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**BIOCHEMICAL, CYTOCHEMICAL LOCALIZATION AND  
PHYSIOLOGICAL STUDIES ON MALPIGHIAN TUBULES OF  
LOCUSTA MIGRATORIA L.**



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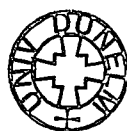
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**(King Abdulaziz University Saudi Arabia)**



**Being a thesis submitted for the degree of Doctor of Philosophy of the  
University of Durham.**

**June 1997**



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**Zarrag I. Al-Fifi**

University of Durham

June 1997

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## CONTENTS

ACKNOWLEDGMENTS.....	i
CONTENTS.....	ii
ABSTRACT.....	iii
GLOSSARY.....	v
<b><u>CHAPTER 1</u></b>	
GENERAL INTRODUCTION.....	1
<b><u>CHAPTER 2</u></b>	
GENERAL MATERIALS AND METHODS.....	29
<b><u>CHAPTER 3</u></b>	
<i>In vitro</i> studies on fluid and ion secretion by the Malpighian tubules of <i>Locusta migratoria</i> .....	32
INTRODUCTION.....	32
MATERIALS AND METHODS.....	37
RESULTS.....	41
DISCUSSION.....	63
<b><u>CHAPTER 4</u></b>	
Biochemical studies on the subcellular distribution of the enzyme activities in Malpighian tubules of <i>Locusta migratoria</i> .....	73
INTRODUCTION.....	73
MATERIALS AND METHODS.....	76
RESULTS.....	89
DISCUSSION.....	97
<b><u>CHAPTER 5</u></b>	
Biochemical studies on the subcellular distribution of the enzyme activities from Malpighian tubules of <i>Locusta migratoria</i> .....	102
INTRODUCTION.....	102
MATERIALS AND METHODS.....	111
RESULTS.....	113
DISCUSSION.....	131

## **CHAPTER 6**

Partial purification and immunocytochemical localization studies on the apical and basal membrane-rich fractions molecular biology technique.....	144
INTRODUCTION.....	144
MATERIALS AND METHODS.....	148
RESULTS.....	158
DISCUSSION.....	168

## **CHAPTER 7**

<u>GENERAL DISCUSSION</u> .....	176
<u>REFERENCES</u> .....	182
<u>APPENDICES</u> .....	Post 222

## ABSTRACT

Specific inhibitors of protein kinase A (PKA), Rp-cAMP, protein kinase C (PKC), chelerythrine and staurosporine, and protein phosphatase 1 and 2A, okadaic acid, inhibited fluid secretion whilst corpora cardiaca (CC) extract and cAMP were found to stimulate fluid secretion. Tubules inhibited by PKC inhibitors, were significantly stimulated when CC extract was included in the bathing medium, but cAMP did not stimulate under these conditions. The inclusion of cAMP in the presence of Rp-cAMP failed to prevent a further reduction in the rate of secretion compared to that in the presence of Rp-cAMP alone. The addition of CC extract in presence of Rp-cAMP failed to stimulate fluid production.

The effect of PKA and PKC inhibitors on the cationic composition of "urine" in the presence and absence of CC extract or cAMP were studied. The composition of the secreted fluid was not significantly changed during stimulation by cAMP. Whilst, CC extract significantly increased  $[Na^+]$ , decreased  $[K^+]$ , increased  $Na^+/K^+$  ratio and increased both  $Na^+$  flux and  $K^+$  flux. However, cAMP caused an increase of both  $Na^+$  flux and  $K^+$  flux, but did not alter the  $Na^+/K^+$  ratio. PKC inhibitors significantly reduced the  $[K^+]$  of the secreted fluid, without affecting its  $[Na^+]$ . CC extract in the presence of chelerythrine or staurosporine increased  $[Na^+]$  but  $[K^+]$  was not reduced below the concentration that already observed in presence of these inhibitors alone. The addition of 1mM cAMP in the presence of chelerythrine or staurosporine did not significant change the ionic composition of the secreted fluid compared to that seen in presence of these inhibitors alone. 1 $\mu$ M chelerythrine or staurosporine caused a significant increase in the  $Na^+/K^+$  ratio. This ratio increased further when tubules bathed in chelerythrine or staurosporine saline were also exposed to CC extract. However, cAMP did not change the level of  $Na^+/K^+$  ratio from than that observed in the presence of PKC inhibitors alone. 0.1mM Rp-cAMP caused a significant fall in  $[Na^+]$  and a rise in  $[K^+]$ . The addition of CC extract appeared to reverse the effects of Rp-cAMP. The  $Na^+$  content of the secreted fluid significantly rose, and whilst the mean  $K^+$  content was somewhat lower, the difference from that observed in the presence of Rp-cAMP alone was not significant. The addition of cAMP to the saline containing Rp-cAMP caused a significant increase in  $[Na^+]$ , restoring it to normal control levels. However, cAMP did not significantly change the  $[K^+]$  compared with that observed in the presence of Rp-cAMP alone. 0.1mM Rp-cAMP caused a significant decrease in the  $Na^+/K^+$  ratio and both  $Na^+$  flux and  $K^+$  flux. In contrast, the addition of CC extract or cAMP to Rp-cAMP saline caused a significant increase in the  $Na^+/K^+$  ratio. Inclusion of either CC extract or cAMP plus Rp-cAMP did not reverse the decrease in  $Na^+$  and  $K^+$  flux observed in the presence of Rp-cAMP alone.

Immunocytochemical localization and cell fractionation methods used in conjunction with biochemical analyses demonstrate the major of  $Na^+/K^+$ -ATPase activity present on basal cell membranes and  $HCO_3^-$ -stimulated and V-type ATPase activities on the apical cell membranes. The biochemical properties of V-type ATPase activity were studied. Immunogold localization study using specific monoclonal antibodies confirmed that V-type ATPase and  $Na^+/K^+$ -ATPase located on the apical and basal cell membranes, respectively.

The results are discussed and a hypothetical model is proposed to account for the role of PKA, PKC and V-type ATPase enzymes in the mechanisms of ion and fluid movement across apical and basal cell surfaces.

## GLOSSARY

ADP	Adenosine 3',5'-diphosphate.
ATP	Adenosine 3',5'-triphosphate
BBMV	Brush border membrane vesicles.
BSA	Bovine serum albumen (Fraction V, Sigma)
cAMP	Cyclic adenosine 3',5'-monophosphate.
cGMP	Cyclic guanine 3',5'-monophosphate.
CC extract	Corpora cardiaca extract.
DAG	1,2-Diacylglycerol.
DCCD	N,N'-dicyclohexylcarbodiimide.
DDSA	Dodecanyl succinic anhydride.
DH	Diuretic hormone.
DHR	Diuretic hormone receptor.
DMP	2, 4, 6, tri [dimethylaminomethyl] phenol.
DMSO	Dimethyl sulfoxide.
E.D.T.A.	Ethylene diamine tetra-acetic acid.
E.G.T.A.	Ethylene glycol bis ( $\beta$ -amino ethyl ether)-N,N'-tetra-acetic acid.
HCO <sub>3</sub> <sup>-</sup> -ATPase	Magnesium-dependent, -bicarbonate-stimulated adenosine triphosphatase.
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.
5-HT	5-Hydroxytryptamine.
Imidazole	1,3-diaza-2,4-cyclopentadiene.
Ins-1,4,5-P <sub>3</sub>	D- <i>myo</i> -inositol 1,4,5-triphosphate.
IP <sub>3</sub>	Inositol triphosphate.
K <sup>+</sup> -ATPase	Magnesium-dependent, potassium-stimulated adenosine triphosphatase.
kDa	Kilo Dalton.
(K <sup>+</sup> +H)-ATPase	Magnesium-dependent, potassium-hydrogen -stimulated adenosine triphosphatase.
Me	Metal.
MOPS	4-Morpholinopropanesulphonic acid.

mV	Millivolts.
Na <sup>+</sup> /K <sup>+</sup> -ATPase	Magnesium-dependent, sodium-potassium -stimulated adenosine triphosphatase.
NEM	N-ethylmaleimide.
NP	Nitrophenol.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
P <sub>i</sub>	Inorganic phosphate.
PIP <sub>2</sub>	Phosphatidylinositol 4,5-biphosphate.
PKA	Protein kinase A.
PKC	Protein kinase C.
Rp-cAMP	Adenosine 3',5'-cyclic monophosphorothioate Rp-isomer.
SDH	Succinate dehydrogenase.
SDS	Sodium dodecyl sulphate.
SEM	Standard error of mean.
TEMED	N,N,N',N'-Tetramethylethylenediamine.
TEP	Transepithelial potential.
Tris	Tris (hydroxymethyl) aminoethane.
Triton X-100	t-Octylphenoxypolyethoxyethanol.
Tween 20	Polyoxyethylenesorbitanmonolaureate.
V <sub>A</sub>	Apical membrane potential.
V <sub>B</sub>	Basal membrane potential.
V-type ATPase	Vacuolar type adenosine triphosphatase.

# CHAPTER 1

## GENERAL INTRODUCTION

Over the past forty years the nature of the mechanisms of ion and fluid secretion has been investigated in a number of different insect species. Ramsay completed the first detailed studies on Malpighian tubules of the insects (Ramsay, 1953; 1954; 1956; 1958). From these studies it was concluded that "potassium is the prime mover in generating the flow of 'urine', and that, in consequence of this secretion, conditions are created which enable water and other constituents to follow". Following this seminal work came investigations into the nature of ion and fluid secretion in a number of different species of insects e.g. *Dysdercus fasciatus*, (Berridge, 1965); *Calliphora vicina*, (was *C. erythrocephala*), (Berridge, 1968; 1969); *Rhodnius prolixus*, (Maddrell, 1969; 1971; 1977); *Locusta migratoria*, (Anstee and Bell 1975; 1978; Anstee *et al.*, 1979; Morgan and Mordue, 1981; 1983); *Glossina austeni*, (Gee, 1975a,b); *Glossina morsitans*, (Gee, 1976a,b). These and other studies confirmed that, water movements were a consequence of ion movements, and that in the majority of the species studied,  $K^+$  was essential for fluid secretion by the tubules therefore, these studies suggested that the active transport of  $K^+$  is the "prime mover" in the generation "urine". However, some other insects, such as *Rhodnius prolixus*, are able to produce "urine" in the presence of  $Na^+$  or  $K^+$  (Maddrell 1969), whilst in others, such as *Glossina morsitans*,  $Na^+$  is the "prime mover" (Gee 1976a,b).

Subsequent studies (Anstee and Bell 1975; 1978; Anstee *et al.*, 1979; Morgan and Mordue 1981; 1983) showed that the Malpighian tubules of *Locusta migratoria*, in common with those of a number of other species (Berridge, 1968; Maddrell, 1969;

1977; Phillips, 1964; Maddrell, 1971; Dalton and Windmill, 1980; Nicholls, 1985; Leyssens *et al.*, 1992a) are able to transport  $K^+$  against a chemical gradient over a wide range of external  $K^+$  concentrations. In addition  $K^+$  are transported in preference to  $Na^+$  even when present at much lower concentrations in the bathing medium (Anstee *et al.*, 1979). These studies lead to the following observations which support a dominant role for  $K^+$  in fluid secretion:

- I) The fluid secreted by the Malpighian tubules of a variety of insects has a higher  $K^+$  concentration than the bathing medium.
- II) Measurements of transepithelial potentials show that  $K^+$  movements into the lumen are thermodynamically uphill i.e.  $K^+$  entry is active.
- III) The rate of fluid secretion is dependent on the  $K^+$  concentration of the bathing fluid.

Two major diffusional pathways are possible in secretory epithelia. A transcellular route in which ions penetrate the apical and basal membranes and a paracellular pathway consisting mainly of "tight" junctions in series with the lateral spaces between epithelial cells. Initially, it was thought that such junctions presented a barrier to paracellular transport (Di Bona, 1972). However, in the early 1970s, it was recognized that such epithelial "tight" junctions were not tight enough to preclude paracellular transepithelial transport (DiBona, 1972; Fromter and Diamond, 1972). Consequently, the term "tight" and "leaky" (Fromter and Diamond, 1972) became common descriptions in epithelial classification (Schneeberger and Lynch, 1984). In "leaky" epithelia, transepithelial diffusion of ions occurs paracellularly through the tight junctions and lateral intercellular spaces (Fromter and Diamond, 1972). Examples are gallbladder of *Necturus* (Fromter, 1972), kidney proximal tubule of *Necturus* (Guggino *et al.*, 1982) and intestine of fresh water prawns (Ahearn, 1980) and *Aplysia*

(Gerencser, 1983). In contrast, in tight epithelia, most ionic diffusion and water movements occur transcellularly, because the combined resistances of apical and basal membranes is lower than that of the tight junctions and intercellular spaces. Examples of this second category where "tightness" has been quantified electrophysiologically include toad urinary bladder (Reuss and Finn, 1974), fundic gastric mucosa (Spenny *et al.*, 1974), rabbit urinary bladder (Lewis *et al.*, 1976) and locust rectum (Hanrahan *et al.*, 1982).

Studies on the Malpighian tubules of insects indicate that in some species secreted "urine" was slightly hypo-osmotic e.g., *Dixippus morosus*, (Ramsay, 1954), *Dysdercus fasciatus*, (Berridge, 1965) but in the majority of insects, the secreted "urine" was found to be slightly hyperosmotic to the bathing medium over a wide range of osmotic concentrations (*Locusta migratoria*, Bell, 1977; Anstee *et al.*, 1979, *Calliphora vicina*, Berridge, 1968, and *Rhodnius prolixus*, Maddrell, 1969; 1971). On this basis one would have predicted that insect Malpighian tubules would be "leaky". However, other studies by O'Donnell and Maddrell (1983) and O'Donnell *et al.*, (1984) suggested that water probably moves through the cells during fluid secretion by Malpighian tubules of *Rhodnius* and that water movements are coupled to salt transport across the basal and apical cell membranes. These workers argue that water movements via a paracellular route are unlikely to represent a significant component of total transepithelial tubule secretion in this species. Similarly, from measurements on transepithelial concentration gradients and resistance, Williams and Beyenbach (1984) have concluded that the Malpighian tubule of *Aedes aegypti* should be classified as a moderately "tight" epithelium. However, in other insect epithelia, there is good evidence that some of the fluid moves through the paracellular compartment

(O'Donnell and Maddrell, 1983). Indeed, paracellular fluid flow has been suggested in the rectal pads of *Periplaneta americana*, and *Calliphora*, (Wall *et al.*, 1970; Gupta and Hall, 1983) and the salivary glands of *Calliphora*, (Gupta *et al.*, 1978).

Several theories have been proposed to explain how solute transport effects iso- or near iso-osmotic secretion across epithelia. Diamond and Bossett (1967; 1968) proposed the so-called "standing-gradient" osmotic flow hypothesis for fluid transporting epithelia in liver and kidney. This model is based on the functional geometry of the tissue and depends upon the "channels" produced between infoldings of the basal cell membrane and between the elongated microvillar projections of the apical cell membrane being structurally or functionally enclosed at one end. At equilibrium, a standing osmotic gradient is established along the length of each extracellular canal at both the basal and apical surfaces. This osmotic gradient is achieved by the pumping of solute across the region of cell membrane at the closed ends of each canal, such that a local difference in osmotic pressure is developed between the end space and the adjacent cytoplasm, and along the canal itself. As a result, water moves into the space from the adjacent cytoplasm so that towards the open end of canal the fluid is iso-osmotic to the cytoplasm. However, difficulties arise when this model is applied to insect Malpighian tubule secretion because the basal cell membrane infoldings and the apical microvilli are too short to permit development of solute gradients along their lengths (Taylor, 1971; Maddrell, 1977). Indeed, the ion gradients predicted by the above model were not observed in the Malpighian tubules of *Rhodnius prolixus*, determinations made by electron-probe X-ray microanalysis (Gupta *et al.*, 1976; 1977). It is also suggested that the osmotic permeabilities of cell

membranes, required for near iso-osmotic secretion, are impossibly high (Hill, 1975a,b; 1977).

Another theory which has been suggested to explain the mechanism of ion and water movements across epithelia is a process of electro-osmosis (Hill, 1975a,b). Maddrell (1977), when discussing this theory in relation to insect Malpighian tubules, suggested that apical electrogenic cation pumps produce an electrical potential difference across the membrane. The resulting electrochemical gradient would draw  $\text{Cl}^-$  out from the cell through the membrane, and in crossing the membrane  $\text{Cl}^-$  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 the apical cell membrane. This and the standing-gradient model are not mutually exclusive (Taylor, 1971; Maddrell, 1977).

Other observations for water movement have proposed and elaborated in some form of "local osmosis" (Taylor, 1971; Maddrell, 1971) as a theory (Diamond, 1964) for insect Malpighian tubule function. This theory proposes that, as a result of solute pumping across the basal and apical cell membranes, the cytoplasm becomes marginally hypertonic to the bathing medium due to active solute transport, and similarly, the lumen content becomes marginally hypertonic to the cytoplasm. However, Maddrell (1980) argued against the paracellular route for water movement across the Malpighian tubules of *Rhodnius prolixus* which, when stimulated, have one of the highest rates of fluid transport yet reported (see Phillips, 1981). Unlike vertebrate gallbladder and proximal renal tubule, intercellular channels in tubules of *Rhodnius prolixus* are relatively rare, comprising only 0.034% of the total surface area (Maddrell, 1980). Furthermore, septate junctions extend along almost the whole length of these

intercellular channels (Maddrell, 1980; Phillips, 1981). Fluid secretion by a paracellular route should also be indicated by the passive movement of large solutes, e.g., xylose, sucrose and inulin, which presumably also take this pathway. However, Maddrell (1980) found that the net flux of these sugars remained unchanged when fluid secretion by tubules of *Rhodnius prolixus* was increased approximately 7-fold by the application of the diuretic hormone mimic, 5-hydroxytryptamine (5-HT). Indeed, if inulin could pass through these intercellular junctions, it would be difficult to imagine how these structures could have reflection coefficients for monovalent ions high enough to create the osmotic gradients required for water movement (Maddrell, 1980; Phillips, 1981). Furthermore, if ion movements did take place via a paracellular route, it would be most difficult to explain the difference in composition between haemolymph and luminal fluid. Maddrell (1980) concluded that water movement across the tubules of *Rhodnius prolixus* occurs largely through the cells, and that the static fluid between the apical microvilli need only be 2-3% hyperosmotic to the haemolymph to cause normal rates of fluid secretion by simple osmosis. As a result of these findings, the Malpighian tubules of *Rhodnius prolixus* would be classified as a "tight" epithelium. Therefore, these differences of osmotic pressure, water would move passively across the epithelial cell. The apical and basal surfaces of Malpighian tubule cells consist of microvilli and basal infoldings respectively which increase the surface area of the cell exposed to extracellular fluids. These amplifications increase the passive permeability of the cells and so increase the driving force for fluid secretion (Taylor, 1971).

Whatever the exact mechanism for coupling of ion and water movements, the various models agree, that fluid secretion by insect Malpighian tubules is a

consequence of solute transport. Ion transport across cell membranes requires the presence of various cell membrane proteins (Scoble *et al.*, 1986) such as:

- I) Uniporters, which facilitate the movement of an ion down its concentration gradient.
- II) Symporters which facilitate the movement of an ion down its concentration gradient associated with the movement of a second ion against a concentration gradient, the latter ion undergoing secondary active transport.
- III) Antiporters, which link the diffusion of ion down its concentration gradient with that of a second ion in the opposite direction.
- IV) ATPases, which link the movement of ions to ATP hydrolysis.
- V) Channels, which form pores through the membrane, allowing passive diffusion of ions down their electrochemical gradients.

A variety of workers have suggested an electrogenic cation pump in the apical membrane of Malpighian tubules of *Glossina morsitans*, Gee, 1976a,b; Nicolson and Isaacson, 1996; *Calliphora vicina*, Berridge, 1968; 1969; *Rhodnius prolixus*, Maddrell, 1971; 1977; O'Donnell and Maddrell, 1984; *Locusta migratoria*, Anstee *et al.*, 1986; Baldrick 1987; Fogg *et al.*, 1991. This apical pump was believed to be relatively non-specific in nature because, although  $K^+$  is the principal ion transported, other cations can be transported under appropriate conditions (Harvey *et al.*, 1983a). For example, Berridge (1968) found that in Malpighian tubules of *Calliphora vicina*,  $Rb^+$  also maintained a high rate of fluid production when it replaced  $K^+$  in the bathing medium. Calcium and sodium could also support fluid secretion but at a lower rate. However, studies carried out by Marshall (1995) found a large amount of  $K^+$  ( $61.9 \pm$

16.9mM) was still being secreted by the tubule cells of *Locusta* even after they had been pre-incubated in  $\text{Rb}^+$ -( $\text{K}^+$ -free) saline for 45min. This is a significantly lower amount than that found in the secreted fluid after 45 min incubation in  $\text{K}^+$ -saline ( $111.6 \pm 7.5\text{mM}$ ), and than the amount ( $115.9 \pm 9.6\text{mM}$ ) secreted by the tubule cells after 5 min pre-incubation in  $\text{Rb}^+$ -( $\text{K}^+$ -free) saline. A similar result has also been reported by Anstee *et al.*, (1979), Fathpour (1980) and Bell (1977), who recorded  $\text{K}^+$  still being secreted (at levels of approximately 40mM) when tubules were bathed in  $\text{K}^+$ -free saline. Therefore, although the amount of  $\text{K}^+$  being secreted fell with respect to the length of incubation in  $\text{Rb}^+$ -( $\text{K}^+$ -free) saline, as would be expected as no  $\text{K}^+$  was entering the cell, a substantial amount of  $\text{K}^+$  was still being secreted. Furthermore, as shown by X-ray microanalysis (Pivovarova *et al.*, 1994a) the cells contained only approximately 10% of the  $\text{K}^+$  found in control cytoplasm. This suggests that the ion pump at the apical surface must have a high affinity for  $\text{K}^+$ . At the same time the amount of  $\text{Na}^+$  being secreted remained fairly constant even though its intracellular content rose considerably when tubules had been incubated in  $\text{Rb}^+$ -saline. This suggests that if there is a common exit pump for  $\text{K}^+$  and  $\text{Na}^+$  then that pump must have a much greater affinity for  $\text{K}^+$ . Alternatively  $\text{Na}^+$  leaves by a different apical mechanism (Wessing and Zierold, 1996).

The tubules of *Rhodnius prolixus* could also secrete at impressive rates (40% of the rate recorded in control saline) in an ammonium solution containing no  $\text{Na}^+$  or  $\text{K}^+$  (Maddrell, 1969). These results along with the fact that *Rhodnius* is able to secrete at the same rate in  $\text{K}^+$ -free solutions led Maddrell (1969) to propose a mechanism whereby at low  $\text{K}^+$  concentrations the pump mechanism could switch from pumping  $\text{K}^+$  to  $\text{Na}^+$ . Maddrell (1977) later presented a model for fluid secretion involving a

common cation pump. He proposed that if the apical pump was non-specific then the cation which would be transported from the cell to the lumen would be the one found at the highest intracellular levels. Using this model it was possible to explain the results obtained using the Malpighian tubules of *Rhodnius prolixus* without postulating that the apical membrane had two separate pumps for  $\text{Na}^+$  and  $\text{K}^+$  and why in the insect *Glossina morsitans*,  $\text{Na}^+$  was the "prime mover". In *Rhodnius prolixus*, it was suggested that the apical pump had a higher affinity for  $\text{Na}^+$ , but the permeability of the basal membrane and the electrochemical gradient of the ions involved would determine the availability of  $\text{Na}^+$  and  $\text{K}^+$  for the apical pump (Maddrell, 1977). Therefore, the secretion of a  $\text{Na}^+$ -rich "urine" by the Malpighian tubules of *Glossina morsitans*, (Gee, 1976a,b) and *Glossina austeni*, (Gee, 1975a,b) would result if the basal membrane had a higher permeability to  $\text{Na}^+$  than  $\text{K}^+$ . However, in species where membrane had a higher permeability to  $\text{K}^+$  then  $\text{K}^+$  would be the dominant cation in the cytoplasm and be transported via the apical pump into the lumen (Maddrell, 1971; 1977).

In the majority of insect tubules secreting a  $\text{K}^+$ -rich fluid,  $\text{K}^+$  presumably exits from the cell via a  $\text{K}^+/\text{H}^+$  antiporter, the energy being provided by a proton gradient generated by a V-type ATPase (Wieczorek *et al.*, 1991; Leyssens *et al.*, 1992a,b; 1994; Nicolson, 1993; Marshall, 1995). In bloodsucking insects, where  $\text{Na}^+$  plays a more important or major role in fluid secretion, a  $\text{Na}^+/\text{H}^+$  antiporter is postulated. Alternatively, the luminal membrane might possess a single type of antiporter mediating both  $\text{K}^+/\text{H}^+$  and  $\text{Na}^+/\text{H}^+$  exchange, but with a preference for  $\text{Na}^+$  (Maddrell and O'Donnell, 1992). Acid secretion will result when  $\text{H}^+$ /cation exchange by the antiporter does not keep pace with the proton pump. This occurs in tubules of

*Drosophila* and *Rhodnius* treated with amiloride an inhibitor of  $\text{Na}^+/\text{H}^+$  antiporters (Bertram, 1989; Maddrell and O'Donnell, 1992).

"Urine" formation depends on the availability of anions to accompany the active transport of  $\text{K}^+$  (Berridge, 1969) in order to maintain electroneutrality. Since the "urine" is rich in KCl, it has generally been accepted that  $\text{Cl}^-$  is the accompanying anion.  $\text{Cl}^-$  transport across the basal membrane is thought to be by secondary active transport, i.e.  $\text{Cl}^-$  moves against an electrical gradient but the energy for this process comes from its linked transport to an ion that is moving down a favourable gradient (Phillips, 1981; O'Donnell and Maddrell, 1984; Hegarty *et al.*, 1991). This type of linked ion transport is carried out by symporters (Scoble *et al.*, 1986). However, basal  $\text{Cl}^-$  channels have been detected in the Malpighian tubules of a desert beetle (Nicolson and Isaacson, 1987) but they are not found in the basal membrane of tubules of the yellow fever mosquito (Sawyer and Beyenbach, 1985).  $\text{Cl}^-$  movement across the apical membrane is believed to be passive in all species as there is a favourable electrical gradient (Nicolson, 1993) and apical  $\text{Cl}^-$  channels have been detected (Wright and Beyenbach, 1987; Wiczorek *et al.*, 1989). However, there is some suggestion that  $\text{Cl}^-$  transport may occur paracellularly in the ant (Dijkstra *et al.*, 1994a) and the tsetse fly (Isaacson and Nicolson, 1994).

Apical  $\text{Cl}^-$  channels have been identified in Malpighian tubules of *Aedes aegypti* (Wright and Beyenbach, 1987). The electrical gradient favours passive exit of  $\text{Cl}^-$  across the apical membrane in all species. Whether the main pathway for chloride movement is transcellular or paracellular is not yet clear, however, Leysens *et al.*, (1992a) suggested, on the basis of the very high apical membrane resistance in *Formica*, that much of the  $\text{Cl}^-$  current may pass through a paracellular shunt rather

than through apical channels. In *Aedes* the  $\text{Cl}^-$  pathway appears to be either paracellular or via a second cell type (Pannabecker *et al.*, 1993).

Relative ionic permeabilities of the basal membrane can be assessed in unperfused tubules: changes in bath  $\text{K}^+$  concentration have an almost Nernstian effect on  $V_b$  in several species (reviewed by Nicolson, 1993; Beyenbach, 1995). The existence of basal  $\text{K}^+$  channels has been confirmed indirectly by the effects of barium ions on tubules of *Onymacris*, (Nicolson and Isaacson, 1987) and *Formica*, (Weltens *et al.*, 1992), and directly by a patch clamp study on the basal membrane of *Onymacris* tubules stripped of their surrounding connective tissue (Nicolson and Isaacson, 1990).

Using ion-sensitive microelectrodes, Leyssens *et al.*, (1991) measured surprisingly variable  $\text{K}^+$  activities in cells of *Formica* tubules: 82mM when bath  $\text{K}^+$  was 41mM, but only 29 mM when bath  $\text{K}^+$  was reduced to 4mM. Yet the  $\text{K}^+$  concentration in the secreted fluid is independent of bath  $\text{K}^+$  (Van Kerkhove *et al.*, 1989). Many epithelial cells display a large basal  $\text{K}^+$  conductance and, in common with other cells also possess a  $\text{Na}^+/\text{K}^+$ -ATPase on their basal surface which pumps  $\text{Na}^+$  out of the cell in exchange for  $\text{K}^+$ . The large basal  $\text{K}^+$  conductance allows  $\text{K}^+$  entering the cell through the  $\text{Na}^+/\text{K}^+$ -ATPase to return to the serosal side (Hanrahan *et al.*, 1986). This permeability to  $\text{K}^+$  has an effect on the membrane potential which in turn influences ion flux through channels and transporters (Hanrahan *et al.*, 1986). Passive movements of ions can occur across cell membranes in response to a concentration or electrical gradient driving this flux. Ions can pass through a membrane via carriers or through pores/channels. Almost all channels display gating, that is, they can exist in more than one conformation (open or closed) and the stimulus for the transition between open and closed states can be a change in voltage across the membrane or by the use of

chemicals that can open or block channels. Channels have been discovered that allow the movement of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  (Van Driessche and Zeiske, 1985).

It has been suggested that ion movement into the cell across the basal membrane occurs via a coupled  $\text{Na}^+/\text{K}^+$  pump (Berridge, 1967; Berridge and Oschman, 1969; Anstee *et al.*, 1986)). In the majority of tissues examined, the enzyme "pump" responsible for such exchange is a  $\text{Mg}^{2+}$ -dependent ( $\text{Na}^+/\text{K}^+$ )-stimulated ATPase, first demonstrated by Skou (1957). Further studies revealed that this enzyme is ubiquitous in animal cells and established some of its major features, it is located in cell membranes and inhibited by ouabain (for review see Skou, 1965; Schuurmans Stekhoven and Bonting, 1981; Skou and Esmann, 1992). Indeed,  $\text{Na}^+/\text{K}^+$ -ATPase activity has been demonstrated in microsomal preparations of Malpighian tubules and hindgut of *Locusta migratoria* (Anstee and Bell, 1975; 1978; Anstee *et al.*, 1979; 1986; Peacock, 1976; Kalule-Sabiti, 1985; Fogg *et al.*, 1991; Marshall, 1995). Other studies have provided evidence for the existence of  $\text{Na}^+/\text{K}^+$ -ATPase in the rectal tissue of *Periplaneta americana* (Tolman and Steele, 1976), the hindgut and Malpighian tubules of *Jamaicana flava*, *Schistocerca gregaria* and *Homorocoryphus nitidulus vicinus* (Peacock *et al.*, 1972; 1976), the rectum of *Schistocerca gregaria* (Lechleitner and Phillips, 1988), Malpighian tubules of *Rhodnius prolixus* (Maddrell and Overton, 1988) and the Malpighian tubules of *Aedes aegypti* (Hegarty *et al.*, 1991). Its occurrence is restricted to cell membranes, with hydrolysis of ATP at the inner side of the membrane resulting in the transfer to the extracellular fluid of  $3\text{Na}^+/\text{ATP}$  molecule. This occurs in exchange for  $2\text{K}^+$  in the majority of cases (for review see Schwartz *et al.*, 1975; Schuurmans Stekhoven and Bonting, 1981; Cantley, 1981; Skou and Esmann, 1992). In support of the proposal by Berridge and Oschman (1969) that a

basal electrogenic  $\text{Na}^+/\text{K}^+$ -ATPase enzyme is responsible for the cytoplasm of most cells having a high (100-160 mM)  $\text{K}^+$  concentration, but relatively low (3-30mM)  $\text{Na}^+$  concentration (Bonting, 1970; Rodriguez and Edelman, 1979; Ernst *et al.*, 1980; Schuurmans Stekhoven and Bonting, 1981; Anstee and Bowler, 1984; Lechleitner and Phillips, 1988; Lebovitz *et al.*, 1989; Fogg *et al.*, 1991; Skou and Esmann, 1992).

O'Donnell and Maddrell (1984) reported that  $\text{Na}^+$  and  $\text{Cl}^-$  were co-transported at the basal cell membrane of Malpighian tubules of *Rhodnius prolixus*, this was a rational idea as electrochemical gradients favoured  $\text{Na}^+$  but not  $\text{Cl}^-$  entry, and that blockers of  $\text{NaCl}$  co-transport (Palfrey and Greengard, 1981; Palfrey and Rao, 1983), furosemide and bumetanide, had effects consistent with the presence of a  $\text{NaCl}$  co-transport mechanism in the Malpighian tubules. O'Donnell and Maddrell (1984) proposed that, during rapid fluid secretion stimulated by 5-HT or the naturally occurring diuretic hormone, a  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  co-transporter, located on the basal membrane, was responsible for the movement of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  into the cell, thus allowing the movement of  $\text{K}^+$  against its concentration gradient. A catholic apical cation pump was proposed for the extrusion of  $\text{K}^+$  and/or  $\text{Na}^+$  from the cell, with  $\text{Cl}^-$  exit being passive. Thus, the fluid secreted would contain approximately equal concentrations of  $\text{K}^+$  and  $\text{Na}^+$  (Maddrell, 1969). However, the fluid secreted by non-stimulated tubules contained only low levels of  $\text{Na}^+$  (Maddrell and Overton, 1988). Williams and Beyenbach (1984) also produced evidence of coupled cation- $\text{Cl}^-$  movement during stimulated fluid secretion by the Malpighian tubules of *Aedes aegypti*.

Baldrick *et al.*, (1988), from microelectrode studies on Malpighian tubules of *Locusta migratoria*, found that the basal cell membrane potential ( $V_B$ ) was largely

accounted for by the selective permeability of this membrane to  $K^+$  and its relative impermeability to  $Na^+$  and  $Cl^-$ . These workers suggested some passive  $Na^+$  entry would occur due to large concentration and electrical gradients and proposed that  $Cl^-$  transport was linked to this  $Na^+$  movement as indicated by using furosemide and bumetanide in electrophysiological and fluid secretion studies (Baldrick, 1987). However, the evidence for  $Na^+/K^+/2Cl^-$  co-transport in tubules of this insect was not as conclusive as for *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). Indeed, it was suggested that  $Cl^-$  entry was not necessarily dependent on co-transport with  $Na^+$ , and the possibility of  $2K^+-2Cl^-$  co-transport was not ruled out (Baldrick *et al.*, 1988).

Later Fogg (1990), continuing the work of Baldrick, suggested that there was no conclusive evidence for the presence of a  $Na^+/K^+/2Cl^-$  co-transporter on the basal surface of the Malpighian tubules of *Locusta migratoria* since furosemide and bumetanide did not have strong electrophysiological effects. However, from flux studies it was demonstrated that  $Cl^-$  transepithelial transport was stimulated by high external  $K^+$ , in  $Na^+$ -free conditions. Hence it was proposed that  $Cl^-$  entry may be dependent on the external  $K^+$  concentration but not on the presence of  $Na^+$  and so may enter by a  $K^+-Cl^-$  transport mechanism.  $K^+-Cl^-$  transport is relatively insensitive to loop diuretics compared to  $Na^+/K^+/2Cl^-$  transport (O'Grady *et al.*, 1987).  $K^+$  stimulated  $Cl^-$  transport had been reported in locust rectum by Hanrahan and Phillips (1982; 1983a,b). However, in control saline the electrochemical gradient for  $K^+-Cl^-$  transport was unfavourable. Therefore it was concluded that there must be some  $Cl^-$  entry by other, possibly furosemide sensitive, mechanisms (Figure 1.1).

