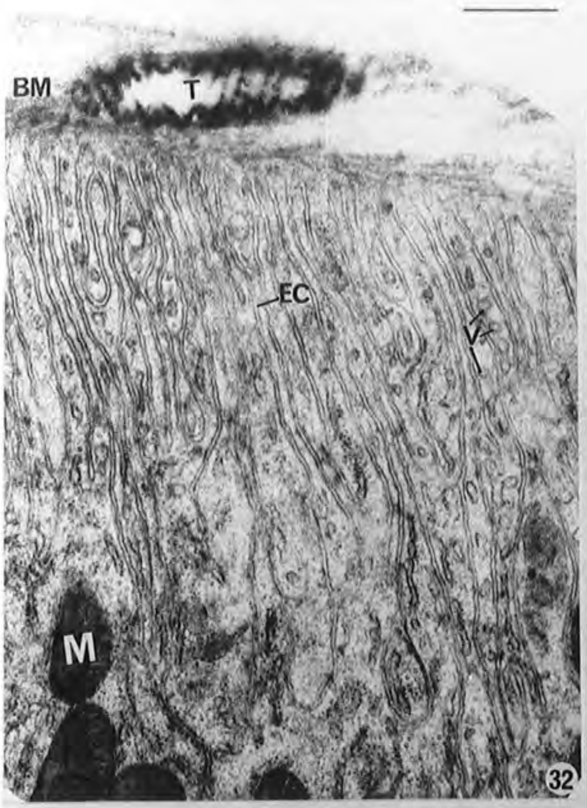


31

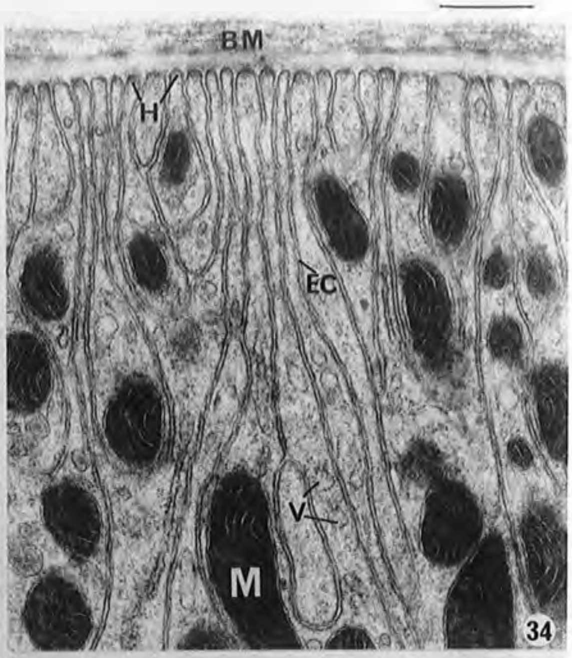
Plates 32-34

High power transverse sections through the basal region of the primary cells at different stages of development. In newly moulted 5th instar locusts (Plate 32), the mitochondria are confined to a region below the basal infolds. With increasing age the mitochondria begin to be found in the cytoplasmic compartments alongside the extracellular channels (Day 5, Plate 33). Eventually, as on Day 9 (Plate 34), the mitochondria are located along the length of the extracellular channels (EC).

Scale = 0.5µm



32



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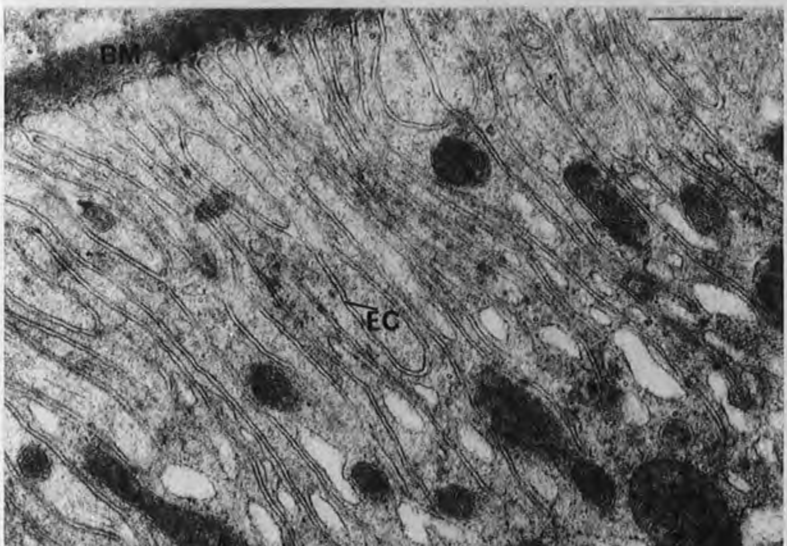


33

Plates 35-39

Transmission electronmicrographs of transverse sections through the primary cells to illustrate the various structures observed.

Plate 35

Note the vacuoles (Va) and the numerous coated vesicles (V) which are found in the intermediate region and at the apical surface.

Scale = 1μm

Plate 36

Numerous concretions (C) of varying electron densities are found in the intermediate region. Some are composed of concentric layers of material. Others have formed dense bodies (D).

Scale = 3μm

Plate 37

Electronmicrograph showing the detailed structure of the multi-vesicular bodies (MB) and residual bodies (RB).

Scale = 1μm

Plate 38

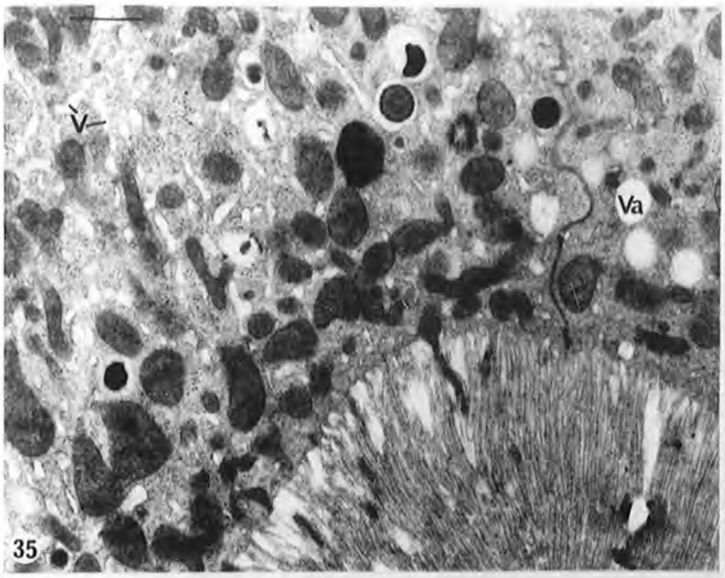
Electronmicrograph showing a double-membrane bound vacuole (Vo).

Scale = 1μm

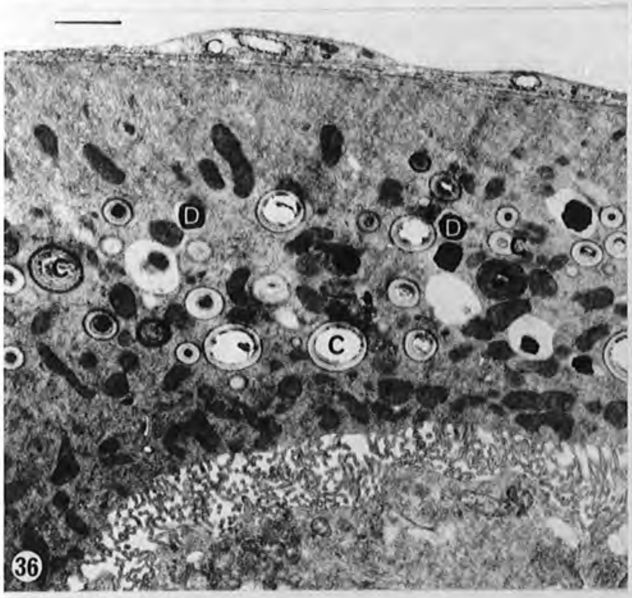
Plate 39

Note the presence of numerous coated vesicles in the intermediate region and in the cytoplasmic compartments between the basal infoldings.

Scale = 1μm


V
Va
35


Va
38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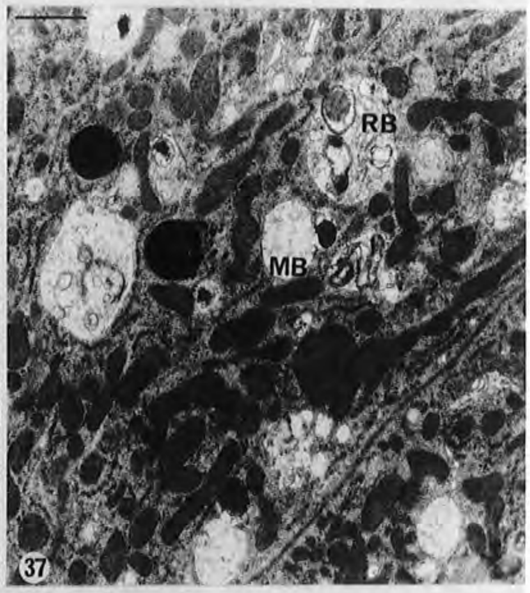
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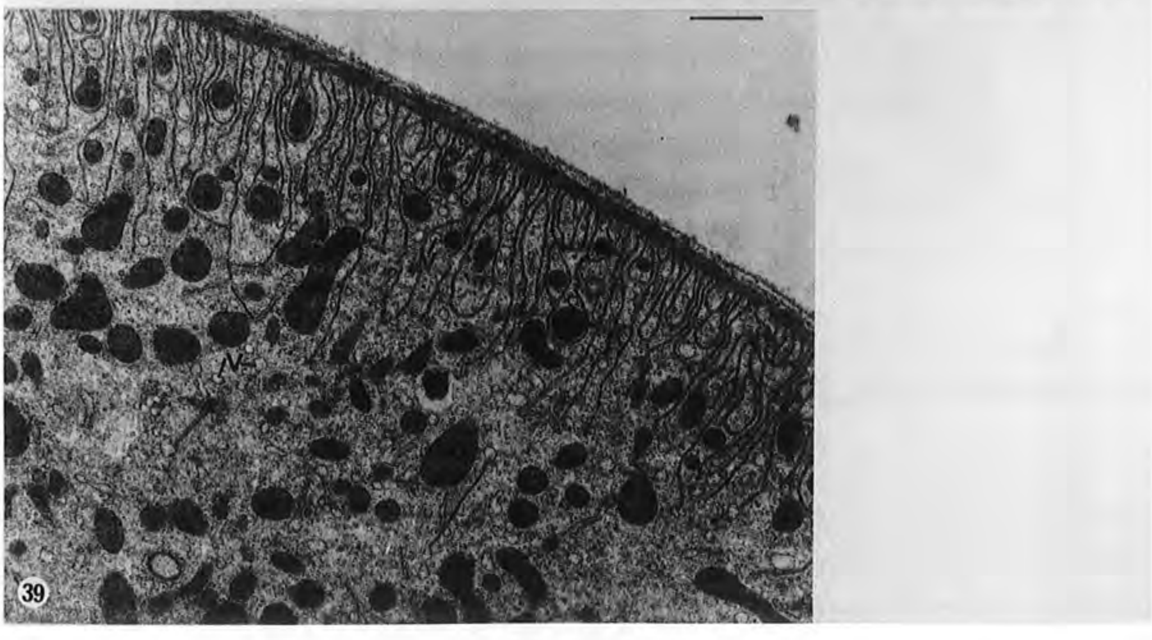
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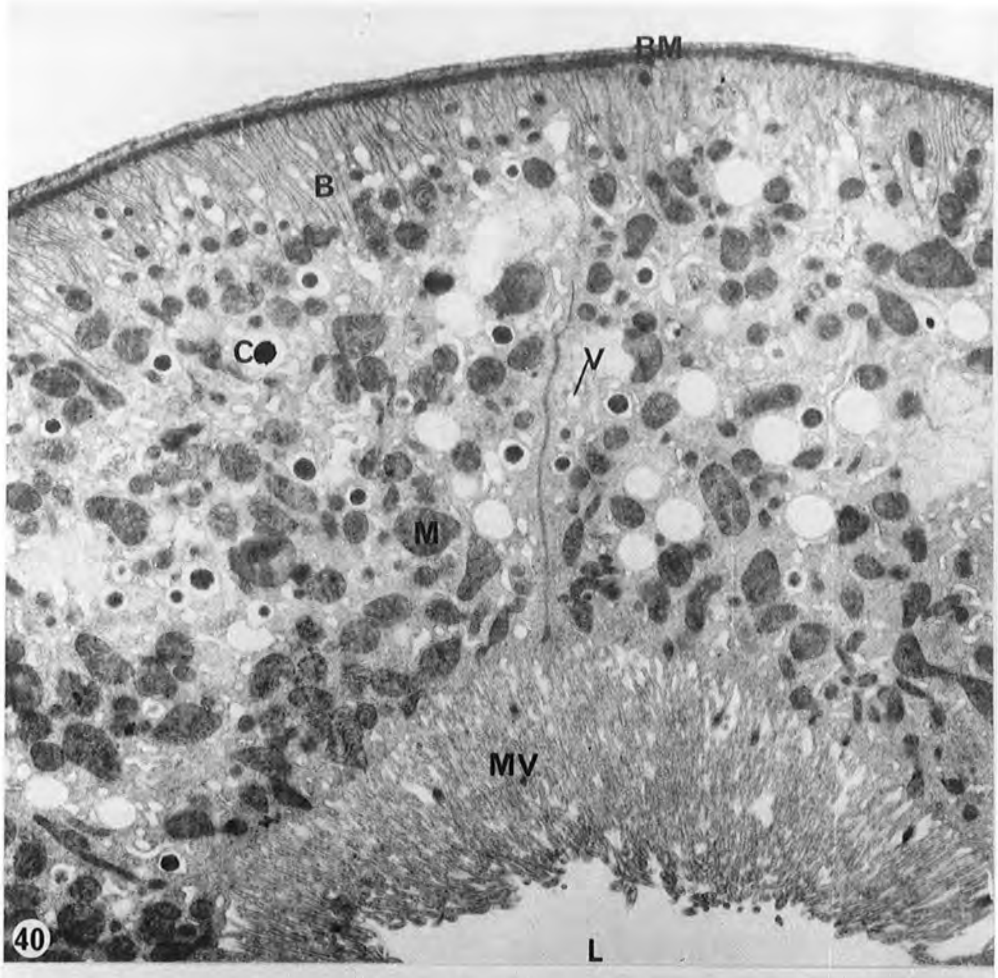
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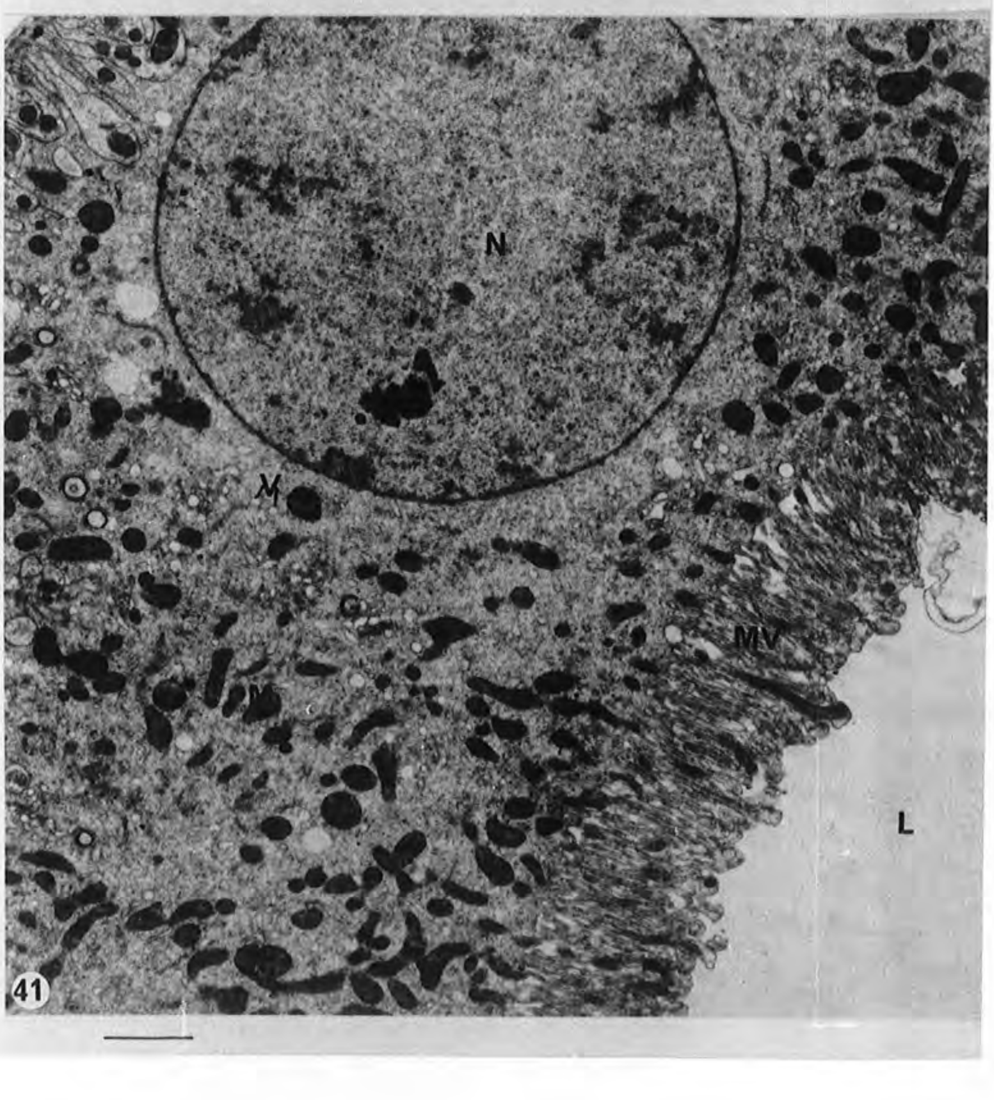
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Plates 40,41

Transmission electronmicrographs showing transverse sections through primary cells from 2 hrs old (Plate 40) and 1 day old (Plate 41), 5th instar locusts. It can be seen that there is a dramatic reduction in the number of small coated vesicles (V) over the first 24 hrs of the instar

Scale = 3μm

CHAPTER 4

THE EFFECT OF OUABAIN AND ETHACRYNIC ACID ON MALPIGHIAN TUBULE FUNCTION IN *LOCUSTA*

Introduction

As mentioned earlier (see Chapter 1), Berridge (1968) and Berridge and Oschman (1969) have proposed a model to explain fluid secretion by the Malpighian tubules of *Calliphora* in which the basal surface of the tubule cells possesses a coupled Na^+/K^+ exchange pump, extruding Na^+ from the cell into the haemolymph in exchange for K^+, whilst on the apical surface there is an electrogenic pump transporting K^+ into the tubule lumen. Furthermore it was pointed out that if this model is correct and a Na^+/K^+ exchange pump is involved, it is to be expected that Malpighian tubule fluid secretion would be inhibited by the cardiac glycoside ouabain, a specific inhibitor of Na^+, K^+-activated ATPase. However the results reported in the literature concerning the effect of ouabain on fluid secretion are in conflict. 'Urine' formation by Malpighian tubules has been reported to be ouabain-insensitive in several insect species (Maddrell 1969; Pilcher 1970; Gee 1976; Rafaeli-Bernstein and Mordue 1978). Although other studies report Malpighian tubule function to be inhibited by ouabain (Anstee and Bell 1975; Anstee *et al.* 1979; Atzbacher *et al.* 1974; Gooding 1975).

It is difficult to understand why such differing results have been obtained using ouabain. It may be that the mechanism of fluid secretion is different in some insect species, but it must also be considered that differing results may reflect differences in the experimental conditions employed. Examination of the literature shows that the experimental conditions vary considerably in two respects, viz., the temperature at which the experiments are carried out and the composition of the Ringer solution used to bathe the tubules.

Ouabain inhibition of the Na^+, K^+-activated ATPase has been reported to be extremely temperature sensitive, both from mammalian (Charnock *et al.* 1975) and insect (Peacock *et al.* 1976) sources. The inhibitory effect of ouabain on insect Na^+,K^+-activated ATPase has been shown to decrease substantially as the temperature decreases below 30°C (Peacock *et al.* 1976).

The effect of ouabain on fluid secretion by *in vitro* preparations of Malpighian tubules seems to have been studied by many workers at temperatures at or below 25°C (Maddrell 1969; Gee 1976; Rafaeli-Bernstein and Mordue 1978) and in some cases the temperature is not precisely stated (see review by Anstee and Bowler 1978). These workers have all failed to show any effect of ouabain on fluid secretion, whereas Anstee and Bell (1975) and Anstee *et al.* (1979), working at 30°C, found ouabain to have an inhibitory effect. It would seem important then to establish whether the temperature at which the experiments are carried out affects ouabain inhibition of fluid secretion.

The composition of the Ringer solution used to bathe the tubules may also be important. Jungreis (1977) suggests that the high K^+ concentration in the bathing medium used by several workers, studying fluid secretion in a variety of epithelia, may not be suitable for demonstrating ouabain inhibition. High K^+ concentrations have been found to antagonize ouabain inhibition of the Na^+,K^+-activated ATPase from a variety of tissues (Kinsolving *et al.* 1963; Judah and Ahmed 1964; Matsui and Schwartz 1968; Akera 1971) and this may indeed help to explain some of the reported lack of sensitivity of some insect tissues to ouabain.

However, the comment by Jungreis (1977) seems to be based on his statement that insect tissues known to be sensitive to ouabain have Na^+,K^+-activated ATPases which are maximally stimulated by $5mMK^+$ (Jungreis and Vaughan 1977). Whereas it has been shown that the Na^+,K^+-activated ATPase from a variety of insect tissues is maximally stimulated by $20mMK^+$ and is still inhibited by ouabain (Grasso 1967; Peacock *et al*. 1976; Tolman and Steele 1976; Piccione and Baust 1977; Anstee and Bell 1978).

In view of the apparent confusion over the effect of K^+, it would seem important to determine the effect of the K^+ concentration of the bathing medium in relation to ouabain inhibition.

In most insects K^+ is generally regarded as being the important transported cation. However in *Glossina*, Na^+ has been found to be actively transported in order to generate 'urine' flow (Gee 1976a,b) Further work by Gee (1976b) has shown the Malpighian tubules of *Glossina* to be insensitive to ouabain although fluid secretion was completely inhibited by $10^{-3}M$ ethacrynic acid. Ethacrynic acid has been shown to be a potent diuretic in mammals although its exact mode of action has not yet been determined. Several workers have demonstrated an effect of ethacrynic acid on Na^+ transport (Whittembury and Fishman 1969). Gee (1976) suggests that transport of Na^+ in the Malpighian tubules of *Glossina* may be by an electrogenic sodium pump. The fact that the tubules were found to be insensitive to ouabain would suggest that a Na^+/K^+ exchange pump was not involved. However the suggestions by Gee (1976) are based on the assumption that ethacrynic acid is a specific inhibitor of Na^+ transport and that Na^+/K^+ exchange pumps are unaffected by ethacrynic acid. There is in fact much evidence to show that this is not so and ethacrynic acid does inhibit the Na^+,K^+-activated ATPase from a variety of tissues (Duggan and Noll 1965; Charnock *et al.* 1970; Proverbio *et al.* 1970; Davis 1970; Daniel *et al.* 1970; Peacock *et al.* 1976).

One of the suggestions that has been put forward to explain the lack of inhibition by ouabain reported by some workers is that the sites of Na^+,K^+-activated ATPase may not be readily accessible to topically applied ouabain (Irvine and Phillips 1971). Irvine and Phillips (1971) found that water uptake and Na^+ absorption, by isolated preparations of the rectum of *Schistocerca*, was inhibited by 10^{-2}M but not by 10^{-3}M ouabain. Because of the relatively high concentration of ouabain necessary to effect inhibition, Irvine and Phillips (1971) suggested that either the inhibition was not a specific effect on an ion pump but was due to a general metabolic inhibition or permeability change, or, that the high concentration of ouabain required for inhibition may have been necessary to overcome a long diffusion path to the active site.

Rafaeli-Bernstein and Mordue (1978) have shown that ouabain is excreted by the Malpighian tubules of *Locusta migratoria* and *Zonercerus variegatus*. This would suggest that in these two insects, at least, the sites of Na^+,K^+-activated ATPase should be accessible to ouabain.

Histochemical methods have been employed in the past in attempts to localise the Na^+,K^+-activated ATPase in a variety of tissues (Ashworth *et al.* 1963; Farquhar and Palade 1966; Kaye *et al.* 1966; Berridge and Gupta 1968). Using a lead precipitation technique, Berridge and Gupta (1968) demonstrated a Mg^{2+}-dependent ATPase in the rectal papillae of *Calliphora*. The histochemical study showed that the Mg^{2+}-dependent ATPASE was specifically located on the intracellular surface of the lateral plasma membranes which from ultrastructural studies appeared the likely sites of ion secretion (Berridge and Gupta 1967). However, using biochemical studies in conjunction with the histochemical technique, Berridge and Gupta (1968) could show only a slight stimulation of ATPase activity after addition of Na^+ and K^+ and no inhibition with ouabain. Similarly, Farquhar and Palade (1966), using a histochemical technique to study ATPase in amphibian epidermis, also located a Mg^{2+}-dependent ATPase but could observe no change in localisation when Na^+ and K^+ and ouabain were added.

There are several problems of interpretation associated with histochemical techniques as is pointed out by Berridge and Gupta (1968). It is possible that lead ions used in the technique may cause non-enzymatic hydrolysis of ATP and consequently the deposition of lead salts would bear no relation to the localisation of ATPase. In view of this, and the fact that it is impossible to localise a ouabain-sensitive, Na^+,K^+-activated ATPase as distinct from other ATPases by histochemical techniques, a more promising method might be the autoradiographic localisation of specifically bound ^3H-ouabain. This technique has been used in the localisation of Na^+,K^+-activated ATPase in frog choroid plexus (Quinton *et al.* 1973) and in the chloride cells of teleost gills (Karnaky *et al.* 1976).

In view of the conflict in the literature, concerning the effect of ouabain on fluid secretion in insects, the present study has been carried out to re-examine the role of Na^+, K^+-activated ATPase in Malpighian tubule function in *Locusta*.

MATERIALS AND METHODS

Sexually mature locusts, *Locusta migratoria* L., of both sexes, were used in all experiments.

1. To determine the effect of ouabain and ethacrynic acid on fluid secretion

The Malpighian tubule preparation was set up as described in Chapter 2. The diameter of the secreted droplet was measured at 5 minute intervals for 35 minutes with the insect in 'normal' Ringer solution. This was then replaced with either fresh 'normal' Ringer solution (the control) or Ringer solution containing ouabain (10^{-5}M - 10^{-3}M) or ethacrynic acid (10^{-7}M - 10^{-3}M). The tubule preparation was then allowed to soak for 30 minutes before redetermining the rate of secretion over a second 35 minute period.

2. Excretion of ouabain

A Malpighian tubule preparation was set up as described in Chapter 2 and the 'normal' Ringer solution surrounding the preparation replaced with 'normal' Ringer solution containing ^{3}H-ouabain (250μCi ^{3}H-ouabain (specific activity 19μCi/mmol) contained in 250μl ethanol was diluted to 2.5mls with deionised water - 10μl of this diluted ouabain solution was then added to each 1ml of 'normal' Ringer solution). The fluid secreted by the Malpighian tubules in the presence of ^{3}H-ouabain was collected, using a 1μl microcap, at 20 minute intervals and transferred to glass vials containing 10mls of 260 scintillation cocktail (Nuclear Enterprises). The samples were counted in a Beta/Gamma scintillation counter (ne 8312, Nuclear Enterprises).

3. Autoradiography

250μCi ^{3}H-ouabain (specific activity 19μCi/mmol) contained in 250μl ethanol was diluted to 10mls with 'normal' Ringer solution.

The Malpighian tubules attached to a 'collar' of gut were quickly dissected from four locusts. The mass of tubules from each insect was divided in half, half being soaked in 10mls Ringer solution containing

^{3}H-ouabain and half in 10mls 'normal' Ringer solution to act as a control. Both sets were soaked for 60 minutes at 30°C.

After 60 minutes the tubules were removed, washed in Ringer solution containing 'cold' ouabain (10^{-3}M) or in 'normal' Ringer solution and rapidly frozen in a 50 : 50 mixture of liquid nitrogen and 2-methylbutane (isopentane). Fresh frozen sections were cut at 12μm on a cryostat. The sections were transferred to slides, fixed in formol saline (10mls formalin, 7mls 10% NaCl, 83mls distilled water), air-dried and coated with Ilford K5 emulsion in a darkroom fitted with an Ilford S safelight. The emulsion was prepared by mixing 24mls of molten Ilford K5 emulsion with 23.5mls distilled water and 0.5mls glycerol at 43°C. The slides, held vertically, were dipped individually into the emulsion, then transferred to a cooled plate and air-dried (approximately 60 minutes). They were then stored in light-proof boxes at 4°C for 6-8 weeks.

The slides were developed in Kodak D19 developer for 3.5 minutes at 21°C, washed quickly in distilled water and fixed for 4 minutes in 1-5 Kodak Amfix. After washing in running tap water for 15 minutes the slides were left to dry, then stained with toluidine blue and mounted in D.P.X.

RESULTS

1. The effect of ouabain on fluid secretion

The rate of fluid secretion by *in vitro* preparations of the Malpighian tubules was determined in the presence of ouabain at concentrations from 0-10^{-3}M. The results are shown in Table 4.1.

Table 4.1 The effect of ouabain on fluid secretion by the Malpighian tubules of *Locusta*

Treatment	n	Mean rate of secretion % original rate ± S.E.	p
Control	10	102.6 ± 7.3	not sig.
Ouabain 10^{-3}M	17	44.0 ± 9.3	<0.001
Ouabain 10^{-4}M	18	62.2 ± 8.4	<0.001
Ouabain 10^{-5}M	15	71.3 ± 12.1	not sig.

(Values for P were obtained by comparing rates 1 and 2 in a paired 't' test. The 100% rate of secretion was 2.4 ± 0.3nl/min.)

It can be seen that ouabain substantially inhibits fluid secretion at a concentration of 10^{-3}M (56% inhibition when Rate 2 is compared with Rate 1). The inhibitory effect of ouabain is decreased as the concentration of ouabain is decreased.

2. The effect of temperature on the inhibition of fluid secretion by ouabain

Rates of fluid secretion by the Malpighian tubules were determined, as described previously, in the presence and absence of 10^{-3}M ouabain at 30°C, 20°C and 15°C.

The results (Table 4.2) show that at temperatures below 30°C ouabain inhibition of fluid secretion is decreased. At 30°C there is 56% inhibition which is reduced to 28% at 20°C. At 15°C ouabain was found to have no inhibitory effect.