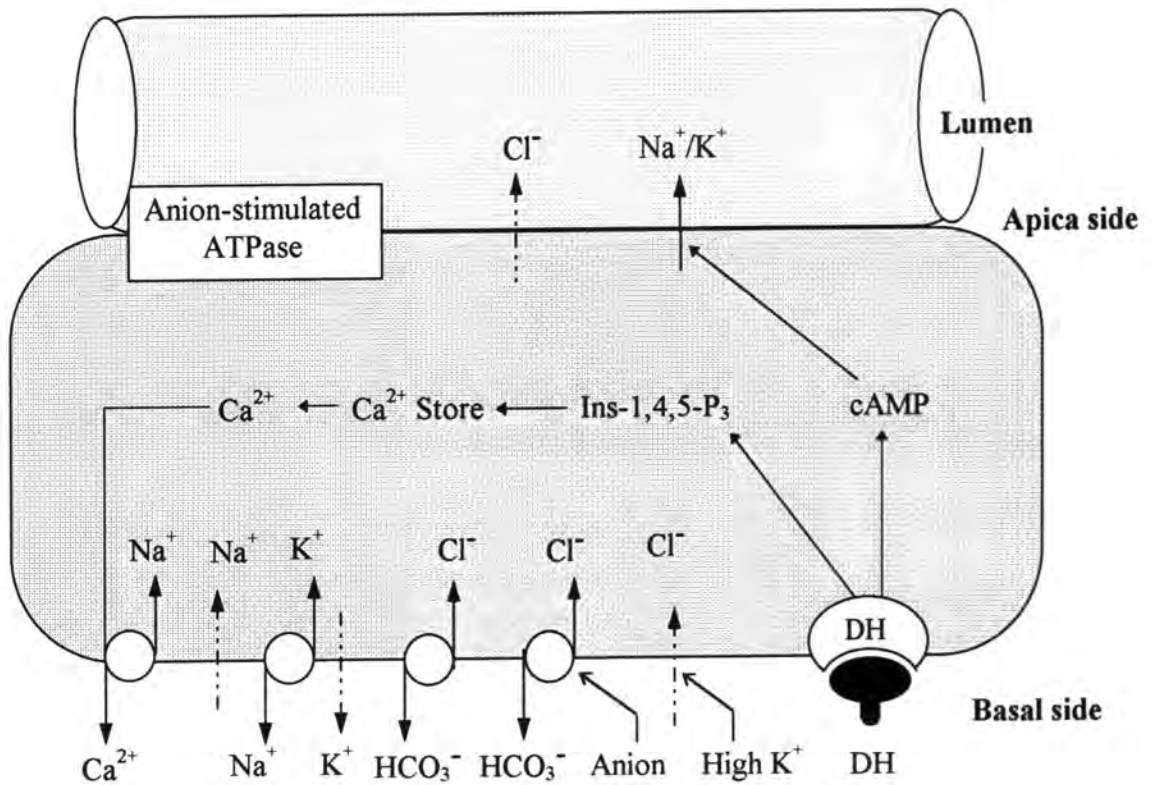


Figure 1.1 (Fogg, 1990)

Cl<sup>-</sup> entry was also suggested to occur in exchange for HCO<sub>3</sub><sup>-</sup>, as proposed for the rectal gland of *Aedes dorsalis* (Strange and Phillips, 1984) and by the activity of (Cl<sup>-</sup>+HCO<sub>3</sub><sup>-</sup>)-ATPase, as proposed for the intestinal epithelium of *Aplysia* (Gerencser and Lee, 1985) or for Malpighian tubules of *Locusta migratoria* (Fogg, 1990). Previous ion substitution experiments using Cl<sup>-</sup>-free and HCO<sub>3</sub><sup>-</sup>-free saline containing acetazolamide gave similar electrophysiological results, suggesting that Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> transport were linked (Baldrick, 1987). Furosemide has been suggested to have a relatively non-specific effect (Palfrey *et al.*, 1980). It has been shown to effect Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange (Chipperfield, 1986). Additionally, Fogg (1990) detected an anion-stimulated ATPase on the basal membrane and this enzyme has been related to Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange (Herrera *et al.*, 1978) and active Cl<sup>-</sup> transport (Lechleitner and

Phillips, 1988). Finally, Fogg (1990) and Fogg *et al.*, (1991), were unable to demonstrate a  $K^+$ -stimulated ATPase on the apical membrane, suggested that the anion-stimulated ATPase which could be detected was a possible candidate for the cation pump.

During fluid secretion, Fogg (1990), suggested that diuretic hormone (DH) appears to interact with its basal membrane receptor (DHR) resulting in the elevation in levels of two intracellular second messengers, cAMP and Inositol 1,4,5-triphosphate (Ins-1,4,5- $P_3$ ). cAMP, in turn, stimulates the apical electrogenic cation pump. This pump was suggested to transport  $K^+$  predominantly, but is relative unspecific and may transport some  $Na^+$ . Ins-1,4,5- $P_3$  effects the release of  $Ca^{2+}$  from intracellular stores.  $Ca^{2+}$ , in turn, when was proposed to cause an increase in  $Cl^-$  conductance across both the basal and apical membranes. Increased  $Cl^-$  conductance across the basal membrane could be brought about by stimulation of both the  $Cl^-/HCO_3^-$  exchanger and the anion-stimulated ATPase.  $Cl^-$  passively follows  $K^+(Na^+)$  into the lumen.

In a recent study on the Malpighian tubules of *Locusta migratoria*, Marshall (1995) proposed that a basal  $Na^+/K^+$ -ATPase is responsible for  $K^+$  entry into the tubule cells and for maintaining low intracellular  $Na^+$ .  $K^+$  channels in the basal membrane allow passive movement of  $K^+$ , under control conditions these channels would allow the passive exit of  $K^+$  from the cell. When the basal membrane hyperpolarizes  $K^+$  channels known as anomalous or inward rectifiers open and allow passive  $K^+$  entry. Passive entry of  $Na^+$  was suggested to occur at the basal surface down concentration and electrical gradients. Co-transporter action at the basal membrane allowing linked  $Na^+$ ,  $K^+$  and  $Cl^-$  entry, although it is thought possible that under  $Na^+$ -free conditions the co-transporter could operate as a KCl transporter (see Figure 1.2).

At the apical membrane Marshall (1995), suggested that  $K^+$  exit is postulated to occur via an antiporter which is energized by a V-type ATPase (Figure 1.2). The antiporter can accept  $K^+$  and  $Na^+$  but has a much higher affinity for  $K^+$ . The exit of  $Cl^-$  occurs passively down a favourable electrical gradient.

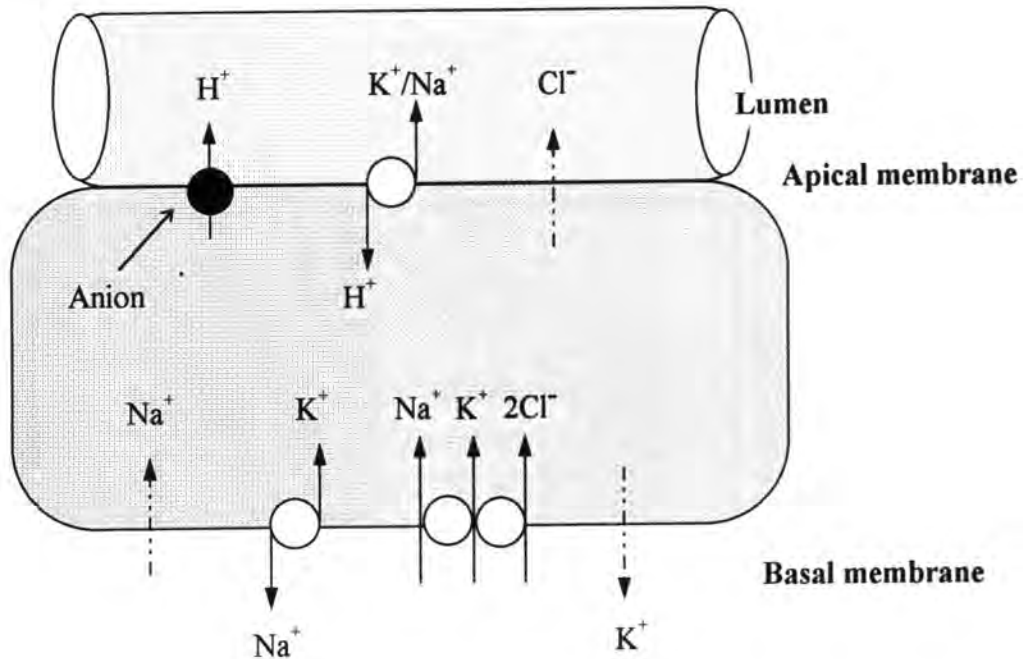


Figure 1.2 (Marshall, 1995)

The existence of a  $K^+$ -ATPase pump in an insect epithelium was first deduced by Ramsay (1953; 1954; 1956; 1958) from *in vivo* measurements on Malpighian tubules of *Dixippus morosus*, and later demonstrated by Harvey and Nedergaard (1964) in isolated, short-circuited larval lepidopteran midgut. Evidence for a similar pump in salivary and labial glands followed (Berridge and Patel, 1968; Kafatos, 1968). More recently, evidence has been presented that a similar  $K^+$ -pump is responsible for generating the receptor current in a variety of insect sensilla (Kuppers and Thurm, 1979; Wiczorek, 1982). The  $K^+$ -pump in all of these epithelia shared many similar

characteristics and was therefore tacitly assumed to be similar at a molecular level (see reviews by Thurm and Kuppers, 1980; Harvey, 1982).

Malpighian tubules are not the only secretory structures where active transport of  $K^+$  has been demonstrated. Harvey and Nedergaard (1964) and Harvey *et al.*, (1967) used radioactive tracer methods to show that the short circuit current across the midgut of *Hyalophora cecropia* was mainly due to the active transport of  $K^+$ . Harvey *et al.*, (1968), later demonstrated this transport was electrogenic and was responsible for generating a midgut potential in excess of 100mV. Ultrastructural evidence linked this active electrogenic step to the apical membrane (Anderson and Harvey, 1966) and Blankemeyer and Harvey (1978) confirmed this suspicion with microelectrode studies. Insect sensilla cells also contain an apical  $K^+$  pump (Thurm and Küppers, 1980) and studies by Berridge and Prince (1972a), Prince and Berridge (1972), Berridge *et al.*, (1976) produced evidence for an apical pump in the salivary glands of *Calliphora*. The presence of an apically located  $K^+$  pump in various insect tissues is reviewed by Harvey (1980) and Harvey *et al.*, (1983b), where evidence is also presented for an apical  $K^+$  pump in *Manduca sexta* midgut.

The first evidence for the presence of a V-type ATPase in Malpighian tubules came from the work of Bertram *et al.*, (1991) working on *Drosophila hydei*. They discovered that fluid secretion was inhibited by NEM and bafilomycin  $A_1$ , and suggested that the V-type ATPase produced a pH gradient which would provide energy for extrusion of  $K^+$  by an electroneutral antiporter (see Figure 1.3.). Weltens *et al.*, (1992) suggest a similar apical exit mechanism for  $K^+$  in the Malpighian tubules of *Formica polyctena*.

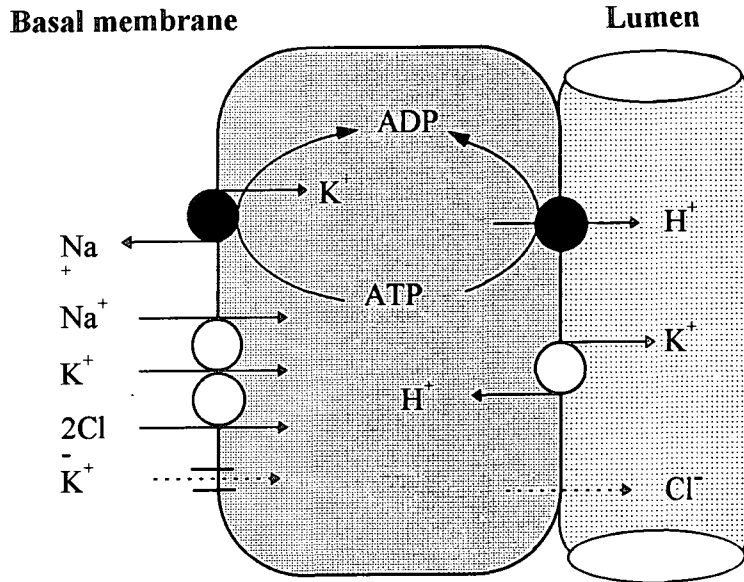


Figure 1.3 (Bertram *et al.*, 1991).

Zeiske (1992) proposed a model to explain fluid secretion by Malpighian tubules of *Manduca sexta*. He suggested that insects with low haemolymph  $\text{K}^+$  possessed both the antiport and a  $\text{Na}^+/\text{K}^+$ -ATPase on their basal membrane, but insects with rich haemolymph  $\text{K}^+$  possessed  $\text{K}^+$  channels in the basal membrane which provided the main entry mechanism for  $\text{K}^+$ , a  $\text{Na}^+/\text{K}^+$ -ATPase being undetectable. At the apical surface, he proposed that there would be a V-type ATPase and an antiporter, with chloride channels as shown above. This has become the standard model to explain fluid secretion across the Malpighian tubules of many insects (Leysens *et al.*, 1992b; Nicolson, 1993).

Maddrell and O'Donnell (1992) suggested a model to explain ion movements underlying secretion by insect Malpighian tubules prior to the discovery that an  $\text{H}^+$ -pumping V-type ATPase was involved (Figure 1.4.A). The transport processes on the

haemolymph-facing membrane illustrate the different ways that ions enter the cell, tubules that transport mainly  $K^+$  and  $Cl^-$ ,  $Na^+$  and  $Cl^-$ , or both. However, they proposed another model for ion movements underlying secretion by insect Malpighian tubules incorporating an  $H^+$ -pumping V-type ATPase on the luminal cell membrane (see Figure 1.4.B)

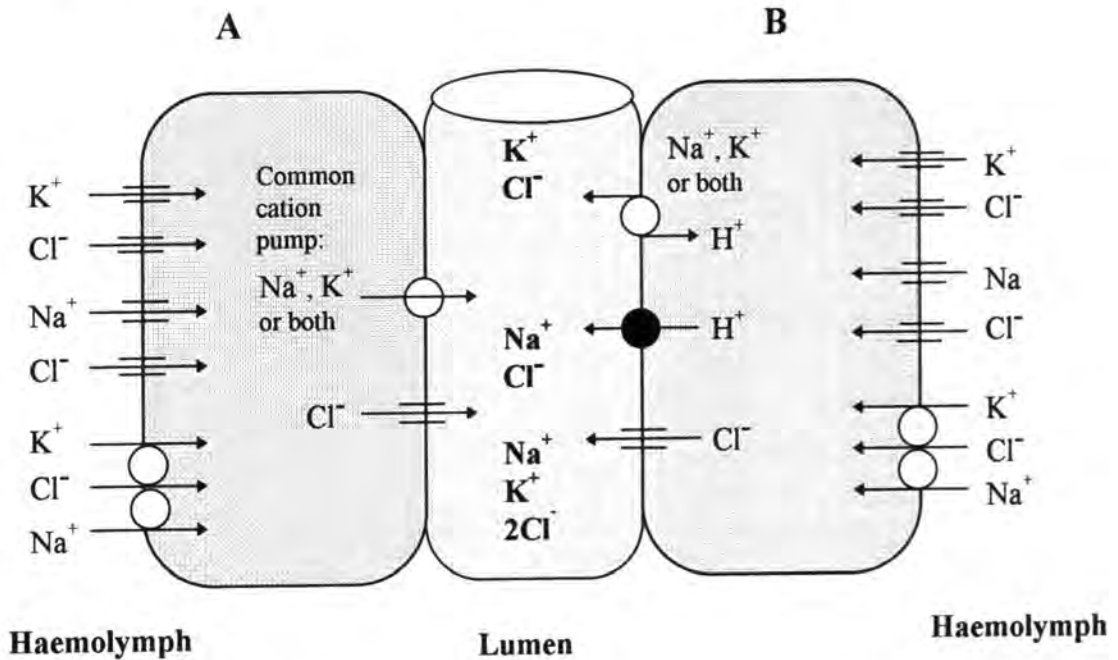


Figure 1.4 (Maddrell and O'Donnell, 1992).

Zhang *et al.*, (1994) working with *Formica polyctena* measured the cellular and luminal pH of Malpighian tubules when the concentration of  $K^+$  in the medium bathing the tubules was varied. In all conditions a cell-inward electrochemical gradient for  $H^+$  was observed so they concluded that the apical  $H^+$  concentration gradient could drive electroneutral  $H^+/K^+$  exchange. Other workers have also produced evidence for the presence of a V-type ATPase and electroneutral antiporter in Malpighian tubules (Hegarty *et al.*, 1992; Wessing *et al.*, 1993; Leyssens *et al.*, 1993a,b).

In searching for the molecular correlate of the insect  $K^+$  pump, Cioffi and Wolfersberger (1983) succeeded in isolating the apical plasma membrane from *Manduca sexta* midgut goblet cells and demonstrated an ATPase activity associated with them (Wieczorek *et al.*, 1986). The ATPase enzyme was a cation-stimulated rather than an anion-stimulated enzyme, this ATPase was insensitive to ouabain and fluoride but inhibited by nitrate. These properties of the "common cation pump" exhibited strong similarities to the class of proton-pumping ATPases which are termed vacuolar-type ATPases (Mellman *et al.*, 1986; Wieczorek *et al.*, 1986) and was subsequently purified and characterised as a vacuolar-type ATPase (Schweickl *et al.*, 1989; Wieczorek *et al.*, 1989; 1991; Klein *et al.*, 1991).

The fact that exogenously applied cAMP can mimic the action of diuretic hormone in many insects and increase the rate of fluid secretion of the Malpighian tubules in, e.g. *Aedes taeniorhynchus*, (Maddrell and Phillips, 1978); *Rhodnius prolixus*, (Maddrell *et al.*, 1971); *Carausius morosus*, (Maddrell *et al.*, 1971); *Pieris brassicae*, (Nicolson, 1976a,b); *Cenocorixa blaisdelli*, (Cooper *et al.*, 1988); *Glossina morsitans*, (Gee, 1976a); *Locusta migratoria*, (Anstee *et al.*, 1980; Morgan and Mordue, 1981); *Schistocerca gregaria*, (Maddrell and Klunswan, 1973; James *et al.*, 1993); *Papilio demodocus*, (Nicolson and Millar, 1983); *Onymacris plana*, (Nicolson and Hanrahan, 1986); *Cenocorixa bifida*, (Sizbo and Scudder, 1979); *Aedes aegypti*, (Petzel and Stanley-Samuelson, 1992) and *Acheta domesticus*, (Kim and Spring, 1992), led to the proposal that it was transducing the action of diuretic hormone in these organs. This is supported by the fact that diuretic hormone was also found to increase intracellular levels of cAMP in the tubules (Aston, 1975; Phillips, 1982; Fogg *et al.*, 1990; Nicolson, 1992; Troetschler and Kramer, 1992; Lehmborg *et al.*, 1993).

cAMP also increased the rate of fluid secretion by salivary glands (Berridge, 1970; Berridge, 1980; Berridge and Patel, 1968) and the rate of fluid absorption in the midgut (Farmer *et al.*, 1981). Additionally, rectal cAMP levels increase 3-fold during exposure to homogenates of corpora cardiaca (Spring and Phillips, 1980). There are some cases where cAMP is reported to have no effect on the rate of fluid secretion, for example, *Musca domestica* (Dalton and Windmill, 1980).

Diuretic hormones have been identified in every insect species that has been studied, and it now appears that insects may have more than one, each operating via a different second messenger system (see review by Spring, 1990). Working with *Locusta migratoria* a set of experiments carried out by Morgan *et al.*, (1987) produced both direct and indirect evidence for the presence of two diuretic peptides in extracts of the corpora cardiaca. When these extracts were analysed by HPLC two peaks were produced but only the peptide corresponding to one of these peaks increased cAMP levels in broken cell preparations. Serotonin also was found to increase the rate of fluid production but had no effect on the intracellular levels of cAMP suggesting a second, cAMP-independent pathway for fluid transport. Other evidence produced by Fogg *et al.*, (1989) demonstrated that corpora cardiaca extract and cAMP exerted different effects on the apical membrane potential, again suggesting the action of diuretic hormone is mediated by more than one messenger. Fogg *et al.*, (1990) went on to show that the levels of the second messenger inositol 1, 4, 5-P<sub>3</sub> were also increased in Malpighian tubules when exposed to diuretic hormone and so suggested that this molecule may also be involved in the response to diuretic hormone, possibly acting through cellular calcium. Evidence from other studies points to a link between inositol 1, 4, 5-P<sub>3</sub> and intracellular calcium release (Berridge, 1983; Streb *et al.*, 1983;

Berridge and Irvine, 1984). Recently, it has been suggested that nitric oxide also has a role in the control of fluid secretion in Malpighian tubules of *Drosophila* where it activates the cGMP signalling pathway and is separate to the  $\text{Ca}^{2+}$  signalling system (Dow *et al.*, 1994a).

Besides cyclic nucleotides, a new generation of phosphoinositide-derived second messengers have been identified (Michell, 1975; Berridge, 1984; Berridge and Irvine, 1984; Nishizuka, 1984; Majerus *et al.*, 1986; Berridge, 1987). Receptor stimulation triggers the phospholipase C catalyzed hydrolytic cleavage of membrane phosphoinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) to yield two second messenger molecules, inositol 1,4,5-triphosphate ( $\text{Ins-1,4,5-P}_3$ ) and sn-1,2-diacylglycerol (DAG), an activator of protein kinase C (Nishizuka, 1984). Once activated by DAG, together with intracellular  $\text{Ca}^{2+}$ , protein kinase C proceeds to phosphorylate specific proteins that are thought to contribute to the final cellular response (for review see Berridge, 1984; 1986; 1987).

The study of the divalent cation  $\text{Ca}^{2+}$  in transport processes is limited in comparison to studies on monovalent ions, especially in insect epithelia. A central role for  $\text{Ca}^{2+}$  in secretory processes was proposed by Douglas (1968), and it has since become evident that changes in intracellular  $\text{Ca}^{2+}$  are necessary for secretion in a wide variety of tissues and that the control of intracellular  $\text{Ca}^{2+}$  is crucial for the regulation of cellular processes (Rasmussen and Goodman, 1977).  $\text{Ca}^{2+}$  plays an important role as a second messenger conveying signals received at the cell surface to the inside of the cell (Berridge, 1980). It is involved in the regulation of such diverse processes as muscle contraction, secretion of hormones, digestive enzymes and neurotransmitters, and the control of glycogen metabolism in the liver. Indeed, this cation is important in

regulation of net transport of ions and water in transporting epithelia (Taylor and Windhager, 1979; Windhager and Taylor, 1983).

The coupling of an extracellular stimulus to a physiological response in various cells, including polar epithelial cells, is mediated by a chain of intracellular "second messenger" molecules (Nishizuka, 1988; Shenolikar, 1988). In tubular cells this transducing mechanism transmits signals from receptors located at the basolateral surface to effector proteins located at the apical surface.  $\text{Ca}^{2+}$ -dependent protein kinases play a central role in signal transduction by phosphorylating, and thereby activating, effector proteins using ATP as a phosphate donor (Nishizuka, 1988; Shenolikar, 1988). Like cAMP-dependent protein kinase (protein kinase A),  $\text{Ca}^{2+}$ -dependent protein kinases specifically phosphorylate serine and threonine residues of target proteins. The two main groups of  $\text{Ca}^{2+}$ -dependent protein kinases include: (1)  $\text{Ca}^{2+}$ - and phospholipid-dependent protein kinase C (PKC), (2)  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (Nishizuka, 1988; Shenolikar, 1988). While  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase is activated by these two ligands, PKC is activated as a part of a quaternary complex which includes the kinase, diacylglycerol (or phorbol ester), phospholipid (usually phosphatidylserine) and  $\text{Ca}^{2+}$  (Nishizuka, 1988).

$\text{Ca}^{2+}$ -dependent protein kinases are known to regulate the activity of several membrane-bound ion channels and carriers in various tissues, including kidney, thereby modulating transmembrane electrolyte transport. PKC-induced phosphorylation of the catalytic subunit of the renal tubular basolateral membrane-bound  $\text{Na}^+/\text{K}^+$ -ATPase inhibits the activity of the pump (Bertorello *et al.*, 1991; Lowndes *et al.*, 1990). The activity of  $\text{Na}^+/\text{K}^+$ -ATPase is known to be modulated by hormones such as insulin, dopamine and prostaglandins E1 and E2, the action of which involves kinase-mediated

protein phosphorylation ((Nishizuka, 1988; Aperia *et al.*, 1991; Taub *et al.*, 1992). PKC inhibits the activity of the tubular luminal membrane-bound  $\text{Na}^+$ -dependent transport mechanisms for glucose (Friedlander and Amiel, 1989) and phosphate (Quamme *et al.*, 1989; Boneh *et al.*, 1989). PKC activation is important in parathyroid hormone-mediated inhibition of the latter transporter (Hruska *et al.*, 1987; Quamme *et al.*, 1989). Weinman and Shenolikar (1986) and Weinman *et al.*, (1988) have demonstrated that the renal apical membrane  $\text{Na}^+/\text{H}^+$  exchanger is stimulated by PKC. In a further study (Horie *et al.*, 1992) showed that long-term activation of PKC leads to a chronic persistent increase in  $\text{Na}^+/\text{H}^+$  exchanger activity that is dependent on transcription and translation.

Isolated brush border membrane vesicles (BBMV) have served as an extremely useful model to explore directly carrier-mediated transport processes (Murer and Gmaj, 1986). Nevertheless, because of the complexities and various cell compounds involved in protein kinase action, the role of these enzymes in membrane transport has been traditionally investigated using whole tissue or cell preparations. A number of studies, successfully probed the effect of various protein kinases on  $\text{Na}^+$ /phosphate (Hammerman and Hruska, 1982) and  $\text{Na}^+/\text{H}^+$  exchange (Weinman and Shenolikar, 1986; Weinman *et al.*, 1987; Rood *et al.*, 1988) in isolated renal BBMV. This was achieved by opening the vesicles or making them permeable to large molecular weight cofactors and nucleotides using “hypoosmotic shock” (Hammerman and Hruska, 1982; Weinman *et al.*, 1987). This allowed access of the phosphorylation “machinery” to the inner cytoplasmic surface.

Using the “hypoosmotic shock” method, Hammerman and Hruska (1982), demonstrated that cAMP-dependent phosphorylation of BBMV proteins was

associated with specific inhibition of  $\text{Na}^+$ -stimulated phosphate transport. Using the same method, (Weinman *et al.*, 1987) showed that cAMP, acting through the endogenous membrane-bound protein kinase A as well as the exogenous catalytic subunit of protein kinase A, inhibited  $\text{Na}^+/\text{H}^+$  exchange in rabbit renal BBMV. In contrast, PKC stimulated the activity of the rabbit renal  $\text{Na}^+/\text{H}^+$  exchanger (Weinman and Shenolikar, 1986).

Wang and Giebisch (1991b) reported that the stimulating effect of ATP on  $\text{K}^+$  channel activity is mediated by phosphorylation of the channel by cAMP-dependent protein kinase A (PKA). In contrast to the effect of PKA, stimulation of PKC inhibits the  $\text{K}^+$  channel by a process that has been shown to be dependent on the intracellular  $\text{Ca}^{2+}$  concentration (Wang and Giebisch, 1991a). Thus, the  $\text{K}^+$  channel is modulated by two different second messenger pathways: the adenylate cyclase system increases channel open probability or recruits additional channels into the apical membrane, whereas stimulation of PKC reduces channel activity.

In a recent study, Wang *et al.*, (1993) produced evidence that apical  $\text{K}^+$  channel activity was linked to basolateral pump turnover rate in rat renal tubule cells. Inhibition of the basolateral  $\text{Na}^+ -\text{K}^+ -\text{ATPase}$  significantly lowered  $\text{K}^+$  channel activity. It has also been found that the application of staurosporine, a potent inhibitor of  $\text{Ca}^{2+}$ -dependent PKC, (Wang *et al.*, 1993) markedly diminished the effect of the pump inhibition on  $\text{K}^+$  channel activity. Therefore, these workers suggested that a  $\text{Ca}^{2+}$ -dependent protein kinase such as PKC plays a key role in the downregulation of apical low-conductance  $\text{K}^+$  channel activity during inhibition of the basolateral  $\text{Na}^+/\text{K}^+ -\text{ATPase}$ .

**The major aims of the work included in this thesis are as the follows:**

- 1) There is a paucity of information on the nature and control of the mechanisms of ion and water transport across the Malpighian tubules of insects. Furthermore, little information is available on the role of protein kinases (PKA and PKC) in ion and fluid secretion by Malpighian tubules of insects. The present study was carried out to address these issues. The effect of diuretic hormone (DH), cAMP, Rp-cAMP (specific inhibitor of PKA), and chelerythrine and staurosporine (potent inhibitors of PKC) on the rate of fluid secretion and cation composition [ $\text{Na}^+$ ] and [ $\text{K}^+$ ] in the “urine” secreted by the Malpighian tubules of *Locusta migratoria* was therefore addressed.
- 2) Isolation of the different membrane fractions from Malpighian tubules of *Locusta migratoria* was carried out to permit their characterization for transport enzymes. The fractions were assured for membrane purity by marker enzyme assays and electron microscopical appearance. In addition, biochemical studies using known transport inhibitors have been used to examine activities of the  $\text{Mg}^{2+}$ -dependent  $\text{HCO}_3^-$ -stimulated ATPase prepared from basal and apical membrane fractions of Malpighian tubules of *Locusta migratoria*.
- 3) The involvement of a V-type ATPase has been demonstrated previously (Marshall, 1995) in fluid secretion by Malpighian tubules of *Locusta migratoria*. Therefore, the main aim of this study was purification of the apical membrane fraction and determination of the characteristics of the V-type ATPase. Immunocytochemical and immunogold localization techniques were used to confirm that the V-type

ATPase and  $\text{Na}^+/\text{K}^+$ -ATPase were located in the apical and basal membrane, respectively, in Malpighian tubules of *Locusta migratoria*.

## CHAPTER 2

### GENERAL MATERIALS AND METHODS

#### Maintenance of Insects:

A stock population of *Locusta migratoria* L. was maintained in an insectary at  $28 \pm 0.5$  °C and a relative humidity of  $60 \pm 0.5\%$  with a constant photoperiod of 12 hours light and 12 hours dark. Continuous air exchange was provided by means of a fan-driven ventilator (Xpelair) and circulation within the room was effected by three wall-mounted electric fans. Humidity was controlled by three humidifiers (Lumatic, Humidifier Group, Bromley, Kent, England). The locusts were reared in perspex-fronted cages (41 cm x 41 cm x 60 cm, supplied by Philip Harris Biological Supplies Ltd., Oldmixon, Weston-super-Mare, Avon, England). Each cage was illuminated by a 40 Watt bulb and consequently the temperature within the cage varied from 28-36°C depending on the distance from the bulb and the photoperiod. Humidity within each cage also varied with the addition of fresh food and water. Locusts were fed daily on fresh grass, water and bran. Animals were reared at sufficiently high population density to prevent reversion to the solitary phase (Joly and Joly, 1953).

#### Glassware:

Pyrex glassware was used throughout. Prior to use it was cleaned by soaking overnight in a 2% Teepol laboratory detergent followed by several rinses in hot tap water and final rinsing in deionized water (six times). All items were then dried in ovens.

**Chemicals:**

All chemicals and drugs were AnalaR grade or the purest commercially available and were largely supplied by Sigma Co., Poole, Dorset, England. Lubrol was gift from I.C.I. Dyestuffs Division. Primary monoclonal antibodies, directed to the V-ATPase from insect plasma membrane (midgut of *Manduca sexta* larvae) were kindly supplied by Prof. Dr. Ulla Klein, Laboratory Wiczorek, Zoological Institute of the University of Munich. Mouse monoclonal antibody IgG  $\alpha$  5 raised against the  $\alpha$ -subunit of the avian sodium pump was generously donated by Prof. D.M. Fambrough, The Johns Hopkins University, Baltimore, Maryland, USA. Chelerythrine chloride was kindly supplied by Dr. Yasmin Weaver, University of Liverpool. Adenosine -3',5'-cyclic monophosphorothioate, Rp- isomer (Rp-cAMP) sodium salt was purchased from BioLog, Life Science Institute, Bremen, Germany.

**Solutions:**

Saline solution buffered with HEPES (pH 7.2) was used throughout, unless otherwise stated. The composition of this saline solution was as in Table 2.1 (Baldrick *et al.*, 1988).

**Table 2.1 Composition of saline solution**

Salt	Concentration (mM)
NaCl	100
KCl	8.6
MgCl <sub>2</sub>	8.5
CaCl <sub>2</sub>	2
NaH <sub>2</sub> PO <sub>4</sub>	4
NaHCO <sub>3</sub>	4
Glucose	34
HEPES	25
NaOH	11

Where necessary bafilomycin A<sub>1</sub> was dissolved in DMSO before adding it to the appropriate saline. The final concentration of DMSO in the experimental saline was <1% and in all studies using this chemical, the same concentration of DMSO was included in the controls.

**Statistics:**

Statistical comparisons of data were carried out using either Student's *t*-test or a paired *t*-test (Snedecor and Cochran, 1967) with reference to the statistical tables of Fisher and Yates (1963). Values where  $p < 0.05$  were taken as significant. Data are presented as mean  $\pm$  SEM unless otherwise stated.

## CHAPTER 3

### *In vitro* studies on fluid and ion secretion by the Malpighian tubules of *Locusta migratoria*.

#### INTRODUCTION

Fluid and ion secretion by Malpighian tubules of numerous insect species has been shown to be under endocrine control (Gee, 1977; Maddrell, 1981; Phillips, 1981; 1982; 1983; Nicholson and Hanrahan, 1986; Wheelock *et al.*, 1988; Dedecker *et al.*, 1994; Patel *et al.*, 1995; Spring and Kim, 1995; Coast, 1995; 1996). Furthermore, there is increasing evidence to suggest that the action of diuretic hormone on Malpighian tubules is mediated by intracellular messengers (Prince *et al.*, 1972; Aston, 1975; Rafaeli *et al.*, 1984; Morgan and Mordue, 1983; 1985; Williams and Beyenbach, 1983; Petzel *et al.*, 1985; 1986; 1987; Rafaeli, 1990; Fogg *et al.*, 1989; 1990; 1993). The role of cAMP as an intracellular second messenger, mediating the action of diuretic hormone on insect Malpighian tubules, has been proposed mainly on the basis that exogenously applied cAMP stimulates tubule secretion, *in vitro*, in a number of different species, including *Locusta* (Anstee *et al.*, 1980; Morgan and Mordue, 1981; Rafaeli and Mordue, 1982). Furthermore, it is generally recognized that the regulation of levels of intracellular cAMP depends upon a balance between the rates of production by adenylate cyclase and degradation by phosphodiesterase. The role of the latter enzyme is indicated by studies which show that fluid secretion by Malpighian tubules of *Locusta* (Morgan and Mordue, 1985) is stimulated by the phosphodiesterase inhibitor IBMX. In addition, various ion transport processes have been shown to respond similarly to treatment with this agent and dibutyryl cAMP (Fogg *et al.*, 1989).

Insect DH's are ; 1) synthesized by the median cells (neurosecretory cells) of the pars intercerebralis and subocellar regions of the brain followed by transportation, via the nervi corpora cardiaca II, to the storage lobes of the CC extract from where they are released into the haemolymph, or 2) they are produced in the ventral suboesophageal and thoracic ganglia and released from adjacent neurohaemal organs in response to abdominal stretching (Phillips, 1982; 1983; Proux *et al.*, 1982). Arginine-vasopressin (AVP) and oxytocin (OT), were first reported as neurohypophyseal hormones in mammals then characterized (for review, see Acher, 1974). AVP has two major effects: water reabsorption and elevation of blood pressure (Proux, 1993). In insects, AVP-like acts as a diuretic hormone by increasing the urine excretion from the Malpighian tubules as demonstrated both *in vivo* (Proux *et al.*, 1982) and *in vitro* (Proux *et al.*, 1988; Proux *et al.*, 1982). Investigations are due to determine the ionic pumps and channels involved in the diuretic activity of the AVP-like. This diuretic action is mediated by cyclic AMP (Proux and Herault, 1988).

A number of studies report differing responses to diuretic hormone and cAMP (Williams and Beyenbach, 1984; Nicholson and Isaacson, 1987; Fogg *et al.*, 1989). These observations have led to the recognition that cAMP, alone, cannot mediate the full effects of diuretic hormone on Malpighian tubules of *Locusta*. Morgan and Mordue (1984) reported that 5-HT stimulation of fluid secretion by locust tubules was only 65% of the diuretic hormone-stimulated rate and that the action of 5-HT was not mediated by cAMP. It was suggested that two spatially distinct membrane receptors were involved in the endocrine control of fluid secretion, one activating adenylate cyclase and leading to increased intracellular cAMP, and the other triggering a different secondary event, possibly leading to increased intracellular  $\text{Ca}^{2+}$  concentration. This

proposal is similar to that suggested for salivary glands of *Calliphora erythrocephala* in which two distinct types of 5-HT receptors have been reported, with one activating adenylate cyclase and the other the calcium-phosphoinositide cascade (Berridge and Heslop, 1981; 1982; Berridge *et al.*, 1984). Further support for such a mechanism in Malpighian tubules of *Locusta* comes from the demonstration that corpora cardiaca extract (CC), with diuretic activity, stimulated an increase in intracellular cAMP and inositol 1,4,5-triphosphate (Ins-1,4,5-P<sub>3</sub>) it was suggested that the latter acted as the calcium-mobilizing intracellular messenger (Fogg *et al.*, 1990). Fogg *et al.*, (1989) and Nicolson and Isaacson (1996) suggested that cAMP stimulated the apical cation pump, whilst a rise in [Ca<sup>2+</sup>]<sub>i</sub> might serve to increase anion conductance across both basal and apical cell membranes. More recently, Davies *et al.*, (1995) and O'Donnell *et al.*, (1996) have reported that a cardioacceleratory peptide, CAP<sub>2b</sub>, present in *Drosophila*, stimulated tubule fluid secretion and increased the transepithelial potential via cGMP production. This suggested that the stimulation of the apical V-type ATPase was involved.

Leucokinins, other neuropeptides, isolated from the head and ganglia of the cockroach *Leucophaea maderae* (Holman *et al.*, 1986; 1987) and were found to stimulate fluid secretion in *D. melanogaster*, are believed to act by the elevation of calcium and not to affect intracellular levels of either cAMP or cGMP (Davies *et al.*, 1995; O'Donnell *et al.*, 1996). Pannabecker *et al.*, (1993) and Beyenbach, (1995) demonstrated that one of the leucokinins, leucokinin-VIII, increases the rates of transepithelial NaCl, KCl and fluid secretion. Furthermore, leucokinin-VIII depolarizes the transepithelial voltage in a dose-dependent manner to values close to 0 mV, an effect which is diminished when the Cl concentration in the peritubular Ringer bath is

reduced (Hayes *et al.*, 1989). The non-selective increase in the secretion of NaCl and KCl suggested that leucokinin-VIII increases the availability of Cl<sup>-</sup> as Na<sup>+</sup> and K<sup>+</sup> are transported (secreted) through the active transport pathway. Examination of the electrical circuit of transepithelial transport indicated that rates of transepithelial NaCl and KCl secretion will increase when leucokinin-VIII increases the Cl<sup>-</sup> permeability of the epithelial shunt.

Both cAMP and cGMP ultimately act by regulating the activity of nucleotide-dependent protein kinase enzymes, A-kinase and G-kinase, respectively. These protein kinases catalyse the phosphorylation of various cellular proteins. Such phosphoproteins may be enzymes which are activated by this process or non-enzymatic proteins such as membrane channels, structural proteins or regulatory proteins. In some processes, cGMP prompts responses opposite to those promoted by cAMP suggesting opposing intracellular actions (Dow *et al.*, 1994a,b). The primary hormonal stimulus is transduced into a regulated functional response. These several demonstrations of the elevation in a number of intracellular messenger molecules associated with tubule fluid stimulation, by hormones or their analogues, suggest a role for the phosphorylation state of proteins closely involved in ion translocation across the apical and/or basal membranes (Fogg *et al.*, 1989; Dow *et al.*, 1994a,b; Davies *et al.*, 1995; O'Donnell *et al.*, 1996). A number of inhibitors of several classes of protein kinases and phosphatases have been identified. For example, chelerythrine and staurosporine have been reported to be specific inhibitors of protein kinase C (PKC), at low concentrations (micromolars) (Tamaoki *et al.*, 1986; Oka *et al.*, 1986; Herbert *et al.*, 1990; 1993; Mizuno *et al.*, 1993; Liu *et al.*, 1994). Rp-cAMP has been reported as

a more specific inhibitor for protein kinase A (PKA) (Rothermel *et al.*, 1984; Richter-Landsberg and Jastorff, 1985).

In the last decade there has been considerable interest in a further group of second messenger molecules derived from membrane phosphoinositides. Appropriate receptor stimulation triggers phosphoinositidase C-catalysed cleavage of membrane phosphatidylinositol 4,5-bisphosphate to yield two second messenger molecules, Ins-1,4,5-P<sub>3</sub> and *sn*-1,2-diacylglycerol (DAG); the latter being an activator of C-kinase (Nishizuka, 1984). There is now general agreement that Ins-1,4,5-P<sub>3</sub> induces the release of Ca<sup>2+</sup> from the endoplasmic reticulum (Berridge and Irvine, 1984; Abdel-Latif, 1986; Berridge, 1986) and thus triggers the biological response to 'calcium-mobilizing' hormones. Intracellular Ins-1,4,5-P<sub>3</sub> levels have been shown to increase in Malpighian tubules of *Locusta* following treatment with corpora cardiaca extracts with established diuretic activity (Fogg *et al.*, 1990). However, a role for DAG and C-kinase in the regulation of tubule function remains to be established.

The present study has been carried out to determine the effect of various inhibitors of nucleotide-dependent protein kinase C (chelerythrine, staurosporine) and protein kinase A (Rp-cAMP) on fluid and cation secretion by Malpighian tubules of *Locusta* under stimulated and unstimulated conditions with a view to further elucidating the endocrine control of tubule secretion.

MATERIALS AND METHODS

Rate of Fluid Secretion:

*In vitro* measurements of rates of fluid secretion by the Malpighian tubules of *Locusta* were carried out using essentially the same technique as that described by (Maddrell and Klunswan, 1973; Anstee and Bell, 1975 and Donkin and Anstee, 1980). Malpighian tubules of adult female were used in all experiments as they were found to give a consistent rate of fluid secretion and furthermore, it was observed that the rate of fluid secretion by female tubules in the presence of corpora cardiaca extract was double of that obtained by male tubules. Locusts were killed by twisting the head, breaking the arthroal membrane between the head and thorax. The extreme tip of the abdomen was cut off allowing the whole gut, bearing the Malpighian tubules, to be carefully drawn out through the thorax with the head still attached. The gut of the experimental animal was immersed in a small volume of control saline in a hollow in a perspex dish. The head remained outside the dish and care was taken to prevent contamination of the bathing media by regurgitated digestive fluid. The entire preparation was covered with liquid paraffin. Up to ten tubules were drawn out of the saline bath into the surrounding liquid paraffin and looped around small stainless steel pegs. Each tubule was then partially severed at a convenient point along its length between two pegs using a fine tungsten needle. At the end of a 15min equilibration period, any secreted 'urine' droplet emerging from the partially severed tubule was removed and discarded. The secretion rate for individual tubule was then determined by measuring the diameter of the secreted 'urine' droplet at 5min intervals over a period of 20min (Rate 1). At the end of this time the bathing medium was replaced by a fresh solution which had either the same

composition (control) or a different (experimental). The rate of secretion was redetermined over the next 20min (Rate 2). A 15min equilibration period, in the appropriate saline, was allowed prior to determination of Rate 1 and Rate 2 secretion.

The rate of fluid secretion was expressed in nl/min. In all experiments controls were used to indicate the extent to which any changes in the rate of secretion over the two periods of measurement were the result of ageing of the preparation. The temperature throughout was maintained at  $30 \pm 0.1^\circ\text{C}$  by placing the perspex dish on a temperature controlled plate.

#### **Corpora Cardiaca Extract Preparation:**

Extracts were prepared immediately prior to use by dissecting corpora cardiaca from the heads of mature male *Locusta migratoria*. The corpora cardiaca were then homogenized in an appropriate ice-cold ( $0-4^\circ\text{C}$ ) buffered saline at 1 gland pair per 0.1ml of saline (Fogg *et al.*, 1989), unless otherwise stated, and stored on ice until required. Homogenization was carried out in a glass homogenizer with a Teflon pestle (clearance 0.1-0.15mm) with 20 passes of the plunger at approximately 1000 rev/min. The resulting homogenate was subsequently diluted with the appropriate saline to a final concentration of 1 gland pair per ml.

#### **Measurement of the $\text{Na}^+$ and $\text{K}^+$ concentrations of the 'urine':**

*In vitro* preparations were set up as described above. Concentrations of  $\text{Na}^+$  and  $\text{K}^+$  in bathing media and secreted fluids were determined by atomic emission spectroscopy using a Perkin-Elmer 5000 spectrophotometer. Following preincubation for 20min in an appropriate saline, fluid secreted by individual tubules was collected over the next 45min (initial rate) and 'pooled' under liquid paraffin.  $0.5\mu\text{l}$  or  $1\mu\text{l}$  were

then transferred by micropipette to a plastic vial containing 1 or 2ml deionized water. The bathing medium was then replaced by a fresh solution which had either the same composition (controls) or a modified composition (experimentals) and following a further 20min equilibration period, the fluid secreted by the same tubules over the next 45min (final period) was collected, 'pooled' and treated as described for fluid collected over the initial period. Samples were stored in plastic vials prior to their analysis.

Emission readings were compared with standard calibration curves constructed with known concentrations of KOH and NaOH (Figures 3.1 and 3.2).

**Determination of the effects of various inhibitors and stimulators on Na<sup>+</sup> and K<sup>+</sup> concentration in the 'urine':**

Malpighian tubule preparations were set up in saline solution as outlined above. Control experiments were carried out in the standard saline solution (as described in Table 2.1 chapter 2). The effect of adding inhibitors or stimulators to the saline was investigated. "Urine" samples were collected at the end of the initial 45min period (Rate 1) and analysed in the manner described above. The saline solution bathing the tubules was then replaced with fresh saline solution containing one of the following: 1μM chelerythrine or 1μM staurosporine, 0.1mM Rp-cAMP, CC extract, 1mM cAMP, CC extract or 1mM cAMP + either 1μM chelerythrine, 1μM staurosporine or 0.1mM Rp-cAMP. Following an equilibration period of 30min, in the presence of a given saline solution, any secreted fluid was discarded. The "urine" collected over the next 45min (Rate 2) was analysed and Na<sup>+</sup> and K<sup>+</sup> concentrations determined.

**Figure 3.1**

Calibration curve for potassium hydroxide concentration against % emission, estimated using a Perkin-Elmer 5000 spectrophotometer wavelength at 766.5nm.

**Ordinate:** % emission

**Abscissa:**  $[K^+]$  mM

**Figure 3.2**

Calibration curve for sodium hydroxide concentration against % emission, estimated using a Perkin-Elmer 5000 spectrophotometer wavelength at 595nm.

**Ordinate:** % emission

**Abscissa:**  $[Na^+]$  mM

Figure 3.1

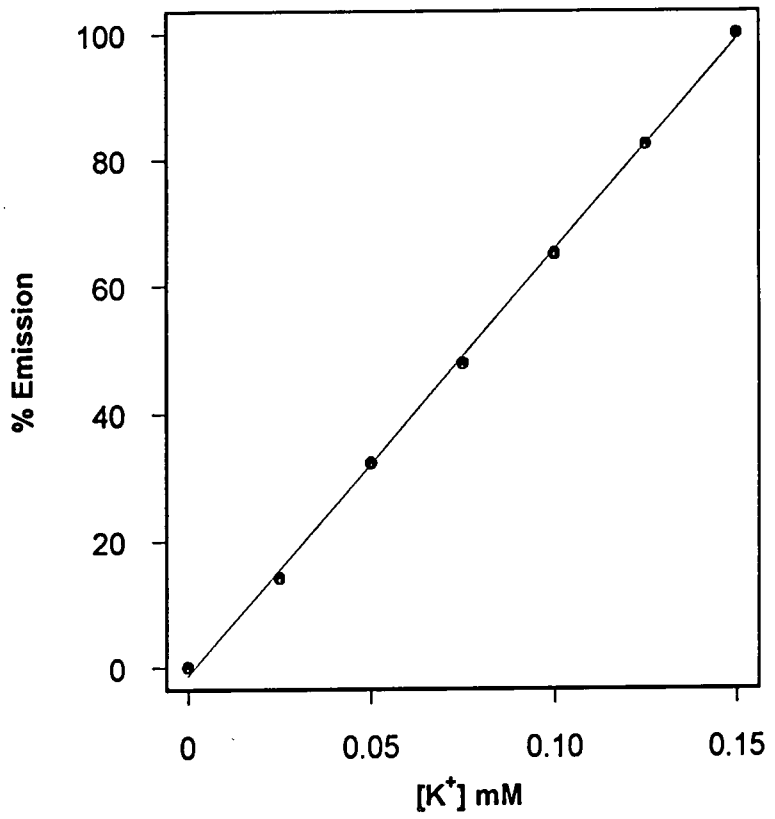
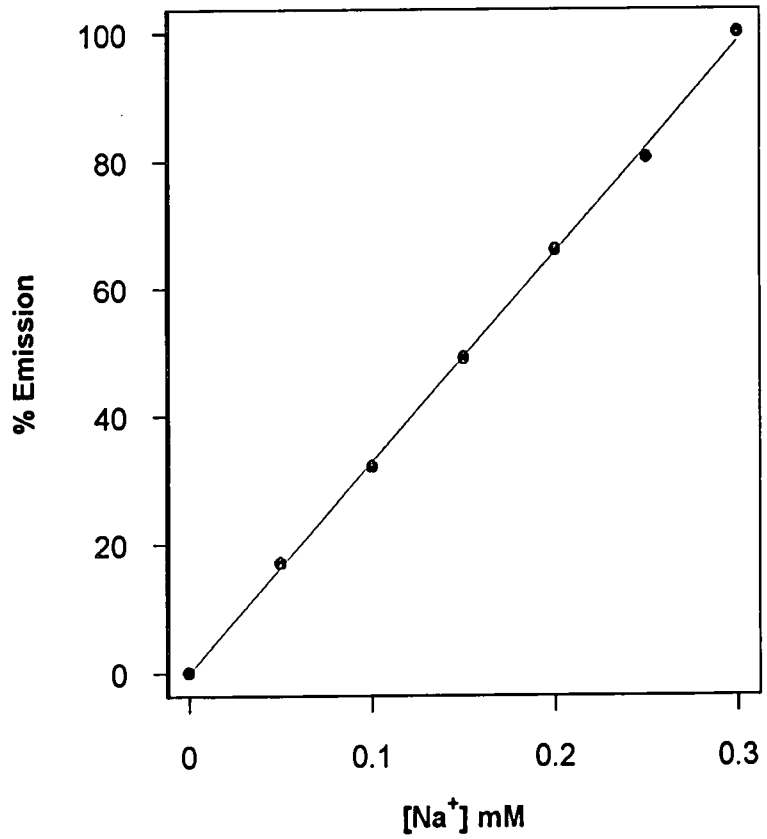


Figure 3.2



RESULTS

Effect of corpora cardiaca (CC) extract and cAMP on fluid secretion by Malpighian tubules:

Table 3.1 shows that under control conditions (both rates being measured in control saline) rate 2 was  $95.4 \pm 0.9\%$  of the rate measured during the initial period. When the data were compared using a paired *t*-test no significant difference was seen between the initial ( $4.6 \pm 0.3$ nl/min) and final ( $4.3 \pm 0.3$ nl/min) measurement periods ( $n=30$ ). Addition of corpora cardiaca (CC) extract (final concentration 1 gland pair/ml) to the bathing medium, increased the rate of fluid secretion from  $4.2 \pm 0.4$ nl/min to  $8.3 \pm 0.7$ nl/min ( $n=35$ ), representing a mean increase of approximately 100% ( $p < 0.0001$ ) over the unstimulated rate. Similarly, the inclusion of 1mM cAMP in the bathing saline increased the rate of fluid secretion from  $4.4 \pm 0.5$ nl/min (rate 1) to  $6.6 \pm 0.7$ nl/min (Rate 2) ( $n=27$ ), representing a mean increase of approximately 49%. Comparison of obtained values by paired *t*-test showed that inclusion of 1mM cAMP in the bathing medium produced a significant ( $p < 0.0001$ ) increase in the rate of fluid secretion.

**Table 3.1 The effect of CC extract or 1mM cAMP on fluid secretion of Malpighian tubules of *Locusta*.**

Bathing media	n	Rate of Fluid secretion (nl/min)	<i>p</i>
Rate 1 normal	30	$4.6 \pm 0.3$	
Rate 2 normal	30	$4.3 \pm 0.3$	n.s.
Rate 1 normal	35	$4.2 \pm 0.4$	
Rate 2 + CC extract	35	$8.3 \pm 0.7$	$< 0.0001$
Rate 1 normal	27	$4.4 \pm 0.5$	
Rate 2 + cAMP	27	$6.6 \pm 0.7$	$< 0.0001$

*p* values obtained by comparison of Rates 1 and 2 by paired *t*-test.

Mean values  $\pm$  SEM.

n.s.: Not significant.

**Effect of Chelerythrine on fluid secretion by Malpighian tubules:**

The mean rate of fluid secretion was  $4.6 \pm 0.3$  nl/min ( $n=30$ ), when the tubules were bathed in control saline during the first 20 min period of measurement. Results presented in Figure 3.3a show that under these control conditions (both rates being measured in control saline) rate 2 was  $95.4 \pm 0.9\%$  of the rate measured during the first 20 min period. The dose dependency of fluid secretion by Malpighian tubules in the presence of chelerythrine at concentrations of 0.07-60  $\mu$ M was investigated. At the lowest concentration used (0.07  $\mu$ M) fluid secretion was inhibited by 18.1% ( $p < 0.005$ ) whereas 60  $\mu$ M chelerythrine caused 84% inhibition (see also Appendix 2, Table 2.1).  $IC_{50}$  for fluid secretion was 2.9  $\mu$ M as determined by probit analysis Figure 3.3b.

**Effect of Chelerythrine and Staurosporine on fluid secretion by Malpighian tubules in the absence and presence of corpora cardiaca (CC) extract or cAMP:**

Table 3.2 shows the effect of 1  $\mu$ M chelerythrine or 1  $\mu$ M staurosporine on fluid secretion, in the presence and absence of cAMP and CC extract. 1  $\mu$ M chelerythrine caused significant inhibition of fluid secretion; the rate fell from  $4.5 \pm 0.6$  nl/min to  $2.4 \pm 0.3$  nl/min ( $p < 0.0001$ ,  $n=24$ ). When both rates were measured in the presence of chelerythrine, a further significant inhibition was observed; the rate decreased from  $3.3 \pm 0.4$  nl/min to  $2.3 \pm 0.3$  nl/min ( $p < 0.0001$ ,  $n=26$ ). Similarly, 1  $\mu$ M staurosporine caused a significant decrease in fluid secretion from  $5.3 \pm 0.5$  nl/min to  $2.7 \pm 0.3$  nl/min ( $p < 0.0001$ ,  $n=20$ ). Also incubation of the tubules in saline containing staurosporine (both rates being measured in the presence staurosporine) caused a further significant inhibition in fluid secretion from  $3.2 \pm 0.3$  nl/min to  $2.4 \pm 0.2$  nl/min ( $p < 0.0001$ ,  $n=22$ ). These data show that both inhibitors cause a progressive inhibition of fluid secretion.

**Figure 3.3a**

Effect of varying concentrations of chelerythrine on fluid secretion by Malpighian tubules of *Locusta migratoria*.

The rate of fluid secretion was measured over two time periods. For controls, both measurements occurred in control saline. However, to determine the effect of chelerythrine on fluid secretion the first measurement of rate was recorded in control saline over a period of 20min whilst the second measurement of rate of fluid secretion was carried out over a subsequent 20 min period but in saline containing a range of concentrations of chelerythrine. This second rate was normalized with respect to that recorded over the first time period in control saline by expressing rate 2 as a % of rate 1 in the absence of chelerythrine.

**Ordinate:** Normalized Rate 2.

**Abcissa:** Chelerythrine concentration in  $\mu\text{M}$ .

**Figure 3.3b.**

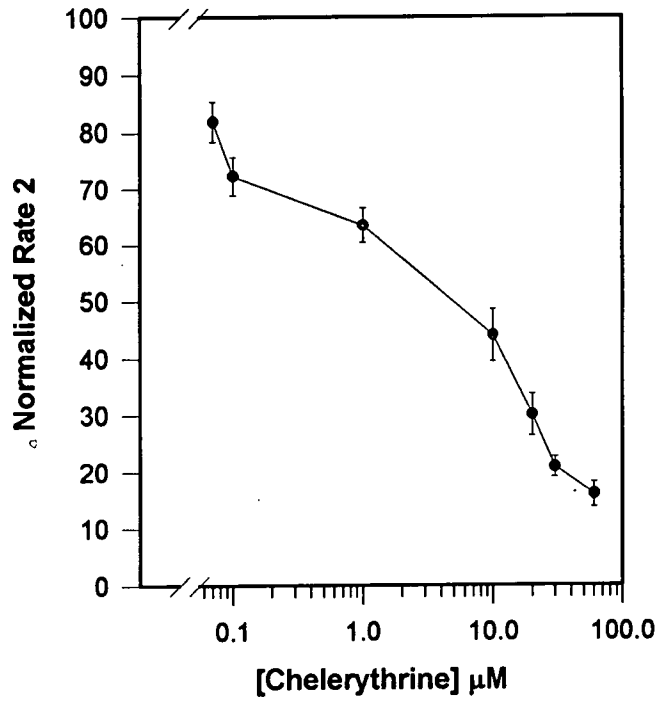
Probit analysis of effect of different concentrations of Chelerythrine on the rate of fluid secretion.

The percentage of inhibition data presented in Appendix 2, Table 2.1 were converted into probits and lines of best fit, calculated according to the method shown in Appendix 1.1.

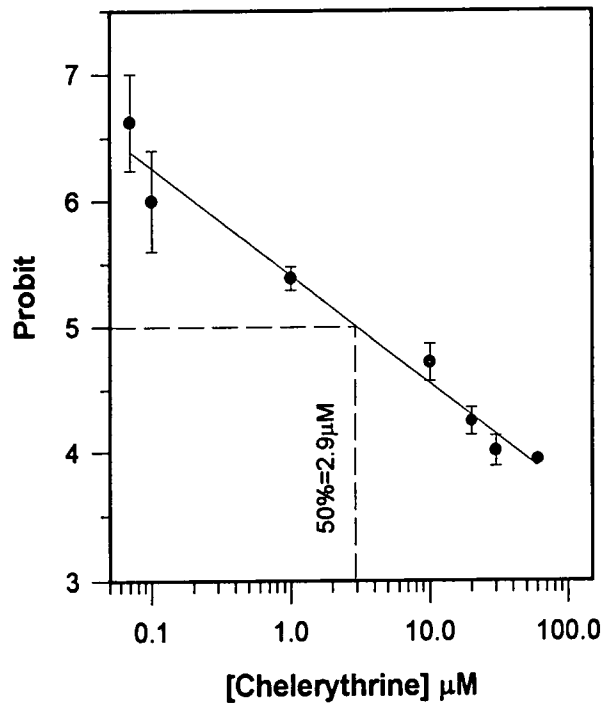
Each point represents the mean  $\pm$  S.E.M.

**Figure 3.3a**

Effect of different concentrations of chelerythrine on the rate of fluid secretion.



**Figure 3.3b** Probit analysis.



**Table 3.2** The effect of protein kinase C inhibitors, chelerythrine (1 $\mu$ M) and staurosporine (1 $\mu$ M) on fluid secretion by Malpighian tubules in the presence and absence of CC extract or 1mM cAMP.

Bathing media	n	Rate of Fluid secretion (nl/min)	<i>p</i>
Rate 1 normal	24	4.5 $\pm$ 0.6	
Rate 2 + Chel	24	2.4 $\pm$ 0.3	<0.0001
Rate 1 + Chel.	26	3.3 $\pm$ 0.4	
Rate 2 + Chel.	26	2.3 $\pm$ 0.3	<0.0001
Rate 1 + Chel.	30	2.9 $\pm$ 0.3	
Rate 2 + Chel. + CC extract	30	3.7 $\pm$ 0.4	<0.0001
Rate 1 + Chel.	20	2.8 $\pm$ 0.3	
Rate 2 + Chel. + cAMP	20	3.0 $\pm$ 0.3	n.s.
Rate 1 normal	20	5.3 $\pm$ 0.5	
Rate 2 + Staur.	20	2.7 $\pm$ 0.3	<0.0001
Rate1 + Staur.	22	3.2 $\pm$ 0.3	
Rate 2 + Staur.	22	2.4 $\pm$ 0.2	<0.0001
Rate1 + Staur.	22	3.4 $\pm$ 0.3	
Rate 2 + Staur. + CC extract	22	4.2 $\pm$ 0.4	<0.002
Rate 1 + Staur.	18	2.8 $\pm$ 0.3	
Rate 2 + Staur. + cAMP	18	3.3 $\pm$ 0.3	n.s.

*p* values obtained by comparison of Rates 1 and 2 by paired *t*-test.

Chel. = Chelerythrine.

Staur. = Staurosporine.

Mean values  $\pm$  SEM.

n.s.: Not significant.

The effect of CC extract and cAMP on fluid secretion must also be considered in the light of the progressive nature of the inhibition of fluid secretion by both chelerythrine and staurosporine during the second 20min incubation period (Rate 2). The paired *t*-test showed that rate 1 was not stimulated by cAMP (Rate 2) in the presence of either chelerythrine or staurosporine. Comparison of rate 2 for staurosporine (2.4 $\pm$ 0.2nl/min) with that for rate 2 for staurosporine + cAMP

( $3.3 \pm 0.3$  nl/min) showed that cAMP did cause stimulation ( $p < 0.0001$ ). With chelerythrine however, these differences were also similar to that in the presence of staurosporine.

Making the same comparisons with stimulation with CC extract shows that the mean rate 2 in presence of CC extract was significantly higher than rate 2 in the presence of either chelerythrine ( $p < 0.001$ ) or staurosporine ( $p < 0.001$ ) alone.

#### **Effect of Rp-cAMP on fluid secretion by Malpighian tubules:**

Figure 3.4a shows that the protein kinase A inhibitor (Rp-cAMP) inhibits fluid production over the concentration range  $10 \mu\text{M}$  to  $1000 \mu\text{M}$ , inhibition increasing with increasing concentration (see also Appendix 2, Table 2.2). In the presence of  $1000 \mu\text{M}$  Rp-cAMP, approximately 62% of fluid production was inhibited ( $p < 0.0001$ ). Probit analysis (Figure 3.4b) gives an  $\text{IC}_{50}$  of  $264 \mu\text{M}$ .

#### **Effect of Rp-cAMP on fluid secretion by Malpighian tubules, in the absence and presence of CC extract or cAMP:**

Table 3.3 shows the effect of the protein kinase A inhibitor, Rp-cAMP on the rate of fluid secretion by Malpighian tubules in the presence and absence of CC extract or cAMP.  $0.1 \text{ mM}$  Rp-cAMP caused significant inhibition of the rate of fluid secretion. The rate decreased from  $4.3 \pm 0.5$  nl/min (in control saline) to  $2.6 \pm 0.3$  nl/min ( $p < 0.0001$ ,  $n=20$ ). When  $0.1 \text{ mM}$  Rp-cAMP was included in the bathing medium during both rates, the rate of fluid secretion was significantly ( $p < 0.001$ ) further reduced from  $3.1 \pm 0.4$  nl/min to  $2.4 \pm 0.4$  nl/min ( $n=15$ ) again emphasizing the progressive nature of the inhibition. The inclusion of cAMP in the presence of Rp-cAMP failed to stimulate fluid secretion or

indeed to prevent a further reduction in the rate to 2.3nl/min compared to 2.4nl/min (rate 2) in the presence of Rp-cAMP alone. The addition of CC extract in presence of Rp-cAMP failed to stimulate fluid secretion (Rate 1 compared with Rate 2), however, it did prevent further decline in rate 2 which was observed in the presence of Rp-cAMP alone.

**Table 3.3** The effect of the protein kinase A inhibitor, Rp-cAMP, on the fluid secretion by *Locusta* Malpighian tubules, in the presence and absence of the stimulation by CC extract or cAMP.

Bathing media	n	Rate of Fluid secretion (nl/min)	p
Rate 1 normal	20	4.3±0.5	
Rate 2 + Rp-cAMP	20	2.6±0.3	<0.0001
Rate 1 + Rp-cAMP	15	3.1±0.4	
Rate 2 + Rp-cAMP	15	2.4±0.4	<0.001
Rate 1 normal	20	4.7±0.4	
Rate 2 + CC	20	9.4±0.9	<0.0001
Rate 1 normal	20	4.3±0.4	
Rate 2 + cAMP	20	6.1±0.5	<0.001
Rate 1 + Rp-cAMP	20	3.0±0.3	
Rate 2 + Rp-cAMP + CC	20	2.7±0.3	n.s.
Rate 1 + Rp-cAMP	20	3.2±0.4	
Rate 2 + Rp-cAMP + cAMP	20	2.3±0.3	<0.001

p values obtained by comparison of Rates 1 and 2 by paired t-test.

Mean values ± SEM.

n.s.: Not significant.

**Figure 3.4a**

Effect of varying concentrations of Rp-cAMP on fluid secretion by Malpighian tubules of *Locusta migratoria*.

The rate of fluid secretion was measured over two time periods. For controls, both measurements occurred in control saline. However, to determine the effect of Rp-cAMP on fluid secretion the first measurement of rate was recorded in control saline over a period of 20 min whilst the second measurement of rate of fluid secretion was carried out over a similar period but in saline containing a range of concentrations of Rp-cAMP. This second rate was normalized with respect to that recorded over the first time period in control saline by expressing rate 2 as a % of rate 1 in the absence of Rp-cAMP.

**Ordinate:** Normalized Rate 2.

**Abscissa:** Rp-cAMP concentrations in  $\mu\text{M}$ .

**Figure 3.6b**

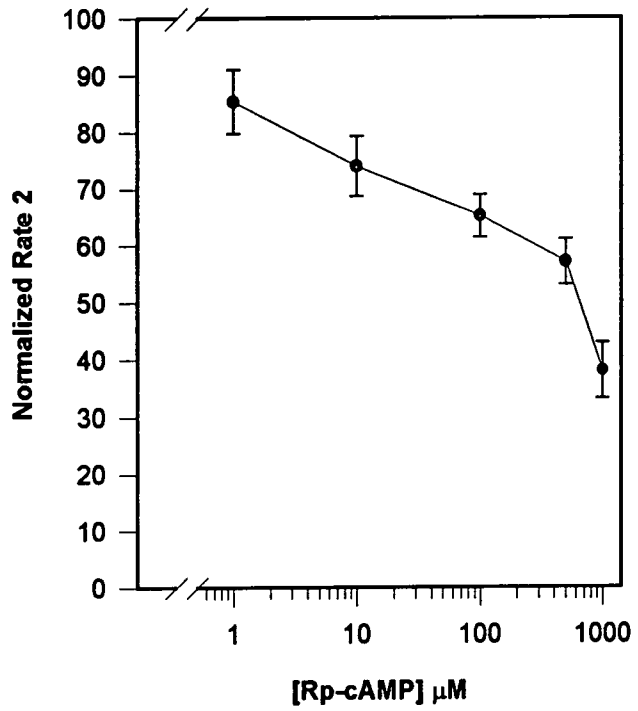
Probit analysis of effect of different concentrations of Rp-cAMP on the rate of fluid secretion.

The percentage of inhibition data presented in Appendix 2, Table 2.2 were converted into probits and lines of best fit, calculated according to the method shown in Appendix 1.1.

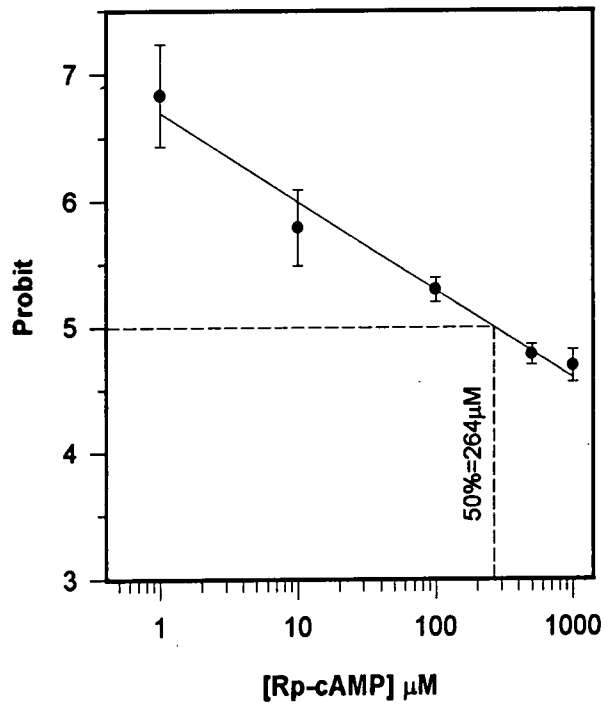
Each point represents the mean  $\pm$  S.E.M.

**Figure 3.4a**

Effect of different concentrations of Rp-cAMP on the rate of fluid secretion.



**Figure 3.4b** Probit analysis.



**Effect of NEM on the fluid secretion:**

Figure 3.5a and Appendix 2, Table 2.3 show the effects of NEM on the rate of fluid secretion at concentrations ranging from 0.1 $\mu$ M to 1000 $\mu$ M. Inclusion of NEM in the bathing saline for the measurement of rate 2 caused a significant inhibition of fluid secretion in a dose dependent manner. Comparing normalized control and experimental values by Student's *t*-test indicates that 1000 $\mu$ M NEM effected significant ( $p < 0.0001$ ) inhibition of the rate of fluid secretion by ~92%. Figure 3.5b shows the probit analysis which indicates that the  $IC_{50}$  for NEM was 9.05 $\mu$ M.

**Effect of Okadaic acid on the fluid secretion:**

Effect of various concentrations of Okadaic acid, ranging from 0.01nM to 1000nM, on the rate of fluid secretion were investigated and the results are shown in Figure 3.6a. It can be seen that very low concentrations of okadaic acid (0.5 to 1000nM) had a marked inhibitory effect on the rate of fluid secretion.

Comparison between normalized control and experimental values, using Student's *t*-test, established that including 1000nM okadaic acid in the bathing saline caused a significant inhibition of the rate of fluid secretion ( $p < 0.0001$ ); the mean rate decreasing by approximately 70%. Similarly, 1nM okadaic acid effected a significant decrease in the rate of fluid secretion ( $p < 0.01$ ), reducing the rate by approximately 25%. However, at 0.01nM concentration, okadaic acid did not significantly affect the rate of fluid secretion. Probit analysis Figure 3.6b gives the  $IC_{50}$  of 91nM. Data are summarized in Appendix 2, Table 2.4.

**Figure 3.5a**

Effect of varying concentrations of NEM on fluid secretion by Malpighian tubules of *Locusta migratoria*.

The rate of fluid secretion was measured over two time periods. For controls, both measurements occurred in control saline. However, to determine the effect of NEM on fluid secretion the first measurement of rate was recorded in control saline over a period of 20 min whilst the second measurement of rate of fluid secretion was carried out over a subsequent 20 min period in saline containing a range of concentrations of NEM. This second rate was normalized with respect to that recorded over the first time period in control saline by expressing rate 2 as a % of rate 1 in the absence of NEM.