3. The effect of K^+ concentration on the inhibition of fluid secretion by ouabain

The rate of fluid secretion by *in vitro* preparations of the Malpighian tubules was measured in Ringer solution with K^+ concentrations of 10mM, 20mM and 40mM. The Na^+ concentration of these solutions was reduced accordingly to maintain the cation concentration (by altering concentrations of KCl and NaCl). The effect of ouabain was determined at a concentration of 10^{-3}M.

The rate of fluid secretion was determined over a first 35 minute period in Ringer solution containing one of the above K^+ concentrations. This was replaced with either fresh Ringer solution or Ringer solution

Table 4.2

The effect of temperature on the inhibition of fluid secretion by ouabain

Temperature °C	n	CONTROL mean rate of fluid secretion % original rate ± S.E.	P	n	10^{-3} OUABAIN mean rate of fluid secretion % original rate ± S.E.	P
30	25	102.1 ± 11.7	not sig.	28	44.3 ± 8.0	< 0.001
20	28	118.7 ± 16.0	not sig.	30	72.0 ± 6.6	< 0.01
15	29	107.7 ± 10.2	not sig.	27	95.9 ± 11.2	not sig.

P values were obtained by comparing rate 1 and rate 2 values in a paired 't' test. The 100% rates were 4.2 ± 0.5nl/min at 30°C, 2.2 ± 0.2nl/min at 20°C and 2.2 ± 0.3nl/min at 15°C.

containing ouabain, both of the same K^+ concentration as Rate 1. The preparation was then allowed to soak for 30 minutes before determining the second rate of fluid secretion over a further 35 minute period.

The results are shown in Table 4.3. It can be seen that changing the K^+ concentration of the bathing medium (up to 40mMK^+) has no effect on the inhibition of fluid secretion by ouabain; the level of inhibition remains more or less constant at 57%.

It was observed during these experiments that as the K^+ concentration of the bathing medium was increased, the rate of tubular secretion increased. The mean rate of secretion at 10mM was 3.4nl/min; this increased to 4.4nl/min at 20mM and 7.3nl/min at 40mM. The rates increased correspondingly when ouabain was present, maintaining the level of inhibition at around 57%.

4. To determine the effect of ouabain on fluid secretion by the Malpighian tubules using an alternative Ringer solution

Insect Ringer solution with the following composition was used: NaCl 168mM, KCl 6.4mM, $MgCl_2.6H_2O$ 3.4mM, $CaCl_2.6H_2O$ 2.1mM, $NaH_2PO_4.2H_2O$ 6mM, $NaHCO_3$ 0.46mM, glucose 16.6mM (Mordue 1969).

Rates of fluid secretion were determined over a 35 minute period in the above Ringer solution; this was then replaced with either fresh Ringer solution (as above) or with the above Ringer solution containing 10^{-3}M ouabain. The tubule preparation was then allowed to soak for 30 minutes before redetermining the rate of secretion over a second 35 minute period. The results are shown in Table 4.4.

It can be seen that the rate of fluid secretion determined after the addition of ouabain was found to be only 51.5 ± 8.4% of the

Table 4.3

The effect of K^+ concentration on inhibition of fluid secretion by ouabain

$[K^+]$	n	CONTROL mean rate of secretion % original rate ± S.E.	P	n	OUABAIN (10^{-3}M) mean rate of secretion % original rate ± S.E.	P
10mM	15	98.5 ± 6.2	not sig.	22	43.1 ± 7.8	< 0.001
20mM	10	102.7 ± 7.0	not sig.	25	45.1 ± 5.2	< 0.001
40mM	17	83.3 ± 5.5	not sig.	21	39.7 ± 7.0	< 0.001

P values were obtained by comparing rate 1 and rate 2 values in a paired 't' test. Application of students 't' test indicated that the mean results obtained with the different treatments were non-significant. Therefore the effect of 10mM K^+ on ouabain sensitivity was not different from that of 20mM K^+ or 40mM K^+ and the result with 20mM K^+ was not different from that obtained with 40mM K^+.

previous rate and the two rates were significantly different from one another. This would seem to suggest that ouabain was having an inhibitory effect. However, examination of the control data reveals that here too there is substantial inhibition : rate 2 being only 46.0 ± 8.8% of rate 1.

It would seem from these results that this Ringer solution is unsuitable for maintaining fluid secretion in *Locusta*. In view of this the secreted droplets were collected after the two 35 minute periods and analysed for Na^+ and K^+ concentrations (see Chapter 6 for method). It was found that a reduced amount of K^+ was being secreted during the second 35 minute period. The K/Na ratio was reduced from a mean value of 2.5 ±0.2 (first 35 minutes) to 1.24 ±0.2 (second 35 minutes). The change in K/Na ratio was due mainly to a decrease in the amount of K^+ secreted although there was a slight increase in Na^+. This

Table 4.4 : <u>The effect of ouabain on fluid secretion using an alternative Ringer solution</u>

Treatment	n	mean rate of fluid secretion % original rate ± S.E.	P
Control	21	46.0 ± 8.8	< 0.001
10^{-3}M ouabain	18	51.5 ± 8.4	< 0.001

P values were obtained by comparing rate 1 and rate 2 in a paired 't' test. The 100% rate of secretion was 2.7 ± 0.3nl/min. Students 't' test applied to the control data and that obtained with 10^{-3}M ouabain indicates that the two results are not significantly different from one another.

decrease in the concentration of K^+ secreted may be related to the inhibition of secretion observed in the controls.

5. The effect of ouabain on fluid secretion by the Malpighian tubules of *Schistocerca gregaria*

Rates of fluid secretion by *in vitro* preparations of the Malpighian tubules were determined, as for *Locusta*, in'normal' Ringer solution and in 'normal' Ringer solution containing 10^{-3}M ouabain. The results are shown in Table 4.5.

It can be seen that after the addition of 10^{-3}M ouabain the rate of fluid secretion was only 52.2 ± 9.2% of the previous rate, giving approximately 48% inhibition. However, in the control the second rate of fluid secretion determined was also lowered, being reduced to 72.6 ± 8.7% of the rate 1, an inhibition of approximately 28%. The amount of inhibition that could be said to be due to ouabain, therefore, was some 20% (comparing the effect of ouabain with the control), and in fact a students 't' test performed on the control and experimental data indicated that the two sets of data were not significantly different.

Table 4.5 : The effect of ouabain on fluid secretion by the Malpighian tubules of *Schistocerca gregaria*

Treatment	n	mean rate of fluid secretion % original rate ± S.E.	P
Control	24	72.6 ± 8.7	< 0.002
10^{-3}M ouabain	25	52.2 ± 9.2	< 0.002

P values were obtained by comparing rate 1 and rate 2 in a paired 't' test. The 100% rate of fluid secretion was 4.6 ± 0.8nl/min.

6. The effect of ouabain on Na^+, K^+-activated ATPase activity

(i) Varying ouabain concentration

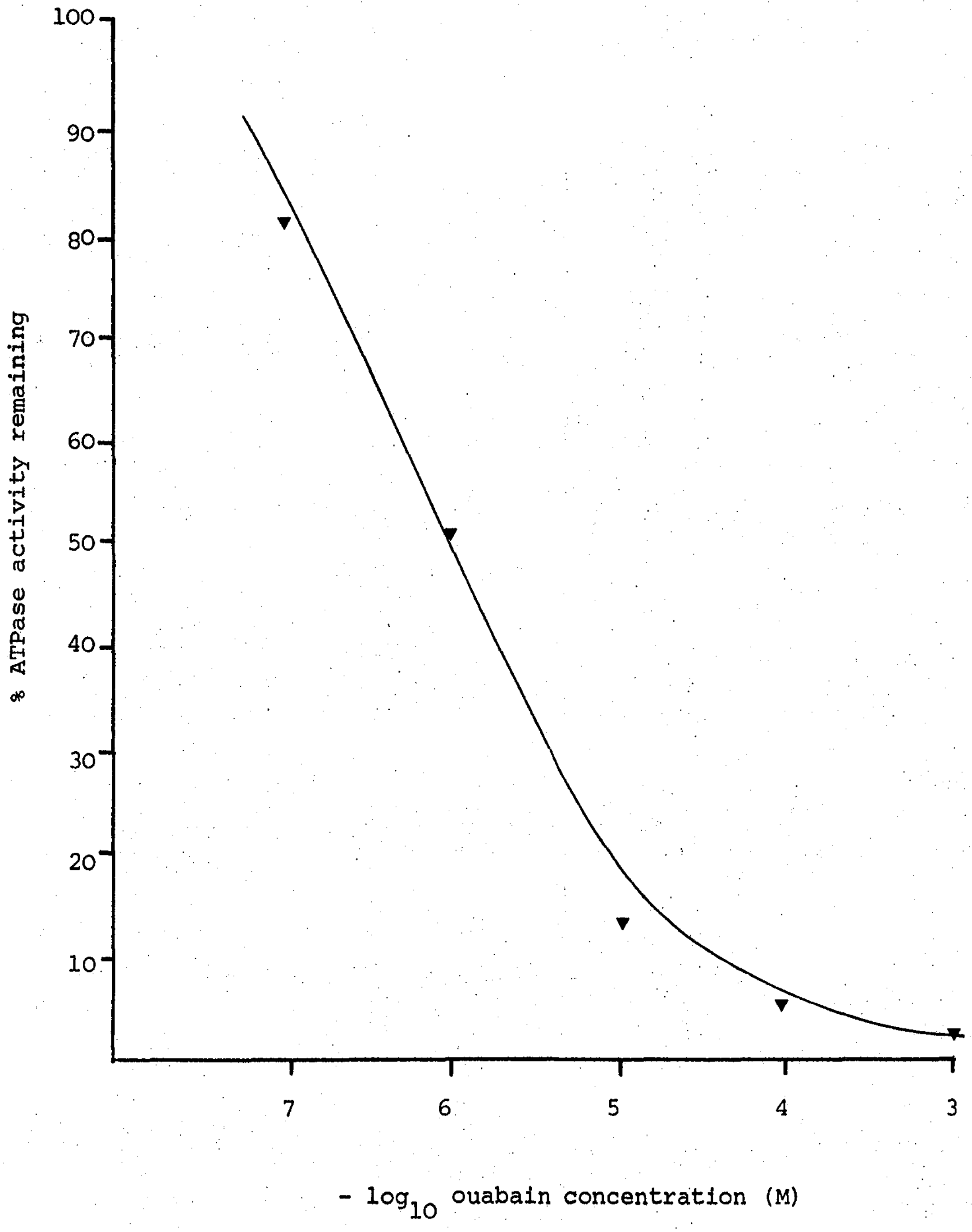
Na^+, K^+-activated ATPase activity from microsomal preparations of the Malpighian tubules was assayed as described previously (see Chapter 2) in reaction media containing concentrations of ouabain from 0 - 10^{-3}M. The results of a typical experiment (Table 4.6) show that the inhibition of Na^+, K^+-activated ATPase increased as the ouabain concentration increased (see Appendix 4.1 for further results).

Table 4.6 : The effect of ouabain on Na^+, K^+-activated ATPase activity

ouabain concentration (M)	enzyme activity (n moles Pi/mg protein/min)
0	276.2
10^{-7}	222.6
10^{-6}	142.2
10^{-5}	34.3
10^{-4}	1.6
10^{-3}	8.5

Figure 4.1 shows a graph of % activity plotted against the negative logarithm of the ouabain concentration. It can be seen from this that the pI_{50} (i.e. the -Log. of the ouabain concentration that gives 50% inhibition) is 5.8.

Figure 4.1 The effect of ouabain on Na^+, K^+-activated ATPase activity



(ii) The effect of temperature on the inhibition of Na$^+$, K$^+$-activated ATPase by ouabain

Na$^+$, K$^+$-activated ATPase activity was assayed at temperatures of 30°C, 20°C and 15°C. The effect of ouabain was determined using 10^{-6}M ouabain in the reaction media.

The results in Table 4.7 show that the inhibition of enzyme activity by ouabain decreased as the temperature is decreased. At 30°C there is approximately 50% inhibition, at 20°C 32% and at 15°C only 15% inhibition.

Table 4.7 : The effect of temperature on the inhibition of Na$^+$, K$^+$-activated ATPase by ouabain

Temp. °C.	enzyme activity n moles Pi/mg protein/min		% Activity remaining
	CONTROL	10^{-6}M OUABAIN	
30	1. 285.7	1. 130.2	1. 45.5
	2. 292.1	2. 159.1	2. 54.4
20	1. 138.6	1. 86.5	1. 62.4
	2. 155.0	2. 112.3	2. 72.4
10	1. 51.7	1. 43.3	1. 83.7
	2. 67.4	2. 57.9	2. 85.9

1\. and 2. refer to the data obtained in 2 separate experiments.

7. The effect of ethacrynic acid on fluid secretion

The rates of fluid secretion by *in vitro* preparations of the Malpighian tubules of *Locusta* were determined in 'normal' Ringer solution and in Ringer solution containing 0 - 10^{-3}M ethacrynic acid.

The results are shown in Table 4.8. It can be seen that ethacrynic acid substantially inhibits fluid secretion over the range 10^{-7}M - 10^{-3}M.

Using ouabain and ethacrynic acid together results in a greater inhibition of fluid secretion than either of them produce alone. 10^{-4}M ouabain gives a mean inhibition of 37.8%, 10^{-4}M ethacrynic acid gives 36.5% while together they produce 62.1%.

Table 4.8 : The effect of ethacrynic acid on fluid secretion

Treatment	n	mean rate of fluid secretion % original rate ± S.E.	P
control	25	102.1 ± 11.7	not sig.
10^{-7}M etha a.	17	72.6 ± 8.0	0.001
10^{-6}M	18	77.3 ± 7.5	0.01
10^{-5}M	19	77.3 ± 9.1	0.02
10^{-4}M	20	63.5 ± 6.3	0.001
10^{-3}M	9	28.4 ± 4.3	0.001
10^{-4}M ouabain	20	62.2 ± 8.4	0.001
10^{-4} ouabain +10^{-4} etha a.	9	37.9 ± 7.6	0.001

P values were obtained by comparing rate 1 and rate 2 in a paired 't' test. The 100% rate was 3.7 ± 0.5nl/min.

8. The effect of ethacrynic acid on Na^+, K^+-activated ATPase activity

Na^+, K^+-activated ATPase activity in microsomal preparations of the Malpighian tubules was determined in reaction media containing 0 - 4mM ethacrynic acid. The results are shown in Table 4.9.

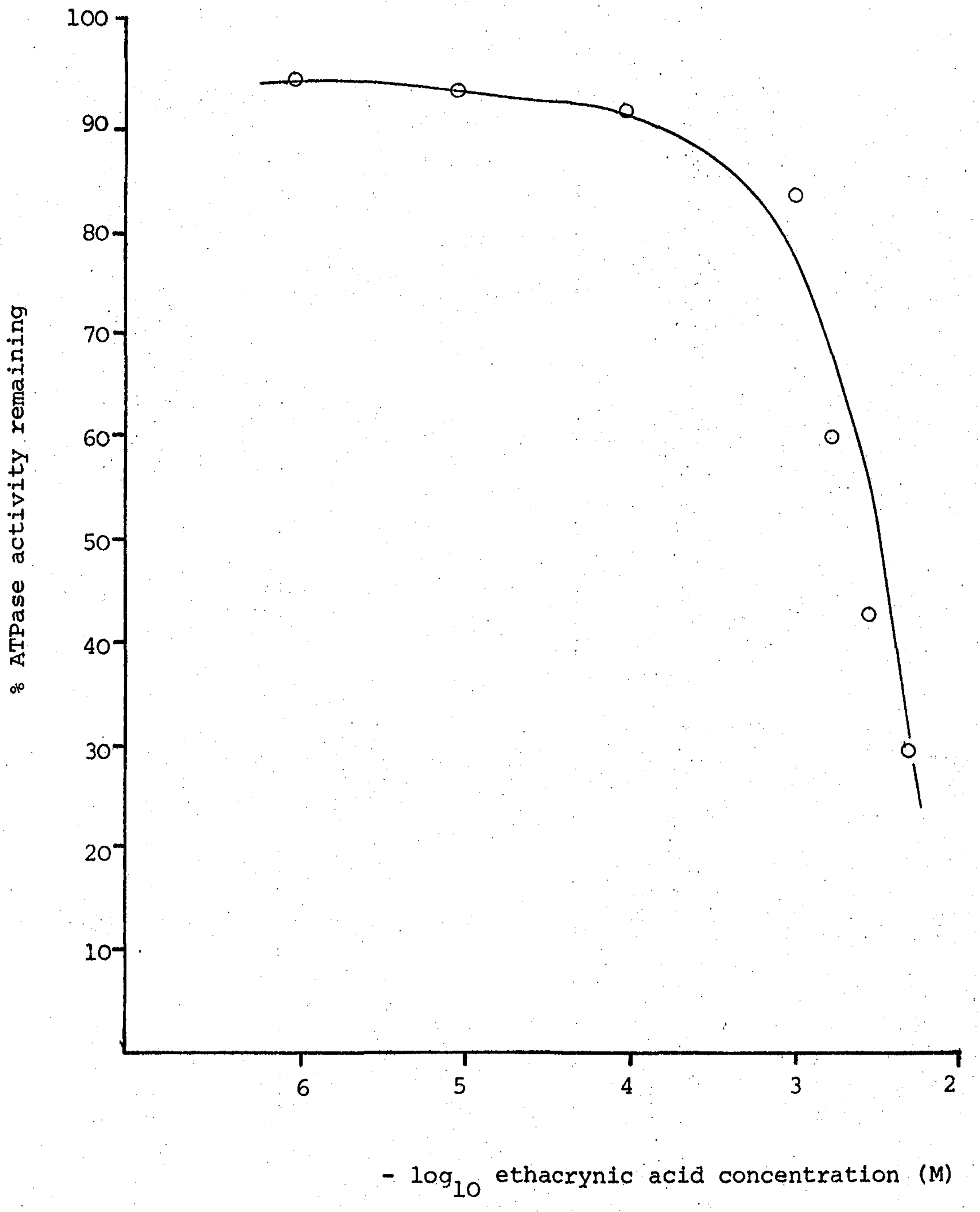
Table 4.9 : The effect of ethacrynic acid on Na^+, K^+-activated ATPase activity

Treatment	% ATPase activity remaining
control	100%
ethacrynic acid 10^{-6}M	1. 95.5% 2. 96.4%
10^{-5}M	1. 95.5% 2. 96.0%
10^{-4}M	1. 93.8% 2. 95.6%
10^{-3}M	1. 83.1% 2. 86.5%
2×10^{-3}M	1. 63.2% 2. 61.3%
3×10^{-3}M	1. 43.1% 2. 44.5%
4×10^{-3}M	1. 34.2% 2. 26.3%

1. and 2. refer to the results of 2 separate experiments.

It was found that ethacrynic acid at concentrations of 10^{-6}M - 10^{-4}M had no effect on the ATPase activity. However 10^{-3}M ethacrynic acid effected 17% inhibition of the Na^+, K^+-activated ATPase activity and the inhibition was found to increase as the concentration of ethacrynic acid was increased above 10^{-3}M. It is obvious that ethacrynic acid

Figure 4.2 The effect of ethacrynic acid on Na^+, K^+-activated ATPase activity



is less potent than ouabain as an inhibitor of Na^+, K^+-activated ATPase activity, 10^{-3}M ouabain effects almost total inhibition of Na^+, K^+-activated ATPase activity (see 6. above). The pI_{50} for ethacrynic acid was found to be 2.5mM (Figure 4.2) which is similar to the result obtained by Peacock *et al.* (1976) for *Homorocoryphus nitidulus vicinus*.

9. Excretion of ^{3}H-ouabain by the Malpighian tubules

As was mentioned in the introduction, one suggestion which has been put forward to explain lack of ouabain sensitivity in some species is that the sites of Na^+, K^+-activated ATPase might not be accessible to topically applied ouabain (Irvine and Phillips 1971). The present, preliminary study was carried out to determine whether ouabain was able to cross the walls of the Malpighian tubules of *Locusta,* a fact which would give some indication as to the likelihood that ouabain was accessible to the sites of ATPase.

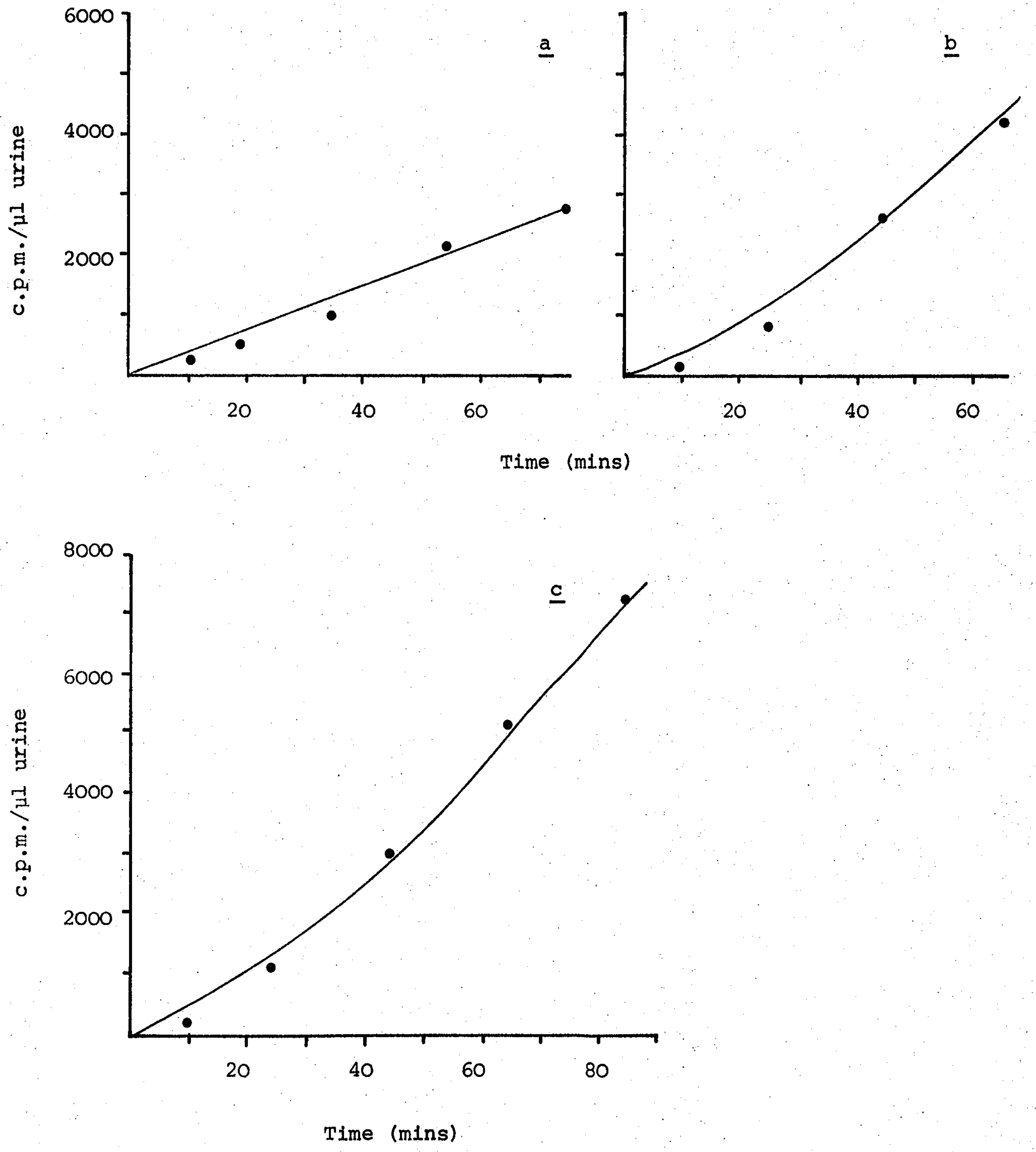
The results showed that ^{3}H was present in the secreted droplets. However, it is not possible to say whether this was in fact due to the presence of ^{3}H-ouabain or one of its labelled metabolites. Figure 4.3 shows graphs of c.p.m./µl urine plotted against time. It can be seen that after the first 20-25 minutes, which may be regarded as an equilibration period, the amount of ^{3}H excreted increased linearly with time.

Preliminary experiments performed as above but with ^{3}H-Inulin showed that this too was found in the secreted fluid. This was also found by Farquharson (1974) for the Malpighian tubules of the pill millipede *Glomeris marginata*.

10. The localisation of Na^+, K^+-activated ATPase using an autoradiographic technique

The present study has confirmed the presence of a ouabain-sensitive, Na^+, K^+-activated ATPase in microsomal preparations of the Malpighian tubules of *Locusta,* and that fluid secretion is inhibited by ouabain. This would tend to support the suggestion (Berridge and Oschman (1969) that there is a Na^+, K^+-activated ATPase present in the tubules and associated with fluid

Figure 4.3 The excretion of ^{3}H-ouabain by the Malpighian tubules

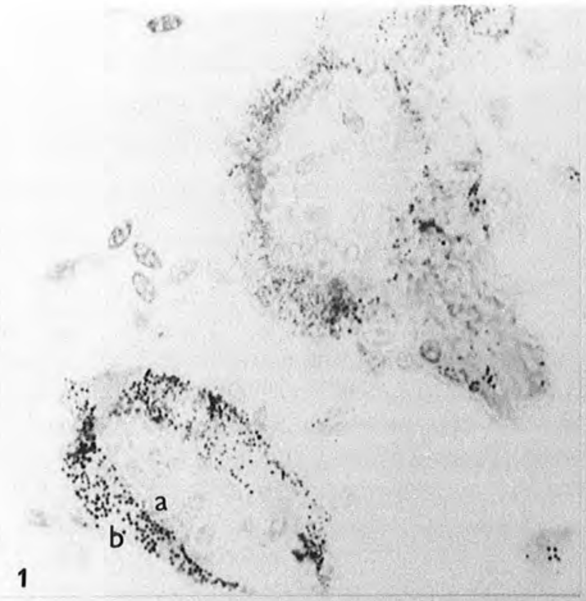


Plates 4.1 - 4.3

Autoradiographs of frozen sections through the Malpighian tubules showing accumulation of silver grains (black dots) corresponding to bound ouabain. The silver grains appear to be associated with both the basal (B) and the apical (A) surfaces of the tubules but it is impossible using frozen sections to localise the bound ouabain any further.

Plate 4.4

Autoradiograph of section through a control Malpighian tubule i.e. one which was soaked in 'cold' ouabain. There seems to be a 'speckly' appearance to the tubules but this is due to granules of the stain used on top of the emulsion.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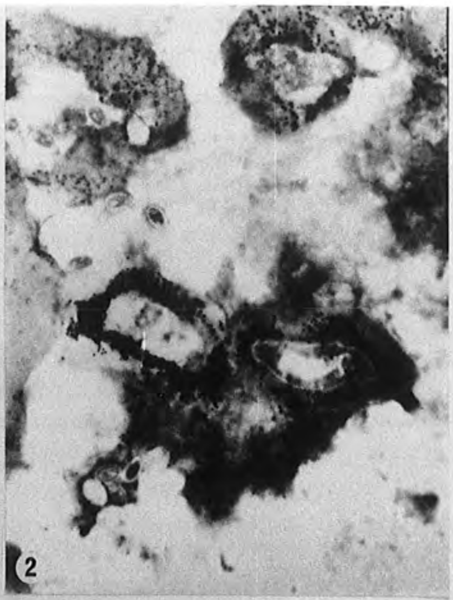
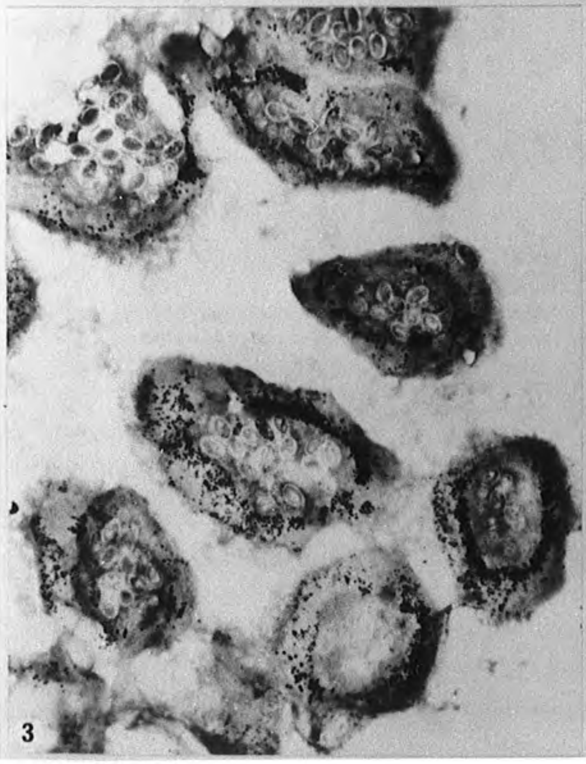

2


3


4

secretion. In view of the difficulties associated with the localisation of ATPase by histochemical techniques, referred to in the introduction to this Chapter, the present study has employed the specific binding of ^{3}H-ouabain in association with autoradiography, in an attempt to localise Na$^+$, K$^+$-activated ATPase in the Malpighian tubules of *Locusta*.

The autoradiographs (Plates 4.1 - 4.4) show accumulations of silver grains, corresponding to bound ouabain, associated with both the basal and apical surfaces of the tubules. Using frozen sections and light microscopy it is impossible to localise the bound ouabain to any particular organelle or cellular membrane. This could be achieved in future studies by combining autoradiography with electron microscopy.

Discussion

The cardiac glycoside ouabain is known to be a specific inhibitor of the Na$^+$, K$^+$-activated ATPase, an enzyme which has been implicated in ion and water transport across epithelia (Dunham and Glynn 1961; Bonting *et al.* 1962; Kinsolving *et al.* 1963; Schwartz *et al.*1963; Whittam and Wheeler 1970; Skou 1972). Consequently ouabain has been shown to inhibit active Na$^+$ and K$^+$ transport in a variety of tissues (Glynn 1964; Skou 1965; Podevin and Boumendil-Podevin 1972). As was outlined in the introduction, a model has been proposed (Berridge and Oschman 1969) to explain fluid secretion in insects, which involves a Na$^+$/K$^+$ exchange pump and depends on active transport of K$^+$ to generate 'urine' flow. However, reports that ouabain does not effect fluid secretion by the Malpighian tubules have led a number of workers to question seriously this model.