**Ordinate:** Normalized Rate 2.

**Abscissa:** NEM concentrations in  $\mu\text{M}$ .

**Figure 3.5b**

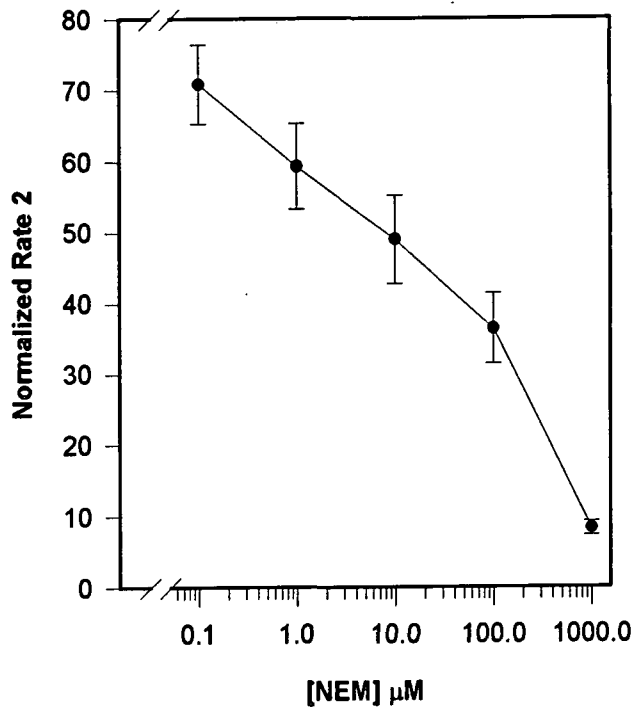
Probit analysis of effect of different concentrations of NEM on the rate of fluid secretion.

The percentage of inhibition data presented in Appendix 2, Table 2.3 were converted into probits and lines of best fit were calculated according to the method shown in Appendix 1.1.

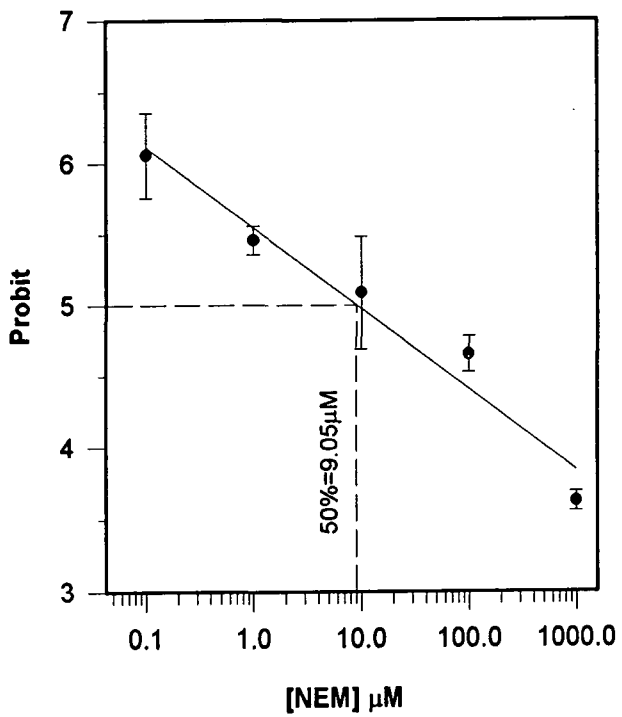
Each point represents the mean  $\pm$  S.E.M.

**Figure 3.5a**

Effect of different concentrations of NEM on the fluid secretion.



**Figure 3.5b** Probit analysis.



**Figure 3.6a**

Effect of varying concentrations of Okadaic acid on *Locusta* Malpighian tubules *in vitro* fluid secretion.

The rate of fluid secretion was measured over two time periods. For controls, both measurements occurred in control saline. To determine the effect of Okadaic acid on fluid secretion the first measurement of rate was recorded in control saline over a period of 20 min whilst the second measurement of rate of fluid secretion was carried out over a subsequent 20 min period in saline containing a range concentrations of Okadaic acid. This second rate was normalized with respect to that recorded over the first time period in control saline by expressing rate 2 as % of rate 1 in absence of okadaic acid.

**Ordinate:** Normalized Rate 2.

**Abscissa:** Okadaic acid concentrations in nM.

**Figure 3.6b**

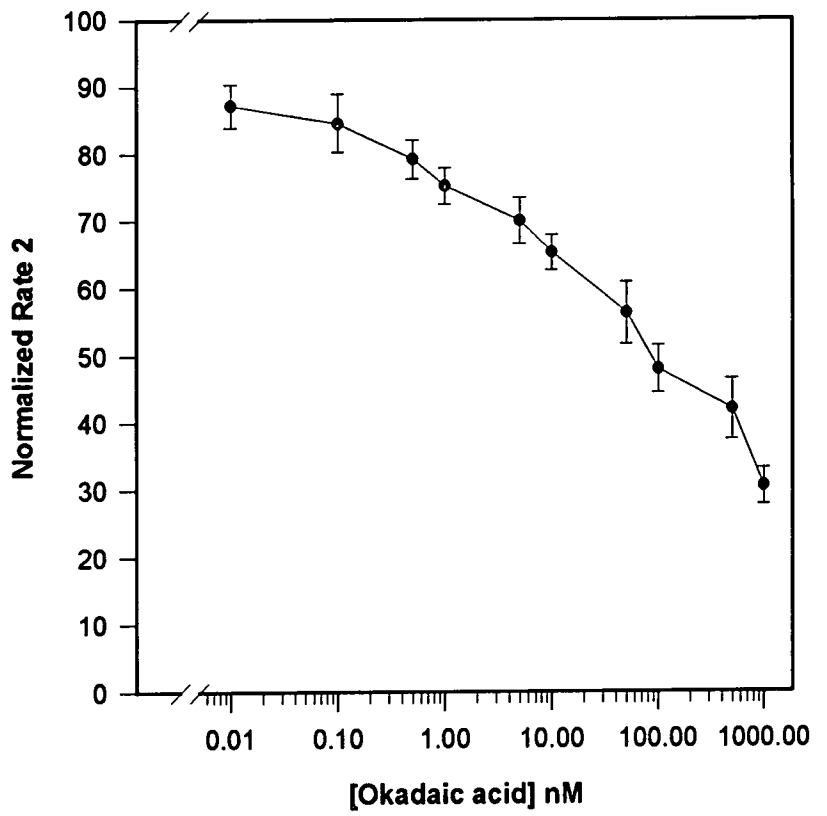
Probit analysis of effect of different concentrations of okadaic acid on the rate of fluid secretion.

The percentage of inhibition data presented in Appendix 2, Table 2.4 were converted into probits and lines of best fit were calculated according to the method shown in Appendix 1.1.

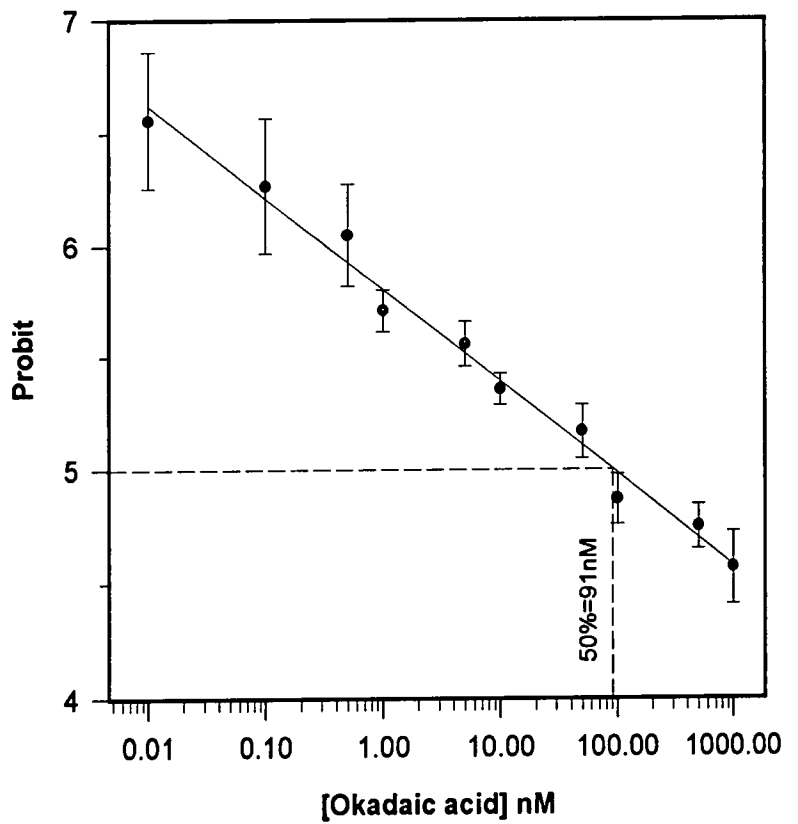
Each point represents the mean  $\pm$  S.E.M.

**Figure 3.6a**

Effect of different concentrations of okadaic acid on the rate of fluid secretion.



**Figure 3.6b** Probit analysis.



### **Effect of collection period on $[Na^+]$ and $[K^+]$ in the "urine":**

The "urine" was collected and analysed as described previously (see Materials and Methods). Tubules were pre-incubated in control saline solution. Original saline was then replaced with fresh saline of the same composition and "urine" collected over the following 60 min period. Table 3.4 shows the monovalent cation composition of the secreted fluid collected over the 60 min period. Values were compared using paired *t*-test. In the case of control saline (8.6mM  $K^+$ , 119mM  $Na^+$ ) Table 3.4 shows that there was no significant difference in the ionic composition of the secreted fluid over the two measurement periods, which varied between  $41.5 \pm 0.9$ mM (Rate1) and  $40.7 \pm 1.2$ mM (Rate2) for  $Na^+$  and between  $125.5 \pm 2.3$ mM (Rate1) and  $126.9 \pm 3.2$ mM (Rate2) for  $K^+$ . Also, the final  $Na^+/K^+$  ratio ( $0.34 \pm 0.01$ ) was not significantly different from the initial  $Na^+/K^+$  ratio ( $0.34 \pm 0.01$ ).

### **Effect of CC extract and cAMP on $[Na^+]$ and $[K^+]$ in the "urine":**

Table 3.4 shows that the inclusion of CC extract in the bathing medium significantly affected the ionic composition of the secreted fluid, the level of  $[Na^+]$  increased from  $41.2 \pm 1.3$ mM to  $59.3 \pm 2.0$ mM ( $p < 0.0001$ ), whereas that of  $[K^+]$  decreased from  $126.8 \pm 2.9$ mM to  $107.2 \pm 2.4$ mM ( $p < 0.001$ ). However, the total monovalent cation content of the secreted fluid was similar to that of control tubules at about 167mM. In contrast, the inclusion of 1mM cAMP in the bathing medium had no significant effect on the  $Na^+$  and  $K^+$  composition of the secreted fluid. Figure 3.7 shows that 1mM cAMP did not alter the ratio of  $Na^+/K^+$  in the secreted fluid. In contrast, CC extract effected a significant ( $p < 0.0001$ ) increase in  $Na^+/K^+$  ratio.

**Table 3.4 Effect of CC extract or cAMP on the Na<sup>+</sup> and K<sup>+</sup> concentrations in the fluid secreted by Malpighian tubules of *Locusta*:**

Bathing media	n	[Na <sup>+</sup> ] mM	<i>p</i>	[K <sup>+</sup> ] mM	<i>p</i>
R1 normal	21	41.5 ± 0.9		125.5 ± 2.3	
R2 normal	21	40.7 ± 1.2	n.s.	126.9 ± 3.2	n.s.
R1 normal	15	41.2 ± 1.3		126.8 ± 2.9	
R2 + CC extract	15	59.3 ± 2.0	<0.0001	107.2 ± 2.4	<0.001
R1 normal	15	41.4 ± 1.9		123.5 ± 4.1	
R2 + 1mM cAMP	15	40.5 ± 1.9	n.s.	124.3 ± 3.2	n.s.

*p* values were obtained by comparing rate 1 and rate 2 by paired *t*-test.

R1: Rate 1.

R2: Rate 2.

n.s.: Not significant.

An estimation of the changes in the flux of Na<sup>+</sup> and K<sup>+</sup> caused by the incubation of either CC extract or cAMP in the bathing medium can be made by calculating the product of the volume secreted and its cation concentration. Table 3.5 shows such values calculated from the mean data in tables 3.2 and 3.6. In control media the mean flux of Na<sup>+</sup> was between 190.9 (Rate 1) and 175.0 pmole/nl/min (Rate 2), and between 545.7 (Rate 1) and 545.7 pmole/nl/min (Rate 2) for mean K<sup>+</sup> flux. The inclusion of CC extract in the bathing medium was found to increase the mean Na<sup>+</sup> flux from 173.0 to 492.2 pmole/nl/min, and K<sup>+</sup> flux from 532.6 to 889.8 pmole/nl/min. The addition of 1mM cAMP to the control saline caused a smaller increase in the mean Na<sup>+</sup> flux from 182.2 to 267.3 pmole/nl/min, and mean K<sup>+</sup> flux from 543.4 to 820.4 pmole/nl/min.

**Table 3.5 The effect of CC extract or 1mM cAMP on mean Na<sup>+</sup> and K<sup>+</sup> flux:**

Bathing media	Na <sup>+</sup> pmole/nl/min	K <sup>+</sup> pmole/nl/min
R1 normal	190.9	577.3
R2 normal	175.0	545.7
R1 normal	173.0	532.6
R2 + CC extract	492.2	889.8
R1 normal	182.2	543.4
R2 + cAMP	267.3	820.4

R1: Rate 1.

R2: Rate 2.

**Effect of chelerythrine on [Na<sup>+</sup>] and [K<sup>+</sup>] in the “urine” in the absence and presence of CC extract or cAMP:**

The effect of chelerythrine on the ionic composition of the secreted fluid is shown in Table 3.6. The fluid secreted over the initial period in the absence of chelerythrine contained approximately 42.4±1.8mM Na<sup>+</sup> and 125.7±3.9mM K<sup>+</sup> (n=15). Bathing tubules in saline containing 1µM chelerythrine had no significant effect on [Na<sup>+</sup>], whilst, the level of K<sup>+</sup> significantly (p<0.001) decreased from 125.7mM to 107.7mM.

The effect of adding CC extract in the presence of chelerythrine is also shown in Table 3.6. As can be seen, the effect of CC extract on the composition of the secreted fluid was similar to that observed in absence of chelerythrine; Na<sup>+</sup> concentrations rose to 51.1mM, but the concentration of K<sup>+</sup> was not reduced significantly below the concentration that had already been observed in presence of chelerythrine alone. The total monovalent cation concentration of the secreted fluid was 157.8mM and whilst not significantly different from that in the presence of chelerythrine alone 151.0mM, it was lower (p<0.005) than that in the presence of CC extract alone (166.5mM). The addition of 1mM cAMP in the presence of chelerythrine did not significant change the ionic

composition of the secreted fluid compared to that seen in presence of chelerythrine alone.

**Table 3.6** Effect of 1 $\mu$ M chelerythrine on the Na<sup>+</sup> and K<sup>+</sup> concentrations in the fluid secreted by Malpighian tubules of *Locusta* in the presence and absence of CC extract or cAMP:

Bathing media	n	[Na <sup>+</sup> ] mM	<i>p</i>	[K <sup>+</sup> ] mM	<i>p</i>
*R1 normal	21	41.5 ± 0.9		125.5 ± 2.3	
*R2 normal	21	40.7 ± 1.2	n.s.	126.9 ± 3.2	n.s.
R1 normal	15	42.4 ± 1.8		125.7 ± 3.9	
R2 + 1 $\mu$ M Chel.	15	43.3 ± 0.8	n.s.	107.7 ± 1.5	<0.001
*R1 normal	15	41.2 ± 1.3		126.8 ± 2.9	
*R2 + CC extract	15	59.3 ± 2.0	<0.0001	107.2 ± 2.4	<0.002
R1 + 1 $\mu$ M Chel.	15	41.5 ± 1.6		107.9 ± 2.5	
R2 + Chel. + CC extract	15	51.1 ± 3.1	<0.0001	106.7 ± 5.4	n.s.
R1 + 1 $\mu$ M Chel.	14	42.5 ± 1.6		107.0 ± 2.8	
R2 + Chel. + cAMP	14	43.9 ± 2.4	n.s.	102.7 ± 4.4	n.s.
*R1 normal	15	41.4 ± 1.9		123.5 ± 4.1	
*R2 + 1mM cAMP	15	40.5 ± 1.9	n.s.	124.3 ± 3.2	n.s.

*p* values were obtained by comparing rate 1 and rate 2 by paired *t*-test

Chel. = Chelerythrine.

R1: Rate 1; R2: Rate 2.

n.s.: Not significant.

\*Data already presented in table 3.4

From the results in Figure 3.7 and Appendix 2, Table 2.5 it can be seen that 1 $\mu$ M chelerythrine caused a significant ( $p < 0.0001$ ) increase in the Na<sup>+</sup>/K<sup>+</sup> ratio. This ratio (0.38 $\pm$ 0.01) increased further when tubules bathed in chelerythrine saline were also exposed to CC extract (0.48 $\pm$ 0.01). However, cAMP did not change the Na<sup>+</sup>/K<sup>+</sup> ratio from than that observed in the presence of chelerythrine alone.

**Figure 3.7**

Effect of 1 $\mu$ M chelerythrine on the Na<sup>+</sup>/K<sup>+</sup> ratio in the presence and absence of CC extract (1 gland pair /ml ) or 1mM cAMP.

**Ordinate:** Na<sup>+</sup>/K<sup>+</sup> ratios in the presence or absence of inhibitor or stimulator.

**Abscissa:** Inhibitor and/or stimulator

Ch. = Chelerythrine

C.C.= Corpora cardiaca extract.

Ch. + C.C. = R1 in the presence of chelerythrine alone.

R2 in the presence of chelerythrine + corpora cardiaca extract

Ch. + cAMP = R1 in the presence of chelerythrine alone.

R2 in the presence of chelerythrine + cAMP

Each point represents the mean  $\pm$  S.E.M.

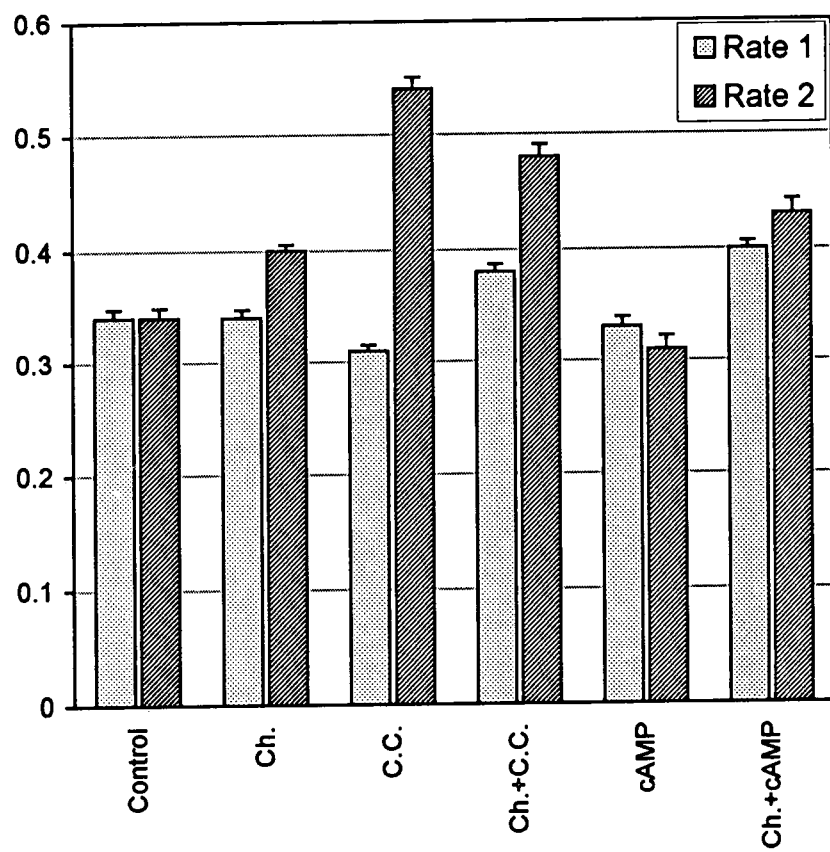


Table 3.7 showed that inclusion of 1 $\mu$ M chelerythrine in the bathing medium caused a decrease in both Na<sup>+</sup> and K<sup>+</sup> flux by approximately 46% and 54.3%, respectively. The addition of CC extract in the presence of chelerythrine caused an increase in Na<sup>+</sup> flux and did not alter K<sup>+</sup> flux compared to that observed in the presence of chelerythrine alone. When 1mM cAMP was included in the bathing medium in the presence of chelerythrine it did not alter the effect seen in the presence of chelerythrine alone on Na<sup>+</sup> and K<sup>+</sup> flux.

**Table 3.7 Effect of 1 $\mu$ M Chelerythrine on the mean Na<sup>+</sup> and K<sup>+</sup> flux in the presence and absence of CC extract or cAMP:**

Bathing media	Na <sup>+</sup> pmole/nl/min	K <sup>+</sup> pmole/nl/min
R1 normal	190.8	565.7
R2 + chelerythrine	103.9	258.5
R1 + chelerythrine	120.4	312.9
R2 + chelerythrine + CC extract	189.1	394.8
R1 + chelerythrine	119.0	299.6
R2 + chelerythrine + cAMP	131.7	308.1

R1: Rate 1; R2: Rate 2.

**Effect of staurosporine on [Na<sup>+</sup>] and [K<sup>+</sup>] in the “urine” in the presence and absence of CC extract or cAMP:**

The effect of 1 $\mu$ M staurosporine on the Na<sup>+</sup> and K<sup>+</sup> composition of the fluid secreted in the presence and absence of CC extract or cAMP is shown in Table 3.8. It can be seen that the effect of staurosporine was similar to that observed with chelerythrine. Staurosporine alone significantly ( $p < 0.001$ ) reduced the K<sup>+</sup> concentration in the secreted fluid from 125.2mM to 108.9mM without significantly affecting the Na<sup>+</sup> concentration. Addition of CC extract in the presence of staurosporine significantly ( $p < 0.0001$ ) increased [Na<sup>+</sup>] from 40.4mM to 50.3mM, but [K<sup>+</sup>] was not significantly

reduced below the concentration observed in the presence of staurosporine alone. As previously described, cAMP did not significantly affect  $\text{Na}^+$  or  $\text{K}^+$  concentrations in the secreted fluid, nor did cAMP significantly modify the effect of staurosporine alone on the concentrations of these two cations.

**Table 3.8** Effect of  $1\mu\text{M}$  staurosporine on the  $\text{Na}^+$  and  $\text{K}^+$  concentrations in the fluid secreted by Malpighian tubules of *Locusta* in the presence and absence of cc extract or cAMP:

Bathing media	n	$[\text{Na}^+]$ mM	<i>p</i>	$[\text{K}^+]$ mM	<i>p</i>
R1 normal	21	$41.6 \pm 1.1$		$125.5 \pm 2.3$	
R2 normal	21	$40.7 \pm 1.2$	n.s.	$126.9 \pm 3.2$	n.s.
R1 normal	13	$42.4 \pm 1.6$		$125.2 \pm 2.3$	
R2 + $1\mu\text{M}$ Staur	13	$41.5 \pm 1.5$	n.s.	$108.9 \pm 1.8$	<0.001
R1 + $1\mu\text{M}$ Staur.	15	$40.4 \pm 2.7$		$110.9 \pm 3.1$	
R2 + Staur. + CC extract	15	$50.3 \pm 3.8$	<0.0001	$104.1 \pm 3.5$	n.s.
R1 + $1\mu\text{M}$ Staur.	19	$41.7 \pm 0.7$		$110.1 \pm 1.7$	
R2 + Staur. + cAMP	19	$40.5 \pm 0.8$	n.s.	$108.4 \pm 2.7$	n.s.

*p* values were obtained by comparing rate 1 and rate 2 by paired *t*-test.

Staur. = Staurosporine.

R1: Rate 1, R2: Rate 2.

n.s.: Not significant.

The effect of staurosporine on the  $\text{Na}^+/\text{K}^+$  ratio in the presence and absence of CC extract or cAMP is shown in Figure 3.8. It can be seen that  $1\mu\text{M}$  staurosporine caused a significant ( $p < 0.0001$ ) increase in the  $\text{Na}^+/\text{K}^+$  ratio from  $0.33 \pm 0.01$  to  $0.38 \pm 0.01$  ( $n=13$ ). Bathing tubules in staurosporine saline in the presence of CC extract effected a further significant ( $p < 0.0001$ ) increase in the  $\text{Na}^+/\text{K}^+$  ratio from  $0.38 \pm 0.01$  to  $0.47 \pm 0.01$  ( $n=15$ ). However, cAMP had no significant effect on the  $\text{Na}^+/\text{K}^+$  ratio compared with that observed in the presence of staurosporine alone (see also Appendix 2, Table 2.6).

**Figure 3.8**

Effect of  $1\mu\text{M}$  staurosporine on the  $\text{Na}^+/\text{K}^+$  ratio in the presence and absence of CC extract (1 gland pair/ml) or  $1\text{mM}$  cAMP.

**Ordinate:**  $\text{Na}^+/\text{K}^+$  ratios in the presence or absence of inhibitor or stimulator.

**Abscissa:** Inhibitor and/or stimulator.

St. = Staurosporine.

C.C. = Corpora cardiaca extract.

St. + C.C. = R1 in the presence of staurosporine alone.

R2 in the presence of staurosporine +  
corpora cardiaca extract.

St. + cAMP = R1 in the presence of staurosporine alone.

R2 in the presence of staurosporine  
+ cAMP.

Each point represents the mean  $\pm$  S.E.M.

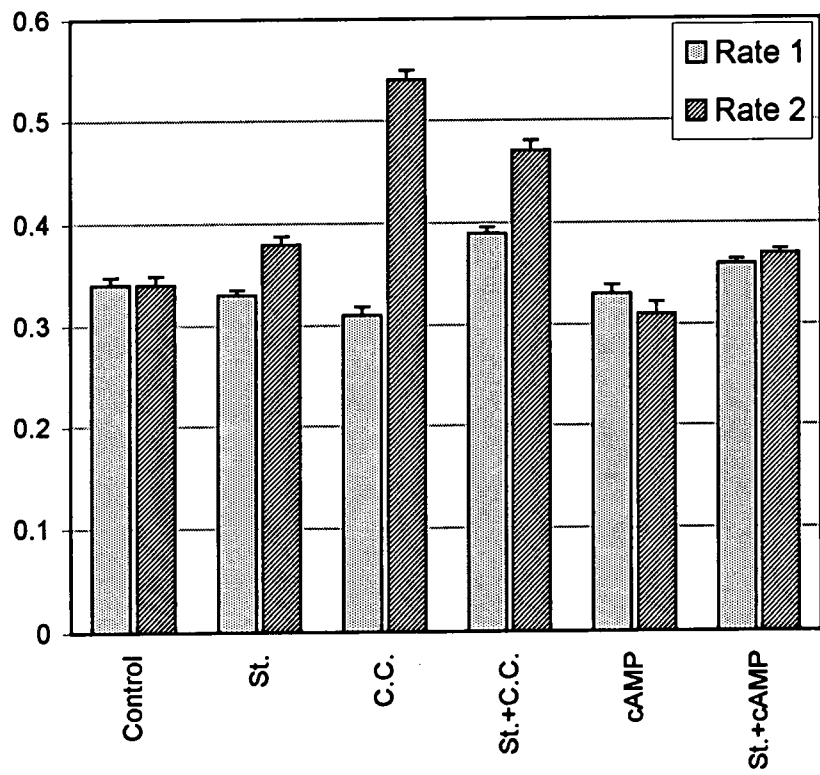


Table 3.9 showed that inclusion of  $1\mu\text{M}$  staurosporine in the bathing medium caused a decrease in the mean  $\text{Na}^+$  flux from 224.7 to 112.1 pmole/nl/min and in  $\text{K}^+$  flux from 663.6 to 294.0 pmole/nl/min. The addition of CC extract in the presence of staurosporine caused an increase in  $\text{Na}^+$  flux and did not change the  $\text{K}^+$  flux compared to that observed in the presence of staurosporine alone. However, when 1mM cAMP was included in the presence of staurosporine it did not alter the mean  $\text{Na}^+$  and  $\text{K}^+$  flux from than that observed in the presence of staurosporine alone.

**Table 3.9 Effect of  $1\mu\text{M}$  staurosporine on the mean  $\text{Na}^+$  and  $\text{K}^+$  flux in the presence and absence of CC extract or cAMP:**

Bathing media	$\text{Na}^+$ pmole/nl/min	$\text{K}^+$ pmole/nl/min
R1 normal	224.7	663.6
R2 + staurosporine	112.1	294.0
R1 + staurosporine	137.4	377.1
R2 + staurosporine +CC extract	211.3	437.2
R1 + staurosporine	120.9	319.3
R2 + staurosporine + cAMP	133.7	357.7

R1: Rate 1, R2: Rate 2.

**Effect of Rp-cAMP on  $[\text{Na}^+]$  and  $[\text{K}^+]$  in the “urine” in the presence and absence of CC extract or cAMP:**

Table 3.10 shows the effect of Rp-cAMP on the  $\text{Na}^+$  and  $\text{K}^+$  composition of the secreted fluid in the presence and absence of CC extract or cAMP. The ionic composition of the fluid secreted control tubules showed that  $\text{K}^+$  concentration during rate 2 was approximately 126.9mM whilst  $\text{Na}^+$  concentration was approximately 40.7mM. The addition of Rp-cAMP caused a significant ( $p<0.005$ ) fall in  $[\text{Na}^+]$  from 37.1mM to 28.4mM and a significant ( $p<0.002$ ) rise in  $[\text{K}^+]$  from 123.7mM to 147.7mM, increasing the total  $\text{Na}^+ + \text{K}^+$  content from 160.8mM to 176.1mM.

The addition of CC extract appeared to reverse the effects of Rp-cAMP. The  $\text{Na}^+$  content of the secreted fluid significantly ( $p < 0.02$ ) rose from 26.5 to 35.9 mM, and whilst the mean  $\text{K}^+$  content was somewhat lower, the difference from that observed in the presence of Rp-cAMP alone was not significant. The total monovalent cation content was unchanged by the addition of CC extract compared with Rp-cAMP alone.

**Table 3.10** Effect of 0.1 mM Rp-cAMP on the  $\text{Na}^+$  and  $\text{K}^+$  concentrations in the fluid secreted by Malpighian tubules of *Locusta* in the presence and absence of CC extract or cAMP.

Bathing media	n	$[\text{Na}^+]$ mM	<i>p</i>	$[\text{K}^+]$ mM	<i>p</i>
R1 normal	21	$41.6 \pm 1.1$		$125.5 \pm 2.3$	
R2 normal	21	$40.7 \pm 1.2$	n.s.	$126.9 \pm 3.2$	n.s.
R1 normal	15	$37.1 \pm 1.6$		$123.7 \pm 3.6$	
R2 + Rp-cAMP	15	$28.4 \pm 1.4$	$<0.005$	$147.7 \pm 3.9$	$<0.002$
R1 + Rp-cAMP	12	$26.5 \pm 2.1$		$138.4 \pm 1.7$	
R2 + Rp-cAMP + CC extract	12	$35.9 \pm 2.2$	$<0.02$	$129.1 \pm 7.0$	n.s.
R1 + Rp-cAMP	13	$26.8 \pm 2.8$		$140.2 \pm 6.0$	
R2 + Rp-cAMP + cAMP	13	$38.5 \pm 2.1$	$<0.02$	$138.0 \pm 3.8$	n.s.

*p* values were obtained by comparing rate 1 and rate 2 by paired *t*-test

R1: Rate 1.

R2: Rate 2.

n.s.: Not significant.

The addition of cAMP to the saline containing Rp-cAMP caused a significant ( $p < 0.02$ ) increase in  $\text{Na}^+$  concentration from 26.8 to 38.5 mM, restoring it to normal control levels. However, cAMP did not significantly change  $[\text{K}^+]$  compared with that observed in the presence of Rp-cAMP alone, producing a fluid with an increased monovalent cation composition of 176.1 mM.

Figure 3.9 shows the effect of 0.1 mM Rp-cAMP on the mean  $\text{Na}^+/\text{K}^+$  ratio in the presence and absence of CC extract or cAMP. It can be seen that the inclusion of 0.1 mM Rp-cAMP caused a significant ( $p < 0.0001$ ) decrease in the mean  $\text{Na}^+/\text{K}^+$  ratio

from  $0.30 \pm 0.01$  to  $0.19 \pm 0.01$ . In contrast, the addition of CC extract or cAMP to Rp-cAMP saline caused a significant increase ( $p < 0.0001$  for both cases) from  $0.19 \pm 0.01$  to  $0.28 \pm 0.01$  and from  $0.19 \pm 0.01$  to  $0.28 \pm 0.02$  respectively, (see also Appendix 2, Table 2.7).

Table 3.11 shows that 0.1 mM Rp-cAMP decreased  $\text{Na}^+$  flux from 159.5 to 73.8 pmole/nl/min and  $\text{K}^+$  flux from 531.9 to 384.0 pmole/nl/min. Inclusion of either CC extract or cAMP plus Rp-cAMP did not reverse the decrease in  $\text{Na}^+$  and  $\text{K}^+$  flux observed in the presence of Rp-cAMP alone.

**Table 3.11 Effect of 0.1mM Rp-cAMP on the mean  $\text{Na}^+$  and  $\text{K}^+$  flux in the presence and absence of CC extract or cAMP.**

Bathing media	$\text{Na}^+$ pmole/nl/min	$\text{K}^+$ pmole/nl/min
R1 normal	159.5	531.9
R2 +Rp-cAMP	73.8	384.0
R1 + Rp-cAMP	79.5	415.2
R2 + Rp-cAMP + CC extract	96.9	348.6
R1 + Rp-cAMP	85.8	448.6
R2 + Rp-cAMP + cAMP	88.6	317.4

R1: Rate 1.

R2: Rate 2.

**Figure 3.9**

Effect of 0.1mM Rp-cAMP on  $\text{Na}^+/\text{K}^+$  ratio in the presence and absence of CC extract (1 gland pair /ml) or 1mM cAMP.

**Ordinate:**  $\text{Na}^+/\text{K}^+$  ratios in the presence or absence of inhibitor or stimulator.

**Abscissa:** Inhibitor and/or stimulator.

C.C.= Corpora cardiaca extract.

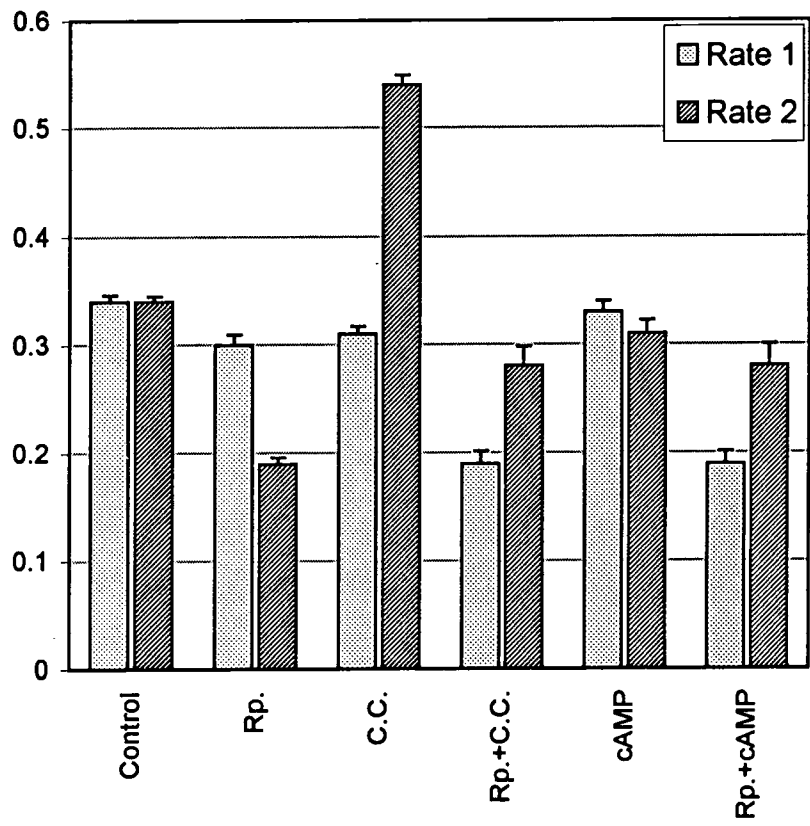
Rp-cAMP + C.C. = R1 in the presence of Rp-cAMP alone.

R2 in the presence of Rp-cAMP + corpora cardiaca extract.

Rp-cAMP + cAMP = R1 in the presence of Rp-cAMP alone.