Berridge (1968); Maddrell (1969); Pilcher (1970); Gee (1976) and Rafaeli-Bernstein and Mordue (1978) have all failed to demonstrate an inhibitory effect of ouabain, at a concentration of 10^{-3}M, on fluid secretion by Malpighian tubules from *Calliphora, Rhodnius, Carausius, Glossina, Locusta* and *Zonocerus* respectively. However, Anstee and Bell (1975) and Anstee *et al.* (1978) have shown that 'urine' production by the Malpighian tubules of *Locusta* is inhibited by ouabain over the concentration range 10^{-6}M - 10^{-3}M. Evidence of ouabain inhibition of Malpighian tubule function is also supplied by Atzbacher *et al.*(1974) who found that the rate of excretion of the dyes azocarmine and indigocarmine was diminished by ouabain (3×10^{-4}M) in *Drosophila hydei*. Also, Gooding (1975) showed that diuresis in *Glossina* was inhibited by ouabain (20µg/ml) ingested in a saline solution. Similarly, Farquharson (1974) found that fluid secretion by the Malpighian tubules of the pill millipede, *Glomeris marginata,* was inhibited by ouabain at a concentration of 5×10^{-6}M. In ixodid ticks the salivary glands play a role in fluid secretion similar to the Malpighian tubules of insects and they require specific ratios of Na^+ and K^+ for maximal salivary secretion (Kaufman and Phillips 1973). Kaufman and Phillips (1973) have shown that in adult female *Dermacentor andersoni* fluid secretion was completely inhibited by 10^{-6}M ouabain.

Whilst Rafaeli-Bernstein and Mordue (1978) failed to demonstrate ouabain-inhibition of fluid secretion by *Locusta* tubules, Mordue and Rafaeli-Bernstein (1978) have shown that Na^+ transport by the Malpighian tubules of *Locusta* was increased after addition of 10^{-7}M ouabain. Other secretory epithelia in insects have also been shown to be sensitive to ouabain. Irvine and Phillips (1971) showed that 10^{-2}M ouabain reduced the rectal transepithelial potential to zero and Goh and Phillips (1978) report that ouabain (10^{-3}M) substantially reduces water reabsorption by *in vitro* rectal sacs of *Schistocerca*. Also, Kafatos (1968) found that labial gland secretion in *Antherea pernyi* was decreased by 50% at

5×10^{-4} ouabain and by 66% at 8×10^{-4}M. Berridge and Schlue (1978) report that ouabain affects membrane potential and internal potassium levels in unstimulated (i.e. in absence of 5-HT) salivary glands of *Calliphora*. Clearly then many secretory epithelia from insects are sensitive to ouabain. Although it must be mentioned that some authors that have obtained effects with ouabain (Kafatos 1968; Irvine and Phillips 1971) have concluded that the ouabain inhibition is not specific to a Na^+/K^+ exchange pump because of the high concentration used (8×10^{-4}M and 10^{-2}M respectively). However, in mammals there is a considerable difference in ouabain sensitivity reported, according to the species studied. The pI_{50} of ouabain on microsomal enzyme from rat kidney is 6×10^{-3}M as compared to 1.6×10^{-6}M in canine kidney enzyme assayed under identical conditions (Nechay 1974). The fact that high concentrations of ouabain may be necessary to cause inhibition does not then mean that the effect is not specific.

Apart from the composition of the Ringer solution used, this present work was carried out under the same conditions as described by Anstee and Bell (1975) and similar results were obtained. Fluid secretion by the Malpighian tubules of *Locusta* was inhibited by ouabain over the concentration range 10^{-5}M - 10^{-3}M. The degree of inhibition was somewhat lower than that reported by Anstee and Bell (1975), 56% compared with 93%, but was still substantial and agrees well with the value of 65% reported by Anstee *et al.* (1979).

In the present study the rates of fluid secretion were found to vary considerably from tubule to tubule and it was therefore important that each tubule acted as its own control; rate 1 being compared with rate 2 for each individual tubule. In some of the work reported in the literature it is not clear how the effect of ouabain was assessed.

Comparing the mean rate of secretion by several tubules before treatment with the mean rate of secretion after treatment may not give the same result as the present approach due to the high variation in secretion rate between one tubule and another. Certainly if this method is not used very large numbers of tubules for each treatment would need to be examined.

Present results confirm the presence of a Na^+, K^+-activated ATPase in microsomal preparations of the Malpighian tubules of *Locusta*. This Na^+, K^+-activated ATPase is classically inhibited by ouabain giving a pI_{50} of 5.8. This pI_{50} value is similar to that reported by Bell (1977) for *Locusta* tubule preparations, and by other workers on a variety of tissues (see Nakao 1975).

In the present study fluid secretion by the Malpighian tubules of *Schistocerca gregaria* was inhibited by 20% compared with the control, at a concentration of 10^{-3}M ouabain, although this difference was not statistically significant. This result is totally different from that obtained with *Locusta* but is, nevertheless, in agreement with the findings of Maddrell (1977) who reports no effect of ouabain on fluid secretion with *Schistocerca* tubules. However, the reduction in secretion rate shown by the controls for rate 2 suggests that the experimental conditions may not be suitable and this may be masking any inhibitory effect of ouabain.

Although it is possible that some insect species are insensitive to ouabain it must also be considered that discrepancies in results may reflect differences in experimental conditions. One of the more obvious methodological differences is the temperature at which fluid secretion by the Malpighian tubules has been studied. Rafaeli-Bernstein and Mordue (1978) who reported no effect of ouabain on fluid secretion in *Locusta* carried out their experiments at 24-25°C. Other workers perform the experiments at room temperature, 19-22°C (Gee 1976b). The inhibition of the Na^+, K^+-activated ATPase by ouabain has been shown to be extremely temperature sensitive (Charnock *et al*. 1975; Peacock *et al*.1976). Present results confirm this for *Locusta* Na^+, K^+-activated ATPase: at 30°C, 10^{-6}M ouabain caused 50% inhibition of the Na^+, K^+-activated ATPase activity in microsomal preparations of the Malpighian tubules, at 20°C there was only 32.3% inhibition and at 15°C only 19.1% inhibition.

The present results on fluid secretion by the tubules show that here too the temperature is important. At 30°C, 10^{-3}M ouabain caused 56% inhibition of fluid secretion, at 20°C this was reduced to 28% and at 15°C ouabain was found to have no inhibitory effect on fluid secretion.

It may be possible then that temperature is one factor which may account for differences in results reported in the literature, as clearly the effectiveness of ouabain as an inhibitor of fluid secretion is reduced at temperatures below 30°C. This may be expected if a Na^+, K^+-activated ATPase is involved in the mechanism of fluid secretion (as results so far would tend to confirm) as ouabain inhibition of the Na^+, K^+-activated ATPase has been shown to be extremely temperature sensitive.

Another factor which may explain the lack of inhibition reported by some workers is the composition of the Ringer solution bathing the tubules. Some workers use 'stimulants' in the Ringer solution bathing the insect preparations in order to increase the rates of fluid secretion. Work on *Rhodnius* (Maddrell 1969) and *Glossina* (Gee 1976b) shows that diuretic hormone and cyclic AMP were used to stimulate high rates of fluid secretion. In this situation it may be possible that any effect of ouabain may be masked. This is supported to some extent by the recent studies of Berridge and Schlue (1978). They report that ouabain affects membrane potential and internal K^+ levels in unstimulated glands but has no effect on glands stimulated by 5-HT.

Apart from the use of 'stimulants' the ionic composition of the Ringer solution may be important. Present results show that using the Ringer solution of Mordue (1969) ouabain inhibition could not be demonstrated in *Locusta*. This was due to the fact that both the control and experimental tubules showed around 50% inhibition of fluid secretion. Analysis of the secreted droplets showed that a decreased amount of K^+ was being secreted over the second set of determinations and this may be related to the decrease in fluid secretion observed in the controls. It would seem then that the ionic composition of this Ringer solution is unsuitable for maintaining fluid secretion by the Malpighian tubules of *Locusta* in the absence of any inhibitor.

Rafaeli-Bernstein and Mordue (1978) were unable to demonstrate ouabain inhibition of fluid secretion in *Locusta* using a Ringer solution containing 20mM K^+ and they suggest that the low concentration of K^+ (8.6mM) used by Anstee and Bell (1975), who report ouabain inhibition, may account for the difference in the two results. Jungreis (1977) also comments that the K^+ concentration of the Ringer solutions used by several workers may be unsuitable for demonstrating ouabain inhibition. High K^+ concentrations have been shown to affect ouabain inhibition of the Na^+, K^+-ATPase in a variety of tissues (Kinsolving *et al*. 1963; Judah and Ahmed 1964; Matsui and Schwartz 1968; Akera 1971; Akera *et al*. 1974). However, the extent to which K^+ antagonises ouabain inhibition depends on the incubation and assay conditions (Akera 1971). In most studies the ouabain enzyme mixture was preincubated in the presence of Na^+, K^+ and Mg^{2+} and the reaction started by addition of ATP and the amount of inorganic phosphate (P_i) assayed after 5-30 minutes incubation. Akera (1971) has shown that by preincubating the enzyme with ouabain, Na^+, Mg^{2+} and ATP and beginning the reaction with K^+, the amount of ouabain necessary to effect 50% inhibition of ATPase activity was 23.7% of that needed by the more conventional method. Akera (1971) suggests that the previously reported effect of K^+ was on the velocity of the ouabain-enzyme complex formation rather than on that of the ouabain inhibited ATPase reaction. Studies on ouabain binding to a Na^+, K^+-activated ATPase preparation have shown that the amount of ouabain bound in the presence of ATP, Mg^{2+}, Na^+ and K^+ equals that bound in the presence of ATP, Mg^{2+} and Na^+ if the experiment is carried out over a prolonged period of time (Allen and Schwartz 1970). If this is related to the effect of ouabain on fluid secretion it would suggest that the length of time the tubule preparation is in the ouabain-Ringer solution will have an effect on the inhibition observed. Fathpour (personal communication) has in fact shown that allowing the tubule preparation to equilibrate in the ouabain-Ringer solution for times less than 30 minutes, at 30°C, results in a reduction in ouabain inhibition. Temperature has also been shown to have an effect on ouabain binding (Akera and Brody 1971) and, therefore, at temperatures below 30°C it may be necessary to have an equilibration period longer than 30 minutes before any inhibition can be observed.

Present results showed that varying the K^+ concentration of the bathing medium from 10-40mM had no effect on the inhibition of fluid secretion by ouabain, at a temperature of 30°C and with an equilibration period of 30 minutes. In each case there was about 50% inhibition. It seems, therefore, that the effect of K^+ as an antagonist of ouabain inhibition is being over-estimated by some workers.

It is possible, however, that the K^+ concentrations used by some workers may have prevented ouabain inhibition. Berridge (1968) found no effect of ouabain on fluid secretion in *Calliphora* using Ringer solutions containing 140mM K^+, 0mM Na^+; 0K^+, 140mM Na^+; and 56mM K^+, 84mM Na^+. It is not too surprising that ouabain inhibition could not be demonstrated in the first two solutions as the Na^+/K^+ exchange pump could not be operating, and the concentration of 56mM K^+ in the third solution may have been high enough to prevent ouabain inhibition.

Present results show that the K^+ concentration of the Ringer solution bathing the Malpighian tubules cannot account for the difference in results reported for ouabain inhibition in *Locusta* by Rafaeli-Bernstein and Mordue (1978) and those obtained in this present study and by Anstee and Bell (1975). It is, therefore, puzzling why two such different effects should be seen using the same species. It was noticed that in the work reported by Rafaeli-Bernstein and Mordue (1978) there was no indication of how long the tubule preparation was bathed in Ringer solution containing ouabain and the importance of this has already been referred to. It was also not clear just how many tubules had been studied or how the effect of ouabain had been assessed.

The rates of fluid secretion reported by Rafaeli-Bernstein and Mordue (1978) for *Locusta* Malpighian tubules are much higher than those obtained in this present study and those reported by Maddrell and Klunsuwan (1973). In a Ringer solution with zero potassium, Rafaeli-Bernstein and Mordue (1978) reported a secretion rate of c. 3.5nl/min, a rate similar to the mean rate of fluid secretion obtained in this present study (3.1nl/min) using 8.6mM K^+. Fathpour (personal communication) has shown that *Locusta* Malpighian tubules only secrete very slowly in K^+-free Ringer solution (0.7nl/min). In Ringer solution containing 20mM K^+m Rafaeli-Bernstein and Mordue (1978) report a secretion rate of c. 15.0nl/min, a rate that is almost four times higher than that obtained in this present study with 20mM K^+ (4.4nl/min) and almost eight times higher than that reported by Maddrell and Klunsuwan (1973) for *Schistocerca* (1-2nl/min).

Ethacrynic acid (2,3-dichloro-4-(2-methylene butyryl 1)-phenoxyacetic acid) has been found to produce diuresis, similar to that observed with cardiac glycosides, in mammals. How ethacrynic acid exerts this effect is not completely understood. It has been suggested that it affects Na^+ transport by a mechanism which is insensitive to ouabain (Whittembury and Fishman 1969; Hoffman and Kregenow 1966; Lubowitz and Whittam 1969; Dunn 1973).

Ethacrynic acid (10^{-3}M) has been shown to completely inhibit fluid secretion in *Glossina* (Gee 1976), an insect which is rather atypical in that Na^+ is the transported cation rather than K^+. Gee (1976) also found that fluid secretion in *Glossina* was unaffected by ouabain (10^{-3}M). He proposed that Na^+ transport in *Glossina* may be by electrogenic sodium pumps and not by a ouabain sensitive Na^+/K^+ exchange pump. However, this presupposes that ethacrynic acid is a specific inhibitor of Na^+ transport and this has been shown not to be the case. Ethacrynic acid has been shown to inhibit Na^+, K^+-activated ATPase in a variety of tissues (Duggan and Noll 1965; Davis 1970; Charnock *et al.* 1970; Proverbio *et al.* 1970; Peacock *et al.*1976). Present results confirm this for *Locusta* Na^+, K^+-activated ATPase; the Na^+, K^+-activated ATPase activity in microsomal preparations of the Malpighian tubules of *Locusta* exhibiting a pI_{50} of 2.5mM for ethacrynic acid. Thus Na^+, K^+-activated ATPase is inhibited by ethacrynic acid although relatively high concentrations are required as compared with ouabain (pI_{50} c. 10^{-6}mM). The pI_{50} for ethacrynic acid was similar to that obtained by Peacock *et al.* (1976) for *Homorocoryphus* (pI_{50} 3mM).

As well as having an effect on the Na^+, K^+-activated ATPase it has also been suggested (Klahr *et al.* 1971) that ethacrynic acid has a direct effect on metabolism. Klahr *et al.* (1971) found that 1mM ethacrynic acid decreased lactate formation from glucose-6-phosphate by 50% in cell free systems of rat and rabbit renal cortex and medulla, isolated epithelium of turtle bladder and hemolysates of human red blood cells. Inhibition of active transport would lead to a secondary decrease in metabolism (Whittam and Wheeler 1970) but since this was a cell-free system it indicated a direct inhibition of metabolism. Similar results were obtained by Gordon and Hartog (1969) for cell-free preparations of Ehrlich ascites tumour cells. Moreover, Landon and Fitzpatrick (1970, 1972) showed that respiration and glycolysis in kidney slices were inhibited by ethacrynic acid. Similarly, Daniel *et al.* (1971) found that ethacrynic acid affected oxidative phosphorylation and glycolysis in rat uterus.

It would appear, therefore, that ethacrynic acid is having both a direct and indirect effect on ion transport. The direct effect being on Na^+, K^+-activated ATPase activity and the indirect effect being the inhibition of glycolysis which would cause a secondary decrease in cation transport by reducing the supply of energy available to the pump.

In the present study ethacrynic acid was shown to be an extremely effective inhibitor of fluid secretion by the Malpighian tubules; 10^{-3}M ethacrynic acid producing 71.6% inhibition of fluid secretion. When ethacrynic acid (10^{-4}M) and ouabain (10^{-4}M) were applied to the Malpighian tubule preparation together, the inhibition produced was greater than when either was applied alone. There are two possibilities; either the two compounds inhibited different systems or they both acted to increase inhibition of the same system. From the results on Na^+, K^+-activated ATPase inhibition it would seem unlikely that ethacrynic acid causes inhibition of fluid secretion by an effect on the Na^+, K^+-activated ATPase. Concentrations of ethacrynic acid in excess of 1mM were necessary to cause any substantial inhibition of the Na^+, K^+-activated ATPase activity whereas 1mM ethacrynic acid had a very pronounced effect on fluid secretion. It is suggested, therefore, that ethacrynic acid affects fluid secretion by a means other than by affecting the Na^+/K^+ exchange pump. Since the effect of ethacrynic acid has been shown to be complex, it is wrong to conclude as Gee (1976) does that electrogenic Na^+ pumps account for all the ion transport in *Glossina*. Clearly ethacrynic acid could be affecting ion transport in more than one way. In contrast to Gee (1976), Gooding (1975) reported that diuresis in *Glossina* was inhibited by ouabain ingested in a saline solution. Whilst it is possible that the ouabain was acting at a site other than the Malpighian tubules, as suggested by Gee (1976), it may be that a Na^+/K^+ exchange pump is involved in fluid secretion in *Glossina*.

This question cannot be resolved by the use of a drug like ethacrynic acid which has such wide ranging effects.

As was mentioned in the introduction, inaccessibility of the sites of Na^+, K^+-activated ATPase has been suggested as an explanation of the apparent insensitivity of some insect tissues to topically applied ouabain. Present results confirm those of Rafaeli-Bernstein and Mordue (1978), in that ^3H-ouabain, or its labelled metabolies, was found to be secreted by the Malpighian tubules of *Locusta*. In passing across the tubule, ouabain would be readily available to any sites of Na^+, K^+-activated ATPase.

It was interesting to note that ^3H-inulin was also found to be excreted by the Malpighian tubules of *Locusta*. Inulin (m.w. c.5000) has been used in the study of vertebrate and invertebrate excretory systems as a compound which is thought to be neither secreted nor re-absorbed (Riegel 1972). ^{14}C-inulin has also been shown to be excreted by the Malpighian tubules of the pill millipede *Glomeris marginata* (Farquharson 1974) and gel filtration studies showed that there was no detectable alteration of inulin whilst it passed through the tubule. Ramsay and Riegel (1961) showed the permeability of *Carausius* tubules to inulin to be extremely low (tubule fluid : medium ratio of 0.046) whereas the permeability of *Glomeris* tubules was very much higher (tubule fluid : medium ratio of 0.68). Farquharson (1974) suggests that the route of inulin across the tubule is not through the cell but through the intercellular junctions.

Further evidence for the accessibility of the sites of Na^+, K^+-activated ATPase to ouabain has been obtained from autoradiographic studies. This is a method which employs the specific binding of ^3H-ouabain to localise Na^+, K^+-activated ATPase. Autoradiographs obtained in the present study show silver grains, corresponding to bound ouabain, associated with both the basal and apical surfaces of the tubules. In future studies it would be interesting to combine electron microscopy with autoradiography in an attempt to identify the silver grains in association with specific cellular membranes. Karnaky *et al.* (1976) have successfully used this technique to locate Na^+, K^+-activated ATPase at the subcellular level in the chloride cells of teleost gills.

The present results are consistent with the model for fluid secretion proposed by Berridge and Oschman (1969) as they suggest the existence of a Na^+, K^+-activated ATPase at the basal cell surface.

Autoradiography would seem to be a more promising method for localising Na^+, K^+-activated ATPase than the use of histochemical techniques. The disadvantages of the histochemical technique have already been mentioned.

The results presented in this Chapter show that there is a ouabain-sensitive, Na^+, K^+-activated ATPase present in microsomal preparations of the Malpighian tubules and that fluid secretion is also inhibited by ouabain. These facts tend to support the involvement of a Na^+/K^+ exchange pump in the mechanism of fluid secretion in *Locusta*.

CHAPTER 5

AGE DEPENDENT CHANGES IN THE Na^+, K^+-ACTIVATED ATPase ACTIVITY OF *LOCUSTA* MALPIGHIAN TUBULES

INTRODUCTION

Although there have been many studies on the control of fluid secretion by insect Malpighian tubules (Maddrell 1963; Highnam *et al.* 1965; Mills 1967; Cazal and Girardie 1968; Mordue and Goldsworthy 1969; Mordue 1969, 1970, 1972; Pilcher 1970; Goldsworthy and Mordue 1972; Aston and White 1974; Gee 1975), most of these have been carried out on mature adults. The regulation of Malpighian tubule activity during development has, in contrast, been generally neglected. However a study on the skipper butterfly *Calpodes ethlius* (Ryerse 1978) has shown that the ability of Malpighian tubules to transport fluid and the rate of fluid secretion depended on the developmental stage of the insects. The larval tubules were permanently switched on and did not require diuretic hormone. Fluid transport continued at larval - larval moults but was 'switched off' 24hrs before pupal ecdysis. There was no secretory activity during the first half of the pupal stage when the tubules were remodelled for adult function, but fluid transport resumed mid-way through the stage in time for rapid diuresis at adult emergence. Adult Malpighian tubules were capable of very rapid fluid transport after feeding or drinking.

Throughout the development of *Calpodes* the secretory activity of the Malpighian tubules was found to be precisely co-ordinated with feeding activity (Ryerse 1978). A similar co-ordination between feeding and fluid secretion has been demonstrated in the salivary glands of female ixodid ticks (Kaufman *et al.* 1976). Unfed female ticks only secreted fluid at a very slow rate (Kaufman 1976) but there was an enhanced ability to secrete fluid on feeding.

Salivary secretion in female ixodid ticks has been shown to depend on active solute transport (Kaufmann and Phillips 1973). Specific ratios of Na^+ and K^+ were necessary for maximal fluid secretion and the process was inhibited by ouabain, suggesting the involvement of a Na^+, K^+-activated ATPase in the fluid secretory process. Kaufmann *et al.*(1976) have demonstrated Na^+, K^+-activated ATPase activity in preparations of the salivary glands, showing that enzyme activity, along with fluid secretion, increased with the time the ticks spent feeding on the host.

The Na^+, K^+-activated ATPase, an enzyme which has been implicated in the active transport of Na^+ and K^+ in many secretory tissues, has been shown to have a role in the processes of fluid secretion and absorption by the Malpighian tubules and rectum of insects (Anstee and Bell 1975; Peacock 1976; Tolman and Steele 1976; Anstee *et al.* 1979; present study). Peacock (1978) has shown that the Na^+, K^+-activated ATPase activity in preparations of *Locusta* rectum showed developmental changes. Enzyme activity was found to increase with age throughout the last larval stadium until the onset of metamorphosis when the activity fell. In the adult, the Na^+, K^+-activated ATPase activity was low just after the moult, but increased with age.

In view of the evidence from studies on *Locusta* (Peacock 1978) and ixodid ticks (Kaufmann *et al.* 1976) that Na^+, K^+-activated ATPase activity varies with development and that there is a corresponding variation in fluid secretion (Kaufmann *et al.* 1976; Ryerse 1978), the present study has been carried out to determine Malpighian tubule ATPase activity throughout the 5th stadium and in early adult *Locusta migratoria*.

MATERIALS AND METHODS

1. Determination of Na^+, K^+-activated ATPase activity

Na^+, K^+-activated ATPase activity in homogenates of the Malpighian tubules of *Locusta* was determined as described previously (Chapter 2). Equal numbers of male and female locusts (12 in all) were used for each experiment. Enzyme assays were carried out on aged animals for each day of the fifth instar and into the adult stage. Under the conditions of rearing the fifth instar lasted approximately 10 days. The procedure for ageing animals is described in Chapter 2.

2. Measurement of wet weight and dry weight

Wet weight of individual male locusts was measured daily throughout the fifth instar. The animals were killed by decapitation over a weighing boat and any food material removed from the alimentary canal before weighing. The locusts were then dried at 125°C for 24hrs to determine final dry weight.

RESULTS

1. Na^+, K^+-activated ATPase activity at daily intervals throughout the fifth instar of *Locusta*

The results presented in Figure 5.1 show the daily changes in Na^+, K^+-activated ATPase activity in homogenates of the Malpighian tubules. The graph (Fig. 5.1) shows Na^+, K^+-activated ATPase activity expressed as nmoles P_i released/set Malpighian tubules/min plotted against the age in days of the insects, and is typical of 4 separate series of experiments, the data for which can be found in Appendix 5.1.

Figure 5.1 Na$^+$, K$^+$-activated ATPase activity at daily intervals throughout the fifth instar of *Locusta*

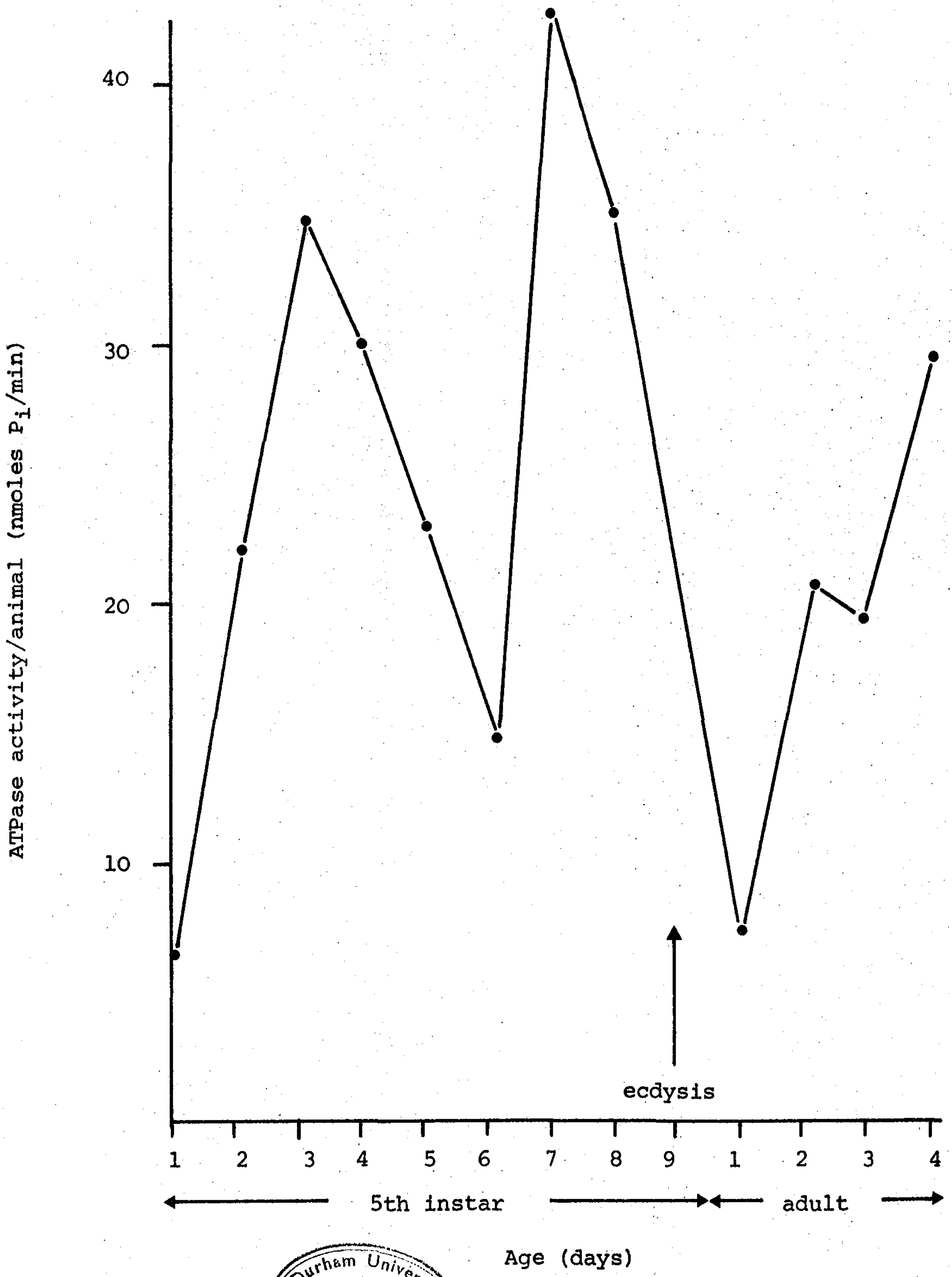
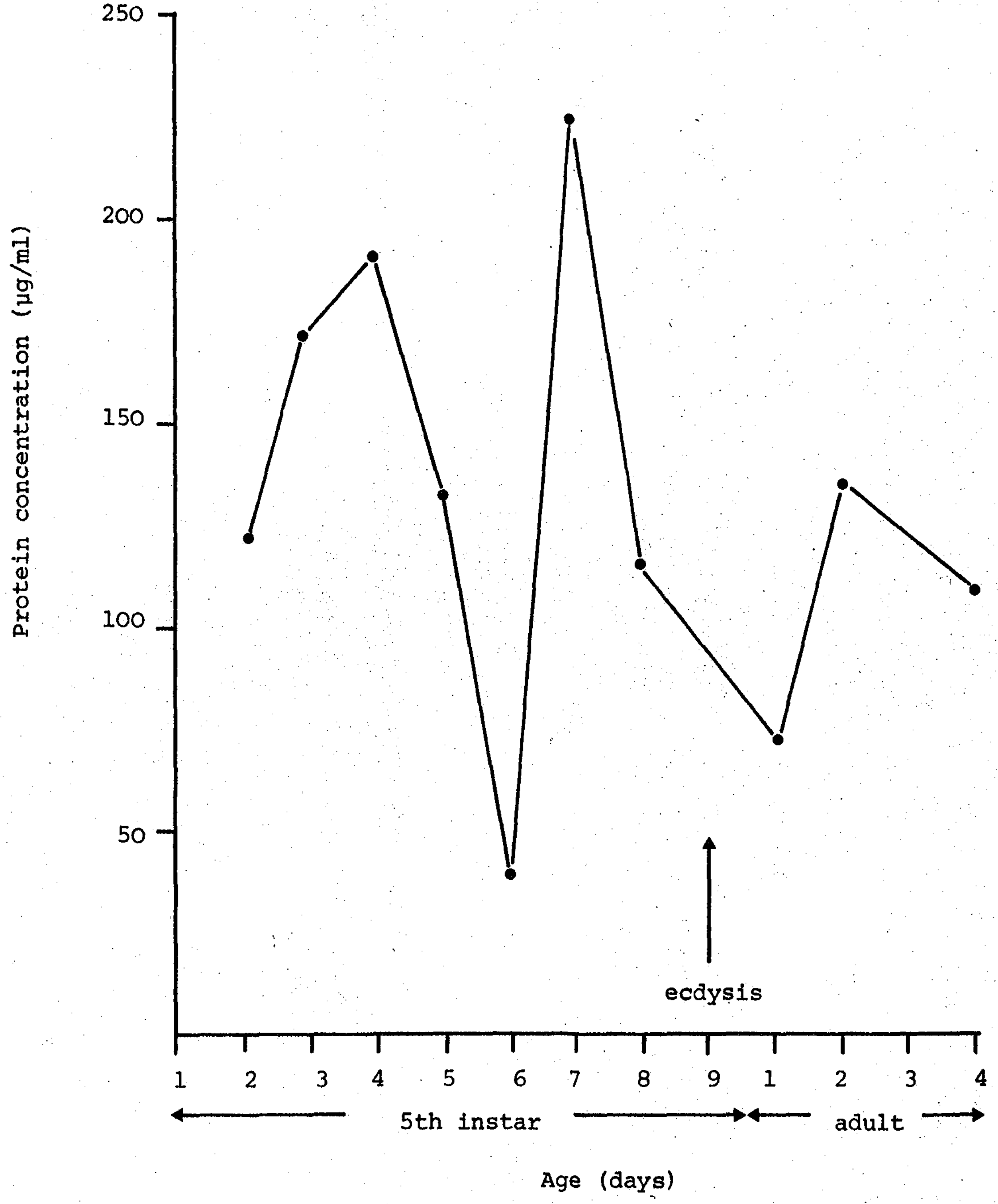


Figure 5.2 Protein concentrations in homogenates of Malpighian tubules at daily intervals throughout the fifth instar



In newly moulted fifth instar locusts the activity of the Na^+, K^+-activated ATPase was very low (6.5nmoles P_i/min), but over the next two days the level of activity increased dramatically to 34.5nmoles/min. During the next 3 days of the instar enzyme activity decreased to around 14.5nmoles/min before rising dramatically once more (42.5nmoles P_i/min) and falling again before the larval-adult moult. Na^+, K^+-activated ATPase activity in newly moulted adult locusts was also very low (7.5nmoles P_i/min) but once again showed a substantial increase with age. In contrast, the Mg^{2+}-dependent ATPase activity varied only slightly (between 2-4nmoles P_i/min) throughout.

2. Variation in protein levels at daily intervals throughout the fifth instar of *Locusta*

The protein content of homogenates of the Malpighian tubules was determined daily using the method of Lowry *et al.* (1951). Figure 5.2 shows a graph of protein concentration plotted against insect age. It can be seen that the protein content varied considerably throughout the fifth instar, the pattern very closely resembling that obtained for Na^+, K^+-activated ATPase activity.

3. Measurement of wet weight and dry weight at daily intervals throughout the fifth stadium of *Locusta*

Figure 5.3 shows the changes in somatic wet and dry weights which occurred during the fifth stadium. Each point on the graph corresponds to the mean of 10 determinations. Throughout the stadium both wet and dry weights increased considerably. The larval-adult ecdysis was accompanied by a considerable loss of water whereas the dry weight remained fairly constant.

Figure 5.3 Changes in wet and dry weights at daily intervals throughout the fifth instar of *Locusta*

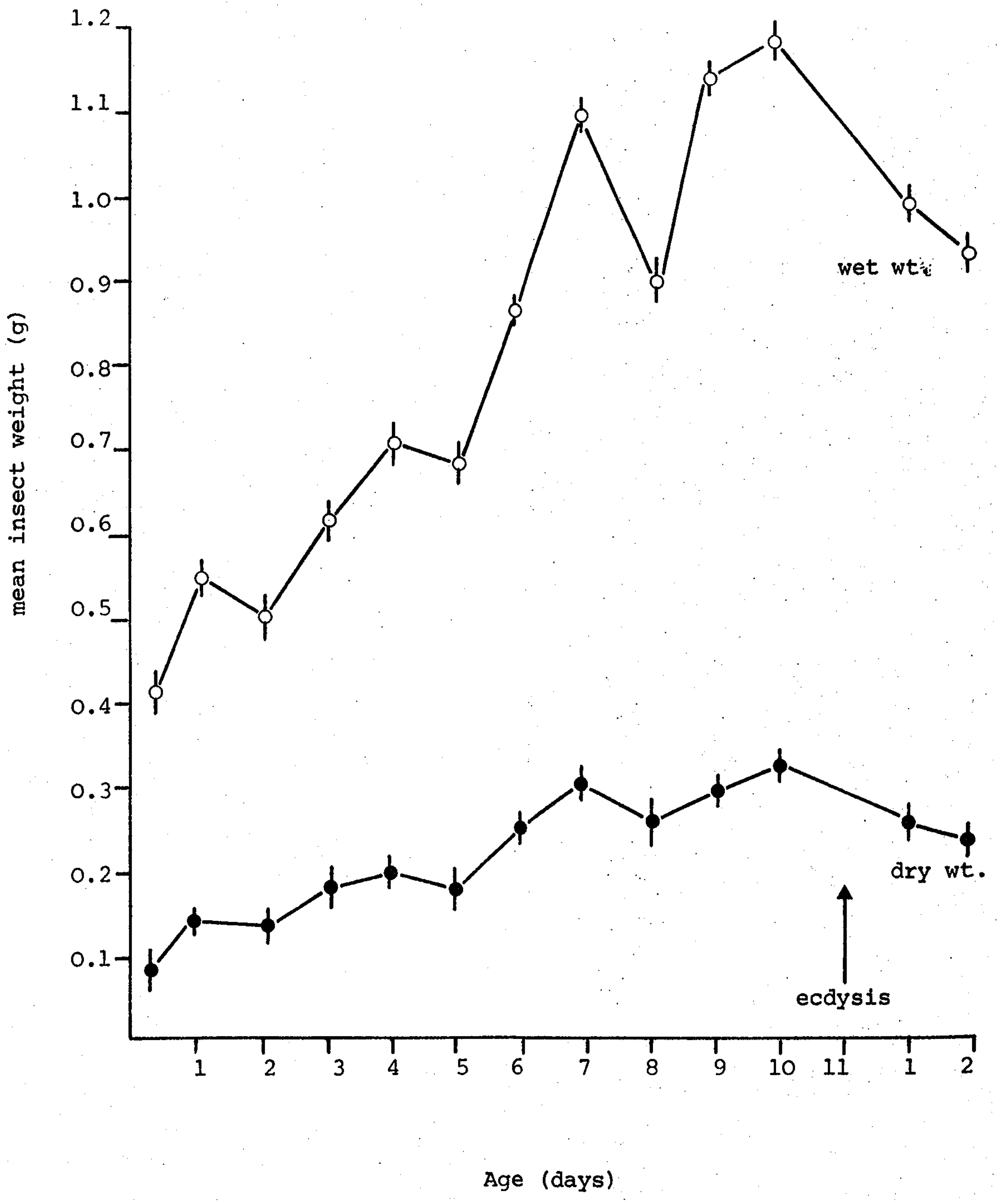
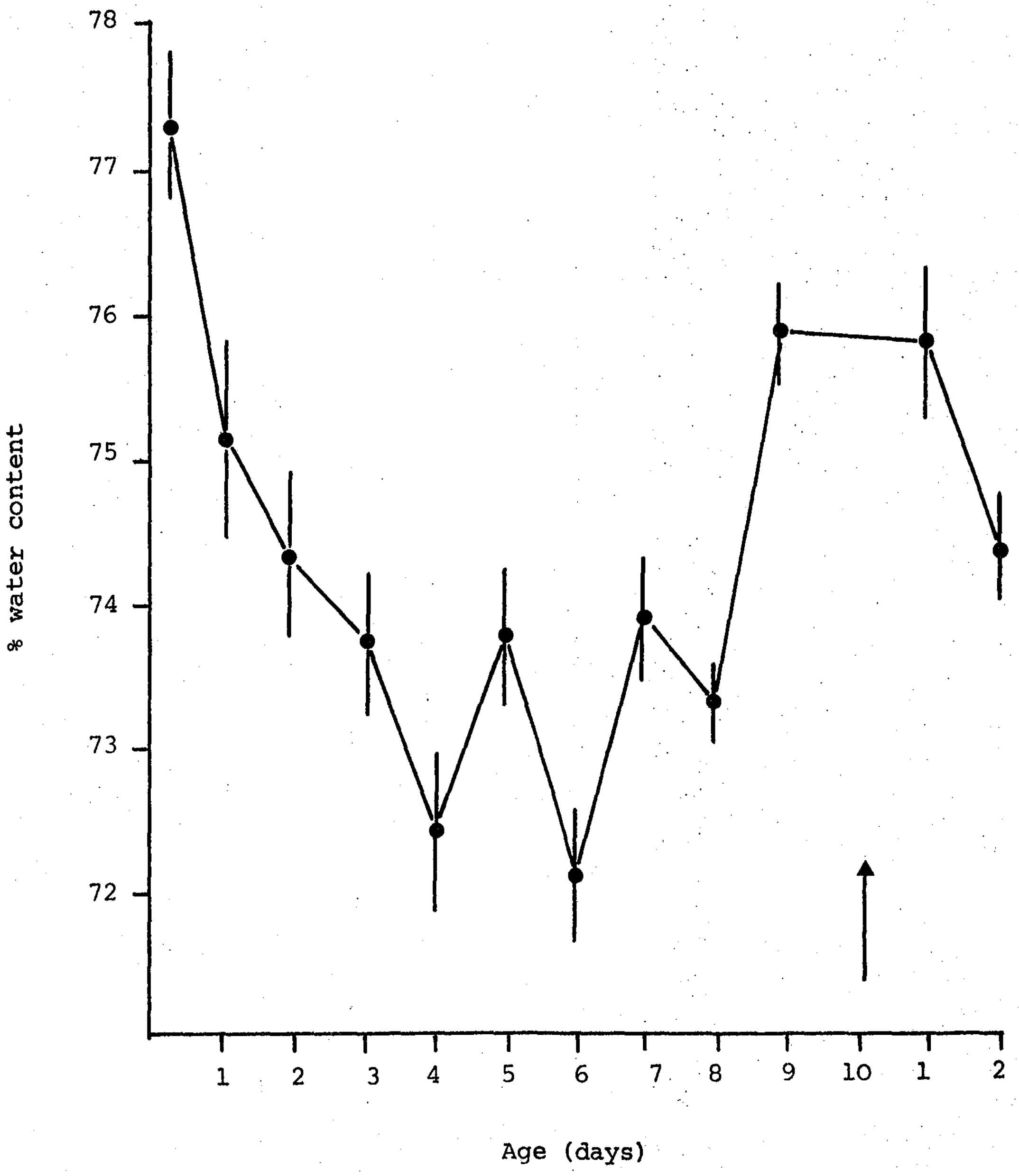


Figure 5.4 Daily changes in relative water content throughout the fifth instar



The results presented in Figure 5.4 show the daily changes in the percentage water content ($\frac{\text{wet wt - dry wt}}{\text{wet wt}}$ x 100) of the locusts. It can be seen that ca. 78% of body weight is due to water in the newly moulted fifth instar locusts. The relative water content then decreased over the next 4 days to 72.4% but was followed by a small but significant increase on day 5. A further decrease in water content was observed on day 6. The relative water content then increased up to the time of the larval-adult moult.

DISCUSSION

In the present study the Na^+, K^+-activated ATPase activity in homogenates of *Locusta* Malpighian tubules has been shown to vary with age throughout the fifth stadium and early adult life. A similar pattern of variation has been reported by Peacock for *Locusta* rectal Na^+, K^+-activated ATPase. Malpighian tubule Na^+, K^+-activated ATPase activity was very low at the beginning of the 5th stage but increased with age during the stadium before falling again just before the larval-adult moult. However, a dramatic decrease in Na^+, K^+-activated ATPase activity was observed mid-instar (days 5-6); the possible significance of this will be discussed later. In preparations from newly moulted adult locusts, enzyme activity was low initially, but increased with age. In contrast, the Mg^{2+}-dependent ATPase activity showed very little variation throughout the period studied.

Kaufmann *et al.* (1976) working on salivary glands from female ixodid ticks have also shown the development of a Na^+, K^+-activated ATPase. They found that the salivary glands from unfed female *Amblyomma hebraeum* exhibited a very low ATPase activity, whilst following feeding the ATPase activity increased steadily and a ouabain-sensitive component appeared.

Maximum Na^+, K^+-activated ATPase activity was observed when the animals fed: unfed weight ratio was approximately 8. Associated with this increased Na^+, K^+-activated ATPase activity on feeding, *in vitro* fluid secretion was enhanced. Once again, fluid secretion was maximal at a fed : unfed weight ratio of 8. This increased ability to secrete fluid can be taken as a further indication of Na^+, K^+-activated ATPase involvement in the mechanism of fluid secretion.

The Malpighian tubules of 5th instar *Locusta* have also been found to show increased secretory activity with development (Aitchison: personal communication). The rate of 'urine' production by *in vitro* Malpighian tubules of newly moulted 5th instar locusts was very low but increased with age until just before the larval-adult moult when the rate of fluid secretion fell. These findings agree well with the changes in Na^+, K^+-activated ATPase activity reported in the present study.

The daily measurements of wet weight and dry weight presented earlier show that there is considerable variation in insect water content throughout the 5th stadium. The larval-adult ecdysis was accompanied by a decrease in insect water content. Similar changes in water content with age have been reported for *Locusta* by Beenakkers and Van Den Broek (1974). Using the daily values for wet weight and dry weight it was possible to determine the percentage water content for each age. The relative water content of 5th instar larvae varied from 72-77.5% with maximal values occurring at the beginning (77.4%) and the end (75.9%) of the stadium. The minimal values occurred on day 4 (72.5%) and day 6 (72%) with an increase on day 5 (73.75%). These daily values for water content plotted against age gave a graph that was almost exactly the converse of the results for Na^+, K^+-activated ATPase activity.

It is impossible to conclude from the present results whether the variation in relative water content resulted from variations in haemolymph volume or tissue water. However, casual observation did suggest that at certain times throughout the 5th stadium (notably just before and after moulting) the haemolymph volume was much increased.

Baehr *et al.* (1979) have also investigated changes in daily water content of 4th and 5th instar *Locusta*. Their findings agree well with those observed in the present study. They showed that the relative water content of 5th instar locusts varied from 72-77% with maximal values at the beginning and the end of the instar, while the minimal values occurred on days 6 and 7.

Beenakkers (1973) has shown that newly ecdysed adult *Locusta* have a high haemolymph volume and that this volume decreases over the following few days. Further evidence for variation in haemolymph volumes in locusts is provided by the study of Lee (1961). Lee (1961) estimated haemolymph volumes at daily intervals throughout the development of *Schistocerca* from the 3rd larval instar onwards. In general the results showed that the blood volume was high just prior to ecdysis, fell over the next few days and then increased again before the next ecdysis. Results for 5th instar locusts showed that the haemolymph volumes were highest at the beginning and end of the instar, whilst the minimum volumes occurred on days 3 and 5 with an increase on day 4.

These patterns of change in haemolymph volumes (Beenakkers 1973; Lee 1961) are very similar to the variations in relative water content observed in the present study and that of Baehr *et al.* (1979) and it therefore seems reasonable to assume that variations in relative water content in 5th instar *Locusta* reflect changes in haemolymph volume. In this context it is perhaps significant that developmental changes in the rates of fluid secretion by the Malpighian tubules of *Locusta*

(Aitchison: personal communication) are consistent with the changes in relative water content described above. Thus, at times when relative water content is high (pre- and post-moult), fluid secretory rates are low and when animal water content is low, there are enhanced rates of fluid secretion.

The results of the present study in conjunction with those of other workers (Lee 1961; Beenakkers 1973; Baehr *et al.* 1979; Aitchison pers. comm.) show that Na^+, K^+-activated ATPase activity, relative water content, haemolymph volume and rates of fluid secretion vary throughout development in a manner which would suggest that they are perhaps related. High Na^+, K^+-activated ATPase activity occurs at times of increased rates of fluid secretion by the Malpighian tubules and low relative water content. Conversely, low enzyme activity is associated with low fluid secretory rates and high relative water content.

As already mentioned, high relative water content/haemolymph volumes have been found to occur immediately pre- and post-ecdysis. Lee (1961) has suggested that the increased blood volume at ecdysis is associated with the expansion of the cuticle and the insect as a whole after ecdysis. The increased relative water content observed in the present study near the middle of the 5th stadium is rather more difficult to explain. This mid-instar increase more or less coincided with the dramatic reduction in Malpighian tubules Na^+, K^+-activated ATPase activity mentioned previously. Lee (1961) also reports an increase in haemolymph volume for 5th instar *Schistocerca* at this time. More recently Morgan *et al.* (1975) showed that in *Schistocerca* apolysis occurred by day 5 (of a 7-9 day instar) in all abdomens studied. Apolysis is the name given by some authors (Jenkin and Hinton 1966) to the process of separation of the old cuticle from the underlying epidermal cells. As the cuticle separates from the epidermis, moulting fluid is secreted into the space between the two. It is tempting to speculate that the mid-5th instar increase in relative water content is related to this process. However further investigation is clearly necessary to establish whether this is the case.

Developmental changes in Malpighian tubule fluid transport have also been reported for the skipper butterfly *Calpodes ethlius*, where fluid transport was found to depend on the physiological state and the developmental stage of the insect (Ryerse 1978). Fluid secretion by the Malpighian tubules increased during periods of feeding and rapid body growth but was 'switched off' 24hrs before the larval-pupal ecdysis when feeding stopped. Similarly, in *Dysdercus* the cessation of feeding prior to the larval-adult moult was associated with the arrest of Malpighian tubule fluid secretion (Berridge 1966).

Beenakkers and Van Den Broek (1974) have studied feeding activity in 5th instar *Locusta* showing that food consumption increased throughout larval development but was low at the beginning and end of the instar. This is in agreement with the observation made in the present study, that the alimentary canal of newly ecdysed 5th instar and adult locusts did not contain food. Thus it would appear that in *Locusta* also relative water content and Malpighian tubule fluid secretion (Aitchison: pers. comm.) are correlated with feeding activity. Fluid secretory rates have been found to be low at the beginning and end of the 5th instar (Aitchison: pers. comm.), as has food consumption (Beenakkers and Van Den Broek (1974).

It has been shown previously that feeding activity in insects can often act as a stimulus for hormone release (Wigglesworth 1934; Clarke and Langley 1963; Maddrell 1964; Mordue 1972). In *Rhodnius* (Maddrell 1964) and *Glossina* (Gee 1975) the diuretic hormone is released in response to a blood meal. Maddrell (1964) has shown that in *Rhodnius* the distension of the abdomen caused by a large intake of fluid is monitored by proprioceptors and their responses result in release of diuretic hormone from thoracic neurosecretory cells. Feeding has also been shown to bring about the release of diuretic hormones in locusts (Mordue 1966, 1969), *Dysderus* (Berridge 1966), *Carausius* (Pilcher 1970) and *Periplaneta* (Mills 1967). In *Dysderus* (Berridge 1966) a high titre of diuretic hormone causes a high secretion of fluid by the Malpighian tubules and a reduction in rectal reabsorption. Non-feeding periods are associated with low rates of fluid secretion by the tubules and very little diuretic hormone circulating in the haemolymph. These results have led several authors to propose similar mechanisms for the control of Malpighian tubule fluid secretion (Maddrell 1964; Berridge 1966; Pilcher 1970; Mordue 1972; Gee 1975). In response to feeding, diuretic hormone is released leading to increased rates of fluid secretion. When the stimulus is removed, diuretic hormone release ceases immediately, the titre in the haemolymph is reduced by a degradative mechanism and the rate of excretion declines. Destruction of diuretic hormone by the Malpighian tubules has been demonstrated in *Rhodnius* (Maddrell 1964), *Dysdercus* (Berridge 1966), *Carausius* (Pilcher 1970) and *Glossina* (Gee 1975).

The release of diuretic hormone in response to feeding has generally been studied in adult insects. Ryerse (1978) has shown that diuretic hormone is not necessary for fluid secretion by the Malpighian tubules of larval *Calpodes ethlius*. This raises the question as to whether diuretic hormone plays the same role in the regulation of fluid secretion in 5th instar *Locusta* as it does in the adult. Studies on adult locusts have shown that the blood volume depends on feeding activity (Mordue 1969). Water accumulates in the haemolymph in the absence of diuretic hormone as a result of the reduced secretory activity of the Malpighian tubules and increased reabsorption through the rectal wall. This could easily explain the changes in relative water content found at the beginning and end of the 5th stadium but it does not explain the increased haemolymph volume mid-instar, a time when the insects are feeding normally.

In the present study the protein content of homogenates of the Malpighian tubules was found to vary throughout the 5th stadium. The protein concentration was low at the beginning of the instar (120μg/ml), then increased over the next 3 days (188μg/ml) before falling dramatically on day 6 (33μg/ml). The protein level then began to rise once again (220μg/ml) but was low just before the larval-adult moult. This pattern is exactly similar to the pattern obtained for Na^+, K^+-activated ATPase activity which tends to suggest that at least some of the protein content which is fluctuating so markedly is due to the production and denaturation of the enzyme.

The mid-instar (c. day 6) of *Locusta* has been shown to be a time of low protein content, low Na^+, K^+-activated ATPase activity and high relative water content. The low protein content at this time may be due to a cessation in protein synthesis which would help to explain the low level of Na^+, K^+-activated ATPase activity also. The changes in enzyme activity and animal water content have been discussed previously but it is difficult to explain how these events should come about.

Other workers have also shown that the mid-instar of *Locusta* is a time of low protein synthesis. Turner and Loughton (1975) have studied *in vitro* protein synthesis by various tissues of 5th instar *Locusta*. They found that protein synthesis varied throughout the instar, with each tissue showing its own characteristic pattern. However, all tissues studied (gut, heart, fat body, haemolymph) produced protein of low specific activity on day 6 and released proteins of extremely high specific activity on day 7. Turner and Loughton (1975) postulated that the low specific activity on day 6 might represent a cessation of protein synthesis.

Baehr *et al*, (1979) have also studied haemolymph protein levels during the 4th and 5th larval instar of *Locusta*. They found that protein concentrations in the haemolymph varied throughout both larval instars. During the 4th instar the level of protein was low for the first 3 days, increased on day 4, fell again between the 4th and 5th day, and rose again between the 5th and 6th day. In the 5th instar, protein concentration in the haemolymph fell at the 4th ecdysis and during the first 24hrs afterwards. Levels remained low until the 3rd day and then rose markedly from the 4th - 7th day. Maximal values were obtained between the 8th and 9th day. During the 2 days preceding the imaginal moult the values fell by 50%.

Phillips and Loughton (1976) have measured the protein content of the cuticle of *Locusta* throughout the 5th stadium. They found that the protein content increased until day 6 (the onset of apolysis) and then began to decline.

These results of Turner and Loughton (1975), Phillips and Loughton (1976) and Baehr *et al.* (1979) in addition to the present study show that several tissues in *Locusta* undergo similar changes in protein content during development. In particular all tissues studied showed low protein levels during the mid-instar. Turner and Loughton (1975) suggest that the tissues are responding to a generalised stimulus during this time. It is probable that the changes in synthetic activity are the result of a hormonal stimulus. Kinnear *et al.* (1971) showed an abrupt cessation of protein synthesis at the end of the feeding stage of *Calliphora stygia* and implicated ecdysone in the control of this event. Baehr *et al.* (1979) have studied haemolymph protein levels in relation to the levels of J.H. and ecdysteroids in the haemolymph. There was found to be some correlation between haemolymph protein and ecdysone levels.

The levels of both ecdysone and Juvenile Hormone have been shown to vary throughout the 5th instar of *Locusta* (Hoffman *et al.* 1974; Baehr *et al.* 1979; Hirn *et al.* 1979). In general, these observations show that the titre of ecdysone rises to a maximum during the second half of the instar and decreases prior to ecdysis. Hoffman *et al.* (1974) found that the ecdysone titre was low in young 5th instar larvae, rising sharply at the time of apolysis (day 6-7) and synthesis of the new cuticle. The hormone level then decreased rapidly and remained low at the time of ecdysis. Baehr *et al.* (1979) have shown that the haemolymph levels of Juvenile Hormone (J.H. I) - immunoreactive substance were high (30.35ng/ml) during the first 5 hours of the 5th instar in *Locusta* and then decreased progressively (3.5ng/ml). J.H. titre remained low throughout the rest of the instar except for a small peak on day 6 (11ng/ml) in females.

It is impossible to relate hormone levels to physiological events in the present study but in view of the results presented above this would clearly be an interesting area for study.

As well as the physiological changes throughout development in the 5th stadium presented above, the Malpighian tubules of *Locusta* also show morphological changes (Chapter 3). The possible significance of these structural changes in relation to functional changes in the Malpighian tubules will be discussed in Chapter 7.

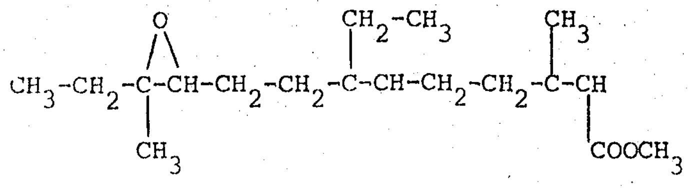
CHAPTER 6

THE EFFECT OF INSECT HORMONES ON MALPIGHIAN TUBULE FUNCTION IN *LOCUSTA*

INTRODUCTION

The 'classical' scheme for the hormonal control of moulting and metamorphosis in insects involves an endocrine system consisting of the brain and associated glands *(corpora cardiaca, corpora allata)* together with the prothoracic glands. Neurosecretory cells in the brain produce brain hormone (prothoracicotrophic hormone) which enters the haemolymph, in many cases via the *corpora cardiaca*. Brain hormone then stimulates the prothoracic glands to synthesise and release the insect moulting hormone. Under direct neural control from the brain the *corpora allata* secrete Juvenile Hormone (see Gilbert and King 1973; Rees 1977).

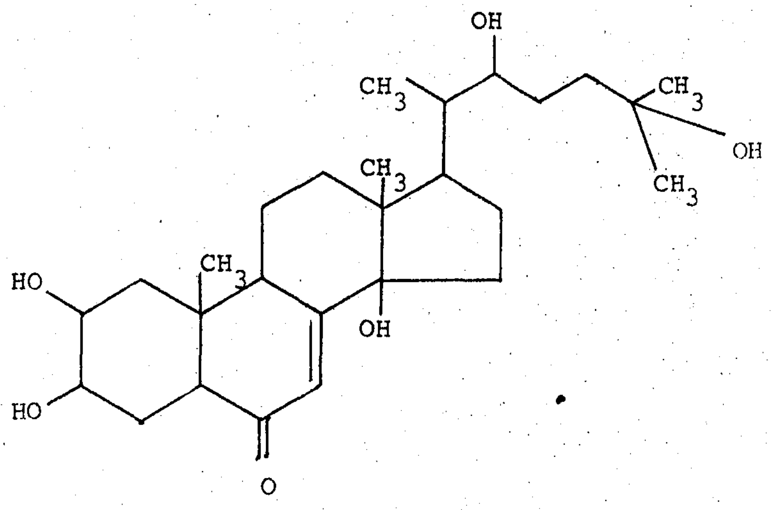
This scheme has been proposed as the result of numerous studies on a variety of insect species (see Novak 1969, 1970; Wyatt 1972; Doane 1972; Gilbert and King 1973; Gilbert 1974). The existence of Juvenile Hormone was first predicted by Wigglesworth (1934) as a result of surgical experiments, but no successful attempts were made to isolate the hormone until Williams (1956) extracted J.H. from the abdomens of male *Hyalophora cecropia*. Although highly purified, the J.H. from *Hyalophora* proved very difficult to characterise until Roller *et al.*(1967) succeeded in isolating, identifying and synthesizing the principal Cecropia Juvenile Hormone (C-18 J.H.I; methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate) with the following structural formula:



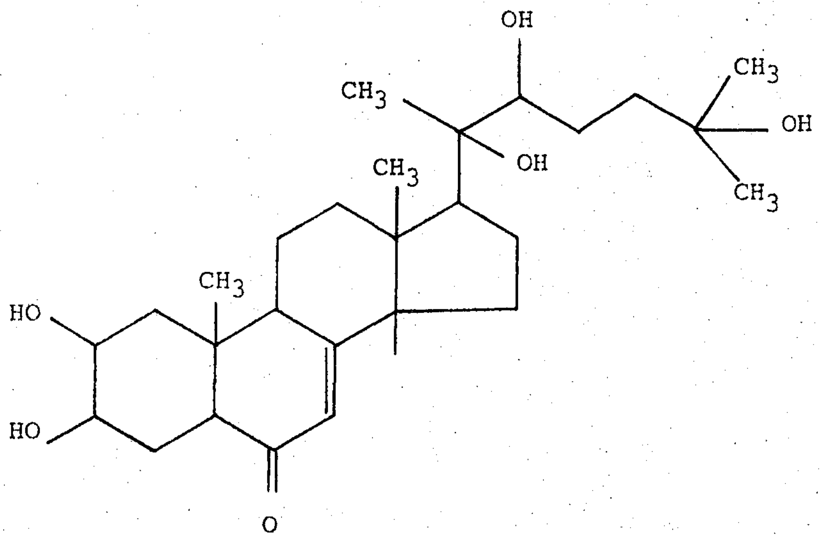
$$CH_3-CH_2-\overset{O}{\overbrace{\underset{CH_3}{\underset{|}{C}}-CH}}-CH_2-CH_2-\overset{CH_2-CH_3}{\overset{|}{C}}-CH-CH_2-CH_2-\overset{CH_3}{\overset{|}{C}}-\underset{COOCH_3}{\underset{|}{CH}}$$

In 1968, Meyer *et al.* showed that the C-18 Cecropia Hormone was accompanied by smaller amounts of a more polar C-17 homologue J.H. II (methyl 10,11-epoxy-3,7,11-trimethyl-2-trans-6-trans tridecadienoate). A third C-16, J.H. III (methyl, 10,11-epoxy-3,7,11-trimethyl-2-trans-6-trans-dodecadienoate) has now been identified in *Manduca sexta* (Judy *et al.* 1973). Sensitive techniques involving gas-liquid chromatography and mass spectrometry for the isolation and identification of J.H. from haemolymph have shown that, not only does the total J.H. titre vary during development, but also that the relative concentrations of the three known Juvenile Hormones change during development (Lanzrein *et al.* 1975), which may suggest a possible variation in function.