R2 in the presence of Rp-cAMP + cAMP.

Each point represents the mean  $\pm$  S.E.M.



DISCUSSION

All of the inhibitors used in this study (chelerythrine, staurosporine, Rp-cAMP, NEM and okadaic acid) inhibited the fluid producing capacity of the Malpighian tubule cells despite having different target sites of inhibition. Chelerythrine and staurosporine are both inhibitors of protein kinase C (PKC) (Tamaoki *et al.*, 1986; Oka *et al.*, 1986). Rp-cAMP is an inhibitor of protein kinase A (PKA) (Rothermel *et al.*, 1984; Richter-Landsberg and Jastorff, 1985), at the concentrations used NEM is a putative inhibitor of V-type ATPase (Bowman *et al.*, 1988; Forgac 1989; Wieczorek *et al.*, 1989; 1991; Bertram *et al.*, 1991; Weltens *et al.*, 1992 Bertram and Wessing, 1994; present study) and okadaic acid is a specific inhibitor of protein phosphatases 1 and 2A (Takai *et al.*, 1987; 1989; Bialojan and Takai 1988; Fujiki and Suganuma, 1993).

The mechanisms of fluid production by Malpighian tubule cells, and their control by hormonal stimulation, are therefore likely to be complex and the purpose of the present work is to establish whether, by the use of specific inhibitors, further insight might be gained.

At the basal surface the primary transporters are likely to be the  $\text{Na}^+/\text{K}^+$ -ATPase (Anstee and Bell, 1975; Anstee *et al.*, 1979; Anstee *et al.*, 1986) and the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  co-transporter (Palfrey and Rao, 1983; Phillips and Hanrahan, 1984; Geck and Heinz, 1986; Maddrell and Overton, 1988). Evidence also exists for the presence of an anomalously rectified  $\text{K}^+$  channel at the basal surface (Marshall, 1995). At the apical surface it is proposed that a V-type ATPase (Wieczorek *et al.*, 1989; 1991; Bertram *et al.*, 1991; and reviews by Nicolson, 1993; Dow, 1994) produces a proton gradient that drives  $\text{Na}^+/\text{H}^+$  and  $\text{K}^+/\text{H}^+$  exchange antiporters (Fogg *et al.*, 1991; Zeiske, 1992; Beyenbach,

1995; Pannabecker, 1995). Chloride ions are thought to exit via  $\text{Cl}^-$  channels. Control of fluid production may involve the regulation of activity of a variety of these channels and transporters. It is proposed that the basal  $\text{Na}^+/\text{K}^+$ -ATPase contributes to  $\text{K}^+$  entry into the cell, in exchange for  $\text{Na}^+$ , thus, maintaining the  $\text{Na}^+$  gradient across the basal membrane. This function for the  $\text{Na}^+/\text{K}^+$ -ATPase had previously been suggested for the Malpighian tubules of *Locusta migratoria* by Fogg (1990) and Baldrick *et al.*, (1988) and also for the tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). The  $\text{Na}^+$  gradient established by the  $\text{Na}^+/\text{K}^+$ -ATPase can be used to drive the basal co-transporter which is likely to be a  $\text{K}/\text{Na}/2\text{Cl}$  (O'Donnell and Maddrell, 1984 ; Phillips and Hanrahan, 1984; Maddrell and Overton, 1988).

In the present study CC extract and cAMP stimulated the rate of fluid secretion by *Locusta* Malpighian tubules by approximately 100 or 50%, respectively. These results confirm earlier reports that fluid secretion by Malpighian tubules is hormonally controlled in the majority of insect species which have been studied, including *Locusta migratoria* (Anstee *et al.*, 1980; Rafaeli and Mordue, 1982; Fogg *et al.*, 1990; Gee, 1977; Schwartz and Reynolds, 1979; Nicolson, 1980; Maddrell, 1981; Phillips, 1981; Williams and Beyenbach, 1983; Rafaeli, 1990; Hegarty *et al.*, 1991; Clark and Spring, 1992; Beyenbach, 1995; Lehmborg, *et al.*, 1993; Nicolson and Isaacson, 1996). Tubular secretion rates are stimulated by a family of neuropeptide diuretic hormones (DH) in insects which are believed to operate via a second messenger system involving cAMP and/or  $\text{IP}_3$  and  $\text{Ca}^{2+}$  (Berridge, 1980; Berridge and Irvine, 1984; Abdel-Latif, 1986; Fogg *et al.*, 1989). Active peptides with DH activity have been partially purified and characterized for some insect species: *Locusta migratoria* (Proux *et al.*, 1988; Schooley *et al.*, 1987), *Manduca sexta* (Kataoka *et al.*, 1989; Blackburn *et al.*, 1991; Audsley *et al.*, 1993), *Acheta* and *Periplaneta* (Kay *et al.*, 1991; 1992), *Rhodnius prolixus* (Aston

1979) and *Formica polyctena* (Dedecker *et al.*, 1993; 1994). The differing characteristics of DH in those species suggested that there are several forms of this hormone involved in the control of primary urine production.

The electrophysiological properties of the basolateral and apical membranes have been reported by Fogg *et al.*, (1989). Corpora cardiaca extract was reported to cause a small hyperpolarisation of the basal membrane potential ( $V_B$ ), whereas cAMP did not effect  $V_B$ . If hormonal stimulation activated  $K^+$  entry via the co-transporter (Na/K/2Cl) alone then no effect should be observed on  $V_B$  as the basal co-transporter is electroneutral. However, stimulation of the electrogenic  $Na^+/K^+$ -ATPase would be consistent with the effect of CC extract on  $V_B$ .

Fogg *et al.*, (1989) also have reported that cAMP and CC extract have different effects on the apical membrane potential ( $V_A$ ). cAMP causes a hyperpolarisation of  $V_A$  that would be consistent with a net extrusion of cations. A likely candidate would be via stimulation of the V-type ATPase. The fact that CC extract causes a depolarisation indicates that other second messenger systems ( $IP_3/Ca$ ) activate a different membrane function that results in an increased anion permeability. This latter effect must outweigh the proposed stimulation of the V-type ATPase involving the cAMP arm of the second messenger system.

Other workers have drawn similar conclusions from work on Malpighian tubules of other insects. For example, Nicolson and Isaacson (1987) concluded, from studies on  $V_A$  and transepithelial potential (TEP) in tubules of *Onymacris plana*, that DH effected changes in TEP as a consequence of changes in  $V_A$ . O'Donnell and Maddrell (1984) and O'Donnell *et al.*, (1996) showed that, in *Rhodnius prolixus*, and *Drosophila*

*melanogaster* respectively, the changes in TEP due to the action of 5-HT represented events at the apical membrane.

These various studies on the effect of hormones and hormone analogues on the membrane potentials of Malpighian tubule cells clearly identifies their physiological effects on fluid secretion as operating via the modulation of ion channels and /or transporters. This hormone stimulation operates through the release of intracellular second messengers (Berridge, 1980; Fogg *et al.*, 1990).

Recently, considerable interest has been shown in a group of second messenger molecules derived from membrane phosphoinositides (see review by Berridge and Irvine, 1989). Appropriate receptor stimulation triggers phospholipase C (PLC, otherwise known as phosphoinositidase C) cleavage of membrane phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to yield two second messenger molecules, inositol 1,4,5-triphosphate (Ins-1,4,5-P<sub>3</sub>) and sn-1,2-diacylglycerol (DAG); the latter being an activator of protein kinase C (Nishizuka, 1984). There is now general agreement that Ins-1,4,5-P<sub>3</sub> induces the release of Ca<sup>2+</sup> from the endoplasmic reticulum (Berridge and Irvine, 1984; Abdel-Latif, 1986; Berridge, 1986) and thus triggers the biological response to 'calcium-mobilizing' hormones and neurotransmitters. Berridge (1986) reported that Ins-1,4,5-P<sub>3</sub> was generated by activation of the 5-HT<sub>1</sub> receptor in *Calliphora* salivary glands; the receptor which appeared responsible for mobilization of intracellular Ca<sup>2+</sup> (Berridge *et al.*, 1984). Fogg *et al.*, (1990) demonstrated the rapid generation of both intracellular cAMP and Ins-1,4,5-P<sub>3</sub> by the Malpighian tubules of *Locusta migratoria* in response to crude CC extracts with diuretic activity.

It has been suggested that increased levels of the second messengers, cAMP and Ca<sup>2+</sup>, effect increased ion and fluid secretion (Berridge, 1980). In *Calliphora* cAMP is

thought to stimulate an apical cation pump whereas calcium increases anion conductance across both the basal and apical cell membranes (Berridge and Prince, 1972b; Berridge *et al.*, 1975a). Morgan and Mordue (1984) proposed a similar model for the hormonal control of fluid and ion secretion by the Malpighian tubules of *Locusta migratoria* in which two spatially distinct receptors exist on the surface of the tubule cells; one which activates adenylate cyclase (R<sub>1</sub>) to increase cAMP synthesis and another (R<sub>2</sub>) which triggers a different cellular event, possibly increasing intracellular Ca<sup>2+</sup>. These second messengers will of course exert their effects via the activation of protein kinases, which together with protein phosphatases, regulate the phosphorylation status of target proteins, and as a consequence the regulation of their activities.

In Malpighian tubules, fluid movement is coupled to the active transport of K<sup>+</sup> and/or Na<sup>+</sup>, with Cl<sup>-</sup> as the major counterion (Nicolson, 1993). Diuretic peptides increase tubule secretion by stimulating ion transport, and there is no evidence of a change in the osmotic permeability of the epithelium (Nicolson, 1993). In the present study, it is of interest that the composition of the secreted fluid was not significantly changed during stimulation by cAMP. However, CC extract significantly increased secreted [Na<sup>+</sup>] from 41.2 to 59.3mM (p<0.0001) and decreased secreted [K<sup>+</sup>] from 126.8 to 107.2mM (p<0.002), leaving the total monovalent cation concentration unchanged (166.5mM) from that of unstimulated tubules (168mM). The Na<sup>+</sup>/K<sup>+</sup> ratio was also significantly increased from 0.31 to 0.54 during stimulation with CC extract (p<0.0001). Results in Table 3.5 show that CC extract effects an increase in both Na<sup>+</sup> flux (184%) and K<sup>+</sup> flux (67%) compared with the unstimulated tubules. However, cAMP caused smaller increase in Na<sup>+</sup> flux and K<sup>+</sup> flux (46%) and (51%) respectively. These data agree with those reported by Fogg (1990) and Fogg *et al.*, (1993).

The increased efflux of both  $\text{Na}^+$  and  $\text{K}^+$  at the apical surface implies that influx of these ions must be increased at the basal surface. The most obvious mechanism would be by activation of the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter. Stimulation by cAMP did not affect  $V_B$  (Fogg *et al.*, 1989) and so it is possible that the co-transporter is activated via protein kinase A. Corpora cardiaca extract does cause  $V_B$  hyperpolarisation and it is possible that this results from activation of the  $\text{Na}^+/\text{K}^+$ -ATPase by a non-cAMP dependent process, presumably via  $\text{IP}_3$  and  $\text{Ca}^{2+}$ . Activation of this enzyme would help maintain the  $\text{Na}^+$  gradient whilst supplying increased  $\text{K}^+$  for secretion at the apical surface.

Ouabain (a specific inhibitor of the  $\text{Na}^+/\text{K}^+$ -ATPase, Skou, 1969) inhibited fluid secretion by Malpighian tubules of *Locusta* (Anstee and Bell, 1975; Anstee *et al.*, 1979; Anstee *et al.*, 1980; Donkin and Anstee, 1980; Anstee *et al.*, 1986; Fogg, 1990; Fogg *et al.*, 1991). Ouabain inhibition of fluid secretion was accompanied by increased  $[\text{Na}^+]_i$  and decreased  $[\text{K}^+]_i$  (Pivovarova *et al.*, 1994b); it is significant that Marshall (1995) reported increased levels of  $\text{Na}^+$  in the "urine". There is evidence from other tissues that  $\text{Na}^+/\text{K}^+$ -ATPase activity is modulated by protein kinase activity. Bertorello *et al.*, (1991) reported that both PKA and PKC can phosphorylate the catalytic subunit of purified shark rectal gland  $\text{Na}^+/\text{K}^+$ -ATPase *in vitro* and that both kinases inhibited  $\text{Na}^+/\text{K}^+$ -ATPase activity of a partially purified preparation from rat renal cortex (Aperia *et al.*, 1987; Satoh *et al.*, 1993). Another possibility is that both kinases interact, directly or through intermediate steps, with other mediators that participate in the signaling cascade in kidney cells, e.g., phospholipase A2 (Hirata, 1981; Nishizuka, 1992; Satoh *et al.*, 1993). These observations could support the present study, where fluid and ion secretion of Malpighian tubules is inhibited by PKA and PKC inhibitors. It has been suggested that pump regulation in this region of the vertebrate nephron is mediated

by the phospholipase C-protein kinase C pathway (Bertorello and Aperia, 1988; Chen *et al.*, 1993; Satoh *et al.*, 1993). Ominato *et al.*, (1996) proposed that short-term regulation of proximal tubule  $\text{Na}^+/\text{K}^+$ -ATPase activity by dopamine and parathyroid hormone occurs via the PLC-PKC signal transduction pathway, which may interact with the pump rather than alter sodium access to it. It has also been reported that PKC-induced phosphorylation of the catalytic subunit of the renal tubular basolateral membrane-bound  $\text{Na}^+/\text{K}^+$ -ATPase inhibits the activity of the pump (Bertorello *et al.*, 1991; Lowndes *et al.*, 1990).

It is possible that fluid production would require activation of the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter to support the stimulated rate. Little work has been carried out on this co-transporter from insect tissues but there are reports that phosphorylation through protein kinase A stimulates, whereas phosphorylation through protein kinase C inhibits the co-transporter (Kaplan *et al.*, 1996). Hegarty *et al.*, (1991) have demonstrated that the bumetamide sensitive  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter is stimulated by exogenous cAMP. The present data showing that cAMP alone can stimulate fluid secretion appreciably (50%) would support activation of the co-transporter via PKA. cAMP stimulates fluid production and  $\text{Na}^+$  and  $\text{K}^+$  efflux but also causes hyperpolarisation of  $V_A$ . This suggests activation of the V-type ATPase, which would steepen the  $\text{H}^+$  gradient so increasing the rate of efflux of both  $\text{Na}^+$  and  $\text{K}^+$  through the counter transporter(s). The hyperpolarisation effect suggests that, in this situation, the efflux of  $\text{Cl}^-$  does not match the net  $\text{H}^+$  together with the  $\text{K}^+$  and  $\text{Na}^+$  efflux. This is consistent with the observation in our laboratory that the amount of chloride present in the cells increased dramatically during stimulation with cAMP (Hopkin, personal communication) implying net  $\text{Cl}^-$  retention during active stimulated secretion. Corpora cardiaca extract doubles the rate of fluid secretion and also changes the  $\text{Na}^+/\text{K}^+$  ratio of the fluid produced (Table 3.1 and

Figure 3.7), co-incident with this is a depolarisation of  $V_A$  (Fogg *et al.*, 1989). There is a clear differential activation of  $\text{Na}^+$  secretion (187%) as compared with  $\text{K}^+$  secretion (67%). The differential effect of CC extract and cAMP at the apical surface suggests that a non-cAMP factor, caused by CC extract stimulation, is also operating to modify ion movements. The increased  $\text{Na}^+$  and  $\text{K}^+$  efflux could be driven by stimulation of the V-type ATPase as argued above. However, anion permeability must also be stimulated, presumably via  $\text{IP}_3$  and/or  $\text{Ca}^{2+}$  stimulation via protein kinase C. The activity of the  $\text{Me}/\text{H}^+$  counter transporters must also be differentially affected because of the changed ratio of  $\text{Na}^+/\text{K}^+$  extruded.

Chelerythrine and staurosporine are specific inhibitors of protein kinase C. The effect of these inhibitors on ion transport by Malpighian tubule cells of *Locusta migratoria* has not previously been reported. The data present in Tables 3.2, 3.6, and 3.8 show that both inhibit fluid production and interestingly significantly reduce the  $[\text{K}^+]$  of the secreted fluid (from 125 to 108mM), without affecting its  $[\text{Na}^+]$ . The inhibition of fluid production in unstimulated tubules by protein kinase C inhibitors may operate via an effect on anion permeability at the apical surface, as well as from effects at the basal surface, presumably by inhibition of the  $\text{Na}^+/\text{K}^+$ -ATPase (Sato *et al.*, 1993). The effects of both staurosporine and chelerythrine on the composition of the secreted fluid also indicate a role for PKC activation/inhibition of the  $\text{Me}/\text{H}^+$  counter transporters, which either differentially inhibits  $\text{K}^+$  extrusion in a binary system or changes  $\text{Na}^+/\text{K}^+$  affinities in a unitary system.

In the present study the rate of fluid secretion by Malpighian tubules of *Locusta migratoria* was significantly inhibited by Rp-cAMP over the concentration range  $1\mu\text{M}$  to  $1\text{mM}$  (~35% inhibition at  $0.1\text{mM}$  Rp-cAMP). Rp-cAMP has been reported as a specific inhibitor for PKA (Rothermel *et al.*, 1984; Richter-Landsberg and Jastorff,

1985). The inhibition effect of Rp-cAMP on fluid secretion by Malpighian tubules emphasizes a role for PKA in the regulation of the secretory process.

In unstimulated Malpighian tubules Rp-cAMP also caused a significant fall in the  $[\text{Na}^+]$  and rise in  $[\text{K}^+]$  of the secreted fluid. The fall in secretion rate could result from effects on transporters at both apical and basal surfaces, the likely candidates are inhibition of the V-type ATPase and the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter respectively. However, this would not explain the effect on ion composition of the secreted fluid, which would suggest an additional effect on the apical  $\text{Me}/\text{H}^+$  counter transporter(s). There is evidence from studies on vertebrate proximal tubule cells that the  $\text{Na}^+/\text{H}^+$  exchanger is inhibited by an increase in cAMP (Felder *et al.*, 1990; Satoh *et al.*, 1993).

In those tubules stimulated with CC extract, it is notable that stimulation of fluid secretion and an increase in "urine"  $\text{Na}^+$  concentration occurred in the presence of PKC inhibitors, but without further associated reduction in  $\text{K}^+$  concentration (Tables 3.6 and 3.8). CC extract in the presence of staurosporine or chelerythrine caused a further significant increase in the  $\text{Na}^+/\text{K}^+$  ratio which resulted from an increase in  $[\text{Na}^+]$ . This suggests that CC extract separately affects  $\text{K}^+$  and  $\text{Na}^+$  extrusion and that elevation of  $[\text{Na}^+]$  in the secreted fluid results from activation of the  $\text{Na}^+/\text{H}^+$  antiporter by PKA. Stimulation with cAMP could not be consistently demonstrated in the presence of PKC inhibitors, nor did cAMP restore the  $\text{Na}^+/\text{K}^+$  ratio in the secreted fluid. This demonstrates the critical role that PKC activation has in  $\text{K}^+$  extrusion at the apical surface. CC extract in the presence Rp-cAMP was able to restore  $[\text{Na}^+]$  of the secreted fluid to the control value without effect on  $[\text{K}^+]$ . Thus, the decrease in the  $\text{Na}^+/\text{K}^+$  ratio, in the presence Rp-cAMP alone, was reversed by the addition CC extract. This effect was evident even though CC extract did not increase fluid secretion in the presence of Rp-cAMP. In Rp-cAMP inhibited tubules, cAMP is able to restore  $[\text{Na}^+]$  of the

secreted fluid to control tubule values without lowering the  $[K^+]$  of the fluid to control levels. Therefore, cAMP in the presence of Rp-cAMP was able to increase the  $Na^+/K^+$  ratio. In addition, Table 3.11 shows that Rp-cAMP decreased  $Na^+$  flux and  $K^+$  flux by ~53% and 28% respectively. However, this decrease was not reversed by addition of CC or cAMP to the medium containing Rp-cAMP.

These data also support the suggestion made by Pivovarova *et al.*, (1994a,b) that the extrusion of  $K^+$  and  $Na^+$  may occur via separate transporters. Those workers showed that the  $Na^+$  and  $K^+$  composition of the secreted fluid was not markedly altered by conditions that dramatically lowered  $[K^+]_i$  and raised  $[Na^+]_i$ . In locust Malpighian tubule cells therefore it is unlikely that  $Na^+$  and  $K^+$  are competing at the same exit route. Thus the changes in ionic composition of the secreted fluid reported here, caused by PKA and PKC inhibitors and CC extract, strongly suggest that the activity or  $Me^+$  affinity of the transporters is determined by phosphorylation state of either or both transporters. The complexity of this process is underlined by the inhibition of fluid production by okadaic acid showing that protein phosphorylation state can be inhibiting as well as stimulatory. In summary, the inhibition of fluid secretion by the specific inhibitors of PKA and PKC indicates that these protein kinases are involved in fluid secretion process. Also, the effect of protein kinases inhibitors on the ionic composition of the fluid secreted and  $Na^+/K^+$  ratios in the presence and absence CC extract or cAMP leads to the suggestion that both PKA and PKC play a role in ion transport across the basal or apical cell membranes (or both) of the Malpighian tubule cells of *Locusta migratoria* in response to DH.

## CHAPTER 4

### **Biochemical studies on the subcellular distribution of the enzyme activities in Malpighian tubules of *Locusta migratoria*.**

#### INTRODUCTION

Cell fractionation has played an important role in our understanding of cellular processes and membrane biochemistry in both vertebrates and invertebrates. In the past insect membranes in particular have received relatively little attention, despite the importance of insects as agricultural pests and vectors of disease (Burgess, 1981; Harvey, 1982). One of the major difficulties encountered in studies on insect membranes was the small amount of tissue that was usually available to insect biochemists (Terra, 1988; 1990).

Several methods have been developed in the past to isolate brush border membrane vesicles (BBMV) from kidney cortex (Berger and Sacktor, 1970; Quirk and Robison, 1972; George, and Kenny, 1973; Booth and Kenny, 1974; Kessler *et al.*, 1978; Biber *et al.*, 1981; Briskin *et al.*, 1987). Most of these methods were based on a differential centrifugation following addition of 10-20mM CaCl<sub>2</sub> or MgCl<sub>2</sub> to the initial homogenate. By these procedures, BBMV could be obtained, which were 10-15 fold enriched as indicated by brush border marker enzymes (e.g., alkaline phosphatase), although the yield of these enzymes was only about 5-20% of the amount present in the initial homogenate (Booth and Kenny, 1974; Biber *et al.*, 1981). However, the first attempts at fractionation of the midgut of *Manduca sexta* (Cioffi, 1979) quickly revealed that the methods which had been successfully used to isolate plasma membranes from vertebrate tissues could not be applied to insect tissues, perhaps

because of difference in cell structure or tissue organization (Berridge and Oschman, 1972). It, therefore, became necessary to devise procedures which would allow the isolation of plasma membranes from an insect epithelium, and which would provide a reasonable yield of material in a condition suitable for biochemical analysis. Ferreira and Terra (1980), identified alkaline phosphatase and other enzymes as present in midgut microvilli of *Rhynchosciara americana* based on the following criteria:

- (a) Presence of most of the enzyme activity in the fraction displaying brush border characteristics under the phase contrast microscopy.
- (b) Increase in the specific activity of the enzymes, in relation to the original homogenates, in the microvilli fraction obtained by the differential calcium (or magnesium) precipitation method (Schmitz *et al.*, 1973).

Several methods have been used to prepare brush border microvilli from invertebrate epithelia. One of these, relied on differential calcium precipitation to purify the microvilli from the columnar cells of the midgut tissue (composed of columnar and goblet cells) of *Philosamia cynthia* larvae (Hanozet *et al.*, 1980). Minor modifications of the procedure of Schmitz *et al.*, (1973) have been used in the preparation of midgut cell microvilli from different insects such as the mosquito *Culex tarsalis* (Houk *et al.*, 1986), and the beetles *Pheropsophus aequinoctialis* (Ferreira and Terra 1989) and *Tenebrio molitor* (Ferreira *et al.*, 1990). The microvillar enzymes in these preparations usually were enriched 5-10 fold with yields of 20-30%.

Brush border microvilli have been prepared from freshly isolated posterior larval *Manduca sexta* midguts (Eisen *et al.*, 1989) by three methods (e. g. differential calcium precipitation, differential magnesium precipitation, and differential

ultrasonication). Comparison of results showed that the yield of BBMV by both differential precipitation methods were 5-6 times greater than that by the differential ultrasonication method. Although alkaline phosphatase,  $\gamma$ -glutamyltransferase, and aminopeptidase enrichments of the BBMV marker enzymes were similar in all preparations.

Fogg *et al.*, (1990) used a modified technique of Rodriguez and Edelman (1979) in an attempt to separate, and partially purify, the basal and apical cell membranes of Malpighian tubules of *Locusta*. Alkaline phosphatase,  $\text{Na}^+/\text{K}^+$ -ATPase and succinate dehydrogenase activities were used as marker enzymes for apical, basal and mitochondria enriched-membrane fractions respectively. Results revealed that the apical rich-membrane fractions were not totally free from  $\text{Na}^+/\text{K}^+$ -ATPase and succinate dehydrogenase activities.

The method used in this study was a modification of that reported by Fogg (1991) which gave greater purity and less mitochondrial contamination of the apical membrane fractions of Malpighian tubules cells of *Locusta migratoria*.

**MATERIALS AND METHODS**

**Membrane Separation for Subsequent Marker Enzyme Assays:**

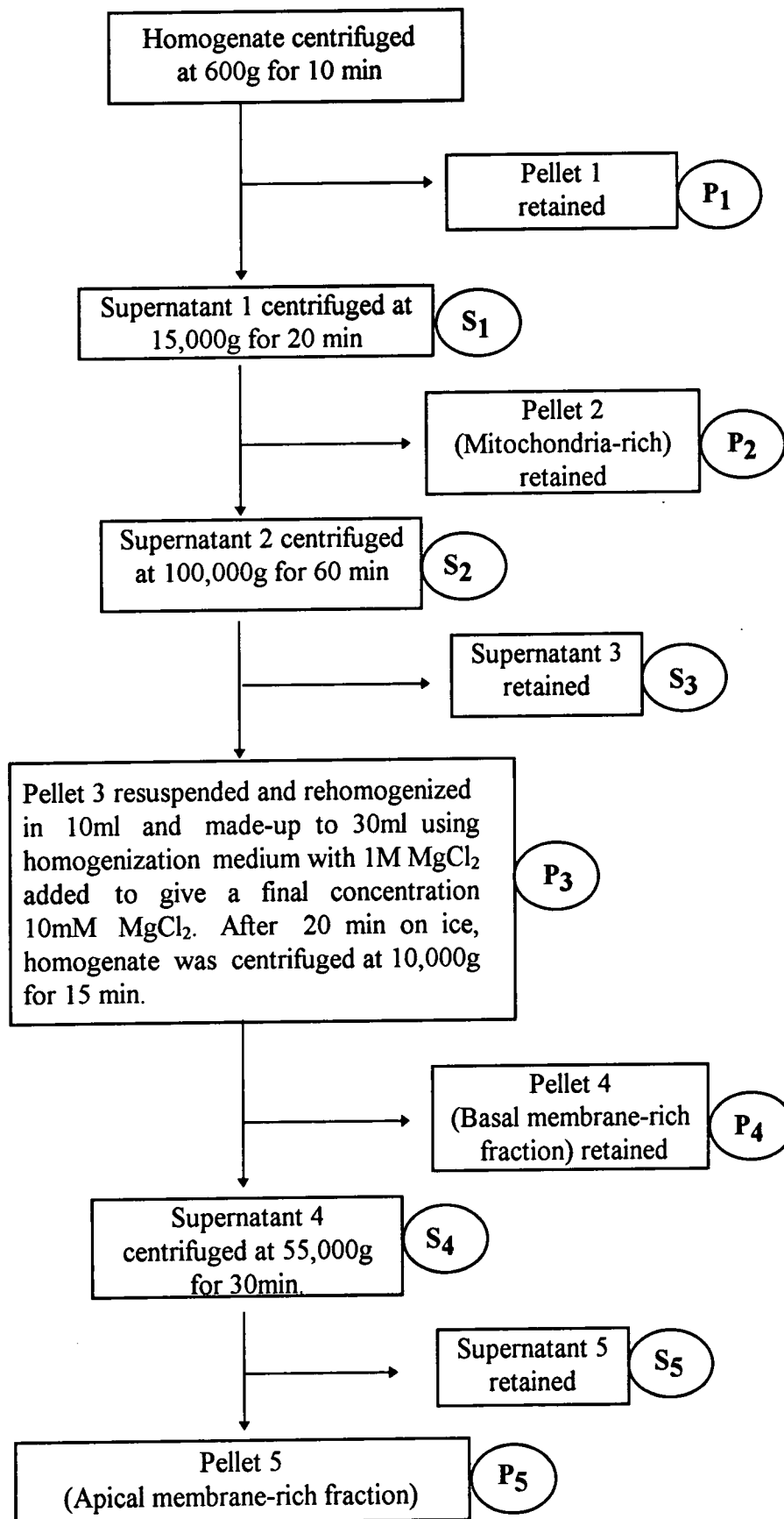
The method employed was a modification of that described by Rodriguez and Edelman (1979) and Fogg *et al.*, (1991). Unless otherwise stated, pellets were resuspended and rehomogenized in a known volume of homogenization medium (5mM imidazole-HCl buffer, pH 7.5, containing 250mM sucrose). The method is summarized in Figure 4.1. All steps were carried out at 4°C.

**Preparation of the Malpighian tubules:**

Approximately equal numbers of mature male and female locusts were used throughout. Animals were killed by decapitation and their Malpighian tubules quickly dissected out and placed ice-cold homogenization medium. Tubules from approximately 15 locusts were added to 10ml of homogenization medium. Homogenization was carried out in a glass homogenizer with a Teflon pestle (clearance 0.1-0.15mm) with twenty passes of the plunger at 1000 rev/min. The homogenization tube was surrounded by ice throughout this procedure as described by Anstee and Fathpour (1979).

The resulting homogenate was centrifuged at 600g for 10 min using a Beckman, J2-HS centrifuge. This pellet was then resuspended and the process repeated. All subsequent centrifugations were carried out using a Beckman J2-HS centrifuge and unless otherwise stated, the 8 x 50 ml fixed angle aluminium rotor was used. The (S<sub>1</sub>) obtained from the previous centrifugation was subsequently diluted

**Figure 4.1** Flow diagram to illustrate the centrifugation steps used in membrane separation.



with homogenization medium (1:1) and centrifuged at 15,000g for 20 min. This resulted in the production of a mitochondria-rich pellet (P<sub>2</sub>). The supernatant (S<sub>2</sub>) produced by this centrifugation was then recentrifuged at 100,000g for 60 min and the pellet obtained (P<sub>3</sub>) resuspended and rehomogenized in 10ml of homogenization medium which was then diluted 3-fold with homogenization medium containing 1M MgCl<sub>2</sub> to give a final concentration of 10mM MgCl<sub>2</sub>. This was then left to stand on ice for 20 min. The resuspended P<sub>3</sub> was then centrifuged at 10,000g for 15 min. The resulting supernatant (S<sub>4</sub>) was then centrifuged at 55,000g for 30 min. The pellet (P<sub>5</sub>) resulting from this spin represents the apical membrane-rich fraction (Rodriguez and Edelman, 1979; Fogg *et al.*, 1991). This pellet was resuspended by homogenization in a known volume of deionized water. All steps were carried out at 4°C.

#### **Preparation of Pellets for Electron Microscopy:**

Pellets obtained from centrifugation were prepared for electron microscopy as described previously for non-treated Malpighian tubules using Karnovsky's fixative (Karnovsky, 1965), which is composed of :

##### **Solution A:**

Paraformaldehyde	2g
Distilled water	40ml
NaOH	2-6 drops

##### **Solution B:**

25% Gluteraldehyde	10ml
0.2M Sodium Cacodylate buffer, pH 7.3	50ml

Solution A and B were mixed in a 1:1 ratio just prior to use. After fixing for 1-1.5 hours on ice, material was post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.5) for 1hr, on ice. Following post-fixation, the tubules were treated to the following steps:

(1) Dehydration through a graded series of alcohol, on ice

(i) 15 min in 70% alcohol, with 3 changes.

(ii) 15 min in 95% alcohol, with 3 changes.

(iii) 15 min in absolute alcohol, with 3 changes.

(2) 30 min in absolute alcohol / propylene oxide in 1:1 ratio, on ice, with 3 changes.

(3) 30 min in propylene oxide, with 3 changes on ice.

(4) 30 min in propylene oxide/Araldite (Araldite CY 212 10ml , DDSA 10ml , dibutyl phthalate 1ml, DMP 30 0.5ml) in 1:1 ratio at 45 °C.

(5) 30 min in Araldite alone at 45 °C.

(6) Embedded in fresh Araldite alone for polymerization, at 45 °C, overnight.

(7) Preparation moved from 45 °C to 60 °C for a further 24 hours.

Silver/silver-gold sections were cut on a Reichart NK microtome using glass knives. Sections were then expanded using chloroform and collected on Formvar coated copper grids. The sections were then stained in uranyl acetate (1% solution in 70% alcohol) followed by lead citrate (Reynolds, 1963) prior to their examination in a Phillips EM 400T electron microscope.

**Assay of ATPase activity and analysis of inorganic phosphate (P<sub>i</sub>):**

Appropriate incubation media consisting of 0.25ml ionic medium and 0.125ml of membrane homogenate (0.05-0.1mg protein/ml), unless otherwise were thermoequilibrated at  $35 \pm 0.1$  °C for 15 min in Eppendorf tubes in a water bath. Reactions were started by adding 0.125ml 12mM ATP (Tris salt) and incubations were carried out at  $35 \pm 0.1$  °C for 30 min, at the end of this time reactions were stopped by the addition of 1 ml of a 1:1 mixture of 1% Lubrol and 1% ammonium molybdate in 0.9M sulphuric acid (Atkinson *et al.*, 1973). Tubes were then allowed to stand at room temperature for 10 min to allow the yellow colour to develop. Controls were used in each experiment to determine the extent of non-enzymatic hydrolysis of ATP.

Following centrifugation at 10,000 rpm for 10 min in a Beckman, Avanti-30 centrifuge at 4°C, to spin down any protein that may have precipitated, the inorganic phosphate released was determined by measuring at 390nm in a Ultraspec 4050 spectrophotometer, the amount of inorganic phosphate (P<sub>i</sub>) released, which was proportional to the yellow colour, was determined by reference to a standard calibration curve Figure 4.2. The latter was produced using a stock solution of 0.6mM Na<sub>2</sub>HPO<sub>4</sub> which was serially diluted to give a concentration range of 0-0.6mM. To 2ml of each concentration, 4ml of the 1:1 Lubrol/acid molybdate was added, this was left to stand at room temperature for 10 min and read at 390nm.

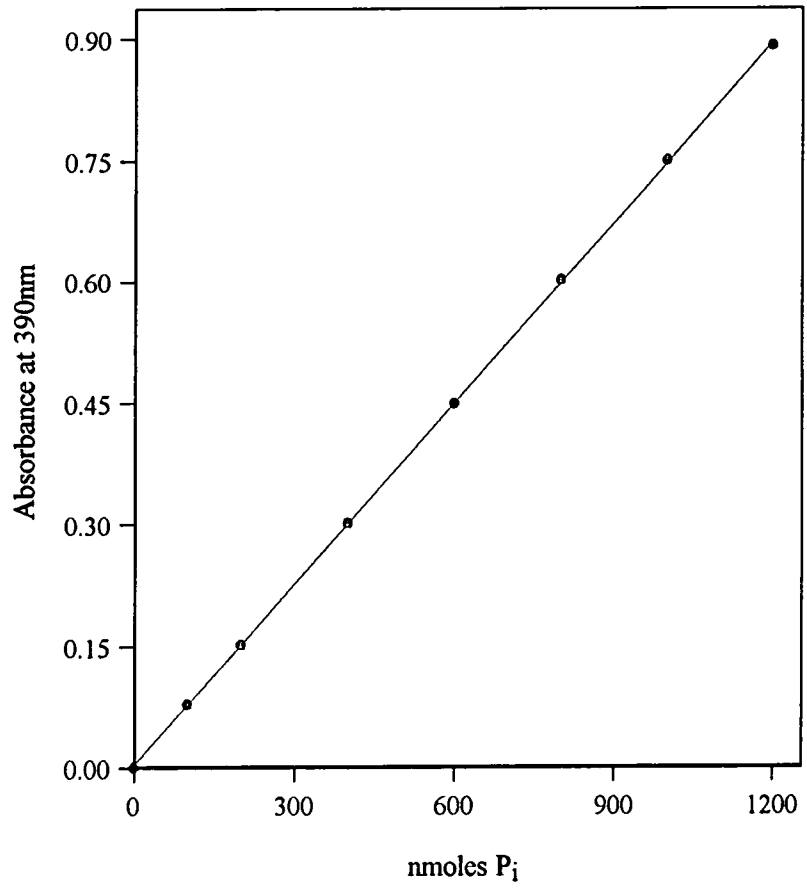
**Figure 4.2**

Standard calibration curve for the determination of inorganic phosphate.