The task of isolation of moulting hormone in pure form was first undertaken by Becker and Plagge (1939) and was finally achieved in 1954 by Butenandt and Karlson. They isolated moulting hormone in a crystalline form from *Bombyx mori* pupae and subsequently this hormone was named ecdysone (Karlson 1956). It was not until 1965 that the structure was determined and the hormone was shown to be a steroid (Huber and Hoppe 1965) with the following structural formula:



This compound was assigned the name α-ecdysone. As well as α-ecdysone, a second steroid was also extracted from *Bombyx* pupae (Karlson 1956) and this became known as β-ecdysone. β-ecdysone was also extracted from the Moroccan locust, *Dociostaurus* (Stamm 1959). Compared to α-ecdysone, β-ecdysone was relatively difficult to isolate and crystallise. The chemical structure remained unknown until 1966 when it was isolated from various sources independently (Hocks and Weichert 1966; Kaplanis *et al.* 1966; Hoffmeister and Grutzmacher 1966), and shown to be identical to α-ecdysone, apart from an extra hydroxyl group at C-20. A variety of names have been used to describe this same hormone. Thus the compound has been named 20-hydroxyecdysone from *Bombyx* pupae (Hocks and Weichert 1966) and *Manduca sexta* pupae (Kaplanis *et al.* 1966) and ecdysterone also from *Bombyx mori* (Hoffmeister and Grutzmacher 1966). These are identical to the β-ecdysone named by Karlson (1956) and have the following structural formula:



Both Juvenile Hormone and ecdysone are now available in both natural and synthetic forms and this has facilitated studies on the effects of these two hormones at both the organismal and sub-cellular levels (Karlson and Sekeris 1964, 1966; Minks 1967; Gilbert 1967; Wyatt 1968; Novak 1970; Gilbert 1974; Slama 1975).

Although a great deal is known about these hormones, the mechanisms by which they act on their target tissues remain obscure. Two main theories have been proposed to explain the primary mode of action of ecdysone : (i) a selective gene derepression hypothesis (Karlson 1963) and, (ii) an ion hypothesis of gene activation (Kroeger and Lezzi 1966). Much of the evidence for both of these theories comes from observations on the giant polytene chromosomes of various tissues of Diptera (Clever and Karlson 1960; Kroeger 1963, 1966; Lezzi and Gilbert 1969; Ashburner 1971; Berendes 1971). At certain times many chromosomal bands produce 'puffs'. This reversible structural modification is thought to be a sign of gene activity and involves some uncoiling of the chromosome fibres to allow synthesis of specific RNA which accumulates at the band site. Most 'puffs' appear at specific stages during development, remain active for a time and then regress. The observation that periods of moulting and the early stages of metamorphosis coincided with intense 'puffing' activity led Karlson (1967) to implicate ecdysone in the control of 'puffing'. Moreover, injection of ecdysone into certain insects induces 'puffing' in polytene chromosomes within 15 minutes (Karlson 1963). This coupled with evidence that 'puffs' represent sites of transcription of the genetic information of the DNA into mRNA led Karlson (1963) to formulate a possible biochemical mechanism for ecdysone action based on the Jacob and Monod model of bacterial gene regression. According to this hypothesis the hormone penetrates to specific chromosomal sites and there interacts with the 'repressor molecule'. This results in derepression so that mRNA synthesis begins at these sites. Following transfer to the cytoplasm the mRNA participates in the biosynthesis of specific proteins. This theory therefore proposes that ecdysone acts directly within the nucleus. In contrast, the ion hypothesis of gene activation (Kroeger and Lezzi 1966) proposes that ecdysone controls gene activity indirectly by acting on the cell membrane to change internal Na^+ and K^+ levels in target cells. According to this theory ecdysone exerts its primary action upon membranes changing their selective permeability. It is proposed that ecdysone stimulates the 'sodium pump' of target cells with a resultant increase in K^+ concentrations in the cell and nucleus. The increased intranuclear K^+ then activates particular genes (Kroeger 1968). Support for this theory of ecdysone action comes from the observation that exposure to appropriate intranuclear ionic concentrations induces the same 'puffs' in *Chironomus* salivary gland polytene chromosomes as does ecdysone (Kroeger 1963, 1966). There are also *in vivo* changes in the Na^+ and K^+ levels in salivary gland cells that correlate with fluctuations in hormone titre during development (Kroeger *et al.* 1973).

Less information is available concerning the mode of action of Juvenile Hormone. In 1957, Wigglesworth, discussing the action of growth hormone in insects, considered that "it is possible to conceive it (J.H.), also, as being concerned in the regulation of permeability relations within the cells - in such a way that the gene controlled enzyme system responsible for the larval characters is brought increasingly into action when the Juvenile Hormone is present." Similarly, Lezzi and Gilbert (1972) suggest that J.H. affects cell permeability. They propose that J.H., like ecdysone, acts on the cell membrane to alter internal cation concentrations, leading to an increase in internal Na^+ concentrations, an effect opposite to that proposed for ecdysone. In support of this suggestion are observations that J.H. specific 'puffs' in polytene chromosomes are induced by ionic concentrations with high

Na^+/K^+ ratios (Lezzi and Gilbert 1972). Results from electrophysiological measurements on salivary glands of *Galleria mellonella* (Baumann 1968) also support this view that J.H. affects cell permeability. It has also been observed that injection of ouabain into *Tenebrio* pupae results in the formation of larval-pupal intermediates as does J.H. application (Chase 1970). Since ouabain is known to inhibit the Na^+/K^+ pump of cell membranes (Schatzmann 1953) it would lead to a decrease in the K^+/Na^+ ratio within the cell which is consistent with the idea of a relationship between J.H. and high intracellular Na^+ concentration.

In a rather different approach to the relationship between ecdysone and cell permeability Gee *et al.* (1977) have examined the function of ecdysteroids by looking at their effect on fluid secretion by the Malpighian tubules of *Glossina morsitans*. It was found that ecdysone and ecdysterone stimulated fluid secretion as did cholesterol and aldosterone. Gee *et al.* (1977) suggest that the ecdysteroids may be increasing the rate of secretion by altering the permeability of the basal membrane of the tubule cells. The rate of fluid secretion by the Malpighian tubules of *Glossina* is thought to be controlled by the permeability of their basal membrane to Na^+ (Gee 1976).

Fristrom and Kelly (1976) have also studied the possible role of Na^+ and K^+ concentrations in hormone action, by determining the effect of β-ecdysone and J.H. on the Na^+, K^+-activated ATPase from homogenates of *Drosophila* imaginal discs. This membrane bound enzyme transports Na^+ and K^+ in a vectorial manner and plays a major role in the regulation of Na^+ and K^+ concentrations in cells (Glynn 1964; Dahl and Hokin 1974). Fristrom and Kelly (1976) found that β-ecdysone had no effect on Na^+, K^+-activated ATPase activity, whereas J.H. increased the activity of the enzyme. Neither of these observations are consistent with the hypothesis proposed by Kroeger and Lezzi (1966) and referred to above. For their hypothesis to be acceptable, one would have expected β-ecdysone to stimulate the Na^+, K^+-activated ATPase and consequently produce high intracellular K^+ concentrations, whilst J.H. would be expected to inhibit Na^+, K^+-activated ATPase activity and increase the intracellular Na^+/K^+ ratio.

It has been shown previously that there is a Na^+, K^+-activated ATPase present in microsomal preparations of the Malpighian tubules of *Locusta* (Anstee and Bell 1975; and see Chapter 4). This enzyme has been implicated in cation and fluid transport across the Malpighian tubules since both of these processes are inhibited by the cardiac glycoside ouabain (Anstee and Bell 1975; Chapter 4). In view of this the Malpighian tubules of *Locusta* would seem to offer a suitable system on which to study the effects of β-ecdysone and Juvenile Hormone on cell permeability and the Na^+, K^+-activated ATPase. The results of this work will be presented in two sections; the effect of insect hormones on fluid secretion by *in vitro* preparations of the Malpighian tubules, and the effect of insect hormones on the Na^+, K^+-activated ATPase in homogenates of the Malpighian tubules.

SECTION 1

Hormonal effects on fluid secretion by the Malpighian tubules of *Locusta*

It is now generally accepted that excretion in insects is regulated by both diuretic and anti-diuretic hormones, which may be peptides or polypeptides, originating from neurosecretory cells (Highnam *et al*. 1965; Mills 1967; Mordue and Goldsworthy 1969; Mordue 1969, 1970, 1972; Aston and White 1974).

In locusts, hormones present within the neurosecretory cell - corpus cardiacum complex exert both diuretic and anti-diuretic effects. The Malpighian tubules and rectum respond to the diuretic hormone, produced in the neurosecretory cells of the brain and stored in the central lobes of the corpora cardiaca, by an increase in fluid secretion by the tubules and a reduction in rectal reabsorption (Highnam *et al.* 1965; Cazal and Girardie 1968; Mordue and Goldsworthy 1969; Mordue 1969, 1970, 1972). The anti-diuretic hormone, present in the dorsal lobes of the corpora cardiaca increases rectal reabsorption (Mordue 1970, 1972; Goldsworthy and Mordue 1972) and Malpighian tubule function (Cazal and Girardie 1968).

Extracts of the protocerebrum and corpora cardiaca have also been shown to have a marked diuretic effect upon the Malpighian tubules of *Carausius* (Pilcher 1970a,b) and *Dysdercus* (Berridge 1966) and diuretic principles have been found in *Rhodnius* (Maddrell 1963) and *Glossina* (Gee 1975b).

The mode of action of the diuretic and anti-diuretic hormones on the excretory system is still under investigation. Maddrell *et al.* (1971) have reported an increase in Malpighian tubule function in response to 5-hydroxytryptamine (5-HT) in *Rhodnius* and *Carausius*, although this compound has no effect on locust tubules (Mordue 1972; Maddrell and Klunsuwan 1973; Anstee *et al.* 1979). In these insects 5-HT may not be sufficiently analogous to the diuretic hormone to mimic its action.

Maddrell *et al.* (1971) have also reported an increase in fluid secretion by the Malpighian tubules of *Rhodnius* and *Carausius* in response to cyclic AMP. Aminophylline, a phosphodiesterase inhibitor, was also found to increase the secretory rate of *Carausius* tubules. Application of cyclic AMP has also been shown to stimulate fluid secretion by the

Malpighian tubules of *Locusta* (Mordue 1969; Anstee *et al.* 1979) and *Schistocerca* (Maddrell and Klunsuwan 1973).

These results suggest that cyclic AMP may be involved in the action of the diuretic hormones and 5-HT (Maddrell *et al.* 1971). Further evidence for this has been provided by Aston (1975) who showed an increase in intracellular cyclic AMP during stimulation of *Rhodnius* tubules by diuretic hormone.

Berridge and Patel (1968) found that fluid secretion by isolated salivary glands from *Calliphora* was stimulated by 5-HT. Application of 5-HT was found to lead to an increase in cyclic AMP and electrophysiological studies suggested that this increased cyclic AMP level may stimulate a cation pump (Berridge 1970; Berridge and Prince 1972a,b; Prince and Berridge 1972, 1973; Prince *et al.* 1972). Berridge (1977) has proposed a model for the stimulation of salivary gland secretion by 5-HT acting through cyclic AMP. This model suggests that cyclic AMP directly stimulates a cation pump (electrogenic) on the apical cell membrane. This may be analogous to the stimulation of Malpighian tubule secretion.

Although a great deal of research has been carried out to isolate the insect diuretic and anti-diuretic hormones and to study their mode of action, very little is known of the effect of ecdysone and Juvenile Hormone on the excretory system. As was mentioned in the introduction to this Chapter, Gee *et al.* (1977) have suggested that ecdysteroids may be involved in the control of Malpighian tubule function, and Wall and Ralph (1964) have reported an 'anti-diuretic principle' found in the corpora allata of *Periplaneta americana*. The following study has been carried out to determine the effects of ecdysone and Juvenile Hormone on fluid secretion by *in vitro* preparations of the Malpighian tubules of *Locusta*. In addition the effects of corpora cardiaca extract, protocerebral neurosecretory cell extract and cyclic AMP, have been re-examined.

MATERIALS AND METHODS

1. To determine the effect of Juvenile Hormone on fluid secretion by the Malpighian tubules

Juvenile Hormone was found to be insoluble in aqueous solution and was therefore dissolved in ethanol prior to its addition to the Ringer solution (50mg J.H. dissolved in 1ml ethanol).

The Malpighian tubule preparations were set up as described in Chapter 2. Rates of fluid secretion were determined over an initial 35 minute period with the preparations bathed in 'normal' Ringer solution. The 'normal' Ringer solution was then replaced with Ringer solution containing J.H. (10µl ethanolic J.H./ml Ringer solution), the preparations allowed to equilibrate for 10 minutes and the rates of fluid secretion redetermined over a second 35 minute period. Two types of control experiments were performed, one in which 'normal' Ringer solution was used throughout and another in which the second rate of fluid secretion was determined with the preparation bathed in 'normal' Ringer solution containing ethanol (10µl ethanol/ml Ringer solution).

2. To determine the effect of Juvenile Hormone on the Na^+ and K^+ concentrations in the 'urine'

Malpighian tubule preparations were set up as described in Chapter 2. After an initial period of 15 minutes the secreted droplets were removed and discarded. This was to ensure that the droplets of 'urine' that were subsequently to be collected and analysed were formed from the Ringer solution and not from the haemolymph prior to dissection. The fluid secreted over the next 35 minute period was collected using a 1μl microcap. The droplets of fluid from several tubules belonging to the same insect preparation were pooled to give a sample of 1μl. This sample was then diluted in 3ml deionised water. The 'normal' Ringer solution bathing the Malpighian tubule preparation was then replaced either with fresh 'normal' Ringer solution or with Ringer solution containing J.H. (500μg/ml). The preparation was equilibrated for 15 minutes and the fluid secreted over the next 35 minute period collected as described above.

The Na^+ and K^+ concentrations of the secreted fluid were determined by atomic emission spectroscopy, using a Pye Unicam SP 90 spectrophotometer. Emission readings were referred to calibration graphs constructed with known concentrations of NaOH and KOH (see Appendix 6.1).

3. Gas chromatography

Samples of J.H. to be injected into the gas chromatograph were dissolved in carbon disulphide. Aqueous samples containing J.H. were extracted in diethyl ether, the ether layer being dried down under a stream of nitrogen and the residue redissolved in carbon disulphide. 5μl samples were analysed in a Pye Unicam Series 104 Gas chromatograph equipped with a flame ionisation detector. The gas chromatograph was fitted with a 3' column packed with 3% polyethyleneglycol adipate on a Gaschrom Q inert support. The assay temperature was 180°C and the carrier gas (nitrogen) flow rate was 45mls/min. Synthetic J.H. was assayed as a standard with which to compare experimental samples.

4. To determine the effect of Juvenile Hormone on the ultrastructure of the Malpighian tubules

Locusts were killed by decapitation and the Malpighian tubules quickly dissected out. The mass of tubules from each insect was divided approximately in half. One half was soaked for 30 minutes in 'normal' Ringer solution and the other in either Ringer solution containing J.H. (500µg/ml) or Ringer solution containing ethanol (10µl ethanol/ml Ringer solution). The tubules were then processed for electron microscopy as described in Chapter 3.

5. Preparation of corpora cardiaca extract and neurosecretory cell extract

Adult male locusts were killed by decapitation and either the corpora cardiaca or the neurosecretory cells of the protocerebrum quickly dissected out and homogenised in ice cold 'normal' Ringer solution to give a concentration of 1 gland pair/ml Ringer solution or n.s.c. from one protocerebrum/ml Ringer solution.

Results

1. The effect of Juvenile Hormone on fluid secretion by the Malpighian tubules of *Locusta*

Rates of fluid secretion by *in vitro* preparations of the Malpighian tubules were determined with the insect preparation bathed in 'normal' Ringer solution and in Ringer solutions containing 5µg/ml - 5mg/ml J.H. The results are shown in Figure 6.1. It can be seen that J.H. has an inhibitory effect on the rate of fluid secretion over the concentration range used. Table 6.1 shows the result of comparing

Figure 6.1 The effect of various concentrations of synthetic J.H. fluid secretion by Malpighian tubules, *in vitro*.

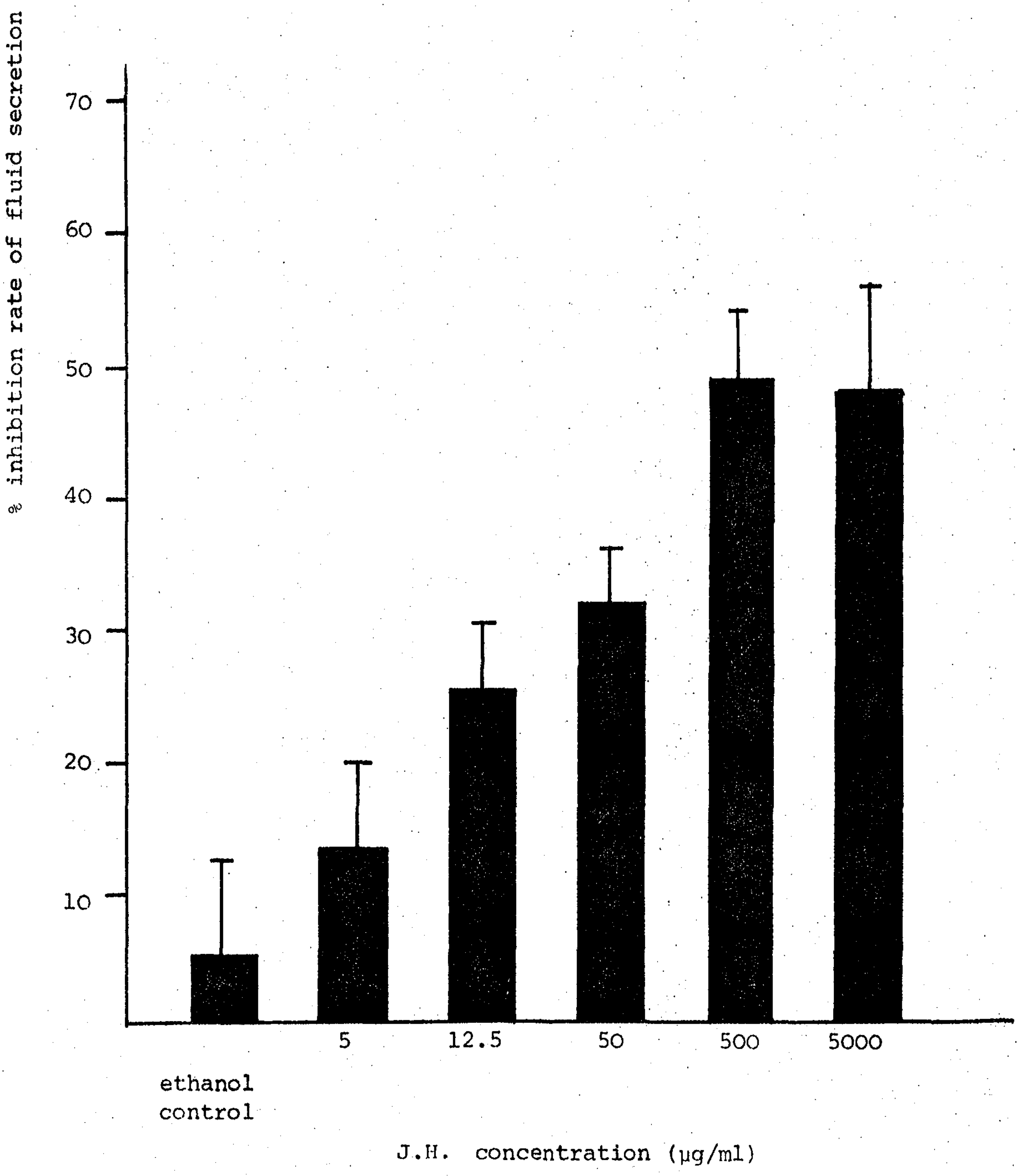


Table 6.1 The effect of Juvenile Hormone on fluid secretion by the Malpighian tubules

Treatment	n	mean rate secretion % original rate±S.E.	P_1 (paired 't' test)	P_2 (Students 't' test)
a. Control	20	91.8 ± 6.0	not sig.	
b. ethanol	32	95.2 ± 7.1	not sig.	a:b not sig.
c. 5µg/ml J.H.	27	86.6 ± 6.5	0.02	b:c not sig.
d. 12.5µg/ml J.H.	30	74.5 ± 5.1	<0.001	b:d <0.001
e. 50µg/ml J.H.	38	68.6 ± 3.7	<0.001	b:e <0.001
f. 500µg/ml J.H.	36	51.3 5.0	<0.001	b:f <0.001 c:f <0.001 d:f <0.001 e:f <0.001
g. 5000µg/ml	17	52.3 ± 8.3	<0.001	f:g not sig.

Values for P_1 were obtained by comparing rate 1 and rate 2 of fluid secretion in a paired 't' test. Values for P_2 were obtained by comparing the mean result for each treatment with the means of all other treatments. The 100% rate of fluid secretion was 3.7 ± 0.4nl/min.

rate 1 and rate 2 of fluid secretion (for each J.H. concentration) in a paired 't' test. It can be seen from these results that ethanol has no effect on the rate of fluid secretion by the tubules.

2. The effect of J.H. on Na^+ and K^+ concentrations in the 'urine'

Na^+ and K^+ concentrations were determined in samples of 'urine' obtained from tubule preparations bathed in 'normal' Ringer solution. The values obtained were compared with the concentrations of Na^+ and K^+ in samples of 'urine' obtained from J.H. (500µg/ml) bathed preparations. To allow for any small variations in sample volume the K^+/Na^+ ratios were compared instead of the actual concentrations of Na^+ and K^+. From the results (Table 6.2) it can be seen that in the control the final K^+/Na^+ ratio was significantly higher ($p < 0.001$) than the initial K^+/Na^+ ratio. The actual concentrations of K^+ and Na^+ in the 'urine' samples (see Appendix 6.1) show that this alteration of the K^+/Na^+ ratio was due to an increase in the amount of K^+ being secreted and not a decrease in Na^+ secretion. When the preparation was bathed in Ringer solution containing J.H. it was found that the initial and final K^+/Na^+ ratios were not significantly different from one another. This would seem to suggest that J.H. is having some effect on K^+ secretion.

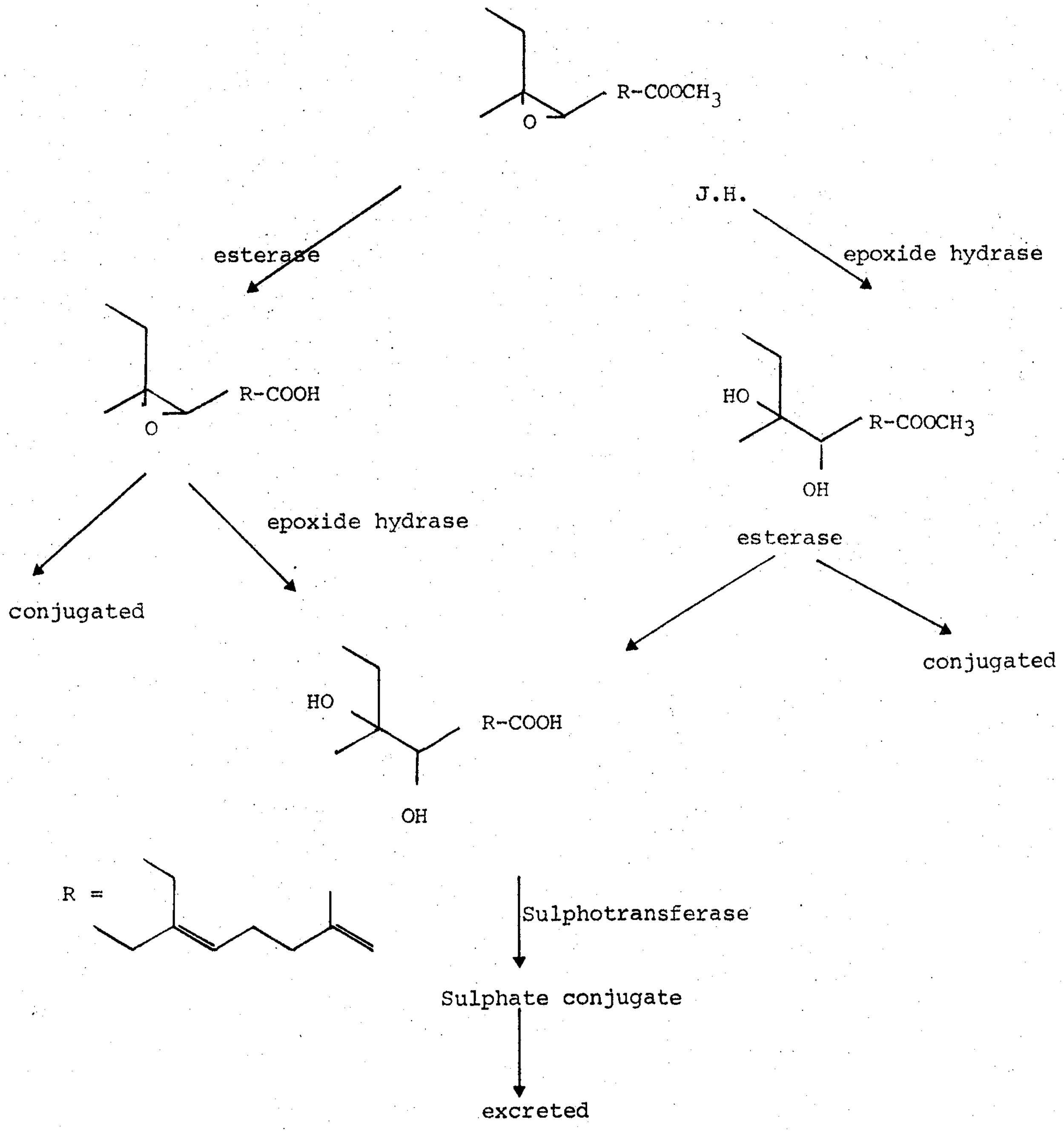
Table 6.2 The effect of Juvenile Hormone on Na^+ and K^+ concentrations in the 'urine'

	Initial $^K/Na$ (a)	Final $^K/Na$ (b)	(b) as % of (a)	P
Control	4.3 ± 0.7	6.2 ± 1.0	141.8± 8.9	<0.001
J.H. 500µg/ml	6.6 ± 0.7	5.4 ± 0.6	88.0±11.3	not sig.

Values for P were obtained by comparing the initial and final K^+/Na^+ ratio for each sample in a paired 't' test.

3. Gas chromatography : To determine whether Juvenile Hormone is being metabolised

It has been well established that Juvenile Hormone is synthesised by the Corpora allata and secreted into the haemolymph in which it is transported to target cells (Wyatt 1972; Doane 1972; Gilbert and King 1973). At some appropriate time it then undergoes inactivation by the action of esterases. Slade and Wilkinson (1974) have shown that in *Prodenia eridania* J.H. is broken down via two major pathways:



Esterase activity was found in all tissues of *P.eridania* and *Hyalophora cecropia* i.e. haemolymph, mid-gut, fat-body, Malpighian tubules, body wall. High epoxide hydrase activity was detected in the fat body and the mid-gut but was also present in all other tissues apart from the haemolymph (Slade and Wilkinson 1974).

In view of the above findings in *P. eridania* and *H. cecropia* it seemed necessary to determine whether the Juvenile Hormone bathing the Malpighian tubule preparations in fluid secretion experiments was being degraded during the course of the experiment. If so, any effects observed could be due to the products of degradation and may not have been attributable to J.H.

Juvenile Hormone (Methyl-10-11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate) is a suitable compound for analysis by gas-liquid chromatography. The following samples were analysed in an attempt to determine whether the J.H. was being metabolised during the course of the experiment.

(i) Synthetic J.H. (standard)

(ii) 'normal' Ringer solution

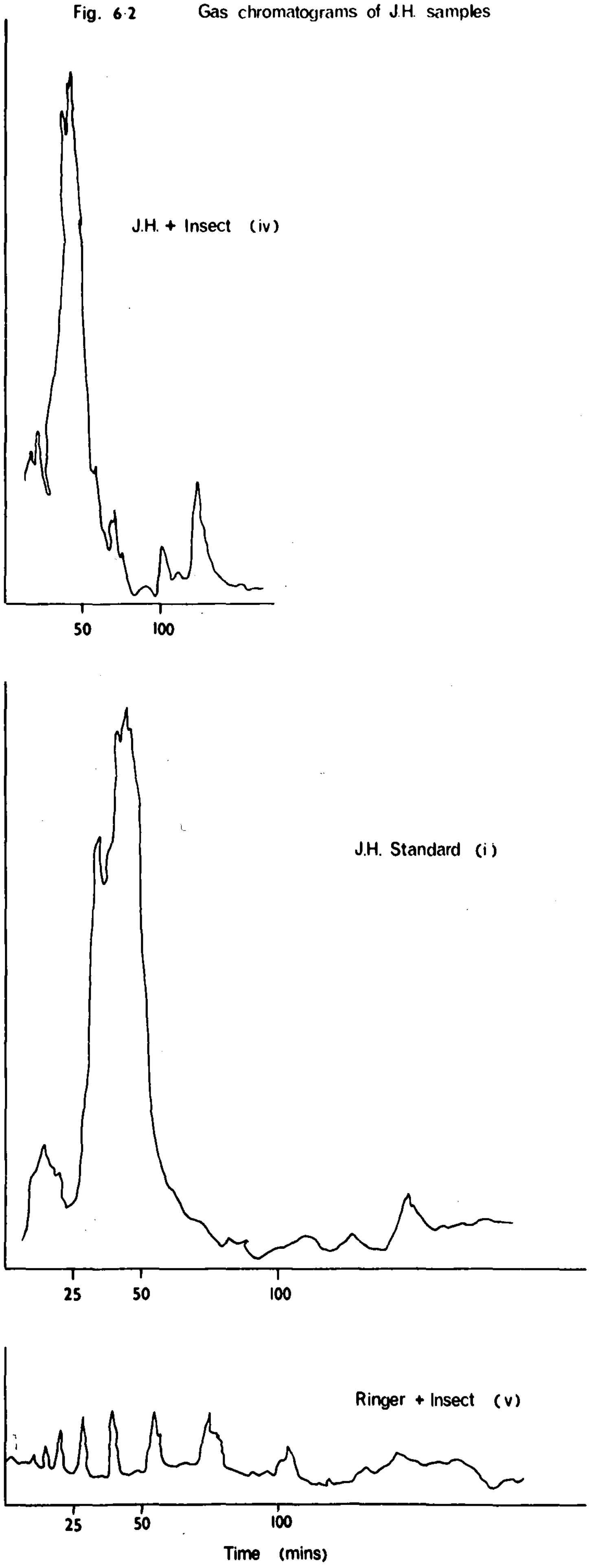
(iii) 'normal' Ringer solution containing J.H. (500µg/ml)

(iv) 'normal' Ringer solution containing J.H. in which a Malpighian tubule preparation had been soaked (30 mins)

(v) 'normal' Ringer solution in which a tubule preparation had been soaked (30 mins).

Analysis of the gas chromatograms showed that the synthetic J.H. standard sample gave 3 major peaks with retention times of 36, 44 and 49 minutes as well as 3 smaller peaks with retention times of 10, 16 and 20 minutes (Fig. 6.2). Analysis of sample (iv) above also showed these same peaks (Fig. 6.2). The result of a typical comparison can be seen

Fig. 6·2 Gas chromatograms of J.H. samples



in Table 6.3. It can be seen from Figure 6.2 that there are no extra peaks corresponding to products with shorter retention times than the J.H. standard, as may have been expected if the J.H. was being degraded. It would seem therefore that there is no evidence to suggest that J.H. is being appreciably broken down, whilst bathing the Malpighian tubules.

Table 6.3 <u>The analysis of J.H. using gas chromatography</u>

Peak retention time (mins)	% total composition	
	J.H. (i.e.(i)&(iii) above	J.H.+ insect (i.e. (v) above
10	1.4	2.1
16	2.5	3.5
20	2.7	5.2
36	23.0	27.7
44	33.0	28.4
49	38.0	32.9

4. The effect of Juvenile Hormone on the ultrastructure of the Malpighian tubules

Malpighian tubules which had been soaked for 30 minutes in 'normal' Ringer solution were compared with tubules which had been soaked for 30 minutes in either Ringer solution containing J.H. (500μg/ml) or Ringer solution containing ethanol (10μl/ml Ringer) using electron microscopy.

It can be seen from Plate 6.1, which is typical of both J.H. and ethanol treatment, that there is no difference between this

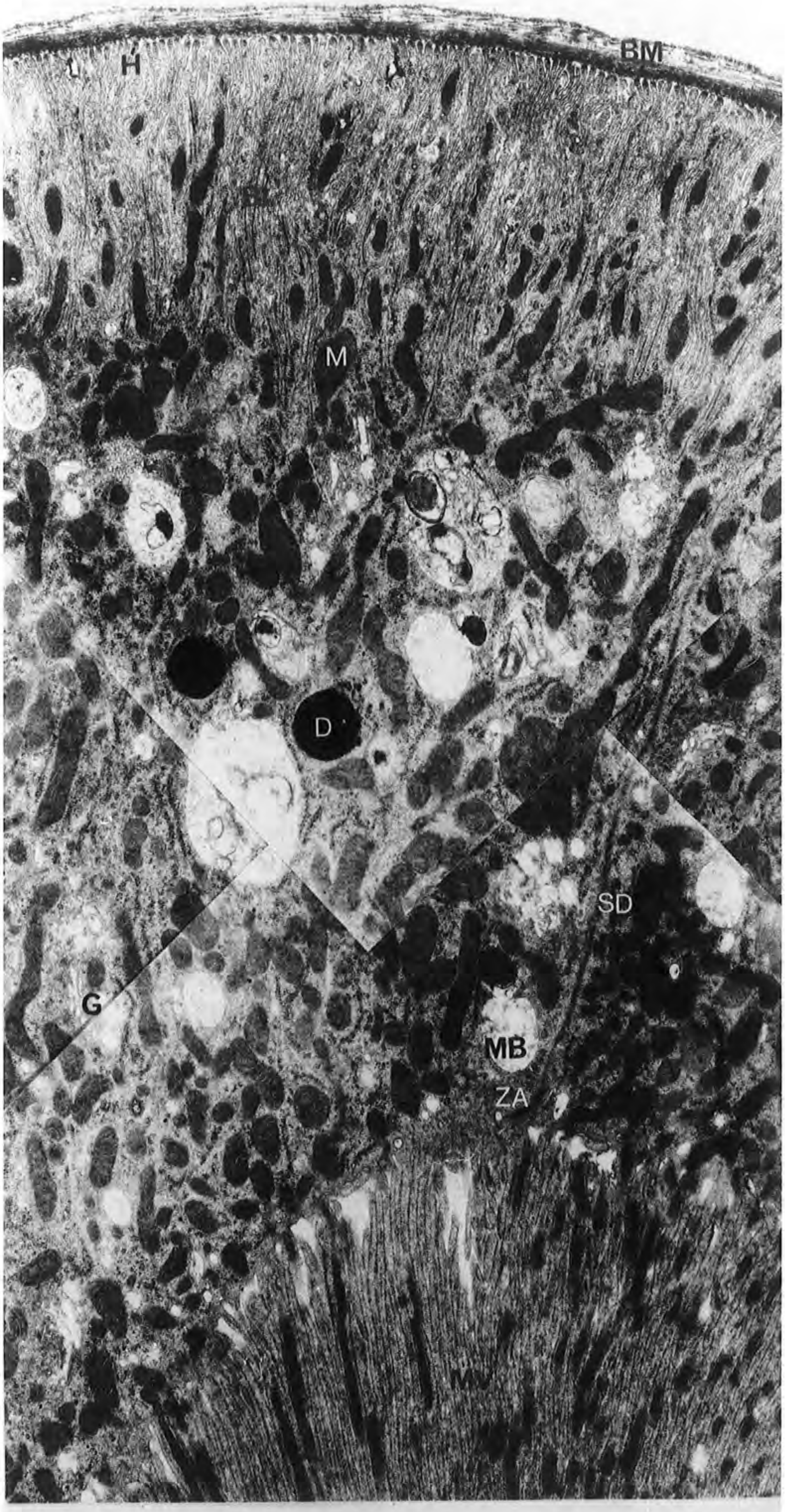

BM
H
M
D
SD
G
MB
ZA
Mv

Plate 6.1

Transmission electronmicrograph of a transverse section through a Malpighian tubule primary cell. This shows the typical appearance of a tubule which has been soaked in either Juvenile Hormone or ethanol. It can be seen that neither of these treatments has any effect on the fine structure of the tubule cells. The appearance is no different from the normal ultrastructure described in Chapter 3. The basal infoldings (Bl) and apical microvilli (MV) have a normal appearance as do the mitochondria (M).

Scale = 1μm

from the normal Malpighian tubule fine structure described in Chapter 3. The effect of J.H. on fluid secretion by the Malpighian tubules cannot therefore be due to hormonally induced changes in cellular fine structure as revealed by electron microscopy.

5. The effect of ecdysone, cholesterol and cyclic AMP on fluid secretion by the Malpighian tubules of *Locusta*

Malpighian tubule preparations were set up as described previously (Chapter 2). The rate of fluid secretion was determined over a first 35 minute period with the insect bathed in 'normal' Ringer solution. This was then replaced with either fresh 'normal' Ringer solution or Ringer solution containing ecdysone (100μg/ml), cholesterol (approx. 5×10^{-6}M), or cyclic AMP (10^{-3}M) and the preparation equilibrated for 15 minutes before determining the second rate of fluid secretion.

To study the effect of cyclic AMP + cholesterol or cyclic AMP + ecdysone, the first rate of fluid secretion was determined with the preparation bathed in 'normal' Ringer solution containing cyclic AMP (10^{-3}M). This was then replaced with either 'normal' Ringer solution + cyclic AMP + cholesterol or 'normal' Ringer solution + cyclic AMP + ecdysone, and the second rate determined.

The results (Table 6.4) show that neither ecdysone nor cholesterol have any effect on fluid secretion by the Malpighian tubules either when applied alone or in the presence of cyclic AMP. It can be seen however that cyclic AMP causes a marked stimulation in the rate of fluid secretion, the rate in the presence of cyclic AMP being 212% of that in 'normal' Ringer solution.

Table 6.4 The effect of ecdysone, cholesterol and cyclic AMP on fluid secretion, *in vitro*

Treatment	n	mean rate fluid secretion % original rate ± S.E.	P
control	20	91.8 ± 6.0	not sig.
ecdysone (100μg/ml)	14	110.4 ± 15.9	not sig.
cholesterol ($5x10^{-6}$M)	16	89.7 ± 8.9	not sig.
cyclic AMP (10^{-3}M)	14	212.7 ± 38.2	<0.01
cyclic AMP + cholesterol	17	102.8 ± 6.0	not sig.
cyclic AMP + ecdysone	17	103.2 ± 7.9	not sig.

Values for P were obtained by comparing Rate 1 and Rate 2 of fluid secretion in a paired 't' test. The 100% rate of fluid secretion was 3.7 ± 0.4nl/min.

6. The effects of corpora cardiaca extract and neurosecretory cell extract on fluid secretion by the Malpighian tubules of *Locusta*

Malpighian tubule preparations were set up as described in Chapter 2. The rate of fluid secretion was determined over an initial 35 minute period with the insect bathed in 'normal' Ringer solution. This Ringer solution was then replaced with either fresh 'normal' Ringer solution (the control) or 'normal' Ringer solution containing crude corpora cardiaca extract (1 gland pair/ml Ringer solution) or 'normal' Ringer solution containing neurosecretory cell extract (N.S.C. from one protocerebrum/ml Ringer solution). The preparation was then equilibrated for 10 minutes before measuring the second rate of fluid secretion over a further 35 minute period. The results are shown in Table 6.5.

It can be seen that both neurosecretory cell extract and corpora cardiaca extract have a significant stimulatory effect on fluid secretion by the Malpighian tubules. However, both the extracts were found to be effective only after immediate preparation. Extracts which had been frozen or were more than 2hrs old had no effect on the rate of fluid secretion.

Table 6.5 The effect of corpora cardiaca extract and neurosecretory cell extract on fluid secretion

Treatment	n	mean rate fluid secretion % original rate ± S.E.	P
control	20	91.8 ± 6.0	not sig.
c. cardiaca extract	13	140.3 ± 7.8	<0.001
N.S.C. extract	17	159.3 ± 23.2	<0.05

Values for P were obtained by comparing Rate 1 and Rate 2 of fluid secretion in a paired 't' test. The 100% rate of fluid secretion was 3.5 ± 0.5nl/min.

Conclusions

The current views on the hormonal control of excretion in insects have been outlined in the introduction to this section. Excretion is thought to be regulated by diuretic and anti-diuretic hormones originating from the neurosecretory cells of the protocerebrum. In addition to this Gee *et al.* (1977) have proposed that ecdysteroids may have regulatory functions in insects over and above that of controlling development. There is no previous evidence to suggest that steroid hormones are involved in the control of excretion in insects, but Gee *et al.* (1977) found that ecdysone (10^{-6}M) and ecdysterone (10^{-6}M) had a stimulatory effect on the rate of 'urine' production by *in vitro* preparations of the Malpighian tubules of *Glossina*. In addition, other biologically active steroids, namely cortisol (10^{-6}M), cholesterol (5×10^{-6}M) and aldosterone (10^{-6}M) also had a stimulatory effect. Gee *et al.* (1977) suggest that the steroids may be increasing the rate of secretion by altering the permeability of the basal membrane of the tubule cells. Fluid secretion by the Malpighian tubules of *Glossina* is thought to be generated by the active transport of Na^+ ions (Gee 1976) and in a speculative model Gee (1976) proposes that the diuretic hormone which controls the rate of secretion by the Malpighian tubules (Gee 1975) stimulates rapid secretion by increasing the permeability of the basal membrane to Na^+, thus allowing Na^+ to flow into the cell down its concentration gradient. The influx of Na^+ initiated by the diuretic hormone would then trigger a Na^+ pump on the apical membrane which would secrete Na^+ ions generating the local osmotic gradients necessary for rapid secretion of fluid.

It has already been suggested that ecdysteroids are able to alter the permeability of cell and nuclear membranes to sodium and to potassium (Kroeger and Lezzi 1966). However, Kroeger and Lezzi (1966) suggest that ecdysteroids stimulate the 'sodium pump' of target cells which would lead to an increase in intracellular K^+ concentrations, a situation which would not be expected to lead to increased fluid secretion by the tubules of *Glossina*.

β-ecdysone, like vertebrate steroid hormones, is synthesized from cholesterol, and cholesterol has also been found to affect the permeability of cell membranes (Szabo 1974). From work on cholesterol-containing monoolein bilayers Szabo (1974) found that cholesterol altered membrane permeability by affecting the potential difference across the membrane-solution interface and by affecting the fluidity of the membrane interior, thereby changing the rate of ionic transfer.

In the present study on *Locusta* neither β-ecdysone (100μg/ml) nor cholesterol (5×10^{-6}M) had any effect on the rate of fluid secretion by *in vitro* preparations of *Locusta* Malpighian tubules.

In *Locusta,* K^+ is the important transported cation for the generation of fluid secretion (Ramsay 1953, 1954; Berridge 1968). In a speculative model (see Chapter 4) for fluid secretion by the Malpighian tubules of *Calliphora,* Berridge and Oschman (1969) propose that a Na^+/K^+ exchange pump on the basal cell membrane supplies K^+ to an electrogenic pump on the apical membrane. The secretion of K^+ into the tubule lumen is then thought to be responsible for generating urine flow. The involvement of a Na^+/K^+ exchange pump in the mechanism of fluid secretion by *Locusta* Malpighian tubules has been shown previously (Anstee and Bell 1975; Anstee *et al.* 1979; and in present study). From the suggestion of Kroeger and Lezzi (1966) that β-ecdysone stimulates the Na^+/K^+ pump it may be expected that application of ecdysone would lead to an increased rate of fluid secretion by the tubules of *Locusta.* However, no such effect was observed and neither this present work nor that of Gee *et al.* (1977) can lend support to the proposal of Kroeger and Lezzi (1966). Gee *et al.* (1977) however quote Bernstein and Mordue (unpublished results) as finding both ecdysterone and cholesterol to have a stimulatory effect on secretion in *Locusta.* It is difficult to understand the difference between these results and those obtained in the present study but they may be due to differences in experimental technique which it is impossible to compare until the results are published.

In vertebrates it is generally agreed that steroid hormones do not act through cyclic AMP (see however Szego and Davis 1967), but there is, in insects, some evidence to connect the action of β-ecdysone and cyclic AMP. The importance of cyclic AMP in hormone action was first recognized by Sutherland and Rall (1958) and since then cyclic AMP has been implicated in cellular control mechanisms in a variety of organisms ranging from bacteria to mammals (Robison *et al.* 1968, 1971). A concept has been developed whereby many hormones act by way of a two messenger system. The hormone, the first messenger, circulates in the blood, binds to the plasma membrane of the target cell and activates adenyl cyclase. Cyclic AMP, the second messenger, is generated on the inner surface of the cell membrane and diffuses through the cell, bringing about the appropriate physiological responses. The concentration of cyclic AMP in most cells is determined by the balance which exists between the synthetic activity of adenyl cyclase and the degradative activity of phosphodiesterase.

Leenders *et al.* (1970) found that cyclic AMP (10^{-3}M), although not inducing ecdysone specific puffs in *Drosophila* salivary glands *in vivo*, did enhance the response that was stimulated by β-ecdysone. A similar enhancement of the effect of ecdysone was demonstrated using theophylline (10^{-2}M), an inhibitor of phosphodiesterase, and thus leading to an increase in cyclic AMP. It has also been shown that salivary glands incubated with β-ecdysone had significantly more cyclic AMP than control glands incubated in the absence of the hormone (see Gilbert and King 1973). Additional work has shown that injection of β-ecdysone into saturniid pupae results in a marked increase in total animal cyclic AMP (see Gilbert 1974). It would seem therefore that in addition to enhancing the effect of β-ecdysone at the chromosomal level, cyclic AMP formation is stimulated by β-ecdysone.

β-ecdysone alone has been found to have no effect on fluid secretion by *Locusta* tubules but it was possible that addition of cyclic AMP may enhance an effect of ecdysone. However, no synergistic effect of cyclic AMP and β-ecdysone was observed. β-ecdysone had no effect on the rate of 'urine' production either in the presence or absence of cyclic AMP (10^{-3}M). However it was found that cyclic AMP, alone, had a marked stimulatory effect on the rate of fluid secretion. This is in accordance with results published by several other workers. Cyclic AMP has been shown to stimulate fluid secretion by Malpighian tubules of both *Carausius* (10^{-4}M) and *Rhodnius* (4×10^{-5}M) (Maddrell *et al.* 1971) as well as *Locusta* (Anstee *et al.* 1979) and *Schistocerca gregaria* (Maddrell and Klunsuwan 1973). In fact it is proposed that the insect diuretic hormone may produce an increased intracellular cyclic AMP level which would then elicit increased fluid secretion (Maddrell *et al.* 1971). In support of this, Aston (1975) has shown an increase in cyclic AMP levels during stimulation of *Rhodnius* Malpighian tubules by the diuretic hormone.

In the present study on *Locusta*, 10^{-3}M cyclic AMP was found to substantially increase the rate of fluid secretion by *in vitro* tubule preparations. In all cases studied, a relatively high concentration of cyclic AMP has been found necessary to activate secretion when applied exogenously (intracellular levels are in the order of 10^{-8}M - 10^{-6}M - Butcher and Sutherland 1962). This is thought to be due in part to the relative impermeability of the cell to cyclic AMP. A high concentration is therefore necessary to raise the intracellular level sufficiently to stimulate secretion. Also, since phosphodiesterase is continually hydrolysing cyclic AMP, sufficient cyclic AMP must enter the cell to combat this process.

Electrophysiological studies on the salivary glands of *Calliphora* have suggested that increasing cyclic AMP levels within the cells causes increased cation transport (Berridge and Prince 1972a,b). Similar studies on the Malpighian tubules of *Locusta* also suggest that cyclic AMP may act by stimulating cation transport (Bell 1977).

β-ecdysone, like the insect diuretic hormone, has been shown to stimulate cyclic AMP formation (Leenders *et al*. 1970; Aston 1975) and this may provide an explanation for the stimulatory effect of β-ecdysone on fluid secretion found by some workers. Instead of directly affecting cell permeability as proposed by Kroeger and Lezzi (1966), β-ecdysone may increase cation transport indirectly by way of increased cyclic AMP levels.

Present results confirm that there are diuretic factors present in the median neurosecretory cells of the protocerebrum and the corpora cardiaca of *Locusta*. Extracts from the neurosecretory cells and the corpora cardiaca produced marked stimulation in the rate of fluid secretion by *in vitro* preparations of the Malpighian tubules. Previously it had been shown that either cautery or removal of the cerebral neurosecretory cells resulted in the retention of water and a decrease in the rate of amaranth excretion by *Locusta* (Highnam *et al*. 1965; Cazal and Girardie 1968; Mordue 1966, 1969). And, Mordue (1969) found that corpora cardiaca extract significantly increased the rate of amaranth excretion through the Malpighian tubules. However, Cazal and Girardie (1968) report a strong anti-diuretic action of extracts of the corpora cardiaca on the Malpighian tubules of *Locusta*. The presence of an anti-diuretic factor within the corpora cardiaca has been confirmed (Mordue 1970, 1972; Goldsworthy and Mordue 1972), but is confined to the glandular lobe. Extracts from the storage lobe have a marked diuretic effect (Mordue 1972).

In the study of Cazal and Girardie (1968) the corpora cardiaca extract was prepared from the whole gland and it is possible that any diuretic effect may have been masked. In the present study too, the corpora cardiaca extract was prepared from the whole gland and it is possible that even greater stimulation of the rate of fluid secretion may have been observed if the storage lobes alone had been used.

There are no previous reports in the literature of the effect of Juvenile Hormone on fluid secretion by *in vitro* preparations of Malpighian tubules although there is some evidence to suggest the presence of an "anti-diuretic principle" in the corpora allata (Wall and Ralph 1964, Beenakkers and Van Den Broek 1974). Present results show that synthetic J.H. inhibits the rate of 'urine' production in *Locusta*. The degree of inhibition was found to increase with increasing J.H. concentration over the range 5µg/ml - 5mg/ml until a maximum of around 50% inhibition was reached. J.H. was found to be only poorly soluble in aqueous solution and this may account for the maximum of 50% inhibition obtained. (Fristrom and Kelly (1976) determined the maximum concentration of J.H. in solution as 15µg/ml.)

Exogenous J.H. has been found to be hydrolysed by esterases in the Malpighian tubules of *Prodenia eridania* and *Hyalophora cecropia*. The metabolism of J.H. by tissues can also be fairly rapid. Chihara *et al.* (1972) have shown that 75% of the J.H. applied exogenously to imaginal discs of *Drosophila* had been metabolised after a period of 3 hours. However, in the present study it was found that the J.H. was not appreciably broken down whilst bathing the Malpighian tubules (30 mins). No degradation products were observed after analysis by gas chromatography. This would seem to suggest, at least at high J.H. concentrations, that a true effect of J.H. and not its metabolites was being observed.

Studies on the ionic composition of the 'urine' secreted in the presence and absence of J.H. showed the K^+ content to be significantly lower after J.H. treatment. This would tend to support the idea that J.H. affects cation transport. Lezzi and Gilbert (1972) have proposed that J.H. acts to increase intracellular Na^+ concentrations by inhibiting the Na^+/K^+ exchange pump of cell membranes. The involvement of such a pump in fluid secretion by *Locusta* Malpighian tubules has already been established from the sensitivity of the secretory process to ouabain (see Chapter 4). A similarity in the actions of J.H. and ouabain has previously been reported by Chase (1970). Injection of ouabain into *Tenebrio* pupae resulted in the formation of larval-pupal intermediates, as did J.H. treatment. Since ouabain is known to specifically inhibit the cell membrane Na^+/K^+ pump (Schatzmann 1953) it would cause an increase in internal Na^+ concentrations which is consistent with the proposed action of J.H. (Lezzi and Gilbert 1972).

In the present study a synthetic J.H. has been used and at this stage it is perhaps unwise to conclude that the observed effect of J.H. on fluid secretion is a true physiological response rather than a pharmacological effect. Nevertheless, Wall and Ralph (1964) have suggested that an 'anti-diuretic principle' may be stored in the corpora allata of *Periplaneta*. And, Beenakkers and Van Den Broek (1974) found that a high titre of corpus allatum hormone resulted in a high water content in *Locusta* whereas allatectomy reduced this content. These physiological responses are certainly consistent with the *in vitro* results reported in the present study.

The results presented above confirm the presence of a diuretic principle in the neurosecretory cells and the corpora cardiaca of *Locusta*. The fact that cyclic AMP also stimulates fluid secretion suggests the possibility that this cyclic compound may be involved as a secondary messenger in the endocrine control of tubule secretion by diuretic hormone. Ecdysone was found to have no effect on tubule fluid secretion whilst the latter was substantially inhibited by J.H. It is possible that J.H. may be exerting this inhibition by acting on the Na^+/K^+ pump in a manner similar to ouabain (Chase 1970).

SECTION II

Hormonal effects on the Na^+, K^+-activated ATPase activity in microsomal preparations of the Malpighian tubules

INTRODUCTION

Most animal cells maintain intracellular K^+ at a relatively high and constant concentration (120-160mM) whereas the intracellular Na^+ concentration is much lower (less than 10mM). A substantial gradient of K^+ and Na^+ exists across the cell membrane since the extracellular fluid contains a relatively high concentration of Na^+ (about 150mM) and a low concentration of K^+ (less than 4mM). The high internal K^+ concentration is maintained by the energy-requiring extrusion of Na^+ from the cell in exchange for K^+, promoted by an active transport system. It has been established that this active transport of Na^+ and K^+ across cell membranes is associated with the splitting of ATP by the membranes and is inhibited by the cardiac glycoside ouabain (Schatzmann 1953). Skou (1957) fractionated a crab nerve homogenate into a microsomal component which exhibited ATPase activity that was stimulated by the addition of Na^+ and K^+ in the presence of Mg^{2+}. Moreover, the stimulation of the ATPase activity by Na^+ and K^+ was inhibited by the cardiac glycoside, ouabain, already known to inhibit the transport of Na^+ and K^+ across cell membrane. Since then cell membrane fractions from many different animal species have been found to contain such a Na^+, K^+-activated ATPase. Nervous tissue is a very rich source of the enzyme (Nakao *et al.* 1965) as is vertebrate kidney (Skou 1962). Na^+, K^+-activated ATPase from insect tissues has been shown in homogenates of cockroach nerve cord (Grasso 1967), cockroach muscle (Koch *et al.* 1969), honeybee C.N.S. (Cheng and Cutkomp 1972), Malpighian tubules and hindgut of *Homorocoryphus* (Peacock *et al.* 1976), cockroach rectum (Tolman and Steele 1976) and *Locusta* Malpighian tubules (Anstee and Bell 1975, 1978).

In order to understand the mechanism of the Na^+ and K^+ active transport system of cell membranes, many workers have studied the reaction sequence of the Na^+, K^+-activated ATPase which represents the activity of the pump in preparations of broken membranes. Studies of this nature have led to the proposal of a model for the reaction sequence of the Na^+, K^+-activated ATPase and its incorporation into a model for Na^+ and K^+ transport.

It has been postulated that the Na^+, K^+-activated ATPase hydrolyses ATP in a stepwise fashion involving Na^+- dependent phosphorylation of the enzyme and K^+-dependent hydrolysis of the phospho-enzyme. Several lines of investigation support the following sequence of reactions (see Schwartz *et al.* 1975):

$$(1)\quad E_1 + ATP + \underset{\text{inside}}{Na} \xrightleftharpoons{Mg^{2+}} E_1 - P(Na^+) + ADP$$

$$(2)\quad E_1 - P\ (Na^+) + Mg^{2+} \rightleftharpoons E_2 - P + \underset{\text{outside}}{Na^+}$$

$$(3)\quad E_2 - P + \underset{\text{outside}}{K^+} \rightleftharpoons E_2\ (K^+) + P_i$$

$$(4)\quad E_2\ (K^+) \rightleftharpoons E_1 + Mg^{2+} + \underset{\text{inside}}{K^+}$$

This model proposes that the enzyme undergoes conformational changes between the two forms designated E_1 and E_2. For if Na^+ and K^+

are moved through the membrane, it is difficult to envisage how such movements could take place without an alteration or series of alterations in the structure of the system catalysing these transmembrane movements (Schwartz *et al*. 1975). It is postulated that the hydrolysis of ATP by the Na^+, K^+-activated ATPase involves the transfer of phosphate to a group or groups in the enzyme before its ultimate transfer to water. Evidence has been presented to show that if membrane fragments are exposed to ^{32}P-ATP in the presence of Na^+ and Mg^{2+}, ^{32}P is incorporated into the membrane and can be released as inorganic phosphate on addition of K^+ (Charnock *et al*.1963; Fahn *et al*.1968; Hokin *et al*.1965). The properties of the bound phosphate were found to be those of an acyl phosphate.

As already mentioned, it has been well established that the Na^+, K^+-activated ATPase is inhibited by cardiac glycosides (Skou 1957) and much work has been carried out to determine the exact mechanism of this inhibition. The study of inhibitory effects on isolated enzyme reactions has been shown to be of great importance in establishing the nature of the free reactants, the nature of their binding site on the enzyme and the specificity and mechanism of the reaction. Study of enzyme kinetics in the presence of inhibitors yields characteristic results according to the type of inhibition. Lineweaver-Burk plots of reciprocal activity $(\frac{1}{V})$ against the reciprocal of the substrate concentration $(\frac{1}{S})$ may show alterations in the slope (competitive inhibition), the intercept (uncompetitive inhibition) or both (non-competitive inhibition). In the case of competitive inhibition the inhibitor can combine with the free enzyme in such a way that it competes with the normal substrate for binding at the active site. Competitive inhibition can be recognized experimentally because the percent inhibition at a fixed inhibitor concentration is decreased by increasing the substrate concentration. In uncompetitive inhibition the inhibitor does not combine with the free enzyme or affect its reaction with the normal substrate, but it does combine with the enzyme-substrate complex to give an inactive enzyme-substrate-inhibitor complex which cannot undergo further reaction to yield the normal products. A non-competitive inhibitor can combine with either the free enzyme or the enzyme-substrate complex, interfering with the action of both. Non-competitive inhibitors bind to a site on the enzyme other than the active site, often to deform the enzyme, so that it does not form the ES complex at its normal rate, and, once formed, the ES complex does not break down at the normal rate to yield the products.

These diagnostic features normally apply to simple, one substrate reactions whereas the Na^+, K^+-activated ATPase is a complex reaction. However, they have been used in the present study, and by other workers (Jenner and Donnellan 1976), to simplify the situation in an attempt to determine the nature of the effects of ouabain and insect hormones on the Na^+, K^+-activated ATPase.

MATERIALS AND METHODS

1. To determine the effect of Juvenile Hormone and β-ecdysone on Na^+, K^+-activated ATPase activity

Na^+, K^+-activated ATPase activity was assayed as described in Chapter 2 using the following ionic media:

(i) 4mM Mg^{2+},

(ii) 4mM Mg^{2+}, 100mM Na^+, 20mM K^+,

all buffered in 50mM Histidine-HCl pH 7.2. The effect of J.H. or β-ecdysone on ATPase activity was determined by addition of 10μl ethanolic J.H. or aqueous ecdysone to the above ionic media.

2. To determine the effect of corpora cardiaca extract on Na^+, K^+-activated ATPase activity

Na^+, K^+-activated ATPase activity was assayed as described above using media (i) and (ii) and a third medium with the following composition: 4mM Mg^{2+}, 50mM Na^+, 5mM K^+ (after Peacock 1976).

Corpora cardiaca were dissected out of freshly killed mature male locusts and homogenised in medium (i) above, immediately before use. The volume of homogenate was such that 10µl added to the reaction media was equivalent to the addition of 1 gland pair.

RESULTS

1. The effect of Juvenile Hormone and β-ecdysone on Na^+, K^+-activated ATPase activity

(i) Synthetic J.H.

Na^+, K^+-activated ATPase activity in microsomal preparations of the Malpighian tubules was assayed as described above. The effect of J.H. was determined by the addition of 0-250µg/ml (final concentration) J.H. to the reaction media. The results of a typical experiment are shown in Figure 6.3, and Table 6.6 shows the mean data of 4 experiments. It can be seen that J.H. inhibits Na^+, K^+-activated ATPase activity; the degree of inhibition increasing as the J.H. concentration increased. Maximum inhibition was ca. 50% and this was obtained with 250µg/ml J.H. Ethanol alone was found to have no effect on the Na^+, K^+-activated ATPase activity at the concentration used.