**Ordinate:** Absorbance at 390nm.

**Abscissa:** Inorganic phosphate ( $P_i$ ) in nmoles.

Standard calibration curve for the determination of inorganic phosphate used in the assay of ATPases activity



**Determination of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity:**

Incubations were carried out as described previously. The reaction media had the following final concentration of ions, unless otherwise stated :

**Medium i)** 4mM MgCl<sub>2</sub> in 20mM imidazole buffer.

**Medium ii)** medium (i) plus 1mM ouabain.

**Medium iii)** medium (i) plus 100mM NaCl and 20mM KCl.

**Medium iv)** medium (iii) plus 1mM ouabain, all at pH 7.2.

The amount of P<sub>i</sub> released from ATP hydrolysis was measured as described previously. Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was determined as the difference in P<sub>i</sub> liberated in reaction medium (iii), containing Mg<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup>, and that released in medium (iv), containing Mg<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> and ouabain. Controls were run where ATP was not added until the reaction had been stopped to determine the extent of non-enzymatic hydrolysis. Activity was expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Estimation of Mg<sup>2+</sup>-ATPase Activity:**

Mg<sup>2+</sup>-ATPase activity was determined as the difference in P<sub>i</sub> liberated in the reaction medium (ii) containing Mg<sup>2+</sup> plus 1mM ouabain and that released non-enzymatically in the controls.

**Determination of Alkaline Phosphatase Activity:**

Alkaline phosphatase activity of the various membrane fractions, produced as described previously, was determined using the method described by Bowers and McComb (1966) with slight modification. Reaction media, consisting of 1ml buffered substrate (1mM MgCl<sub>2</sub> and 25mM *p*-nitrophenyl phosphate in Sigma 221 buffer,

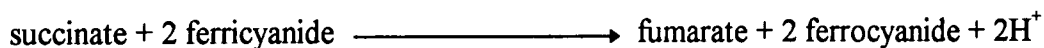
pH 10.3) and 0.5ml deionized water, were thermoequilibrated at  $35 \pm 0.1$  °C for 15 min. The reaction was started by the addition of 0.5ml membrane homogenate. Changes in absorbance at 420nm were measured at 5 min intervals, over a 20 min period using an Ultraspec 4050 spectrophotometer. Controls were run in parallel in which 0.5ml deionized water replaced the 0.5ml homogenate.

The observed changes in absorbancy were converted to nmoles *p*-nitrophenol (NP) liberated by reference to a standard *p*-nitrophenol curve (Figure 4.3) prepared by serial dilution from a 2mM NP stock solution, to give a concentration of 0-800 nmoles NP/ml. To 2ml of each concentration 3ml of 2M tris-buffer solution were added. Each mixture was left to stand for 10 min at room temperature and the absorbance read at 420nm.

#### **Determination of Succinate Dehydrogenase Activity:**

The succinate dehydrogenase (SDH) activity of the various membrane fractions, prepared as described previously, were determined using the method described by King (1967) with slight modification.

SDH can catalyze the oxidation of succinate in the presence of certain artificial electron acceptors. The determination of SDH activity is based on this property. The artificial electron acceptor used in the present study was ferricyanide. In the presence of sodium succinate, ferricyanide is converted to ferrocyanide, and succinate to fumarate. The overall reaction according to Singer and Kearney (1967) is:



The rate of decrease in absorbance at 420nm, due to ferricyanide reduction by succinate, is used as a measure of the enzyme activity.

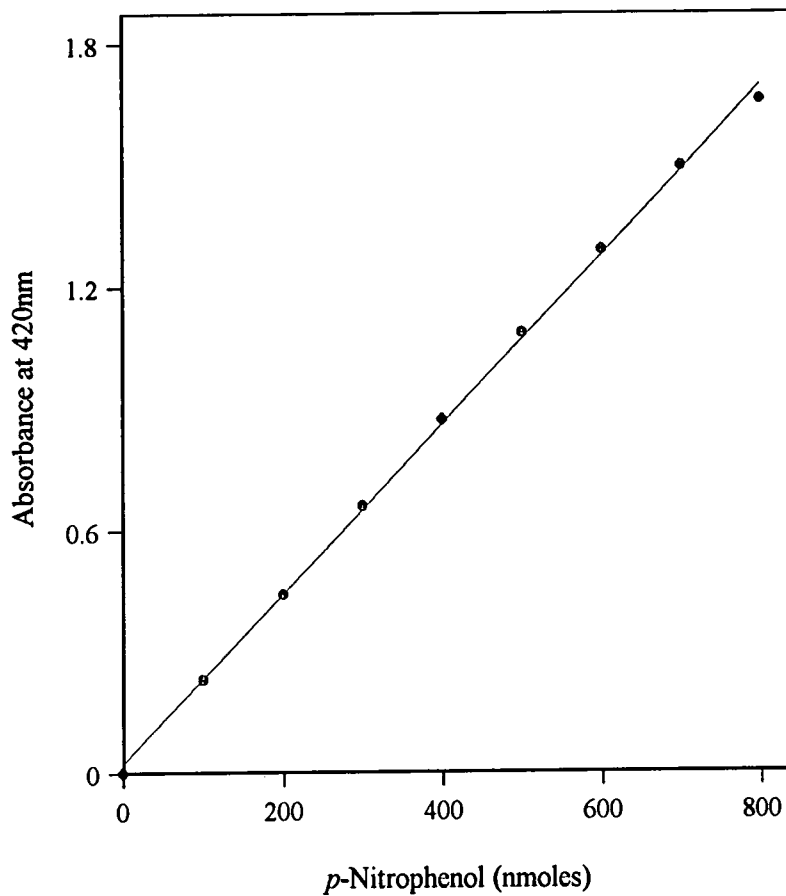
**Figure 4.3**

Standard calibration curve for the determination of *p*-Nitrophenol (NP).

**Ordinate:** Absorbance at 420nm.

**Abscissa:** *p*-Nitrophenol (NP) in nmoles.

Standard calibration curve for the determination of *p*-nitrophenol used in the assay of alkaline phosphatase activity



Reaction media were thermoequilibrated in polypropylene tubes at  $35 \pm 0.1^\circ\text{C}$  for 15 min. These consisted of 1.5ml of 0.2M phosphate buffer (pH 7.8), 0.2 ml of 0.6M sodium succinate, 0.3ml of 1% BSA, 0.1ml of 0.03M potassium ferricyanide (freshly prepared) and 0.5ml deionized water. Reactions were started by addition of 0.5ml membrane homogenate. Changes in absorbance at 420nm were measured at 5 intervals min, over a 20 min period using an Ultraspec 4050 spectrophotometer. Controls were run in parallel in which 0.5ml deionized water replaced the 0.5ml homogenate.

The observed changes in absorbance were converted to units of nmoles succinate oxidized, as described by King (1967), i.e., 0.485 x change in absorbance at 420 nm. The specific activity was expressed as nmoles succinate oxidized/mg protein/min.

#### **Estimation of Protein:**

The method employed was essentially the same as that described by Lowry *et al.*, (1951) using bovine serum albumin (BSA) Fraction V as Standard Reagents:

- (1) 2%  $\text{Na}_2\text{CO}_3$
- (2) 0.5%  $\text{CuSO}_4$
- (3) 1% KNa Tartrate

#### **Folin Solution A:**

Prepared by mixing equal volumes of solution (2) and (3) and to each volume of this mixture adding 50 volumes of solution (1).

**Folin Solution B :**

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

**Method :**

Bovine serum albumin fraction V was serially diluted to give concentrations of 0.4, 0.3, 0.2, 0.1, 0.05, and 0mg protein/ml. 3 ml of Folin Solution (A) were added to 0.2 ml of protein solution and this allowed to stand for 30 min at room temperature. 0.3ml of Folin Solution (B) was then added to each tube and the resulting solutions allowed to stand for a further 40 min at room temperature. The absorbance was measured at 500nm. A standard calibration graph of protein concentration against absorbance was constructed. From this curve the amount of protein in the unknowns could be determined. A new calibration curve was prepared each time an assay was carried out. A typical standard protein calibration curve is shown in Figure 4.4.

**Preparation of 12 mM Tris ATP:**

The disodium salt of ATP was converted into Tris ATP by using an ion exchange Dowex 50-X8 resin ( $H^+$  form) according to Schwartz *et al.*, (1962) and Anstee and Bowler (1984). 100mg Dowex resin was first mixed well with 800 ml 5% HCl for 30 min. The resin was then washed with several changes of deionized water in a Buchner funnel until the effluent had a pH between 3 and 4. At this stage all the residual acid was removed from the resin and it was in its charged ( $H^+$ ) form. It was resuspended in its volume of deionized water and stored at pH 3-4, at room temperature, until required. When required, a vertically held 5ml "Volac" glass pipette

**Figure 4.4**

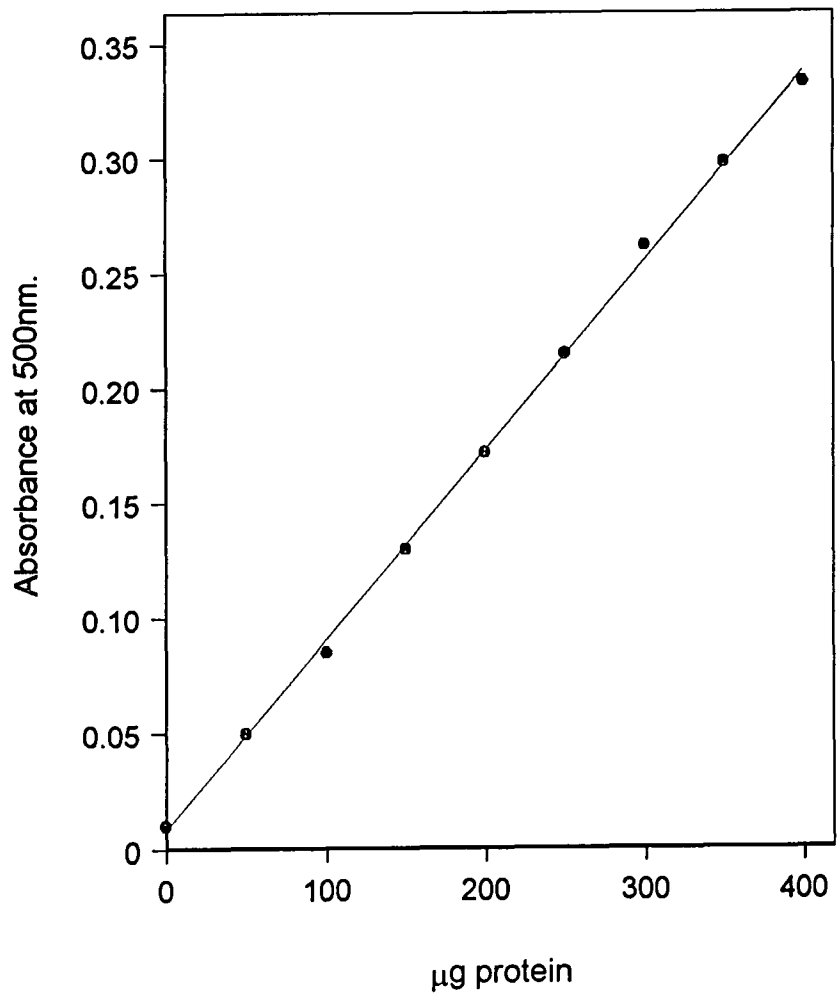
Standard calibration curve for determination of protein using bovine serum albumen (BSA) Fraction V as standard (typical example).

**Ordinate:** Absorbance at 500nm.

**Abcissa:** Protein mg/ml.

**Figure 4.3**

A typical example of a standard protein calibration curve using bovine serum albumen fraction V as standard.



was packed with resin using a wide mouthed Pasteur pipette. The level of fluid was kept above that of the resin at all times to prevent drying. Air pockets were dislodged with a plunger. Glass wool placed inside the pipette, at its base, prevented the loss of Dowex resin from the column, but allowed the passage of fluids. A silicon tube connected to the base of the column, and a "pinch-clip", enabled the flow of fluid from the column to be controlled.

The packed column of resin was then washed with deionized water until the effluent colouration disappeared and its pH was about 7.

A known amount of disodium ATP dissolved in about 15ml deionized water and then slowly passed down the column and collected in an acid-washed beaker. The solution was repassed down the column a further 2 times. Then deionized water was passed through the beads and collected in the same beaker until the collected volume was about 20 ml less than that required. The effluent collected in the beaker was ATP in its  $H^+$  form. This was converted to Tris salt by the addition of 2M Tris until the pH was 7.2. It was then made up to the volume required with deionized water and stored in aliquots at  $-20^{\circ}C$ . The weight of the ATP (sodium salt) taken was that previously calculated to give the required concentration in the final volume.

**RESULTS**

**Biochemical studies on the subcellular distribution of the enzyme activities from Malpighian tubules of *Locusta migratoria*.**

**Use of marker enzyme activity assays to localize membrane fractions:**

Enzyme activity assays were carried out, in order to determine the  $Mg^{2+}$ -dependent ATPase,  $Na^+/K^+$ -ATPase, alkaline phosphatase and succinate dehydrogenase (SDH) activities within each pellet and supernatant obtained during the differential separation procedure (summary of membrane separation Figure 4.1). Their subcellular localization could then be deduced reference to the enzyme activity of basal cell membrane marker ( $Na^+/K^+$ -ATPase), apical cell membrane marker (alkaline phosphatase) and the mitochondrial membrane marker (SDH).

***Distribution of  $Na^+/K^+$ -ATPase:***

Results obtained from five independent preparations are shown in Figure 4.5 and Appendix 3, Table 3.1. It can be seen that the major activity of  $Na^+/K^+$ -ATPase was confined to the microsomal membrane fraction pellets (P<sub>3</sub> and P<sub>4</sub>), with maximal mean specific activity ( $331.9 \pm 11.9$  nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.) being observed in P<sub>4</sub>. This would suggest that the majority of  $Na^+/K^+$ -ATPase was precipitated in P<sub>4</sub> at 10,000g. However, pellet P<sub>5</sub> and the supernatants (S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub> and S<sub>5</sub>) contained low  $Na^+/K^+$ -ATPase activity.

***Mg<sup>2+</sup>-dependent ATPase:***

Previous studies have demonstrated  $Mg^{2+}$ -dependent ATPase activity in microsomal preparations from Malpighian tubules of *Locusta migratoria* (Anstee and

**Figure 4.5**

Measurements of  $Mg^{2+}$ -ATPase and  $Na^+/K^+$ -ATPase activities in the various membrane fractions obtained from the Malpighian tubules of *Locusta migratoria*.

**Ordinate:** ATPases activity expressed as nmoles  $P_i$  liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** Membrane fractions.

H = Crude Malpighian tubules homogenate.

P<sub>1</sub> = Crude membrane pellet produced after 600g spin for 10min.

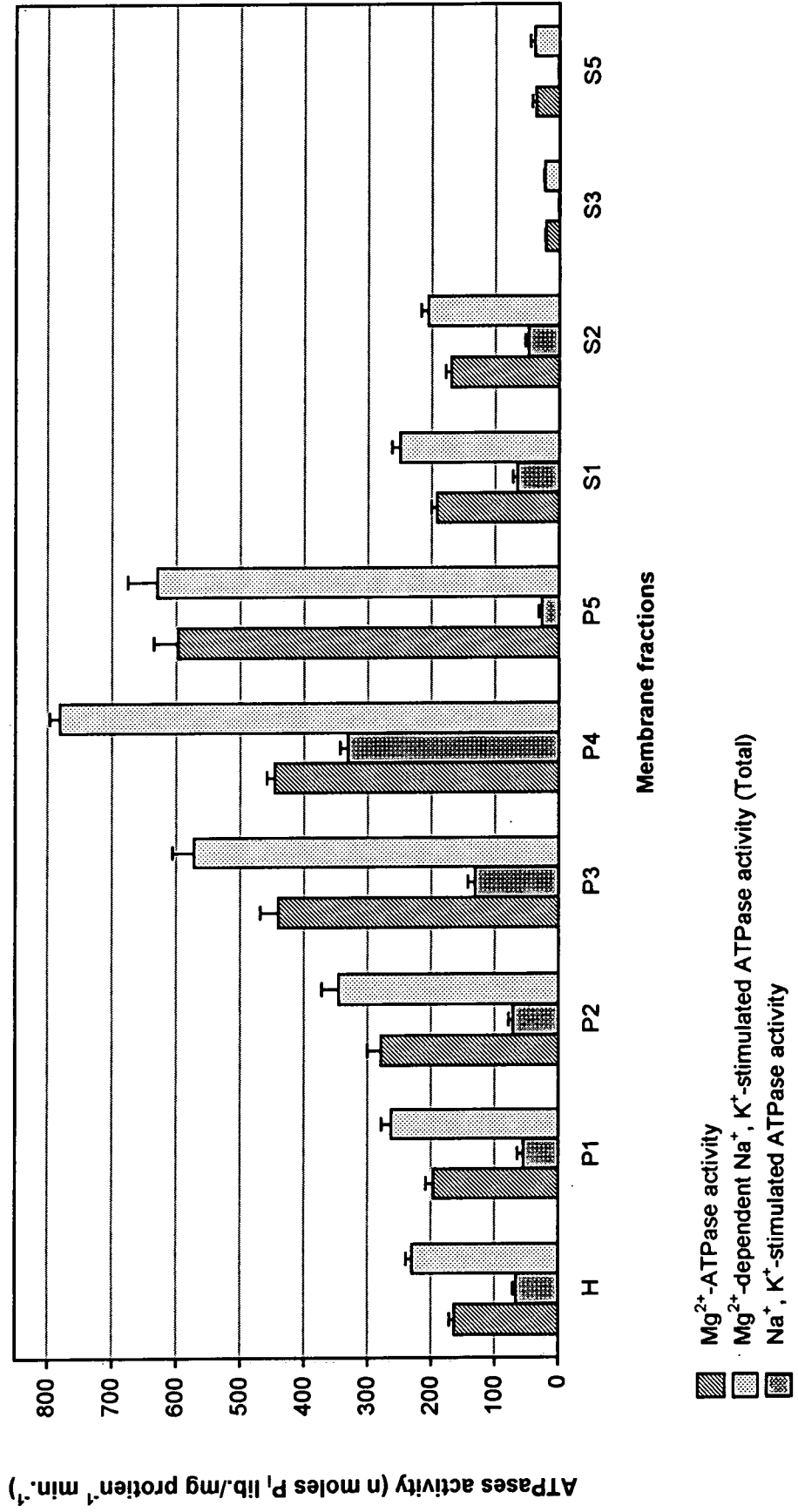
P<sub>2</sub> = Pellet produced after 15,000g spin for 20min (mitochondria-enriched pellet).

P<sub>3</sub> = Pellet produced after 100,000g spin for 60min (basal and apical membrane-enriched pellet).

P<sub>4</sub> = Pellet produced after 10,000g spin for 15min (basal membrane-enriched pellet).

P<sub>5</sub> = Pellet produced after 55,000g spin for 30min (apical membrane-enriched pellet).

S = Supernatants.



Bell, 1975; 1978; Fogg, 1990; Fogg *et al.*, 1991). Results of the present study as shown in Figure 4.5 revealed high levels of  $Mg^{2+}$ -dependent ATPase activity in P<sub>3</sub>, P<sub>4</sub> and P<sub>5</sub>, with relatively low levels of activity being observed in P<sub>2</sub> (see also Appendix 3, Table 3.1).

#### ***Alkaline phosphatase:***

The distribution of alkaline phosphatase activity was examined as described previously by cytochemical localization studies and found to be confined to the microvillar surface of Malpighian tubules of *Locusta migratoria* (Fogg, 1990). In the present study examination of data in Figure 4.6 and Appendix 3, Table 3.2 reveals that the majority of alkaline phosphatase activity was associated with P<sub>5</sub>, with relatively low activity observed in P<sub>3</sub> and P<sub>4</sub>. Thus, the ratio of  $Na^+/K^+$ -ATPase activity (expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>) to alkaline phosphatase activity (expressed in nmoles *p*-nitrophenol liberated mg protein<sup>-1</sup> min<sup>-1</sup>) was 1:1.6 in P<sub>4</sub> and 1:144.7 in P<sub>5</sub>. The differential separation of  $Na^+/K^+$ -ATPase and alkaline phosphatase indicated that the P<sub>3</sub> was successfully separated to produced enriched apical (P<sub>5</sub>) and basal (P<sub>4</sub>) membrane fractions.

#### ***Succinate dehydrogenase (SDH):***

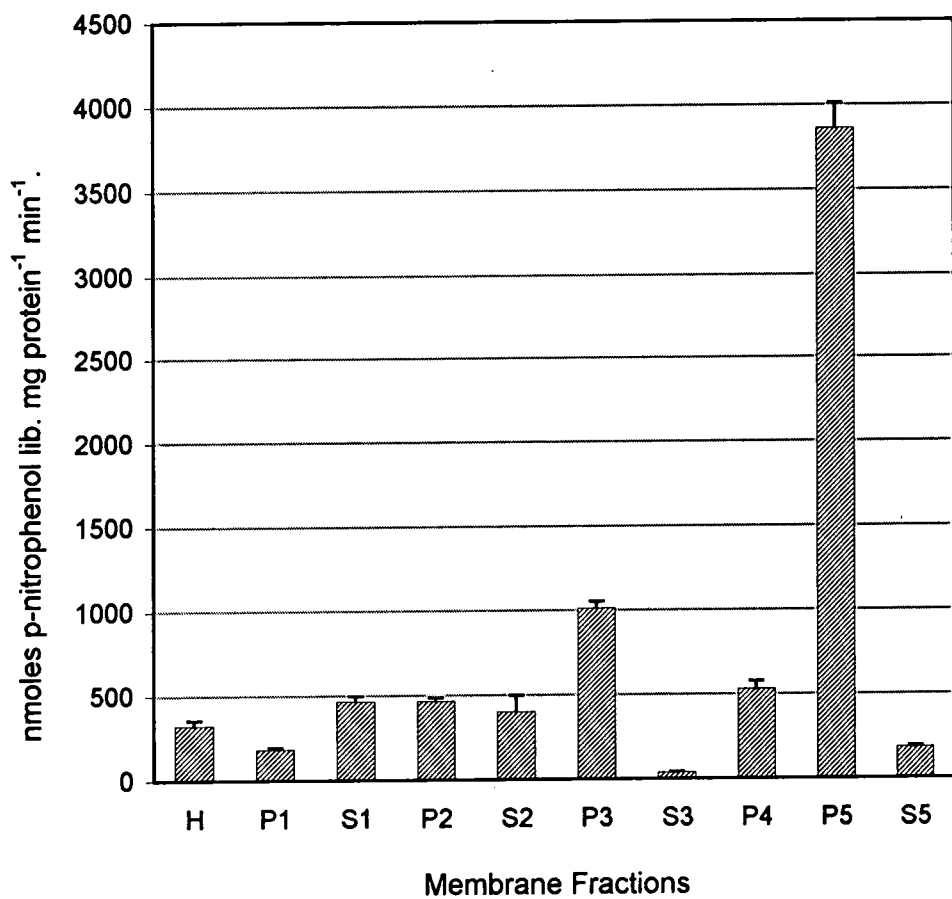
Data in Figure 4.7 and Appendix 3, Table 3.2 reveals that the mean specific activity of SDH present in P<sub>2</sub> was  $287.2 \pm 29.2$  nmoles ferricyanide reduced/mg protein/min, and that significant levels of activities were also present in P<sub>1</sub>, P<sub>3</sub> and P<sub>4</sub>. In contrast, the apical membrane-enriched fraction (P<sub>5</sub>) contained very little SDH activity suggesting minimal mitochondrial contamination.

**Figure 4.6**

Measurements of alkaline phosphatase activity in the various membrane fractions obtained from the Malpighian tubules of *Locusta migratoria*

**Ordinate:** Alkaline phosphatase activity expressed as nmoles *p*-nitrophenol liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** Membrane fractions see Figure 4.5.

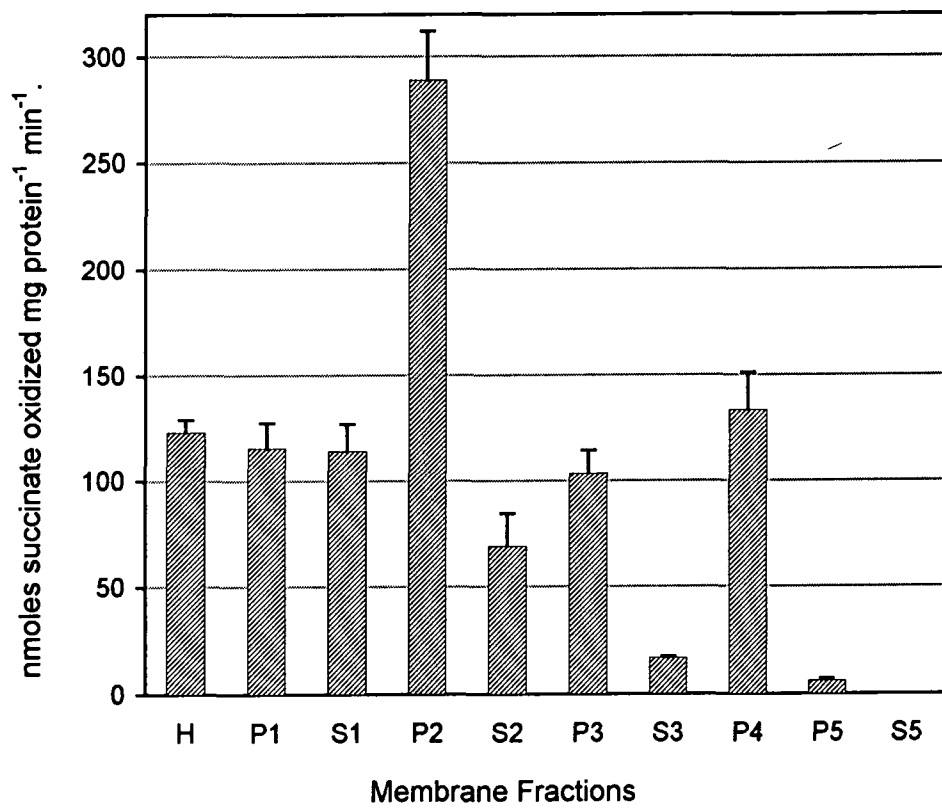


**Figure 4.7**

Measurements of succinate dehydrogenase activity in the various membrane fractions obtained from the Malpighian tubules of *Locusta migratoria*.

**Ordinate:** Succinate dehydrogenase activity expressed as nmoles succinate oxidized mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** Membrane fractions see Figure 4.5.



**Electron microscopic study of membrane pellets produced by differential separation of the Malpighian tubules of *Locusta migratoria*:**

Electron microscopical studies were carried out on the pellets of the different membrane fractions in order to determine their contents.

Pellets (P<sub>2</sub>, P<sub>3</sub>, P<sub>4</sub> and P<sub>5</sub>) were prepared and fixed for electron microscopy as described previously. A series of representative electronmicrographs of sections through these pellets is shown in Plates (4.1, 4.2, 4.3 and 4.4).

It can be seen that P<sub>2</sub> (Plate 4.1 a, b) contained numerous mitochondria. This is consistent with the biochemical distribution study of SDH activity.

P<sub>3</sub> (Plate 4.2 a, b) consisted of a mixture of large and small membrane bound vesicles, and from biochemical studies this pellet represented a mixture of both basal and apical membrane fractions.

A variety of vesicles of different sizes were present in P<sub>4</sub> (Plate 4.3 a, b) together with numerous of mitochondria being observed. P<sub>5</sub> consisted mainly of a large number of small vesicles (Plate 4.4 a, b) with no obvious mitochondrial contamination. The micrographs of P<sub>4</sub> and P<sub>5</sub> fractions confirmed the biochemical studies that these pellets represented the basal and apical enriched membrane fractions respectively.

**Plates 4.1A and B**

These micrographs show a section through pellet 2 which was the mitochondria (M), enriched-membrane fraction (P<sub>2</sub>).

***Scales:***

A = 0.5 $\mu$ m.

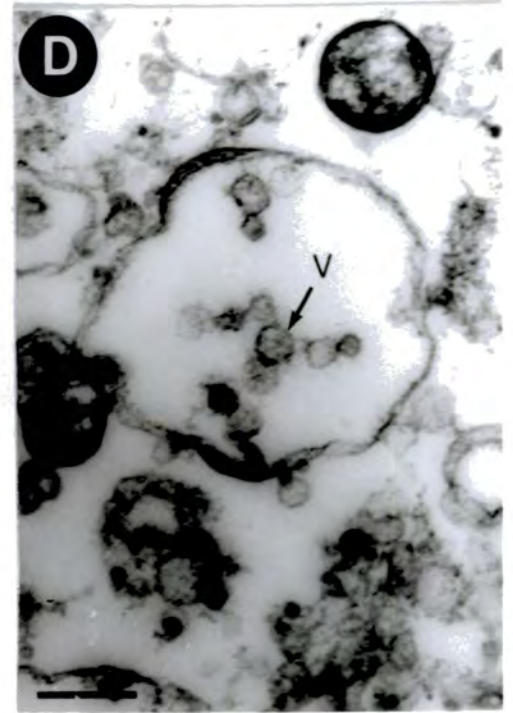
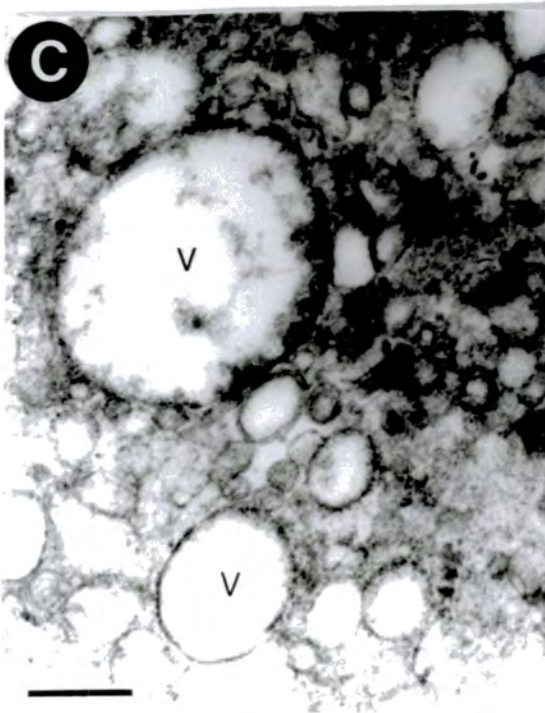
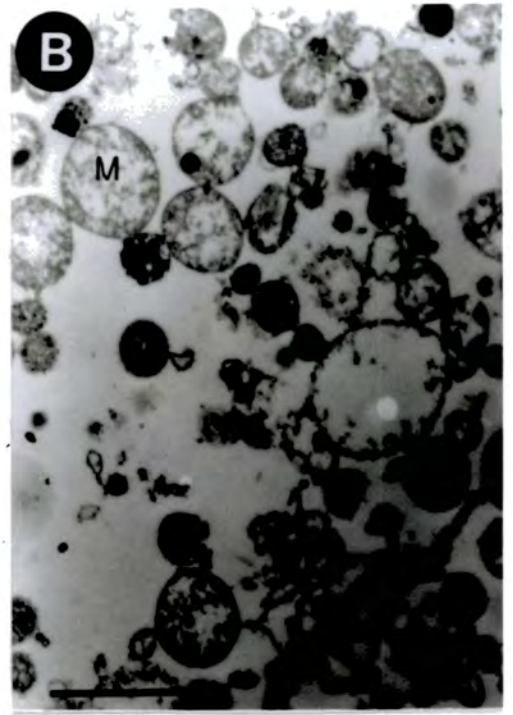
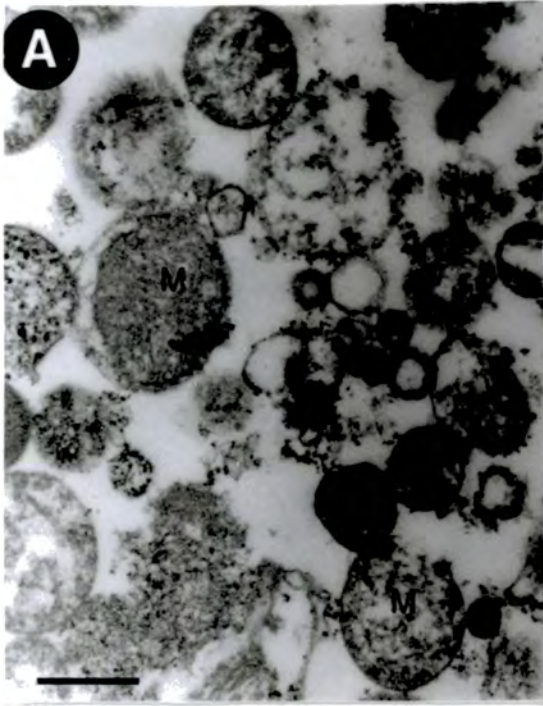
B = 1.5 $\mu$ m.

**Plates 4.2C and D**

These plates showing, a section through pellet 3 (P<sub>3</sub>) which from biochemical studies is believed to represent a mixture of both basolateral and apical cell membranes.

V: Membrane bound vesicles.

***Scales*** = 0.5 $\mu$ m.



**Plates 4.3E and F**

Electron micrograph showing, a section through pellet 4 (P<sub>4</sub>) which from biochemical studies is believed to represent the enriched-basolateral membrane fraction.

M: Mitochondria.

R: Electron dense ribosome particles.