Figure 6.3 The effect of synthetic Juvenile Hormone on Na^+, K^+-activated ATPase activity

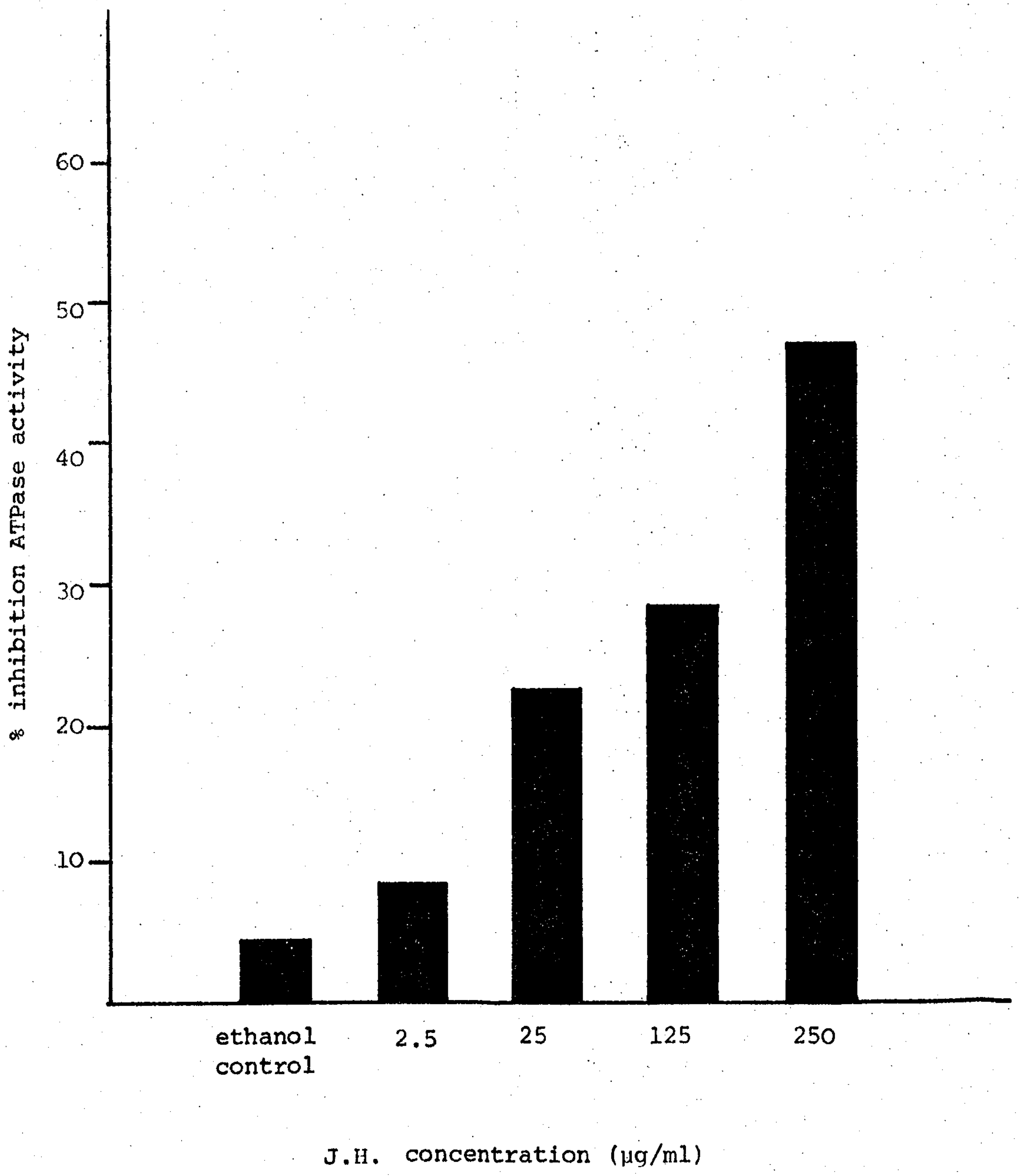


Table 6.6 The effect of Juvenile Hormone and β-ecdysone on Na^+, K^+-activated ATPase activity

Treatment	n	mean % ATPase activity remaining ± S.E.	P
a. control	4	100%	
b. ethanol	4	94.5 ± 3.8	a:b not sig.
c. J.H. 2.5µg/ml	4	90.9 ± 5.1	b:c not sig.
d. J.H. 25µg/ml	4	78.5 ± 2.9	b:d <0.02 c:d <0.05
e. J.H. 125µg/ml	4	65.9 ± 5.0	b:e <0.01 c:e <0.02 d:e <0.05
f. J.H. 250µg/ml	4	48.8 ± 7.2	b:f <0.002 c:f <0.01 d:f <0.01 e:f <0.05
g. β-ecdysone 50µg/ml	3	97.3 ± 1.73	a:g not sig.

Values for P were obtained by comparing the mean result of each treatment in a students 't' test.

(ii) β-ecdysone

The effect of β-ecdysone on the Na^+, K^+-activated ATPase in microsomal preparations of the Malpighian tubules was determined at a concentration of 50µg/ml. The mean result of three experiments is shown in Table 6.6. It can be seen that, unlike J.H. β-ecdysone has no effect on ATPase activity.

(iii) To compare the effects of synthetic J.H., J.H.I., J.H.II and J.H. III

As well as the synthetic J.H. used commonly in the experiments described in this chapter, samples of J.H. I (m.w. 294.4), J.H. II (m.w. 280.4) and J.H. III (m.w. 266.4) were tested. Na^+, K^+-activated ATPase activity was assayed as described previously (Chapter 2) in assay media containing one of the following compounds: synthetic J.H. 250μg/ml; J.H. I 50μg/ml; J.H. II 16.5μg/ml; J.H. III 16.5μg/ml. The results are shown in Table 6.7.

Table 6.7 The effect of synthetic J.H., J.H. I, J.H. II, and J.H. III on Na^+, K^+-activated ATPase activity

Treatment	n	% ATPase activity remaining
Synthetic J.H. 250μg/ml	4	45.8 ± 7.2
J.H. I 50μg/ml	4	68.8 ± 4.9
J.H. II 16.5μg/ml	2	1. 75.7 2. 75.2
J.H. III 16.5μg/ml	2	1. 86.3 2. 92.0

It can be seen that in all cases the J.H. had an inhibitory effect on Na^+, K^+-activated ATPase activity.

2. The effect of varying the K^+ concentration on Juvenile Hormone and ouabain inhibition of the Na^+, K^+-activated ATPase

Na^+, K^+-activated ATPase was assayed as described previously (Chapter 2), in reaction media containing 100mM Na^+ and 4mM Mg^{2+} but with K^+ concentrations ranging between 1.5-20.0mM. The effect of J.H. was determined at a concentration of 250μg/ml and that of ouabain at 10^{-6}M.

(i) Juvenile Hormone

The result of a typical experiment is shown in Figure 6.4 in the form of a Lineweaver-Burk plot of the reciprocal of the activity $(\frac{1}{V})$ against the reciprocal of the K^+ concentration $(\frac{1}{S})$. The lines were drawn by regression analy sis and the values of apparent K_m and V_{max} calculated from the graph. Table 6.8 shows the mean data of 3 experiments. These results show that the presence of Juvenile Hormone in the reaction medium decreases both apparent K_m and V_{max}, a result typical of uncompetitive inhibition.

Table 6.8 K_m and V_{max} values with respect to K^+ for Na^+, K^+-activated ATPase in the presence and absence of J.H.

Treatment	n	K_m (mM) mean ± S.E.	V_{max} (nmoles P_i/mg protein/min
Control	3	4.4 ± 0.2	648.9 ± 58.9
+ J.H. 500µg/ml	3	2.76± 0.4	357.5 ± 38.3
P		0.02	<0.02

(ii) Ouabain

The result of a typical experiment is shown in Figure 6.5 in the form of a Lineweaver-Burk plot and Table 6.9 shows the mean result of 3 experiments. It can be seen that ouabain causes a decrease in V_{max} but has no significant effect on apparent K_m, a result typical of a non-competitive inhibitor.

Figure 6.4 Lineweaver-Burk plot of Na^+, K^+-activated ATPase activity against K^+ concentration

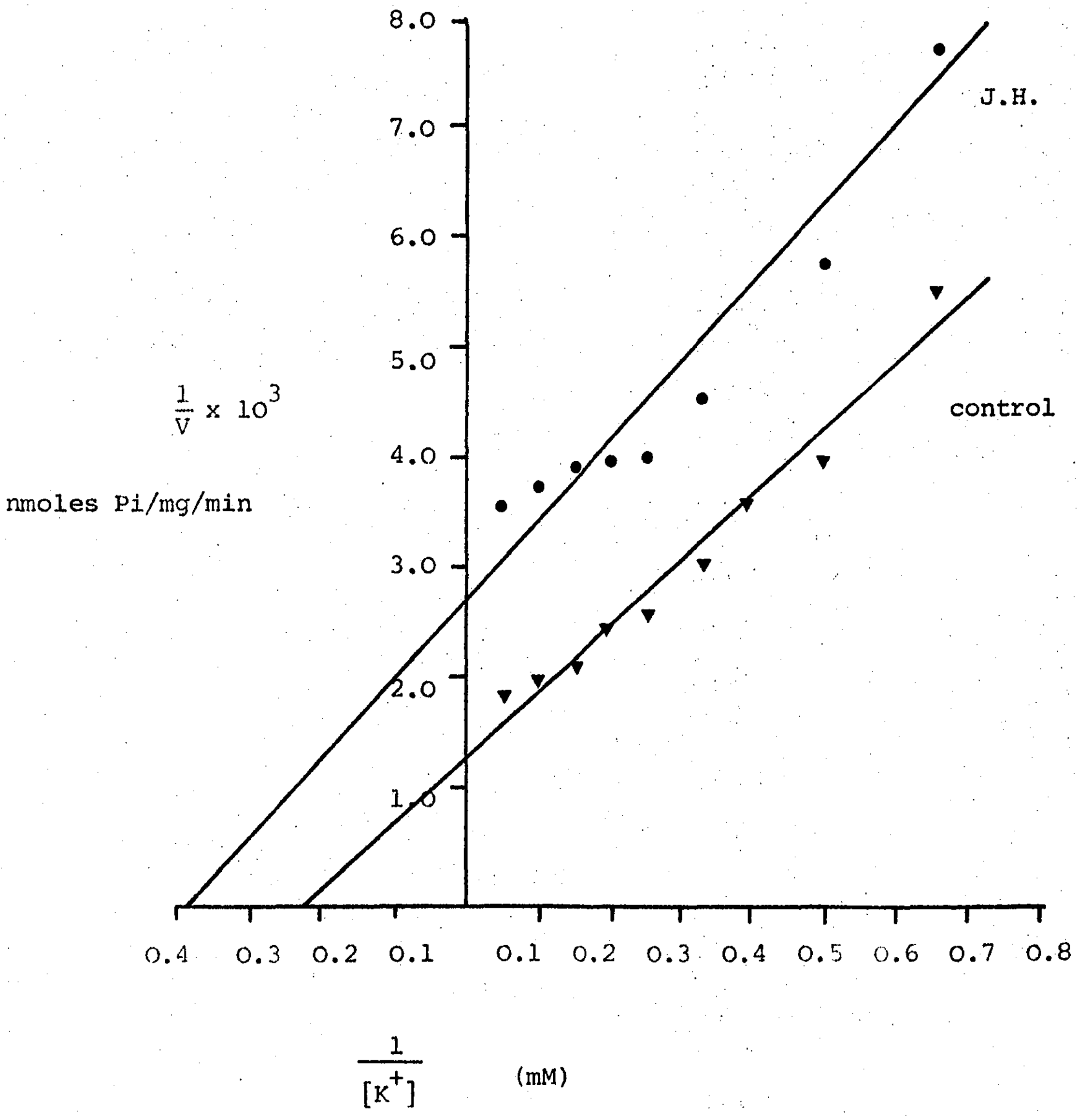


Figure 6.5 Lineweaver-Burk plot of Na^+, K^+-activated ATPase activity against K^+ concentration

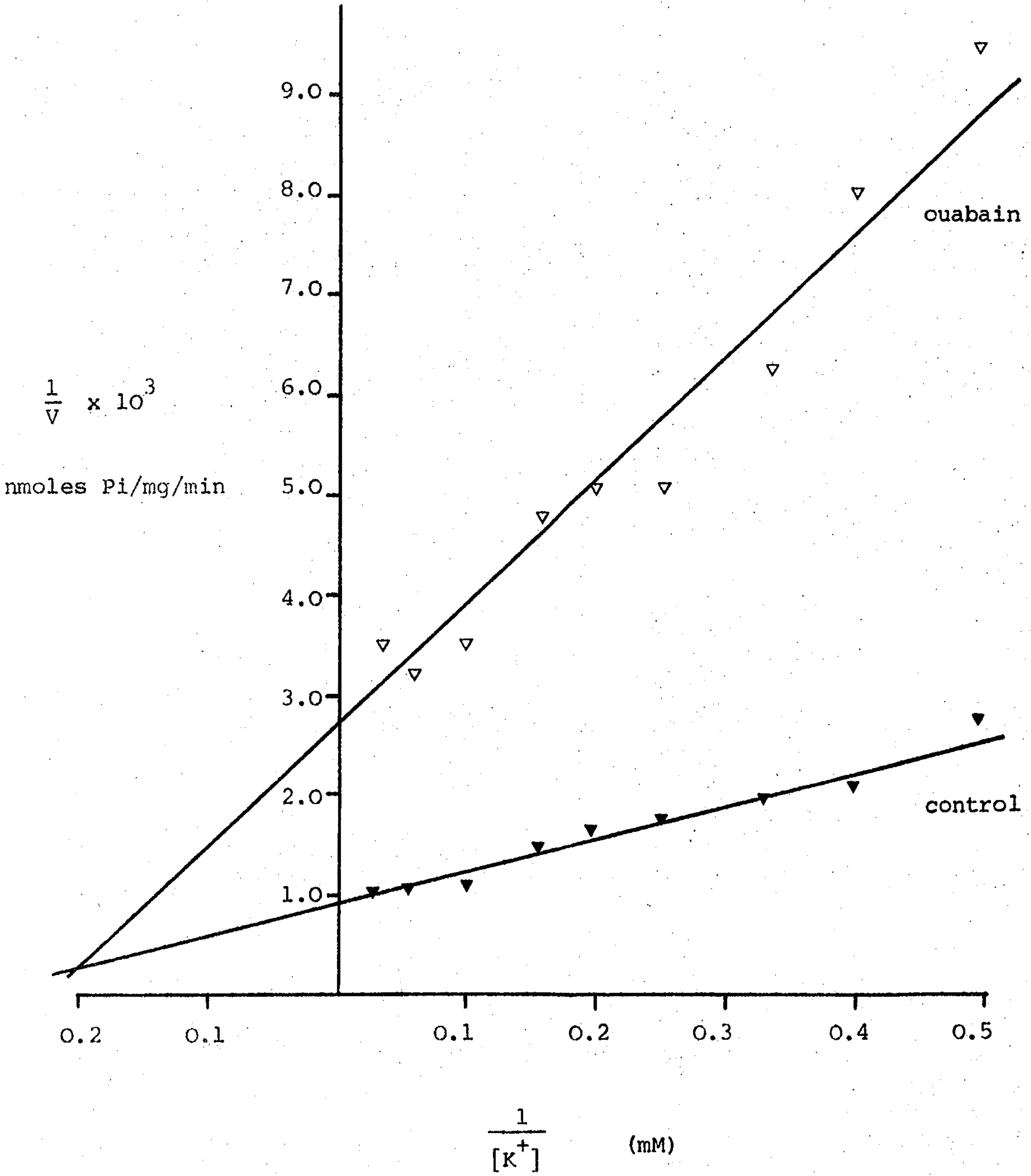


Table 6.9 K_m and V_{max} values with respect to K^+ for Na^+, K^+-activated ATPase in the presence and absence of ouabain

Treatment	n	K_m ± S.E. (mM)	V_{max} ± S.E. nmoles Pi/mg protein/min
Control	3	2.69 ± 0.3	751.2 ± 188.5
+ ouabain 10^{-6}M	3	3.5 ± 0.5	246.6 ± 64.6
P		not sig.	<0.05

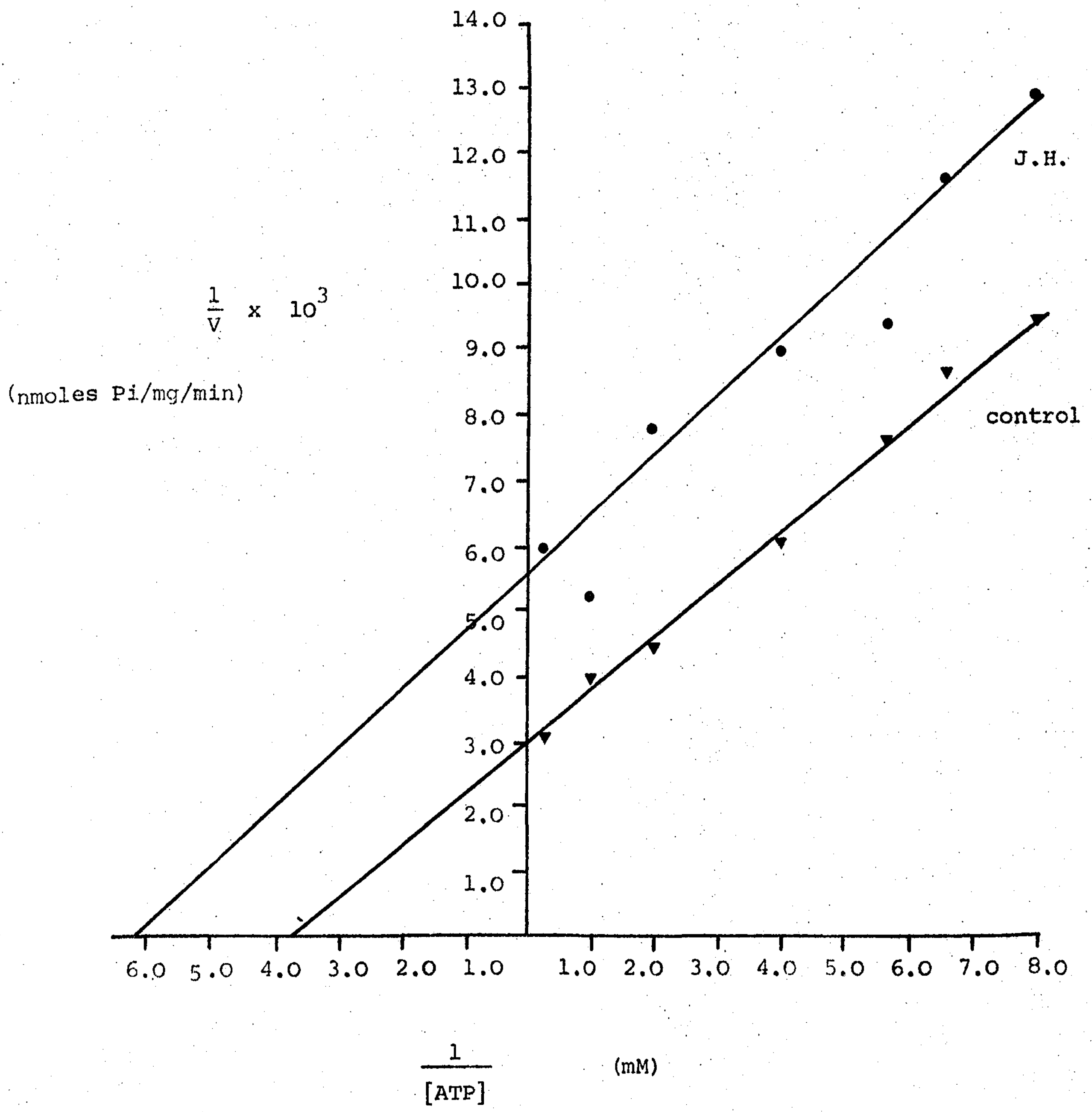
3. The effect of Juvenile Hormone on Na^+, K^+-activated ATPase activity at varying ATP concentrations

Enzyme assays were carried out in reaction media in which the ATP concentration varied from 3mM - 0.125mM. The reaction was allowed to proceed for 15 mins instead of the usual 30 mins to prevent the availability of ATP to the enzyme becoming a rate limiting factor. The results of a typical experiment are shown in Figure 6.6 in the form of a Lineweaver-Burk plot. The lines were drawn by regression analysis and the values of apparent K_m and V_{max} calculated from the graph. Table 6.10 shows the mean result of 4 experiments. It can be seen that the addition of J.H. decreases both the values for apparent K_m and V_{max}, a result typical of an uncompetitive inhibitor.

Table 6.10 Kinetic constants relating to ATP concentration in the presence and absence of J.H.

Treatment	n	K_m (mM)	V_{max} nmoles Pi/mg protein/min
Control	4	0.26 ± 0.04	559.8 ± 126.2
+ J.H. 500μg/ml	4	0.14 ± 0.02	282.0 ± 44.1
P		<0.05	0.05

Figure 6.6 Lineweaver-Burk plot of Na^+, K^+-activated ATPase activity against ATP concentration



4. The effect of temperature on Juvenile Hormone inhibition of Na^+, K^+-activated ATPase

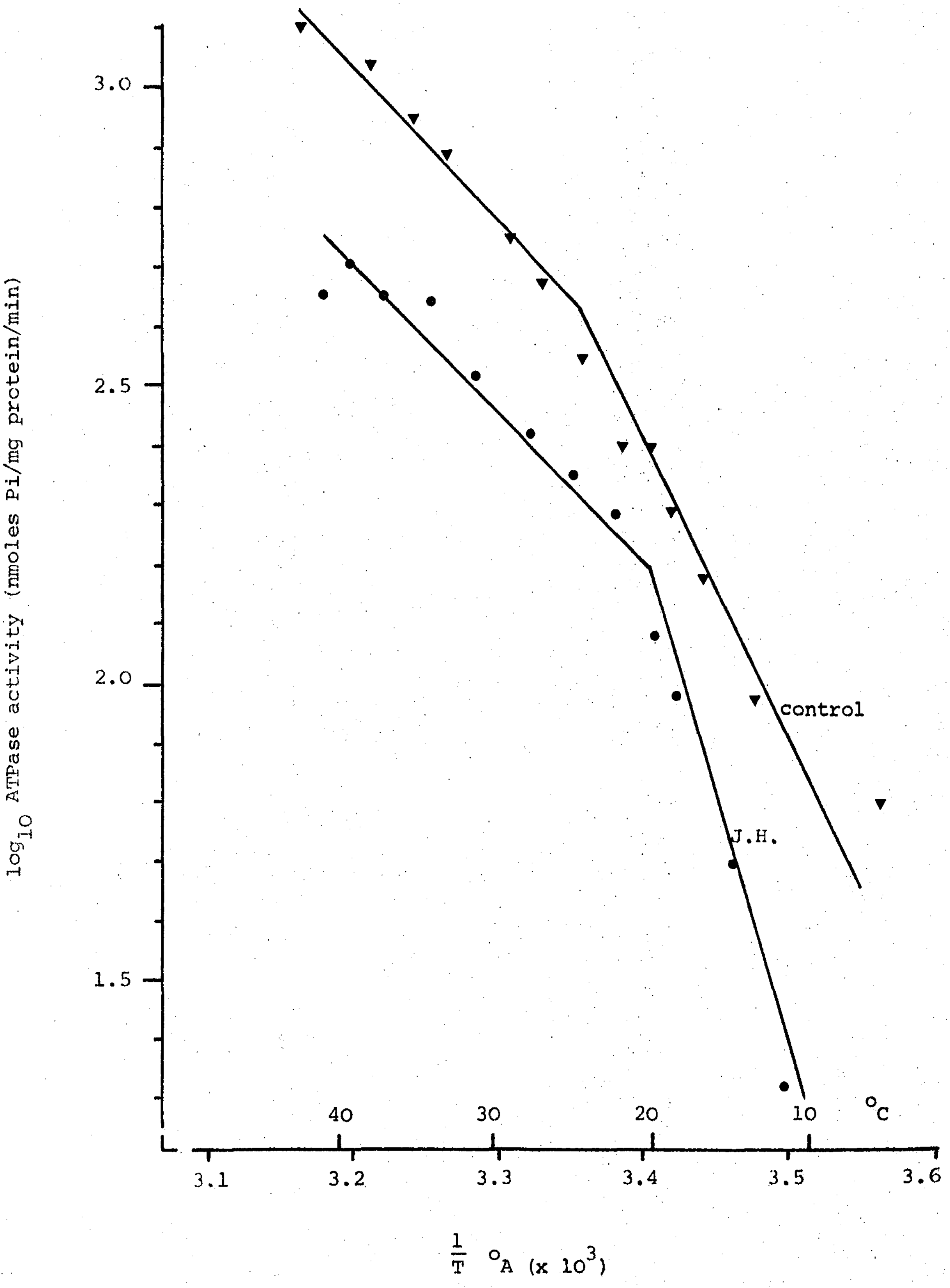
Temperature gradients were set up using a Forbes bar, a thick aluminium bar (1.2 x 0.1 x 0.06M) with a series of water filled holes at short intervals along its length to accommodate the assay tubes. A crushed ice bath at one end and a hot water bath at the other provided a gradient of temperature ranging from 6°C - 44°C. Pairs of tubes, one containing 4mM Mg^{2+}, and 3mM ATP, and the other 4mM Mg^{2+}, 3mM ATP, 100mM Na^+ and 20mM K^+, were arranged along the bar alternately with pairs of tubes containing the same assay media plus J.H. (250µg/ml).

ATPase activity was determined as described previously (Chapter 2) but the reaction was allowed to proceed for differing times depending on the temperature: 60 mins below 17°C; 45 mins from 17°C - 30°C; 30 mins above 30°C.

The results of a typical experiment are shown in Figure 6.7 in the form of an Arrhenius µ plot of Log. activity against the reciprocal of the temperature (°A). It can be seen that the temperature-activity relationship of the Na^+, K^+-activated ATPase is non-linear over the range 10°C - 40°C. The resultant curve has therefore been resolved into 2 straight lines which show a so-called 'break point'. This represents the point around which the Na^+, K^+-activated ATPase undergoes a large change in activation energy. The two lines were drawn by regressing all values above 20°C and drawing a line and then regressing all values below 20°C and drawing a second line. The two lines intersect at the 'break point'.

In the case of the control it can be seen that the critical temperature was 25.3°C whereas when J.H. was present it had shifted to 21.4°C.

Figure 6.7 Arrhenius plot of Na$^+$, K$^+$-activated ATPase activity in presence and absence of J.H.



Activation energies calculated from such a plot are 91.9 and 53.61 K.J. mole^{-1} between 10-21°C and 21-42°C respectively, in the case of the control, and 153.1 and 47.8 K.J. mole^{-1} over the same ranges when J.H. was present.

Activation energies were calculated from the Arrhenius equation:

$$E_a = R \times 2.303 \times \text{slope K.J. mole}^{-1}$$

where R = gas constant, 8.314 K.J./mole/°A.

5. The effect of Corpora cardiaca extract on Na^+, K^+-activated ATPase activity

The effect of crude Corpora cardiaca extract on the Na^+, K^+-activated ATPase activity of microsomal preparations of either the Malpighian tubules or the rectum was determined. This study was approached in two ways:

(1) Under optimal conditions (100 Na^+, 20 K^+, 4 Mg^{2+})
(2) Under sub-optimal conditions (50 Na^+, 5 K^+, 4 Mg^{2+})
(see Peacock 1976).

(1) Optimal conditions

Na^+, K^+-activated ATPase activity was determined as described previously (Chapter 2). The effect of corpora cardiaca extract was determined by the addition of the equivalent of 1 gland pair to the assay medium. The results of experiments on microsomal preparations of the Malpighian tubules and rectum of *Locusta* can be seen in Table 6.11.

Table 6.11 The effect of Corpora cardiaca extract on Na^+, K^+-activated ATPase activity

	Mg^{2+}-dependent ATPase activity		Na^+, K^+-activated ATPase activity			Calculated activity in presence of C.C. extract
			from Malpighian tubules or rectum		from C.C.extract	
	+ C.C.	- C.C.	+ C.C. (a)	- C.C. (b)	(c)	(b + c)
Malpighian tubules (mean of 2 expts)	58.6	48.8	781.0	590.0	158.1	748.1
Rectum (1 expt)	-	15.9	365.0	206.3	126.9	333.2

Values given above are ATPase activity in nmoles P_i/mg protein/min

It can be seen from column (b) that Na^+, K^+-activated ATPase activity in Malpighian tubule homogenates was 590.0nmoles P_i/mg protein/min. When corpora cardiaca extract was present (column (a)) the enzyme activity measured was 781.0nmoles P_i/mg protein/min. The corpora cardiaca extract therefore appears to be stimulating the Na^+, K^+-activated ATPase activity. However, control assays using corpora cardiaca extract but no Malpighian tubule homogenate also showed Na^+, K^+-activated ATPase activity (column (c)). It can be seen then that if the theoretical activity is calculated i.e. Na^+, K^+-activated ATPase activity from Malpighian tubule homogenate plus Na^+, K^+-activated ATPase activity from corpora cardiaca extract (columns (b) and (c) 748.1nmoles P_i/mg protein/min),the resulting total activity is found to be very similar to the actual assayed Na^+, K^+-activated ATPase activity in the presence of corpora cardiaca extract. It would seem then that the corpora cardiaca extract does not stimulate ATPase activity but merely adds another source of enzyme to the reaction media.

Na^+, K^+-activated ATPase in rectal homogenates also appeared to be stimulated by corpora cardiaca extract, but again, by comparing theoretical and actual values for ATPase activity, it can be seen that the increased activity was due to hydrolysis of ATP by the corpora cardiaca extract.

(2) Sub-optimal conditions

Na^+, K^+-activated ATPase was determined at sub-optimal conditions in the presence and absence of corpora cardiaca extract. Under optimal conditions for ATPase activity (100mM Na^+, 20mM K^+) it would be unlikely that any stimulation of ATPase activity could be observed since the enzyme would already be operating maximally. If the corpora cardiaca extract did stimulate enzyme activity this might be more apparent under sub-optimal conditions.

Table 6.12 The effect of Corpora cardiaca extract on Na^+, K^+-activated ATPase activity assayed at sub-optimal conditions

	Mg^{2+}-dependent ATPase activity		Na^+, K^+-activated ATPase activity			Calculated activity in presence or C.C. extract
			from Malpighian tubules or rectum		from C.C. extract	
	+ C.C.	- C.C.	+ C.C.	- C.C.		
			(a)	(b)	(c)	(b + c)
Malpighian tubules (mean of 2 expts)	58.6	48.8	713.2	486.7	190.5	677.2
Rectum (1 expt)	-	15.9	436.5	306.3	111.1	417.4

Values given above are ATPase activity in nmoles P_i/mg protein/min

The results of experiments on microsomal preparations of the Malpighian tubules and rectum are shown in Table 6.12. It can be seen that the corpora cardiaca extract appears to stimulate Na^+, K^+-activated ATPase activity in the Malpighian tubule and rectal homogenates (i.e. comparing columns (b) and (a)). However, control assays also showed that the corpora cardiaca extract was a source of enzyme activity (column (c)). When the theoretical Na^+, K^+-activated ATPase was calculated i.e. column (b) + (c), 677.2nmoles P_i/mg/min, it was found to be very similar to the assayed enzyme activity 713.2nmoles P_i/mg protein/min. As under optimal conditions the apparent stimulatory effect of corpora cardiaca extract would seem to be due to the addition of another source of enzyme with the extract.

Conclusion

The purpose of this study was to investigate the possibility that insect hormones exert their effects on fluid secretion by affecting the Na^+, K^+-activated ATPase. An effect on this enzyme would result in significant changes in the internal Na^+ and K^+ concentrations of the cells which would in turn have an effect on fluid secretion. An effect of insect hormones on the Na^+, K^+-activated ATPase would also, if found, lend support to the hypotheses put forward by Kroeger and Lezzi (1966) and Lezzi and Gilbert (1972) that β-ecdysone and Juvenile Hormone exert their regulatory effects on target tissues by changing the internal Na^+ and K^+ concentrations and ratios.

There are few reports of the interaction between insect hormones and Na^+, K^+-activated ATPase activity but Peacock (1976) has reported that extracts from the corpora cardiaca stimulate the Na^+, K^+-activated ATPase in homogenates of the rectum of *Locusta*. The corpora cardiaca are known to contain a diuretic hormone which accelerates fluid production by the Malpighian tubules and reduces re-absorption from the rectum (Highnam *et al.* 1965; Cazal and Girardie 1968; Mordue and Goldsworthy 1969; Mordue 1969, 1970, 1972). Fluid secretion by the Malpighian tubules and reabsorption of fluid by the rectum is known to involve a Na^+/K^+ pump (Anstee and Bell 1975; Anstee *et al.*1978) and to be correlated with the activity of the Na^+, K^+-activated ATPase enzyme system (Peacock 1975; Anstee and Bell 1975). Peacock (1976) found that crude corpora cardiaca extract stimulated the Mg^{2+}-dependent ATPase by about 549% and that stimulation of the Na^+, K^+-activated ATPase depended upon the concentrations of Na^+ and K^+. Under optimal conditions i.e. 100mM Na^+, 20mM K^+, there was an increase in enzyme activity of about 14% whereas under sub-optimal conditions i.e. 50mM Na^+, 5mM K^+ the Na^+, K^+-activated ATPase activity increased by 205%. In the present study similar experiments were performed using homogenates of the Malpighian tubules and rectum of *Locusta*, but the results obtained by Peacock (1976) could not be confirmed. The corpora cardiaca extract did not stimulate the Mg^{2+}-dependent or Na^+, K^+-activated ATPase activity under either optimal or sub-optimal assay conditions. In all cases it at first appeared that the corpora cardiaca extract had a stimulatory effect but control assays proved that the 'stimulation' was due to ATP hydrolysis by the corpora cardiaca extract itself.

The effect of β-ecdysone on the membrane-bound Na^+, K^+-activated ATPase has been studied previously by Fristrom and Kelly (1976). Fristrom and Kelly (1976) have reported that β-ecdysone (1μg/ml) had no effect on the Na^+, K^+-activated ATPase in homogenates of *Drosophila* imaginal discs. This result has been confirmed in the present study. β-ecdysone (100μg/ml) was found to have no effect on the Na^+, K^+-activated ATPase in homogenates of the Malpighian tubules of *Locusta*. Such results are inconsistent with the hypothesis of Kroeger and Lezzi (1966) that ecdysone acts on target cells by affecting the 'sodium pump'.

Fristrom and Kelly (1976) have also studied the effect of J.H. on the Na^+, K^+-activated ATPase, reporting that J.H. III (100μg/ml) caused an increase in ATPase activity in homogenates of imaginal discs. Similarly, Abu-Hakima and Davey (1979) have found a stimulatory effect of J.H. on the Na^+, K^+-activated ATPase in homogenates of *Rhodnius* follicle cells. However, in the present study J.H. was found to inhibit the Na^+, K^+-activated ATPase activity in microsomal preparations of the Malpighian tubules of *Locusta*. This inhibitory effect was found to increase with increasing J.H. concentration over the range 2.5μg/ml - 250μg/ml. Fristrom and Kelly (1976) report that J.H. III stimulated Na^+, K^+-activated ATPase activity, but in the present study all three homologues of J.H. were found to have an inhibitory effect.

Although this inhibitory effect of J.H. is contradictory to that reported by Fristrom and Kelly (1976) and Abu-Hakima and Davey (1979), the nature of the experiment as well as the tissue used was different. In the study by Fristrom and Kelly (1976) the imaginal discs were incubated with J.H. for 1hr before washing, homogenising and assaying for Na^+, K^+-activated ATPase activity. Similarly the *Rhodnius* follicle cells assayed by Abu-Hakima and Davey (1979) were obtained from ovaries which had been pre-incubated with J.H. for 30 mins before homogenization and enzyme assay. In both these cases J.H. was found to stimulate Na^+, K^+-activated ATPase activity. In the present study J.H. was present only in the reaction media and was found to inhibit enzyme activity. Fristrom and Kelly (1976) found that when J.H. was added to the homogenising medium it did not stimulate Na^+, K^+-activated ATPase activity. In a further study they mixed imaginal discs which had been incubated with J.H. with discs incubated without J.H. (50:50) and assayed for enzyme activity. They would have expected to find ATPase activity intermediate between the J.H. stimulated and control levels if the stimulation of enzyme activity by J.H. resulted from a direct modification of the Na^+, K^+-activated ATPase. However, the results showed that, following mixing, the enzyme activity was still at the stimulated level. They concluded from this that the stimulation of Na^+, K^+-activated ATPase activity by J.H. resulted from the production of some effector molecule and was not a direct effect of J.H. on the enzyme.

In the present study where J.H. was present in the enzyme assay media it seemed probable that the resulting inhibition was due to a direct effect of J.H. on the Na^+, K^+-activated ATPase.

Fristrom and Kelly (1976) also studied the effect of J.H. on ^3H-ouabain binding to imaginal discs. They found that J.H. reduced ouabain binding and Scatchard plots indicated that this was due to J.H. acting as a non-competitive inhibitor of ouabain binding. From the parameters for ouabain binding in the presence and absence of J.H. they proposed that J.H. caused a reduction in the actual or effective number of ouabain binding sites and also reduced the dissociation constant. They suggested that this change in the dissociation constant resulted mainly from an increased stability of the ouabain-enzyme complex in the presence of J.H. The reduction in the number of ouabain binding sites indicated either a loss of Na^+, K^+-activated ATPase molecules or the presence of conformational changes which prevented ouabain binding.

These results obtained by Fristrom and Kelly (1976) for the effect of J.H. on ouabain binding are consistent with the inhibition of Na^+, K^+-activated ATPase activity by J.H. observed in the present study. The reduction in the number of ouabain binding sites could be due to the inhibitory effect of J.H. on the enzyme and might suggest that both J.H. and ouabain were competing for binding sites on the enzyme.

It has already been well established that the Na^+, K^+-activated ATPase hydrolyses ATP in the presence of Na^+ and K^+ by a mechanism inhibited by cardiac glycosides (Skou 1957). And, as a result of numerous studies, a great deal is now known of the ouabain-Na^+, K^+-activated ATPase interaction and how this causes enzyme inhibition (Matsui and Schwartz 1966, 1967, 1968; Post and Sen 1967; Albers *et al.* 1968; Schwartz *et al.* 1975).

Studies on erythrocyte 'ghosts' have shown that in intact membrane preparations cardiac glycosides inhibit the Na^+ pump only when they are in the extracellular fluid (Hoffman 1969; Whittam 1962; Whittam and Agor 1964). This suggests that the receptor for ouabain resides on the external surface of the membrane. From the model proposed to explain Na^+, K^+-activated ATPase action (see Introduction) it can be seen that the binding site for K^+ is also on the external membrane surface whereas the binding sites for Na^+ and ATP are on the internal surface of the membrane. Ouabain and K^+ have been found to be antagonistic to one another with respect to their actions on the Na^+ pump in both intact transporting systems (Glynn 1957) and membrane preparations (Dunham and Glynn 1961). This antagonism was originally thought to reflect a competition between ouabain and K^+ for the K^+ activation site (Ahmed and Judah 1965; Ahmed *et al.* 1966). However, although it is generally accepted that ouabain binds to a phosphorylated intermediate (E_2-P in proposed model) in the reaction sequence of the Na^+, K^+-activated ATPase and thereby prevents the K^+-dependent hydrolysis of E_2-P (Matsui and Schwartz 1967, 1968), this does not necessarily mean that K^+ and ouabain are competing for the same site. Further studies suggest that ouabain interacts with a site that is different from the K^+ activation site on the external surface of the membrane (Hoffman 1966). Studies on the kinetics of ouabain inhibition yield results typical of non-competitive inhibition with K^+ as substrate (Schoner 1971; Jenner and Donnellan 1976).

Since ouabain binds to the phosphorylated intermediate E_2-P, the conformation for binding K^+, increasing the K^+ concentration inhibits glycoside interaction by decreasing the rate of complex formation between ouabain and its receptor (Schwartz *et al.* 1975).

In the present investigation the possibility that J.H. may have caused inhibition of the Na^+, K^+-activated ATPase by a mechanism similar to that of ouabain was examined by comparing the action of the two inhibitors on the enzyme's kinetics.

The effects of both inhibitors on ATPase activity were studied at varying K^+ concentrations. Results typical of non-competitive inhibition were obtained with ouabain as inhibitor. Addition of ouabain to the assay media was found to cause a decrease in V_{max} from 751.2nmoles P_i/mg protein/min to 246.6nnoles P_i/mg/min, but had no significant effect on the value of K_m. Similarly Jenner and Donnellan (1976) found that the inhibition by ouabain of housefly head Na^+, K^+-activated ATPase was non-competitive with respect to K^+.

In contrast, when the same experiment was performed in the presence of J.H., kinetics typical of uncompetitive inhibition were observed. Addition of J.H. to the assay media was found to cause a decrease in the value of K_m from 4.4mM to 2.7mM and to decrease V_{max} from 648.9nmoles P_i/mg protein/min to 357.5nmoles P_i/mg/min. Uncompetitive J.H. inhibition was also observed at different ATP concentrations. Uncompetitive inhibition indicates the formation of an inactive enzyme-substrate-inhibitor complex which cannot undergo further reaction. It would appear therefore that J.H. may act by inhibiting the conformational change of E_2 back to E_1 (see Introduction), thus preventing further phosphorylation of the enzyme.

The mechanism by which J.H. may be stabilising the enzyme-substrate complex is uncertain. However, examination of the results obtained with Arrhenius μ plots in the presence and absence of J.H. may provide a possible explanation.

Arrhenius temperature profiles of Na^+, K^+-activated ATPase activity showed a 'break-point' at around 25°C in the absence of J.H. However, when J.H. was present, the break point occurred at around 21°C i.e. the presence of J.H. effected a shift of some 4°C. in the transition temperature of the Na^+, K^+-activated ATPase from *Locusta* Malpighian tubules.

Previous studies by Grisham and Barnett (1973) have reported that Arrhenius plots of lamb kidney Na^+, K^+-activated ATPase activity against temperature revealed a 'break' at about 20°C. They showed that this temperature corresponded to a transition in the state of the lipids extracted from a Na^+, K^+-activated ATPase preparation. The transition in the state of the extracted lipids reflected a conversion from a more ordered state (below 20°C) to a less ordered state (above 20°C). Barnett and Palazzotto (1974) concluded that the change in the state of the lipids altered a rate-limiting step in the reaction sequence for ATP hydrolysis. Further analysis revealed that the following 'partial reactions' were not altered by the lipid transition: (i) Phosphorylation of the Na^+, K^+-activated ATPase by ATP in the presence of Mg^{2+} and Na^+; (2) K^+-stimulated p-nitrophenolphosphatase; (3) rates of ouabain binding. Barnett and Palazzotto (1974) concluded that the 'partial reaction' affected by the physical state of the lipids was the conversion of a K^+-sensitive form of the Na^+, K^+-activated ATPase to a Na^+-sensitive form ($E_2 \longrightarrow E_1$).

From this work by Grisham and Barnett (1973) and Barnett and Palazzotto (1974) it can be seen that the state of the membrane lipids is important in modulating the conformational changes of the Na^+, K^+-

activated ATPase. Since J.H. clearly alters the transition temperature of the Na^+, K^+-activated ATPase it may in some way be affecting the membrane lipids. J.H. is a terpenoid compound and it is possible that it may have become inserted in the membrane lipids, thereby affecting membrane fluidity. The effect of this would be to prevent the normal conformational changes necessary for ATP hydrolysis, thus bringing about enzyme inhibition. Barnett and Palazzatto (1974) propose that the state of the membrane lipids affects the conversion of $E_2 \rightarrow E_1$ and this is consistent with the results of the Lineweaver-Burk plots which also suggest that J.H. inhibition of Na^+, K^+-activated ATPase activity is due to the prevention of this partial reaction.

Fristrom and Kelly (1976) have also suggested that J.H. may produce conformational membrane changes. The presence of J.H. was found to reduce ouabain binding to *Drosophila* imaginal discs. It was proposed that this reduction in the number of ouabain binding sites may have been due to conformational changes which prevented ouabain binding.

Previous evidence for J.H. interaction with membranes comes from work by Baumann (1968, 1969). It was shown (Baumann 1968) that J.H. increased the conductance of the salivary gland cell memberane of *Galleria mellonella*, suggesting a membrane effect. In a further study on the effect of J.H. on the conductance of bimolecular lipid membranes, Baumann (1969) showed that the presence of J.H. enhanced membrane conductance. Baumann (1969) suggested that J.H. may interact with the lipid molecules to rearrange the structure of the membrane, causing it to become more rigid.

Chapter 7

CONCLUSIONS

As mentioned earlier, the transport of Na^+ and K^+ in many secretory and absorptive epithelia is known to involve a Na^+/K^+ exchange pump (Skou 1965; Whittam and Wheeler 1970). The pump requires ATP for transport and is specifically inhibited by the cardiac glycoside ouabain (Schatzmann 1953). The activity of this cation transport mechanism has been correlated with the activity of a Na^+, K^+-activated ATPase; a membrane-bound enzyme which is synergistically stimulated by Na^+ and K^+ and is inhibited by ouabain (Skou 1957). In common with numerous other tissues which have been studied (Skou 1957, 1969; Nakao *et al.* 1965; Proverbio *et al.* 1970; Whittam and Wheeler 1970), the Malpighian tubules of *Locusta* possess a Na^+, K^+-activated ATPase (Anstee and Bell 1975, 1978; Chapter 4). This enzyme has been implicated in the mechanism of fluid secretion by *Locusta* Malpighian tubules by virtue of the sensitivity of the secretory process to ouabain (Anstee and Bell 1975; Chapter 4). However, as described earlier (Chapter 4), the failure of some workers to demonstrate ouabain-inhibition of fluid secretion has cast doubt on the involvement of a Na^+/K^+ exchange pump in fluid and cation secretion across Malpighian tubules (Maddrell 1969; Pilcher 1970; Gee 1976; Rafaeli-Bernstein and Mordue 1978). In the present study ouabain clearly inhibited fluid secretion by *in vitro* preparations of the Malpighian tubules of *Locusta* although the experimental conditions were found to be extremely important for demonstrating ouabain inhibition (Chapter 4). In particular, the inhibitory effect of ouabain was found to be extremely sensitive to the temperature at which the experiments were carried out. Fluid secretion by *Locusta* Malpighian tubules was far less sensitive to ouabain at 15°C and 20°C than at 30°C. This might be expected if a Na^+, K^+-activated ATPase is involved as the inhibition of ATPase activity by ouabain has been shown to be substantially affected by temperature in the present study, confirming the results of other workers (Charnock *et al*. 1975; Peacock *et al*. 1976). Examination of the literature shows that some workers who report ouabain to have no effect on fluid secretion performed their experiments at 24-25°C (Rafaeli-Bernstein and Mordue 1978) or at room-temperature, ca. 19-22°C (Gee 1976). Since temperature has been shown to be an important factor in determining the extent to which ouabain inhibits Malpighian tubule function in *Locusta*, the failure to demonstrate inhibition of fluid secretion at room temperature should not be taken as evidence against the involvement of a Na^+, K^+-activated ATPase in the fluid secretory mechanism.

It has also been suggested (Jungreis 1977; Rafaeli-Bernstein and Mordue 1978) that the K^+ concentration in the bathing medium may affect the demonstration of ouabain inhibition. High K^+ concentrations have been shown to antagonise the ouabain inhibition of the Na^+, K^+-activated ATPase (Kinsolving *et al*. 1963; Judah and Ahmed 1964; Matsui and Schwartz 1968; Akera 1971). However in the present study varying the K^+ concentration from 10-40mM failed to affect the ouabain sensitivity of the fluid secretory process. It appears then that the K^+ concentration of the bathing medium may vary substantially without affecting a significant reduction in ouabain inhibition.

Whilst it is possible that the mechanism of fluid secretion is different in some insect species it would appear that methodological differences may in fact account for the conflicting results as to the effect of ouabain, that are reported in the literature.

The secretion of fluid by the Malpighian tubules has been shown previously to be under hormonal control (Highnam *et al*. 1965; Mordue and

Goldsworthy 1969; Mordue 1969, 1970, 1972). The present study confirms that extracts of the cerebral neurosecretory cells and the corpora cardiaca cause a marked stimulation in the rate of fluid secretion by *in vitro* preparations of the Malpighian tubules of *Locusta* A diuretic effect of corpora cardiaca or neurosecretory cell extract in *Locusta* has been shown previously as a result of cautery or dye excretion experiments (Highnam *et al.* 1965; Cazal and Girardie 1968; Mordue 1966, 1969). Peacock (1976) has shown that extracts from the corpora cardiaca produced a stimulation of rectal Na^+, K^+-activated ATPase activity in *Locusta*. Stimulating the Na^+/K^+ pump may be expected to produce an increase in the rate of fluid secretion by Malpighian tubules and this may have offered a mechanism for the action of diuretic hormone. However, in the present study, the stimulatory effect of corpora cardiaca extract on Na^+, K^+-activated ATPase activity could not be confirmed (Chapter 6). The results of the experiments were complicated by the fact that addition of crude corpora cardiaca extract was in effect adding another source of enzyme to hydrolyse ATP. Perhaps by using purified diuretic hormone a true effect on Na^+, K^+-activated ATPase could be demonstrated.

It is more probable that diuretic hormone does not directly affect membrane permeability, but acts indirectly through cyclic AMP. Cyclic AMP has been shown previously to increase fluid secretion by the Malpighian tubules of *Rhodnius* and *Carausius* (Maddrell *et al.* 1971), *Schistocerca* (Maddrell and Klunsuwan 1973) and *Locusta* (Mordue 1969; Anstee *et al.* 1979). The effect of cyclic AMP on fluid secretion by *Locusta* Malpighian tubules has been confirmed in this present study (Chapter 4). Aston (1975) has shown that addition of diuretic hormone to the Malpighian tubules of *Rhodnius* leads to an increase in cyclic AMP levels and it has been suggested that increasing cyclic AMP levels may stimulate a cation pump (Berridge 1970; Berridge and Prince 1972).

The effects of diuretic hormone on fluid secretion by the Malpighian tubules of a variety of insects have been well documented (Highnam *et al*. 1965; Mordue 1969; Pilcher 1970; Gee 1975). However, much less is known of the effects of β-ecdysone and Juvenile Hormone. As mentioned earlier (Chapter 6), it has been suggested that β-ecdysone affects cell permeability (Kroeger and Lezzi 1966; Gee *et al*. 1977). Kroeger and Lezzi (1966) propose that β-ecdysone changes internal Na^+ and K^+ concentrations in 'target' cells by stimulating the Na^+ pump. This theory is not supported by the present study nor has it been possible to confirm the findings of Gee *et al*. (1977) that β-ecdysone stimulated Malpighian tubule fluid secretion. β-ecdysone was found to have no effect on the rate of fluid secretion by *Locusta* Malpighian tubules, which may have been expected if ecdysone was stimulating the Na^+/K^+ pump. And, more importantly, β-ecdysone has no effect on the Na^+, K^+-activated ATPase in microsomal preparations of the Malpighian tubules. This confirms the results of Fristrom and Kelly (1976) who also report no effect of ecdysone on Na^+, K^+-activated ATPase activity.

It has also been suggested that J.H. affects cell permeability. Lezzi and Gilbert (1972) propose that J.H. inhibits the Na^+-pump in 'target' cells leading to an increase in internal Na^+ concentrations. In the present study, J.H. was found to have an inhibitory effect on rates of fluid secretion by the Malpighian tubules of *Locusta* (Chapter 6), as would be expected as J.H. does inhibit the Na^+-pump. In addition, in the present study, J.H. was found to inhibit the Na^+, K^+-activated ATPase in microsomal preparations of the tubules and thus confirms the suggestion that J.H. does in fact inhibit the Na^+ pump.

Attempts have been made to explain how J.H. may bring about inhibition of the Na^+, K^+-activated ATPase. Evidence from kinetic studies and from Arrhenius temperature profiles suggests that J.H. produces conformational changes in the membrane structure which then prevent the normal reaction sequence of the ATPase. Further evidence for an effect of J.H. on membrane structure comes from a report by Baumann (1969) who suggests that J.H. produces membrane stability by interacting with the membrane lipids. Fristrom and Kelly (1976) have also suggested that J.H. causes conformational changes in membrane structure. Additional support for an effect of J.H. on cell membranes is provided by the work of Cohen and Gilbert (1972) who showed that J.H. causes swelling and lesions in the plasma membrane of insect cells growing in culture. Also, Steele (1976) suggests that the effect of J.H. on *in vitro* mitochondrial respiration is due to J.H. altering the mitochondrial membrane such that it disrupts electron transport and increases the permeability to succinate.

Whilst J.H. inhibits both fluid secretion by *in vitro* preparations of the Malpighian tubules and the Na^+, K^+-activated ATPase in extracts of the tubules, it is not possible to conclude that J.H. has a similar function in the intact animal. J.H. which is lipid soluble is thought to be carried in the haemolymph bound to a hydrophilic carrier protein. Such a binding protein has been recognised in the haemolymph of *Manduca sexta* (Kramer *et al.* 1974). The Malpighian tubules in an intact animal are therefore unlikely to encounter J.H. in a form similar to that used in the present *in vitro* experiments. This, plus the fact that high concentrations of J.H. were used owing to its relative insolubility in aqueous solution (i.e. effect may have been pharmacological rather than physiological), make it impossible to say whether J.H. has a role in fluid secretion in the intact animal.

However, there is some evidence to show that the corpora allata may contain an 'anti-diuretic' principle (Wall and Ralph 1964; Beenakkers and Van den Broek 1974). Beenakkers and Van den Broek (1974) found that a high titre of corpus allatum hormone results in a considerably higher water content in *Locusta*, whereas allatectomy reduces this content. Also, Strong (1968) has shown that allatectomy results in a lower insect wet weight. These results are certainly consistent with an inhibitory effect of Juvenile Hormone on rates of fluid secretion.

More recently (Ryerse 1980), ecdysone and J.H. have also been implicated in controlling the developmental physiology of Malpighian tubules. Ryerse (1980), working on *Calpodes*, has shown that 20-hydroxyecdysone switches off fluid secretion and initiates cellular remodelling at pupation and also triggers adult development of the Malpighian tubules including completion of cellular remodelling and restoration of fluid secretion. J.H. was found to modify the influence of 20-hydroyecdysone on the Malpighian tubules at moulting in larvae. Ryerse has shown previously (1978, 1979) that *Calpodes* Malpighian tubules undergo extensive changes in cell structure and fluid secretion during development. Larval tubules show high rates of fluid secretion and have deep basal infolds and long, mitochondria containing apical microvilli. Fluid secretion is switched off at pupation and the cells undergo loss of the basal infolds, retraction of the mitochondria from the microvilli and extensive organelle and plasma membrane autophagy. The Malpighian tubules persist through metamorphosis, and mid-way through the pupal stage the basal infolds reform, mitochondria are reinserted into the microvilli and fluid secretion resumes. Ryerse (1980) has shown that ecdysone is responsible for the reduction in basal infold depth and apical microvillar length as well as the retraction of mitochondria from the apical microvilli.

The present study describes similar developmental changes in the Malpighian tubules of a hemimetabolous insect i.e. an insect in which the larval forms are similar in appearance to the adult. At the beginning of the 5th instar of *Locusta* the tubule cells show little invagination of either the basal or apical cell membranes. As the age of the insect increases, so the degree of membrane invagination increases, with the basal infoldings and the apical microvilli becoming longer. At the same time, the number of mitochondria in the tubule cells appear to increase and the mitochondria are increasingly found to lie alongside the extracellular channels formed by the infoldings of the basal plasma membrane. Just prior to the larval-adult moult, the basal and apical surface invaginations decrease in length and the mitochondria disappear from alongside the extracellular channels. Functional changes also appear to accompany these ultrastructural changes. The activity of the membrane-bound Na^+, K^+-activated ATPase is low at the beginning and end of the 5th instar, at times when cell membrane area is reduced. In addition, the animal water content is high at these times, suggesting that the animals are excreting less fluid. It may not be surprising that functional changes accompany ultrastructural changes since mitochondria provide energy for active transport and the degree of invagination of the cell membranes would affect rates of fluid secretion. In the light of the work by Ryerse (1980) it would be interesting in future studies to look at the effects of ecdysone and Juvenile Hormone on the developmental structure and function of the Malpighian tubules of *Locusta*. Haemolymph titres of J.H. and ecdysone throughout the 5th instar have been reported for *Locusta* (Hirn *et al*. 1979; Baehr *et al*. 1979). Hirn *et al*. (1979) found that ecdysteroid levels showed a small peak on Day 3 and a large peak on Day 8 of a 10-11 day instar. J.H. levels were high during the first 5 hrs of the 5th instar. Baehr *et al*. (1979) report 3 peaks of ecdysteroid concentration at 24 hr, 64 hrs and c. 120 hrs of a 144 hr instar. These peaks of ecdysone concentration could be correlated with the switching off of fluid secretion and ultrastructural changes in *Locusta*. Perhaps this may even help provide an explanation for the dramatic decrease in Na^+, K^+-activated ATPase around the mid-instar, observed in the present study. This is a time when ultrastructurally the tubules show well-developed invaginations of the basal and apical cell membranes. It may be that ecdysone is responsible for 'switching off' the enzyme activity at this time.

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Appendix 5.I Na,K-activated ATPase activity at different stages throughout the 5th instar and in early adult Locusta.

Age (days)	Na,K-ATPase activity (nmoles Pi/animal/min)
I	I4.7
2	24.5
4	25.8
5	I6.8
6	34.2
7	34.2
8	46.2
9	9.6
I0	4.I
I adult	6.2
2	I7.8
3	20.3

I	5.4
2	I3.7
3	I4.6
5	6.2
7	24.0
8	6.0
9	5.3
I adult	2.5
2	8.6

I	I.6
2	3.0
4	II.6
5	5.8
6	2.0
7	5.2
8	I2.4
I0	4.8
I adult	3.0
2	5.I
3	9.7
4	I2.4

Appendix 6.1

The effect of J.H. on Na^+ and K^+ concentrations in the 'urine'

Ringer solution		Ringer + J.H.	
$[Na^+]$mM	$[K^+]$mM	$[Na^+]$mM	$[K^+]$mM
30	206.25	33.75	183.75
20.25	281.25	16.8	108.75
24.3	187.5	20.25	90.0
71.25	311.25	63.75	266.1
41.25	270.0	45.0	240.0
41.25	138.75	41.25	93.75
33.75	232.5	33.75	108.75
21.3	243.75	24.3	200.0

Figure A.6.1 Calibration curve for KOH concentration against % emission (at 760nm)

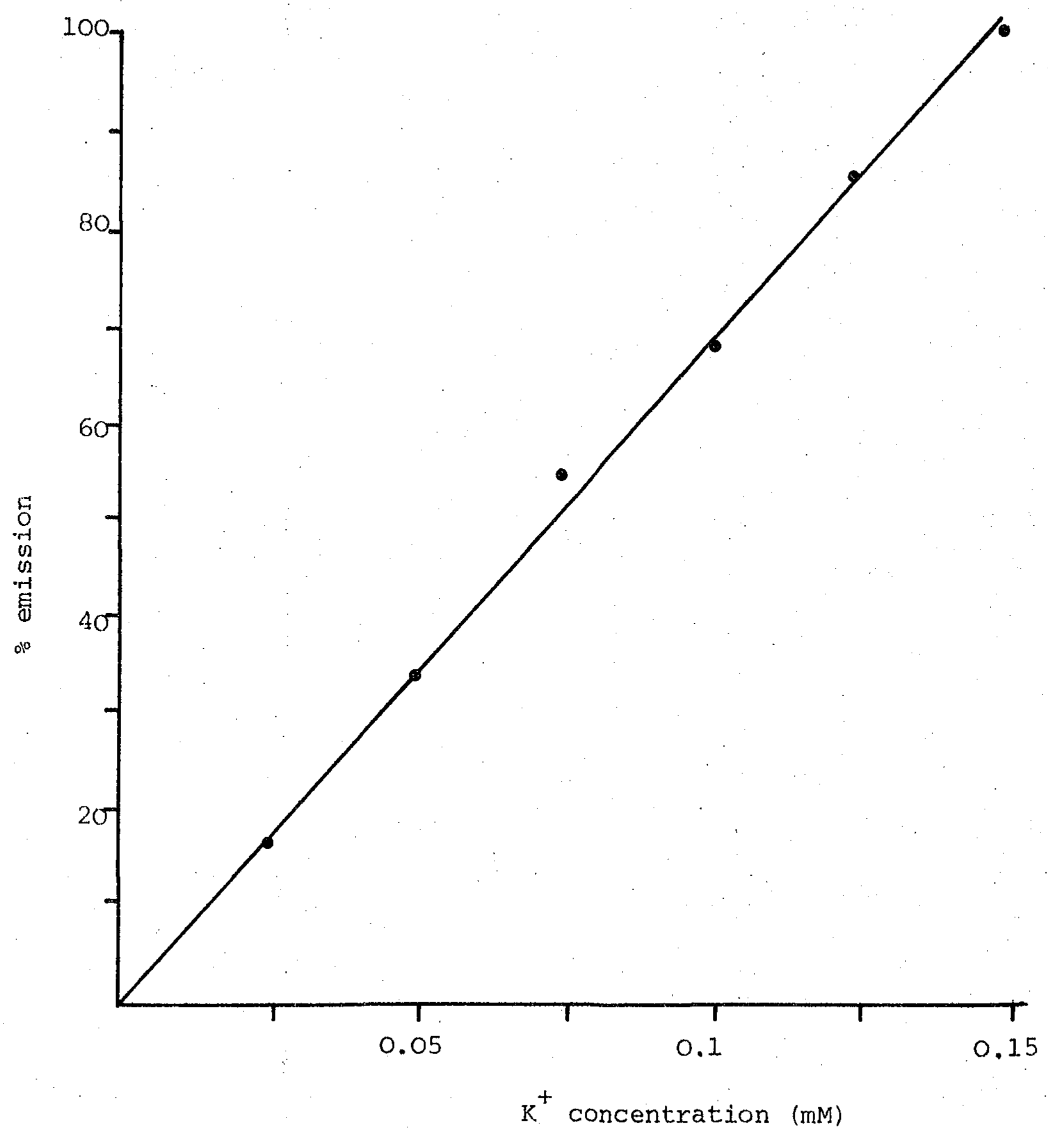

Figure A.6.2 Calibration curve for NaOH concentration against % emission (at 589nm)