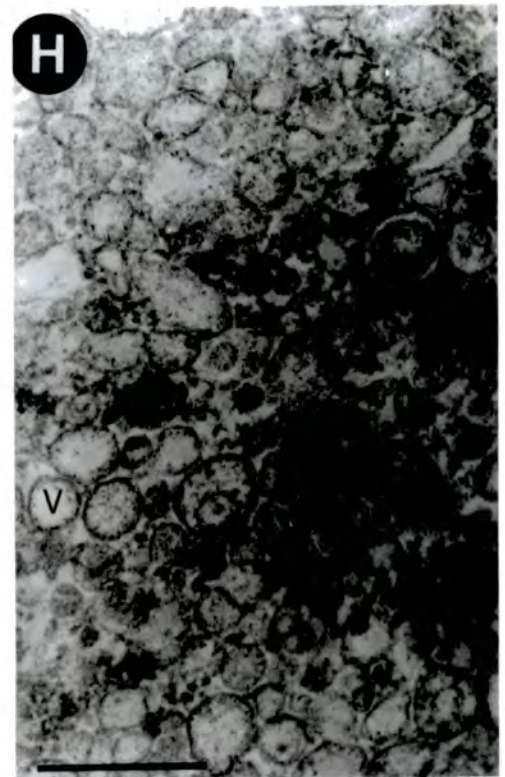
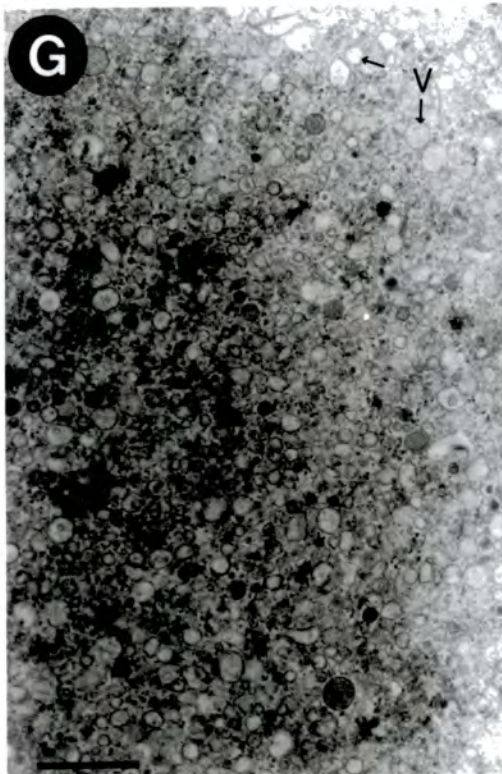
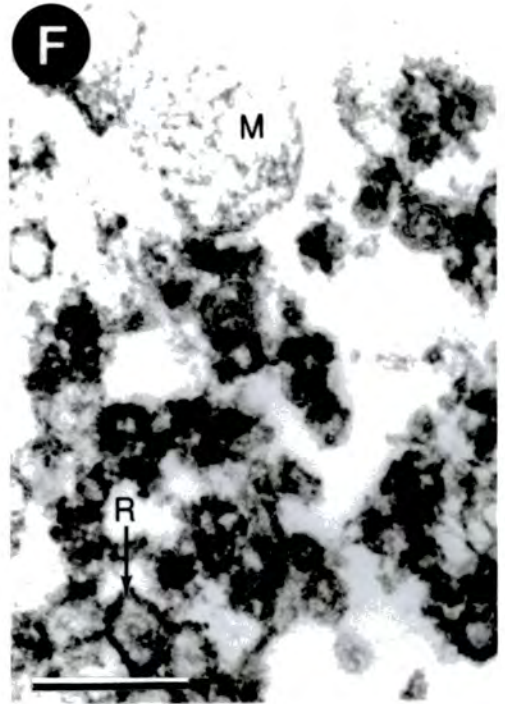
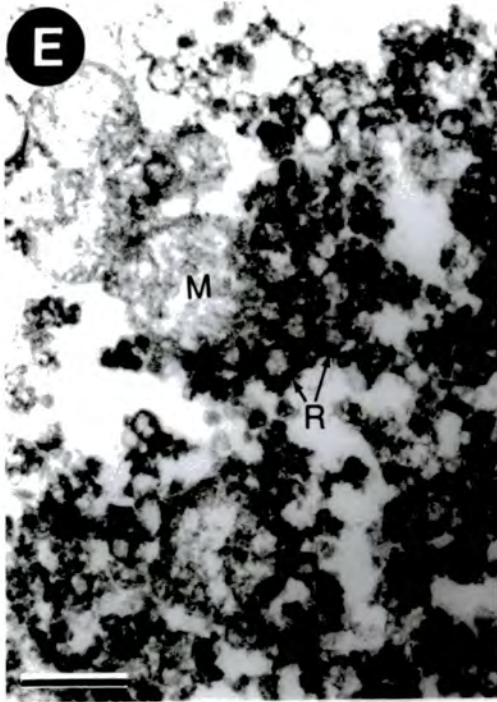
*Scales* = 0.5μm.

**Plates 4.4G and H**

Electron micrograph show a section through pellet 5 (P<sub>5</sub>) which from biochemical studies is believed to represent the enriched-apical membrane fraction.

V: Numerous membrane bound vesicles.

*Scales* = 0.5μm.



## Chapter 4

### DISCUSSION

The localization of enzyme activity is usually done using the assay of marker enzymes in conjunction with inhibitor assays and sometimes electron microscopy. A modified separation method was used to produce membrane fractions in this study which resulted in the production of plasma membrane fractions of high purity as judged from marked enzyme assays.

Marker enzyme assays were carried out on the membrane fractions obtained by differential centrifugation of a crude homogenate of Malpighian tubules from *Locusta migratoria*. These enabled the location of the main membrane fractions to be identified and the degree of cross contamination by other cellular fractions to be assessed.

The results presented here confirm earlier reports of the presence of  $Mg^{2+}$ -ATPase and  $Na^+/K^+$ -ATPase in Malpighian tubules of *Locusta migratoria* (Anstee and Bell, 1975; 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986; Fogg, 1990; Fogg *et al.*, 1991; Marshall, 1995; and present study). Electron microscopy and/or assays for alkaline phosphatase and SDH activities were used to confirm the presence of apical cell membranes and mitochondrial membranes in various cell fractions. After the 100,000xg spin differential separation of the apical cell membrane marker enzyme, alkaline phosphatase, and basolateral membrane marker,  $Na^+/K^+$ -ATPase, were observed. The pellet, P<sub>4</sub>, contained relatively high specific  $Na^+/K^+$ -ATPase activity, indicating that this pellet represents the basolateral membranes, with little apical membrane contamination, reflected by the relatively low alkaline phosphatase activity. Assays on pellet 5 demonstrated the largest alkaline phosphatase activity and negligible succinate dehydrogenase and  $Na^+/K^+$ -ATPase activities, indicating that this represents

the apical membrane-enriched pellet. The results presented in this study agree well with the previous membrane separation works of Fogg *et al.*, (1991) and Marshall (1995) on the same tissue; Rodriguez and Edelman (1979) on toad bladder epithelium, and with reports that the ouabain-sensitive  $\text{Na}^+$  pump is restricted to this cell surface in a variety of different tissues from various animal species (Ernst *et al.*, 1980; Anstee and Bowler, 1984). Lechleitner and Phillips (1988) also provided biochemical and electron microscopic evidence for the concentration of  $\text{Na}^+/\text{K}^+$ -ATPase in the basolateral membranes of locust rectal pad epithelium.

Anstee and Bowler (1984) demonstrated that deoxycholate/NaI-extracted preparations produce microsomes with high  $\text{Na}^+/\text{K}^+$ -ATPase activity and low  $\text{Mg}^{2+}$ -dependent ATPase activity (usually less than 20% of the total ATPase activity). However, the method used in the present study produced a basolateral membrane pellet ( $\text{P}_4$ ) with a  $\text{Na}^+/\text{K}^+$ -ATPase activity (42.5% of total ATPase activity) lower than the  $\text{Mg}^{2+}$ -dependent ATPase activity (58.4% of total ATPase activity). The  $\text{Na}^+/\text{K}^+$ -ATPase :  $\text{Mg}^{2+}$ -ATPase ratio value was 1:1.4. However, the specific activity of  $\text{Na}^+/\text{K}^+$ -ATPase in  $\text{P}_4$  was 331 nmoles  $\text{P}_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$  which was higher than that obtained by the deoxycholate/NaI technique, which was 98 nmoles  $\text{P}_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ .

Mitochondrial contamination has often been a problem when trying to localize the apical membrane fraction. Table 4.1 shows a comparison of the marker enzymes activity in different pellets obtained by the original separation method (Fogg *et al.*, 1991) and the modified method used by this study. The indication from the present study, using marker enzyme assays showed greatest succinate dehydrogenase activity in pellet 2, which corresponds to the mitochondrial pellet in electron microscopic

studies (Plate 4.1). The SDH P<sub>2</sub>:P<sub>4</sub>:P<sub>5</sub> ratio value is 1:0.46:0.02, whilst, it was 1:0.6:0.16 as reported by Fogg *et al.*, (1991), indicating that the modified separation method used in the present study produced insignificant succinate dehydrogenase activity in pellet 5 coupled with the apparent absence of mitochondria from pellet 5 as shown by electron microscopy (Plate 4.4). This suggests that the apical-enriched membrane fraction is relatively free of mitochondrial contamination.

**Table 4.1 Marker enzymes activities in different pellets:**

Marker enzyme	Enzyme activity			References
	P <sub>2</sub>	P <sub>4</sub>	P <sub>5</sub>	
SDH	289.2±29.6	133.1±11.9	6.0±1.0	The present study
	116.0±6.1	71.3±5.4	18.1±5.9	Fogg <i>et al.</i> , 1991
Na <sup>+</sup> /K <sup>+</sup> -ATPase	71.4±6.9	331.9±21.9	26.7±5.6	The present study
	14.3±5.3	187.4±9.4	25.4±8.3	Fogg <i>et al.</i> , 1991
Alkaline phosphatase	466.3±19.4	530.1±45.5	3863.3±145.5	The present study
	38.8±7.8	359.7±34.1	1938.0±45.0	Fogg <i>et al.</i> , 1991

SDH activity expressed in nmoles succinate oxidized mg protein<sup>-1</sup> min<sup>-1</sup>.

ATPase activity expressed in nmoles Pi liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

Alkaline phosphatase activity expressed in nmoles p-nitrophenol liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

The greatest alkaline phosphatase activity was observed in P<sub>5</sub> indicating that this pellet represents the apical enriched-membrane fraction. The minimal succinate dehydrogenase activity in pellet 5 indicated that there was negligible mitochondrial contamination in the apical-enriched membrane fraction. This is similar to findings by

Fogg *et al.*, 1991, and Marshall (1995) working on Malpighian tubules of *Locusta migratoria*.

Marker enzyme studies also point to a plasma membrane location for  $Mg^{2+}$ -dependent ATPase in the rectum of dragonfly larvae (Komnick *et al.*, 1980) and in rumen epithelium (Hegner and Anika, 1975). The localization of enzyme activity is usually done by marker assay enzymes in conjunction with inhibitor assays and sometimes electron microscopy. For example, Wheeler and Harrison (1982) found that not all the  $Mg^{2+}$ -dependent ATPase activity recorded from the mantle of the clam, *Anodonta cataraacta*, could be attributed to mitochondrial contamination as some of the activity was completely distinct from cytochrome oxidase activity (a mitochondrial marker). On the other hand Van Amelsvoort *et al.*, (1977) believed that all of the anion-sensitive activity was of mitochondrial origin and this view was also supported by Izutsu and Siegel (1975). However, Deaton (1984) who demonstrated  $Mg^{2+}$ -dependent ATPase activity in microsomal fractions of insect midgut, found the activity of succinate dehydrogenase in the microsomal fraction was only 12% of that in mitochondrial fractions. Similarly, Anstee and Fathpour (1981) studying the Malpighian tubules of *Locusta migratoria* found microsomal fractions were relatively free of mitochondrial contamination, the succinate dehydrogenase activity being only 16% of the activity recorded from the mitochondrial fraction. A study by Kinne-Saffran and Kinne (1979) isolated an activity from the brush border of rat kidney cortex which was stimulated by  $Cl^-$ ,  $HCO_3^-$  and sulphite, they also isolated a mitochondrial anion-stimulated ATPase. However, these two enzyme activities exhibited different characteristics. Only the  $Mg^{2+}$ -ATPase activity of the mitochondrial fraction was inhibited by atractyloside, a chemical which affects the inner mitochondrial

membrane, whereas filipin, a chemical which affects cholesterol, only inhibited the  $Mg^{2+}$ -ATPase activity of the plasma membrane fraction, as this membrane is cholesterol-rich. Lechleitner and Phillips (1988) produced evidence for a differential distribution of mitochondrial markers in the different cell membrane fractions produced from Locust rectum. Fogg *et al.*, (1991) proposed that since the succinate dehydrogenase and  $Mg^{2+}$ -dependent ATPase showed a different distribution in the different membrane fractions of the Malpighian tubules of *Locusta migratoria*, then this ATPase activity could not be exclusively mitochondrial in origin



## CHAPTER 5

### **Biochemical studies on the subcellular distribution of the enzyme activities from Malpighian tubules of *Locusta migratoria*.**

#### INTRODUCTION

The mechanism of fluid transport by Malpighian tubule cells has been reviewed recently (Maddrell and O'Donnell, 1992; Nicolson, 1993; van Kerkhove, 1994; Dow, 1994; Beyenbach, 1995; Pannabecker, 1995). There is agreement that, in all the species studied to date, the primary active ion pump at the apical surface is V-type H<sup>+</sup>-ATPase. This maintains a proton gradient across the apical membrane that drives movement of alkali cations from cell to lumen through apical Na<sup>+</sup>/H<sup>+</sup> and K<sup>+</sup>/H<sup>+</sup> exchangers (antiporters). The pump also establishes a favourable electrical gradient for movement of Cl<sup>-</sup> from cell to lumen, and water movement that is a secondary consequence of active ion transport, the two processes probably being coupled simple osmosis (O'Donnell *et al.*, 1982; McElwain, 1984), with water movement perhaps facilitated by water channels (Dow *et al.*, 1995). Ultimately, then, transport of both cations and anions into the lumen, and the resultant production of fluid, depends on the activity of the luminal V-type ATPase, and this appears to be generally true for most insect epithelia studied to date (Wieczorek, 1992; Klein, 1992; Dow, 1994). Investigations into the nature of the apical K<sup>+</sup> pump in various insect tissues found ouabain had no effect on K<sup>+</sup> transport (Berridge *et al.*, 1976; Maddrell, 1977; Harvey, 1980), therefore, apical K<sup>+</sup> transport was not believed to occur via Na<sup>+</sup>/K<sup>+</sup>-ATPase. Wolfersberger (1979) and Wolfersberger *et al.*, (1982) used biochemical assays to demonstrate a K<sup>+</sup>-modulated ATPase activity in the midgut of *Manduca sexta*. Similarly, Cioffi and Harvey (1981), were able to detect a K<sup>+</sup> modulated activity in an

enriched apical membrane fraction, which contained portasomes. Portasomes being the name given to the studded particles which had previously been detected on the apical membranes of insect secretory cells (Anderson and Harvey, 1966; Cioffi, 1979; Harvey, 1980). Cioffi and Harvey (1981) suggested that as these particles resembled the proton-translocating  $F_1F_0$  complex of mitochondria/bacteria that they may be involved in ion transport. The specific activity of this  $K^+$ -ATPase in the enriched apical fraction was 6-7 fold greater than in the crude homogenate (Cioffi and Harvey, 1981). Wiczorek (1982) carried out biochemical assays on the proboscis of a fly and discovered a  $K^+$ -sensitive ATPase, this enzyme activity was further characterized by Wiczorek and Gnatzy (1985). Deaton (1984) identified a  $K^+$ -stimulated activity in the microsomes of the midgut of *Manduca sexta* and Wiczorek *et al.*, (1986) also located a  $K^+$ -stimulated ATPase activity in midgut goblet cell apical membranes. The characteristics of this enzyme differed from those of an  $H^+/K^+$ -ATPase (Wiczorek *et al.*, 1986) which had been suggested as a potential candidate for the common cation pump by some authors (Wallmark *et al.*, 1980; Deaton, 1984). The enzyme was inhibited by nitrate.

Nitrate is an inhibitor of V-type ATPases (O'Neill *et al.*, 1983; Lichko and Okorokov, 1984) but has no effect on the gastric microsomal vesicles  $H^+/K^+$ -ATPase (Lee *et al.*, 1980). Nitrate is also an inhibitor of anion-stimulated ATPases (Van Amelsvoort *et al.*, 1977; Anstee and Fathpour, 1981); an ATPase activity that has been reported at the apical surface of Malpighian tubules of *Locusta* (Fogg *et al.*, 1991). Wiczorek *et al.*, (1986) also discovered that the apical  $K^+$ -ATPase activity was not inhibited by ouabain, orthovanadate, azide or oligomycin but 150 $\mu$ M DCCD caused complete inhibition. These inhibitor studies pointed to an ATPase which was more

closely related to the ATPases found in the membrane organelles of the exo- and endocytic pathways than the phosphorylated (P-type) or the mitochondrial/bacterial (F-type or  $F_0F_1$ -) ATPases. Therefore, at that time, evidence was accumulating for the apical cation pump being a  $K^+$ -ATPase, unique to insect transporting epithelia (Harvey *et al.*, 1983b) thought to reside in portosomes on the apical membrane (Harvey *et al.*, 1983a). Fogg *et al.*, (1991), working on the Malpighian tubules of *Locusta migratoria*, were able to detect some  $K^+$ -stimulated, ouabain insensitive ATPase activity in microsomal fractions. However, this activity was associated with the basal rather than apical membranes. Furthermore, despite what other workers (Harvey *et al.*, 1981; Harvey *et al.*, 1983b) have stated, Anstee and Bell (1975) did not demonstrate, nor claim to have demonstrated, an ATPase activity from microsomal preparations of Malpighian tubules which was stimulated by more than 2.5 fold by  $K^+$  alone.

Later, Wieczorek *et al.*, (1989; 1991) presented evidence that the so-called 'common cation pump' or the molecular mechanism of the  $K^+$  pump in the apical membrane is actually a proton ( $H^+$ ) pump, showing the characteristics of the classical V-type ATPases. This  $H^+$  pump can function as the driving force for  $K^+$  extrusion since it maintains a proton gradient, favourable for proton influx via a  $K^+/H^+$  exchanger (Schweickl *et al.*, 1989; Wieczorek *et al.*, 1989; 1991; Wieczorek, 1992; Dow, 1992; Zhang *et al.*, 1992).

As described previously (Maddrell, 1978) active cation transport is believed to be sited at the apical microvilli of the plasma membrane. It has now been shown that antibodies to V-type ATPase selectively bind to the apical plasma membrane of

Malpighian tubules of *Manduca sexta* (Russell *et al.*, 1992) and *Locusta migratoria* (Marshall, 1995).

Schweickl *et al.*, (1989), using membrane bound ATPase prepared from the midgut of *Manduca sexta*, demonstrated an ATPase which was insensitive to azide and vanadate but sensitive to N-ethylmaleimide (NEM) and stimulated by  $K^+$ . This activity corresponded to the ATPase activity of the highly purified goblet cell apical membranes of midgut of *Manduca sexta* studied by Wieczorek *et al.*, (1986). NEM, when used at appropriate concentrations is a specific inhibitor of Vacuolar or V-type ATPases. V-type ATPases are sensitive to much lower concentrations of NEM (1-2 $\mu$ M) than the phosphorylated or P-type ATPases (100 $\mu$ M-1mM) and the F-type ATPases characteristic of mitochondria, chloroplasts and bacteria are virtually resistant (Forgac, 1989). In the study by Schweickl *et al.*, (1989) 1 $\mu$ M NEM caused approximately 50% inhibition of enzyme activity and so provided significant evidence that a V-type ATPase may play a part in ion and fluid secretion. Fogg *et al.*, (1991) suggested that the  $HCO_3^-$ -stimulated ATPase activity from Malpighian tubules of *Locusta migratoria* could represent, in part, an anion-sensitive V-type ATPase.

All characterized energized  $H^+$ -pumps fall into three major categories: F, P, and V-type, based on subunit organization, mechanism and inhibitor sensitivity (Forgac, 1989; Nelson, 1992). They are subject to inhibition by azide, orthovanadate and bafilomycin  $A_1$ , respectively and the enzymes within each category show functionally significant sequence identities, indicating a common ancestry (Nelson and Taiz, 1989; Pederson and Carafoli, 1987a,b). By contrast, the vacuolar  $H^+$ -PPase is not inhibited by any of these type-specific inhibitors (see review Rea *et al.*, 1992).

As explained above, V-type ATPases are a class of proton pumps. The question then arises as to how can they be linked to  $K^+$  transport in insect ion and fluid transporting epithelia. Wieczorek *et al.*, (1989) demonstrated the ATP-dependent development of a pH gradient across vesicular membranes of apical membrane preparations produced from the midgut of *Manduca sexta*. Although this proton transport exhibited the same inhibitor profiles and substrate specificities as ATPase activity recorded from the same tissue, proton transport was not stimulated by  $K^+$ , in fact in the presence of  $K^+$  a proton gradient could not be developed or was dissipated. One conclusion reached from these results was that a proton/potassium antiporter existed, therefore the active transport of  $H^+$  would energize the antiporter and hence extrusion of  $K^+$  out of the cell.

Wieczorek *et al.*, (1989) proposed a model for the extrusion of  $K^+$  at the goblet cell apical membrane of the midgut of *Manduca sexta*. Further experiments were carried out on vesicles of purified goblet cell apical membrane using acridine orange as a pH indicator and oxonol V as a membrane potential indicator (Wieczorek *et al.*, 1991). This study revealed that a positive interior vesicle potential was recorded when vesicles preloaded with  $K^+$  were added to a  $K^+$ -free solution suggesting that  $H^+/K^+$  exchange was not 1:1 during an exchange cycle of the antiporter. This interpretation was confirmed by studies using harmaline which is an inhibitor of the antiporter. Inclusion of harmaline in the extracellular medium prevented the development of a vesicle positive interior voltage. The new model which possessed an electrogenic antiporter on its apical membrane agreed with previous results obtained by Moffett and Koch (1988a,b) and Chao *et al.*, (1991) working on the same material. Moffett and Koch conducted experiments using  $K^+$ -specific microelectrodes and had been unable to

demonstrate a  $K^+$  chemical activity gradient across the goblet cell apical membrane. Chao *et al.*, (1991) discovered that the pH gradient across the apical membrane was in the wrong direction to drive  $K^+$  from the cell to the goblet cavity. These findings were confirmed by Moffett and Koch (1992) who stated that no difference in the  $H^+$  or  $K^+$  concentration across the apical membrane meant that neither ion could drive the movement of the other in electroneutral antiport. The driving force for the antiporter had to be the voltage component of the proton motive force created by the V-type ATPase. Therefore the conclusion of these and other papers was that the antiporter had to be electrogenic (see Wieczorek, 1992; Grinstein and Wieczorek, 1994). Recent work by Azuma *et al.*, (1995) concluded that the stoichiometry of the antiporter is  $K^+/2H^+$ . Work by Lepier *et al.*, (1994) using cholate as a solubilizer of the antiporter has been able to show that the V-type ATPase and the antiporter are separate proteins. Therefore there is strong evidence for the electrogenic nature of the antiporter in the midgut of *Manduca sexta*.

$H^+$ -ATPases as mentioned previously are found in a variety of endomembrane organelles of plants, animals and fungi. In general, their function is to generate a transmembrane  $H^+$ -electrochemical potential difference which, in turn, provides the energy for solute transport or ATP synthesis (see reviews Forgac, 1989; Nelson, 1992). However, plant biochemical studies have revealed that the vacuolar membrane (tonoplast) of plant cells contains an inorganic pyrophosphate (diphosphate,  $PP_i$ )-energized  $H^+$ -pump ( $H^+$ -PPase; EC 3.6.1.1) as well as a more conventional  $H^+$ -ATPase (Rea and Sanders, 1987; Nelson and Taiz, 1989). This enzyme which appears to serve the purpose of electrogenic  $H^+$ -translocation from the cytosol to vacuole

lumen, has the unusual characteristic of exclusively using  $PP_i$  as an energy source (Rea and Poole, 1986; Rea and Sanders, 1987; Hedrich *et al.*, 1989; Rea *et al.*, 1992).

The  $H^+$ -PPase has been demonstrated in vacuolar membrane fractions from all of the major vascular plant types (Rea and Sanders, 1987; Hedrich *et al.*, 1989; Davies *et al.*, 1991; Richter and Schafer, 1992; Sarafian *et al.*, 1992; Oberbeck *et al.*, 1994; Sosa and Celis, 1995). However, the situation in non-plant cells is not so clear. Although Lichko and Okorokov (1991) have reported  $PP_i$ -driven  $H^+$ -translocation in vacuolar membrane-enriched fractions from *Saccharomyces carlsbergensis*. However, attempts to identify an  $H^+$ -PPase homologous either by immunological cross-reactivity to the plant  $H^+$ -PPase or by genomic analyses have yielded negative results.

$HCO_3^-$ -stimulated ATPase activity has been demonstrated in microsomal fractions of various tissues from different species (Blum *et al.*, 1971; Kinne-Saffaren and Kinne, 1974; De Renzis and Bornancin, 1977; and see reviews Schuurmans Stekhoven and Bonting, 1981; Gerencser and Lee, 1983). Indeed, such activity has been reported in the Malpighian tubules of *Locusta migratoria* and was inhibited by  $SCN^-$ , sodium acetazolamide and oligomycin (Anstee and Fathpour, 1979; 1981; Fathpour and Anstee, 1981; Fogg *et al.*, 1991) and in other insect tissues, e.g. midgut of *Hyalophora cecropia* (Turbeck *et al.*, 1968), midgut and integument of *Manduca sexta* (Deaton, 1984), rectum of dragonfly (Komnick, 1978), *Schistocerca gregaria* (Herrera *et al.*, 1978; Lechleitner and Phillips, 1988) and *Periplaneta americana* (Tolman and Steele, 1976). Since this enzyme is also present on the inner mitochondrial membrane, some early studies which located this enzyme activity to the plasma membrane, can now be attributed to mitochondrial contamination (see review

by Schuurmans Stekhoven and Bonting, 1981). However there is good evidence for anion-stimulated ATPases in some tissues with low mitochondrial contamination (Kinne-Saffran and Kinne, 1974; Kinne-Saffran and Kinne, 1979, Lechleitner and Phillips, 1988) including *Locusta migratoria* Malpighian tubules (Fogg *et al.*, 1991).

Lechleitner and Phillips (1988) provided evidence for an apical plasma membrane  $\text{HCO}_3^-$ -stimulated ATPase, which may be responsible for the active transport of  $\text{Cl}^-$  in locust rectum. An active  $\text{Cl}^-$  extension process in the basolateral membrane of *Aplysia* intestinal epithelium has also been reported by Gerencser (1983), and this electrogenic,  $\text{Na}^+$ -independent mechanism may be a  $(\text{Cl}^- + \text{HCO}_3^-)$ -stimulated ATPase (Gerencser and Lee, 1985). Whilst anion-sensitive ATPase has been implicated in  $\text{Cl}^-/\text{HCO}_3^-$  exchange in the rectum of *Schistocerca gregaria* (Herrera *et al.*, 1978),  $\text{H}^+/\text{HCO}_3^-$  transport in rat submandibular gland (Simon *et al.*, 1972a) and  $\text{HCO}_3^-$  transport and  $\text{Na}^+/\text{H}^+$  exchange in the brush border membrane of the renal proximal tubule (Liang and Sacktor, 1976).

Several theories have been proposed to explain the transport of ions and water through various epithelia. These theories have suggested that the anion-stimulated ATPase takes part in the active transport of anions (Komnick, 1978, Gerencser and Lee, 1985; Lechleitner and Phillips, 1988; Fogg, 1990; Fogg *et al.*, 1991). This enzyme has mainly been linked to ion/water transport because thiocyanate, an inhibitor of the anion-stimulated ATPase, also inhibits secretion by some epithelia (Simon and Thomas, 1972; Blum *et al.*, 1971).

Previously, Fogg *et al.*, (1991) had tentatively suggested that an anion-stimulated activity located on the apical membrane of *Locusta* may represent an anion-

sensitive V-type ATPase. In many tissues the common cation pump has been replaced by a parallel arrangement of a V-type ATPase and a  $K^+$  (or  $Na^+$ )/ $nH^+$  antiport (Wessing and Zierold, 1996). However, the involvement of a V-type ATPase and antiporter in fluid secretion by the Malpighian tubules of *Locusta migratoria* has been established (Marshall, 1995). Therefore, one of the aims of this project was to confirm and demonstrate the properties of V-type ATPase activity isolated from apical membrane-rich fractions of Malpighian tubules of *Locusta migratoria*.

MATERIALS AND METHODS

Determination of V-type ATPase:

Unless otherwise indicated, V-type ATPase activity was assayed as described by (Schweickl *et al.*, 1989) in a reaction mixture that contained 0.125ml membrane fraction, 0.125ml of 12 mM ATP (Tris salt) and 0.25ml (final concentrations) of one of the following:

**Medium 1):** 1mM MgCl<sub>2</sub>, 20mM KCl, 50 mM Tris-MOPS, 0.1mM EGTA, 1mM 2-mercaptoethanol, 0.5mM sodium azide, 0.1mM sodium orthovanadate, 0.3mg of BSA/ml, and 0.05% Triton X-100, except in the pH optimum experiments, the pH was adjusted to 7.5 with tris-base.

**Medium 2):** Medium 1 plus 1mM NEM.

The mixture was preincubated in the absence of the substrate for 30 min at 35±0.1°C, and the reaction was started by the addition of 0.125ml of ATP (Tris salt) for 30min. V-type ATPase activity was measured as the difference in P<sub>i</sub> liberated in reaction medium 1, and that released in medium 2, in the presence of NEM. Activity was expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>. Controls were run where ATP was not added until the reaction had been stopped by the addition of 1 ml of a 1:1 mixture of 1% Lubrol and 1% ammonium molybdate in 0.9M sulphuric acid (Atkinson *et al.*, 1973) to determine the extent of non-enzymatic hydrolysis.

**Determination of Mg<sup>2+</sup>-dependent and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated****ATPase:**

Incubations were carried out at  $35 \pm 0.1^\circ\text{C}$  for a fixed time. Appropriate incubation media were thermoequilibrated for 15 min in Eppendorf tubes. These consisted of 0.25ml ionic and medium 0.125ml membrane fraction. Three basic ionic media having the following final concentration of ions were used, unless otherwise stated:

**Medium i):** 4mM MgCl<sub>2</sub> in 20mM imidazole buffer, pH 7.5,

**Medium ii):** (medium i) plus 20mM NaCl,

**Medium iii):** (medium i) plus 20mM NaHCO<sub>3</sub>.

The reaction was started by the addition of 0.125ml of ATP (Tris salt) for 15min. Liberation of P<sub>i</sub> was measured as described previously. HCO<sub>3</sub><sup>-</sup>-stimulation of ATPase activity was calculated as the difference in P<sub>i</sub> liberated in reaction medium (iii), containing MgCl<sub>2</sub> and NaHCO<sub>3</sub>, and that released in medium (ii), containing MgCl<sub>2</sub> and NaCl. Controls were run where ATP was not added until the reaction had been stopped by the addition of 1 ml of a 1:1 mixture of 1% Lubrol and 1% ammonium molybdate in 0.9 M sulphuric acid to determine the extent of non-enzymatic hydrolysis.

Reaction media had to be modified when NEM was included in the assay as it reacts with imidazole. Therefore, in experiments involving NEM the buffer used was HEPES. Activity was expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

RESULTS

Section I

**Biochemical and inhibitor studies on the  $Mg^{2+}$ -dependent ATPase and  $Mg^{2+}$ -dependent  $HCO_3^-$ -stimulated ATPase activities prepared from basal and apical membrane-enriched fraction of Malpighian tubules.**

**Presence of the  $Mg^{2+}$ -dependent ATPase and  $Mg^{2+}$ -dependent  $HCO_3^-$ -stimulated ATPase in the basal and apical membrane-rich fractions:**

The basal-enriched membrane fraction (P<sub>4</sub>), and apical-enriched membrane fraction (P<sub>5</sub>) were prepared as described previously in Chapter 4.

The results obtained from five independent preparations are shown in Figure 5.1 (see also Appendix 4, Table 4.1) as mean  $\pm$  SEM. It can be seen that the  $Mg^{2+}$ -dependent  $HCO_3^-$ -stimulated ATPase activity is present in both pellets P<sub>4</sub> and P<sub>5</sub>. The data were normalized with respect to the activity observed in the presence of 20mM NaCl.  $Mg^{2+}$ -dependent ATPase activity of P<sub>4</sub> and P<sub>5</sub> in the presence of 20mM NaCl was  $435.8 \pm 11.6$  and  $650.3 \pm 33.6$  nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min.<sup>-1</sup>, respectively. However, inclusion of 20mM NaHCO<sub>3</sub> in place of 20mM NaCl in the reaction medium, resulted in increased enzyme activity, the activity of P<sub>4</sub> rose to  $645.0 \pm 29.3$  nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min.<sup>-1</sup> and that of P<sub>5</sub> to  $979.6 \pm 43.7$  nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min.<sup>-1</sup>; representing a significant increase in the normalized activity of  $178.3 \pm 25.5$  and  $282.8 \pm 29.7$  nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min.<sup>-1</sup> respectively compared to that recorded in the presence of 20mM NaCl.

**Figure 5.1**

The  $Mg^{2+}$ -dependent ATPase and  $Mg^{2+}$ -dependent  $HCO_3^-$ -stimulated ATPase activities associated with the basal (P<sub>4</sub>) and apical (P<sub>5</sub>) membrane-enriched fractions of Malpighian tubules from *Locusta migratoria* were measured in the presence of 4mM  $MgCl_2$  and 20mM NaCl or 20mM  $NaHCO_3$ . Reactions were started by adding ATP Tris-salt (final concentration 3mM) and incubations were carried out at 35 °C for 30 min as described in Methods.

$HCO_3^-$ -stimulated ATPase activity was measured as the difference in inorganic phosphate measured in the reaction medium containing 4mM  $MgCl_2$ , 20mM  $NaHCO_3$  and that released in a reaction medium containing 4mM  $MgCl_2$ , 20mM NaCl.

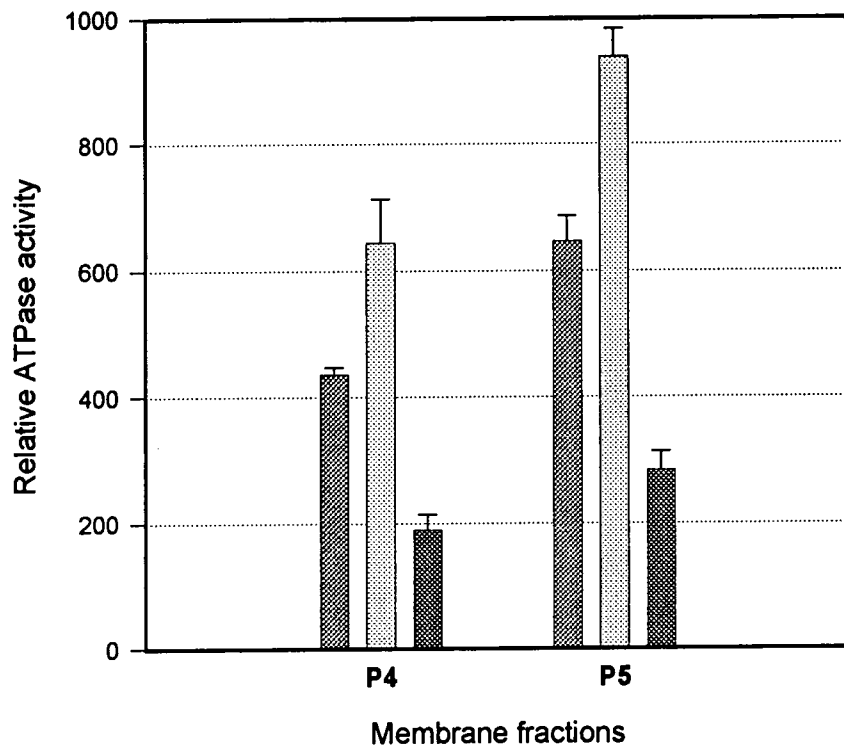
**Ordinate:** ATPases activity expressed in nmoles  $P_i$  liberated  $mg\ protein^{-1}\ min^{-1}$ .

**Abscissa:** Membrane fractions:

P<sub>4</sub> Basal membrane fraction

P<sub>5</sub> Apical membrane fraction

Each point represents the mean  $\pm$  S.E.M. (n=5)



- ▨ Mg<sup>2+</sup>-dependent ATPase activity in the presence of 20mM NaCl.
- ▤ Mg<sup>2+</sup>-dependent ATPase activity in the presence of 20mM NaHCO<sub>3</sub>
- Stimulation due to the presence of NaHCO<sub>3</sub> instead of NaCl.

**Influence of various inhibitors on Mg<sup>2+</sup>-dependent ATPase and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activities:**

The basal- and apical membrane-enriched fractions (P<sub>4</sub> and P<sub>5</sub>) were prepared as described previously and Mg<sup>2+</sup>-dependent ATPase and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activities assayed in presence and absence of various inhibitors.

**Effect of NaSCN on ATPases activity:**

Mg<sup>2+</sup>-dependent ATPase (in the presence and absence of 20mM NaCl) and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity were determined in the presence and absence of 10mM NaSCN in the reaction media. Figures 5.2a and 5.2b (see also Appendix 4, Tables 4.2 and 4.3) show results obtained from three independent experiments. It can be seen that inclusion of 10mM NaSCN in the reaction medium caused a substantial inhibition of both Mg<sup>2+</sup>-dependent and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activities of P<sub>4</sub> and P<sub>5</sub>.

**Effect of NEM and bafilomycin A<sub>1</sub> on ATPases activity:**

1mM NEM and 1μM bafilomycin A<sub>1</sub> had similar effects on the Mg<sup>2+</sup>-dependent ATPase and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity of pellet 4 and 5. Figures 5.2a and 5.2b show results obtained from three independent experiments. It can be seen that NEM and bafilomycin A<sub>1</sub> had no significant effect on either Mg<sup>2+</sup>-dependent ATPase or Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activities of pellet 4. In contrast, the Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity of pellet 5 was inhibited by approximately 55% and 60% in the presence of 1mM NEM and 1μM

bafilomycin A<sub>1</sub>, respectively. However, neither NEM nor bafilomycin A<sub>1</sub> significantly affected the Mg<sup>2+</sup>-dependent ATPase activity of P<sub>5</sub>.

**Effect of sodium azide (NaN<sub>3</sub>) on ATPases activity:**

Inclusion of 1mM NaN<sub>3</sub> in the assay medium caused significant inhibition (p<0.05) of both Mg<sup>2+</sup>-dependent ATPase (~65%) and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity (~50%) of P<sub>4</sub> Figures 5.2a, b; (see also Appendix 4, Tables 4.2 and 4.3). Lower inhibition (~25%) was observed in the activity of Mg<sup>2+</sup>-dependent ATPase and Mg<sup>2+</sup>-dependent HCO<sub>3</sub><sup>-</sup>-stimulated ATPase of P<sub>5</sub> in the presence of 1mM NaN<sub>3</sub>. NaN<sub>3</sub> did not effect on the HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity of P<sub>4</sub> and P<sub>5</sub>.

**Figures 5.2a and 5.2b**

Effect of a various inhibitors on  $Mg^{2+}$ -dependent ATPase and  $Mg^{2+}$  -dependent  $HCO_3^-$  -stimulated ATPase activity associated with the basal (P<sub>4</sub>) and apical (P<sub>5</sub>) membrane-enriched fractions of Malpighian tubules from *Locusta migratoria* were investigated. Reactions were started by adding ATP Tris-salt (final concentration 3mM) and incubations were carried out at 35 °C for 30 min as described in Methods.

$HCO_3^-$  -stimulated ATPase activity was measured as the difference in inorganic phosphate measured in the reaction medium containing 4mM  $MgCl_2$ , 20mM  $NaHCO_3$  and that released in a reaction medium containing 4mM  $MgCl_2$ , 20mM  $NaCl$ .

**Ordinate:** ATPase activity expressed in nmoles Pi liberated mg Protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** Inhibitors:

SCN= 10 mM NaSCN

NEM= 1mM NEM

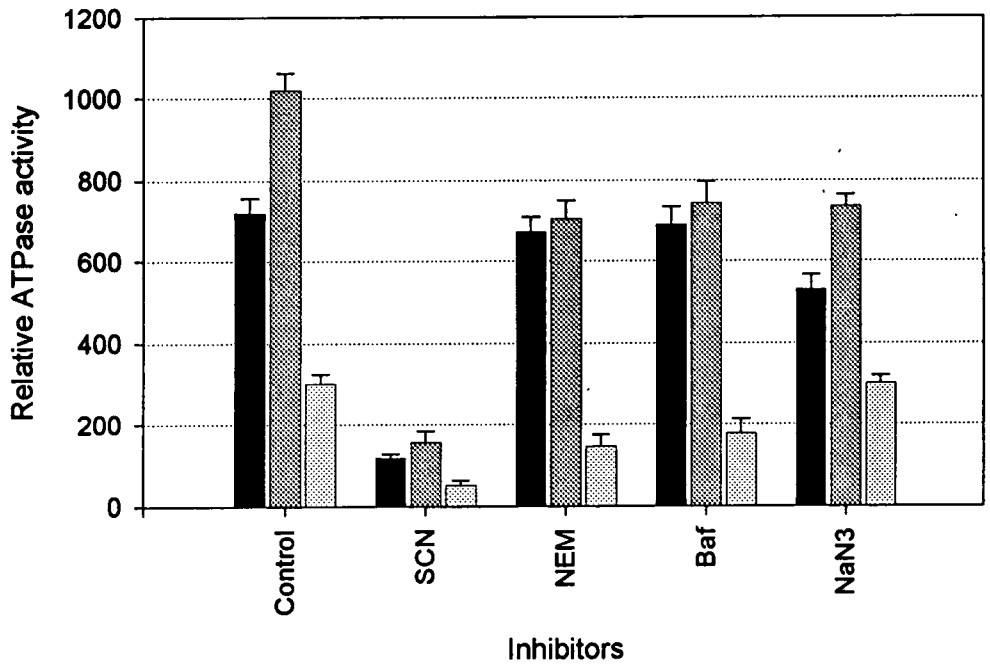
Baf= 1 $\mu$ M Bafilomycin A<sub>1</sub>

$NaN_3$ = 1mM Sodium azide

Each point represents the mean  $\pm$  S.E.M. (n=3).

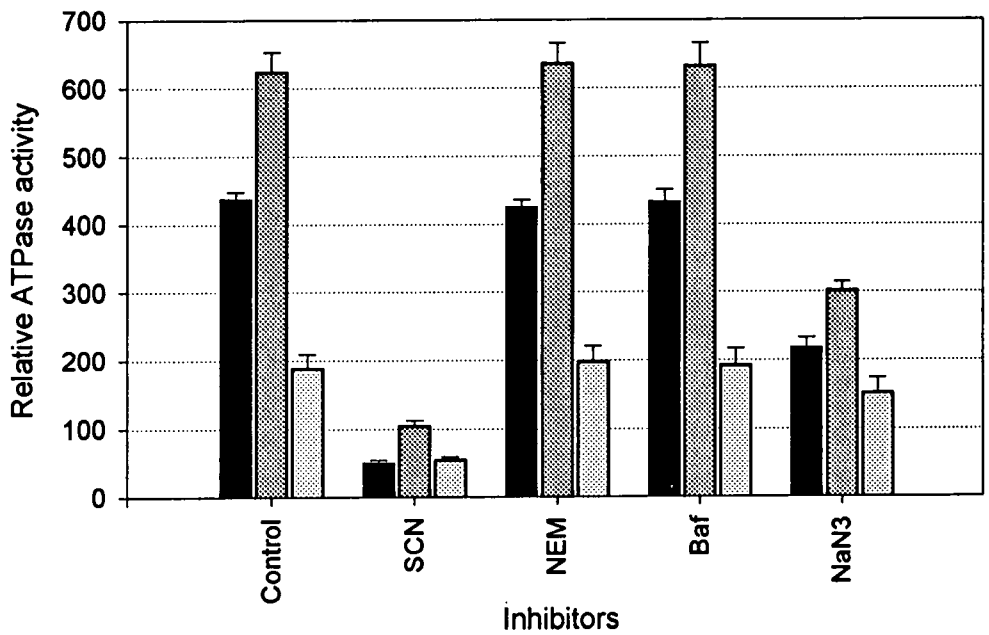
**Figure 5.2a**

**Apical membrane fraction (P<sub>5</sub>)**



**Figure 5.2b**

**Basal membrane fraction (P<sub>4</sub>)**



■ Mg<sup>2+</sup>-dependent ATPase activity in the presence of 20mM NaCl  
▨ Mg<sup>2+</sup>-dependent ATPase activity in the presence of 20 mM NaHCO<sub>3</sub>  
□ HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity

## Section II

### **Characterization study of V-type ATPase of the apical membrane-enriched fraction of Malpighian tubules.**

#### **Effect of pH on V-type ATPase activity:**

V-type ATPase was assayed in the reaction medium (50mM Tris- Mops, 20mM KCl, 1mM EGTA, 0.5mM  $\text{NaN}_3$ , 0.1mM  $\text{Na}_3\text{VO}_4$ , 0.05% Triton X-100 and 0.3 mg/ml BSA, pH 7.5). A 30mM bis-tris propane buffer system, was used to produce a stable pH range from pH 6.0 to 10.0. Reaction media were adjusted to the required pH with HCl. Figure 5.3 (see also Appendix 4, Table 4.4) shows the change observed in V-type ATPase activity in relation to pH. Results obtained from five independent preparations gave maximum activity ( $2153.4 \pm 100.4$  nmoles  $\text{P}_i$  liberated  $\text{mg protein}^{-1} \text{min}^{-1}$ ) at pH 7.5. The activity fell sharply when pH was increased or decreased about this value.

#### **Effect of ATP concentration on V-type ATPase activity:**

Enzyme assays were carried out in reaction media in which ATP (Tris-salt) concentration varied between 0.0 and 6.0mM. The relationship between ATPase activity and ATP concentration is shown in Figure 5.4 and Appendix 4, Table 4.5. It can be seen that the relationship between V-type ATPase activity and increasing ATP concentration is hyperbolic. As expected, in the absence of ATP there was no activity.

#### **Effect of $\text{Mg}^{2+}$ concentration on V-type ATPase activity:**

V-type ATPase was assayed in reaction medium containing concentrations of  $\text{MgCl}_2$  ranging from 0 to 7mM. Figure 5.5 shows the relationship between ATPase activity and  $\text{MgCl}_2$  concentration.

**Figures 5.3, 5.4 and 5.5**

The effect of pH, MgCl<sub>2</sub> and ATP on V-type ATPase activity was investigated. The reaction medium consisted of: 1mM MgCl<sub>2</sub>, 20mM KCl, 50mM Tris-MOPS, 0.1mM EGTA, 0.5mM NaN<sub>3</sub>, 0.1mM Na<sub>3</sub>VO<sub>4</sub> and 0.3mg/ml BSA, pH 7.5. Reactions were started by adding ATP (final concentration 3mM), except when investigating the effect of ATP; incubations were carried out at 35°C for 30 min as described in the Methods.

Each point represents the mean ± S.E.M.

**Figure 5.3**

Effect of pH on V-type ATPase activity.

**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** pH. (n=5).

**Figure 5.4**

Effect of different concentrations of ATP (Tris salt) on V-type ATPase activity.

**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** [ATP] mM. (n=5)

**Figure 5.5**

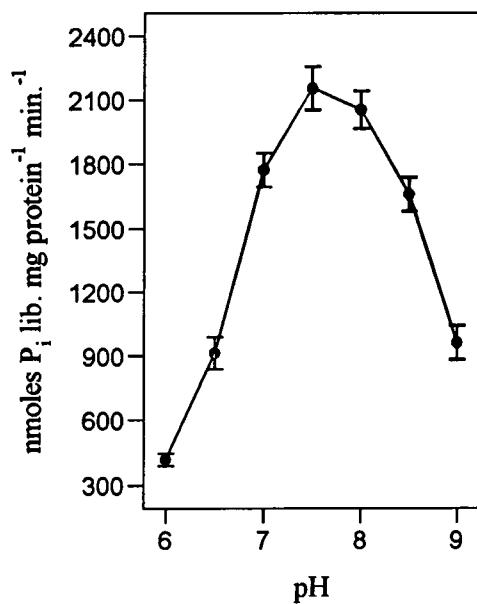
Effect of different concentrations MgCl<sub>2</sub> on V-type ATPase activity.

**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** [MgCl<sub>2</sub>] mM. (n=5)

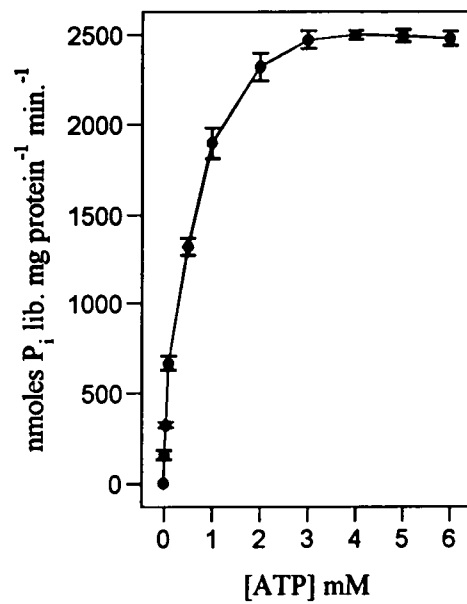
**Fig. 5.3**

Effect of pH on V-type ATPase activity



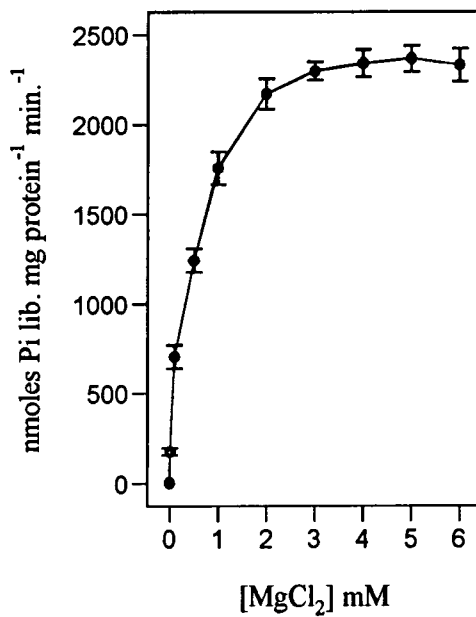
**Fig. 5.4**

Effect of ATP on V-type ATPase activity



**Fig. 5.5**

Effect of  $MgCl_2$  on V-type ATPase activity



It can be seen that in the absence of  $\text{MgCl}_2$  there was no activity. A marked increase in activity was observed with increasing  $\text{MgCl}_2$  concentration up to 5mM for the V-type ATPase. Maximum specific activity was obtained at 5mM ( $2366.5 \pm 72.0$  nmoles  $\text{P}_i$  lib. mg protein<sup>-1</sup> min<sup>-1</sup>) (see Appendix 4, Table 4.6)

#### **Effect of NEM on V-type ATPase activity:**

Figure 5.6a shows the effect different concentrations of NEM (0 - 1000 $\mu\text{M}$ ) on V-type ATPase. It can be seen that V-type ATPase activity was highly sensitive to NEM. At 5 $\mu\text{M}$  NEM activity was reduced significantly ( $p < 0.001$ ) to approximately 30% of the control value (see also Appendix 4, Table 4.7). 100 $\mu\text{M}$  NEM effected almost total inhibition of V-type ATPase activity. The  $\text{IC}_{50}$  of V-type ATPase activity (i.e. the NEM concentration that gives 50% inhibition) as shown in Figure 5.6b was 2.15 $\mu\text{M}$ .

#### **Effect of bafilomycin $\text{A}_1$ on V-type ATPase activity:**

Figure 5.7a (see also Appendix 4, Table 4.8) show the effect of different concentrations of bafilomycin  $\text{A}_1$  (0 - 1000nM) on the V-type ATPase. It can be seen that the enzyme activity was extremely sensitive to bafilomycin  $\text{A}_1$ , at only 0.1nM activity was significantly ( $p < 0.05$ ) reduced by approximately 23% of that of the controls. 10nM bafilomycin  $\text{A}_1$  effected almost total inhibition of V-type ATPase activity.

Figure 5.7b shows probit analysis of the  $\text{IC}_{50}$  of V-type ATPase activity (i.e. the bafilomycin  $\text{A}_1$  concentration that gives 50% inhibition) which was 0.44nM.

**Figure 5.6a**

Effect of different concentrations of NEM on V-type ATPase activity of *Locusta migratoria* Malpighian tubules microsomal preparations.

The V-type ATPase activity associated with the apical membrane-enriched fraction (P<sub>5</sub>) of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of NEM. The reaction medium consisted of: 1mM MgCl<sub>2</sub>, 20mM KCl, 50mM Tris-MOPS, 0.1mM EGTA, 0.5mM NaN<sub>3</sub>, 0.1mM Na<sub>3</sub>VO<sub>4</sub> and 0.3mg/ml BSA, pH 7.5. Reactions were started by adding ATP (final concentration 3mM) and incubations were carried out at 35°C for 30 min as described in the Methods.

**Ordinate:** Activity remaining expressed as a % of original activity in the absence of NEM.

**Abscissa:** [NEM]  $\mu$ M.

Mean value of 100% activity in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup> was 1945.5 $\pm$ 75.5.

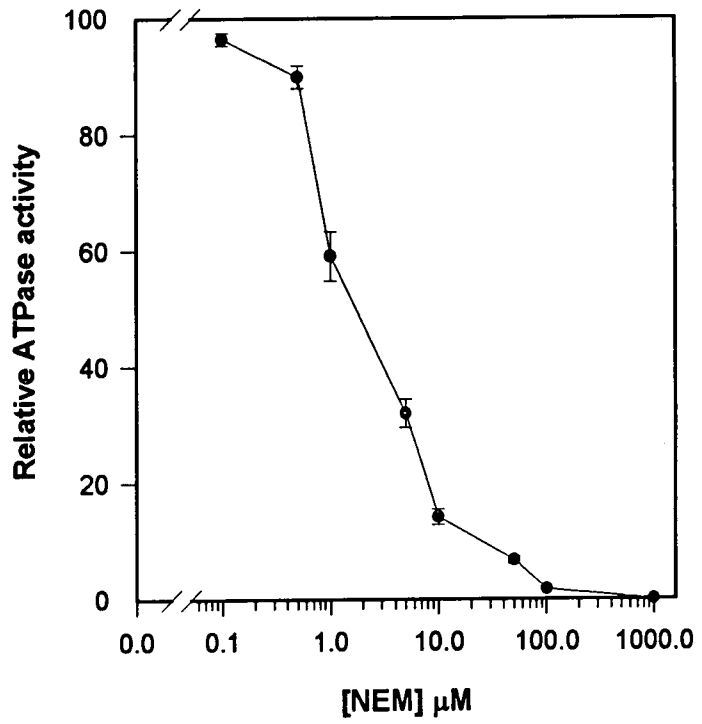
**Figure 5.6b**

The percentage of inhibition data presented in Appendix 4, Table 4.7 were converted into probits and lines of best fit were calculated according to the method shown in Appendix, 1.1.

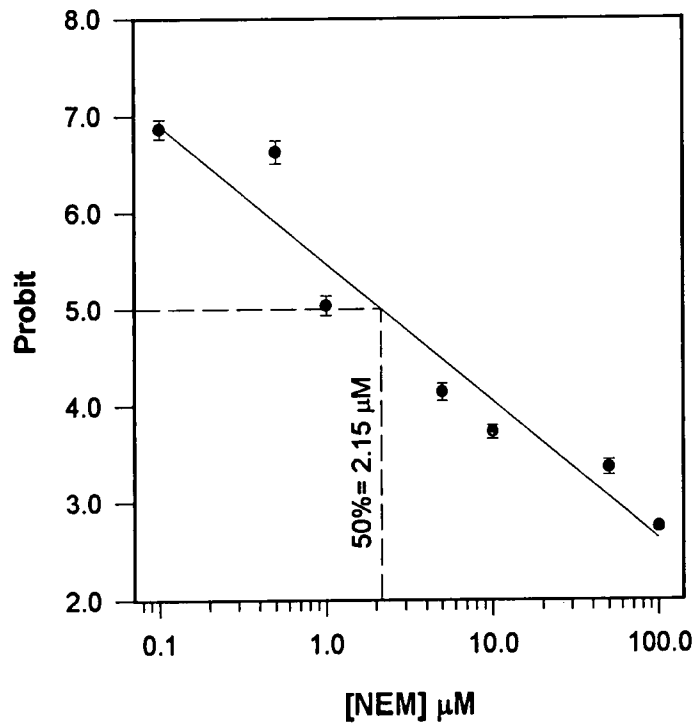
Each point represents the mean  $\pm$  S.E.M. (n=5)

**Figure 5.6a**

Effect of [NEM] on V-type ATPase activity.



**Figure 5.6b** Probit analysis.



**Figure 5.7a**

Effect of varying concentrations of Bafilomycin A<sub>1</sub> on V-type ATPase activity in *Locusta migratoria* Malpighian tubules microsomal preparations.

The V-type ATPase activity associated with the apical membrane-enriched fraction (P<sub>5</sub>) of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of Bafilomycin A<sub>1</sub>. The reaction medium consisted of: 1mM MgCl<sub>2</sub>, 20mM KCl, 50mM Tris-MOPS, 0.1mM EGTA, 0.5mM NaN<sub>3</sub>, 0.1mM Na<sub>3</sub>VO<sub>4</sub> and 0.3mg/ml BSA, pH 7.5. Reactions were started by adding ATP (final concentration 3mM) and incubations were carried out at 35 °C for 30 min as described in the Methods.

**Ordinate:** Activity remaining expressed as a % of original activity in the absence of Bafilomycin A<sub>1</sub>.

**Abscissa:** [Bafilomycin A<sub>1</sub>] nM.

Mean value of 100% activity in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>. was 1640.8±74.0.

**Figure 5.7b**

The percentage of inhibition data presented in Appendix 4, Table 4.8 were converted into probits and lines of best fit were calculated according to the method shown in Appendix, 1.1.

Each point represents the mean ± S.E.M. (n=5)

## **Effect of various salts on V-type ATPase of the apical membrane-enriched fraction.**

### **Effect of KCl on V-type ATPase activity:**

The effect of different concentrations of KCl (0 - 60mM) on V-type ATPase activity is shown in Figure 5.8. It can be seen that increasing KCl concentration resulted in a significant stimulation of enzyme activity. The results obtained from three preparations are shown in Appendix 4, Table 4.9, maximum activity was observed at a concentration of 30mM KCl. However, a marked reduction in the stimulation level was recorded when KCl concentration was increased above 30mM.

### **Effect of NaCl on V-type ATPase activity:**

The effect of different concentrations of NaCl (0-60mM) on V-type ATPase activity is shown in Figure 5.9 (see also Appendix 4, Table 4.10). NaCl stimulated activity of the enzyme, with maximum activity observed in the presence of 40mM NaCl.

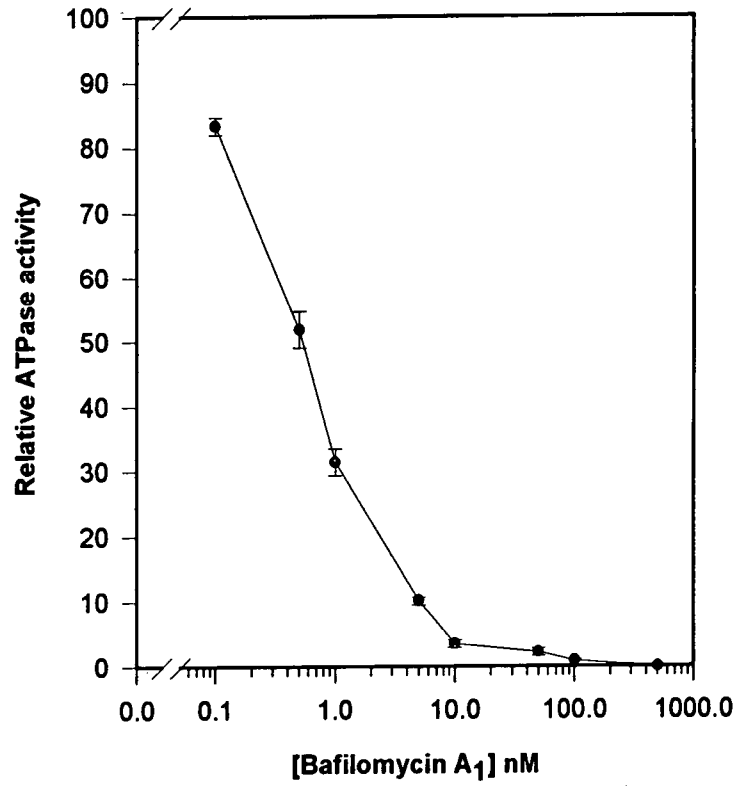
The unstimulated activity of V-type ATPase was  $777.8 \pm 60.1$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . However, inclusion of 40mM NaCl in the reaction medium caused the activity to rise to  $1510.4 \pm 87.5$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . A slight reduction in the percentage stimulation was observed when the concentration of NaCl increased above 40mM.

### **Effect of LiCl on V-type ATPase activity:**

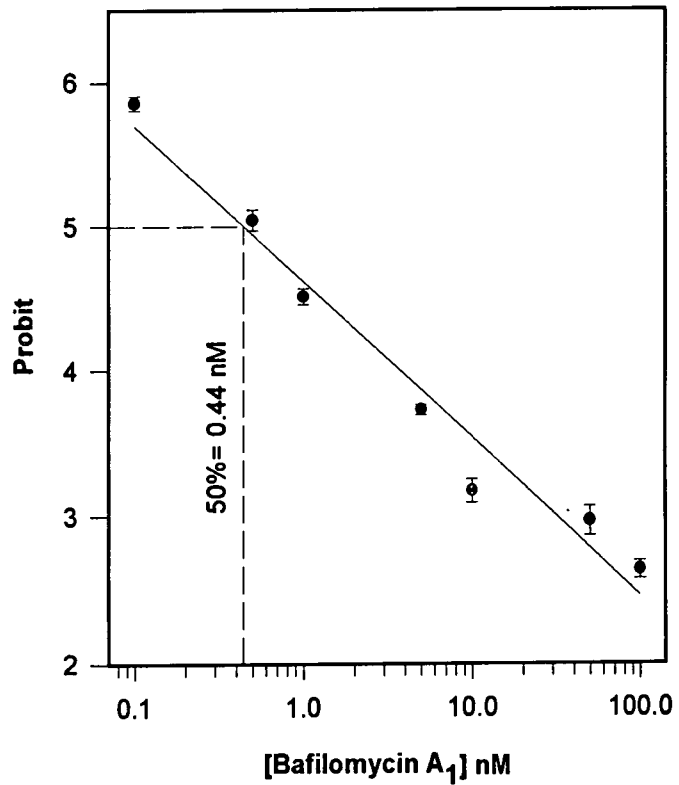
V-type ATPase was assayed in reaction media containing concentrations of LiCl ranging from 0 to 60mM. The results obtained from three independent

**Figure 5.7a**

Effect of bafilomycin A<sub>1</sub> on V-type ATPase activity.



**Figure 5.7b** Probit analysis.



preparations are shown in Figure 5.10 and Appendix 4, Table 4.11. It can be seen that LiCl stimulated V-type ATPase activity, maximum stimulation being observed in the presence of 40mM LiCl. ATPase activity in the absence of LiCl was  $760.3 \pm 35.6$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . In the presence of 40mM LiCl activity increased to  $1165.2 \pm 60.1$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ , representing a stimulation of ~150%.

#### **Effect of Choline Chloride on V-type ATPase activity:**

Effect of different concentrations of choline chloride (0-60mM) on ATPase activity is shown in Figure 5.11 (see also Appendix 4, Table 4.12). It can be seen that inclusion of choline chloride in the assay medium caused an increase in the activity of V-type ATPase. There was a stimulation of ~30% when 10mM choline chloride was included in the assay medium and this increased gradually until it reached a maximum activity at 50mM, a stimulation of approximately 185%.

#### **Effect of Tris-HCl on V-type ATPase activity:**

Enzyme assays were carried out in reaction medium containing concentrations of Tris-HCl ranging from 5 to 80mM. Figure 5.12 (see also Appendix 4, Table 4.13) show the relationship between ATPase activity and concentration of Tris. It can be seen that there was stimulation associated with increasing concentration of Tris-HCl in the reaction medium; maximum stimulation level being achieved at 50mM.

A concentration of 5mM Tris-HCl produced an activity of  $791.6 \pm 68.3$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . However, at 50mM it rose to  $2008.1 \pm 123.6$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . Increasing concentration of Tris-HCl above 50mM in the reaction medium reduced the level of stimulation.

**Effect of RbCl on V-type ATPase activity:**

Enzyme assays were carried out in reaction media in which the RbCl concentration was varied between 0 and 60mM. Figure 5.13 shows the relationship between ATPase activity and RbCl concentration (see also Appendix 4, Table 4.14). In the absence of RbCl activity was  $721.3 \pm 42.0$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . Inclusion of RbCl in the assay medium caused an increase in the V-type ATPase activity. Maximal activity ( $1020.9 \pm 62.1$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ ) was observed in the presence of 40mM RbCl, representing approximately a 140% stimulation of activity. Higher concentrations of RbCl resulted in a small, but not significant, reduction in this level of activity.

**Effect of  $\text{KHCO}_3$  on V-type ATPase activity:**

To investigate the effect of  $\text{KHCO}_3$  on V-type ATPase activity, a reaction medium was used which contained a different concentrations of  $\text{KHCO}_3$  (0-100mM), results are shown in Figure 5.14 and Appendix 4, Table 4.15. It can be seen that significant stimulation was caused by increasing the concentration of  $\text{KHCO}_3$ , maximum stimulation was observed at a concentration of 70mM  $\text{KHCO}_3$ , whereas, the mean basic activity obtained in the presence of 1mM  $\text{MgCl}_2$  alone was  $601.9 \pm 40.2$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ , however, a significant rise to  $2388.9 \pm 172.1$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$  was recorded in the presence of 70mM  $\text{KHCO}_3$ . Further, increases in  $\text{KHCO}_3$  concentration above 70mM resulted a slight reduction in the level of stimulation.

**Figures 5.8.,9, 5.10, 5.11, 5.12, and 5.13**

Effect of varying concentrations of various salts on V-type ATPase activity of *Locusta migratoria* Malpighian tubules microsomal preparations.

The V-type ATPase activity recorded in assay medium: 1mM MgCl<sub>2</sub>, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN<sub>3</sub> and 0.1mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.5. Different concentrations of the salt were included in this medium and stimulation recorded. Reactions were started by adding ATP (final concentration 1mM) and incubations were carried out at 35°C for 30 min as described in the Methods.

**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** [Salt] mM.

**Figure 5.8**

Effect of KCl, (n=5).

**Figure 5.9**

Effect of NaCl, (n=3).

**Figure 5.10**

Effect of LiCl, (n=3).

**Figure 5.11**

Effect of choline chloride, (n=3).

**Figure 5.12**

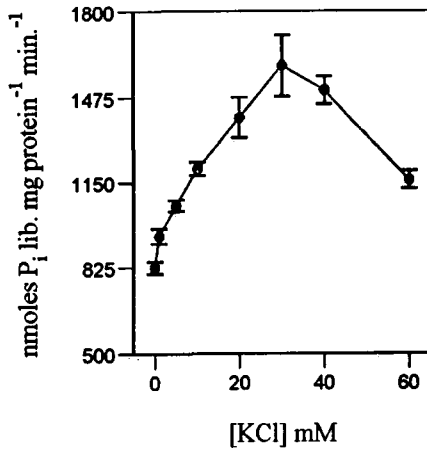
Effect of Tris-HCl, (n=3).

**Figure 5.13**

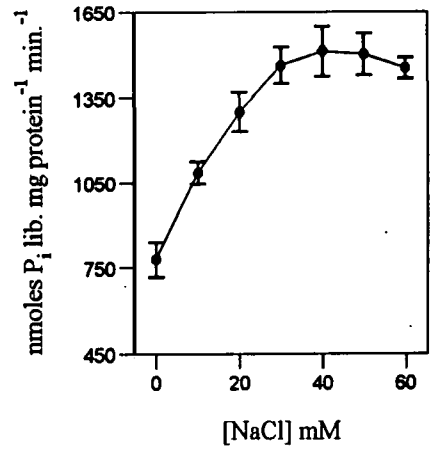
Effect of RbCl, (n=3).

Each point represents the mean ± S.E.M.

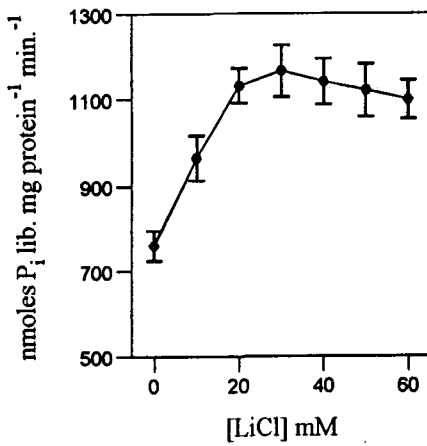
**Fig. 5.8 Effect of KCl on V-type ATPase activity**



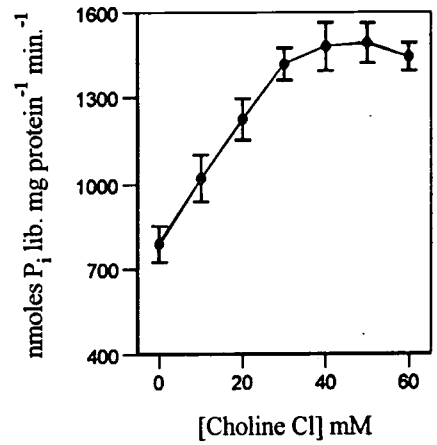
**Fig. 5.9 Effect of NaCl on V-type ATPase activity**



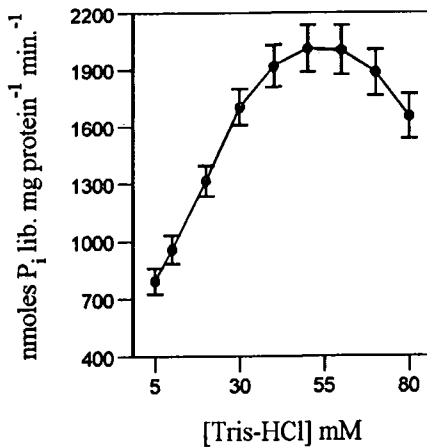
**Fig. 5.10 Effect of LiCl on V-type ATPase activity**



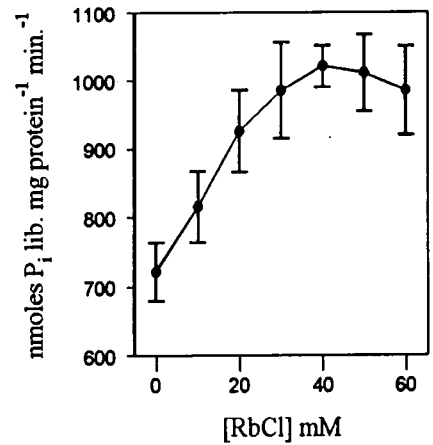
**Fig. 5.11 Effect of Choline Cl on V-type ATPase activity**



**Fig. 5.12 Effect of Tris-HCl on V-type ATPase activity**



**Fig. 5.13 Effect of RbCl on V-type ATPase activity**



**Effect of NaHCO<sub>3</sub> on V-type ATPase activity:**

V-type ATPase activity was determined over a range of concentrations of NaHCO<sub>3</sub> (0 - 100mM). Figure 5.15 shows the relationship between concentrations of NaHCO<sub>3</sub> and enzyme activity (see also Appendix 4, Table 4.16). Inclusion of NaHCO<sub>3</sub> in the assay medium caused a stimulation of the V-type ATPase activity; maximum stimulation was recorded with 70mM.

In the presence of 1mM Mg<sup>2+</sup> alone the activity was 640.2 ± 41.2 nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>. Inclusion of 70mM NaHCO<sub>3</sub> caused the activity to rise to 2210.3±131.6 nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup> representing ~245% stimulation of activity.

**Effect of KSO<sub>4</sub> on V-type ATPase activity:**

ATPase assays were carried out in reaction media in which the concentration of KSO<sub>4</sub> varied between 0 to 60mM. The results obtained are shown in Figure 5.16 (see also Appendix 4, Table 4.17). A marked increase was observed in the V-type ATPase activity with increasing KSO<sub>4</sub> concentration. The maximal stimulation was recorded at a concentration of 30mM (1210.9 ± 80.5nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>). However, this stimulatory effect was reduced when the concentration of KSO<sub>4</sub> rose above 30mM.

**Effect of Potassium Gluconate on V-type ATPase activity:**

V-type ATPase was assayed in reaction medium in which K gluconate was present over the concentration range 0 - 60mM. The results obtained are shown in Figure 5.17 and Appendix 4, Table 4.18. Potassium gluconate stimulated V-type

**Figures 5.14 and 5.15**

Effect of varying concentrations of bicarbonate on V-type ATPase activity in *Locusta migratoria* Malpighian tubules microsomal preparations.

The V-type ATPase activity recorded in assay medium: 1mM MgCl<sub>2</sub>, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN<sub>3</sub> and 0.1mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.5. Different concentrations of the salt were included in this medium and stimulation recorded. Reactions were started by adding ATP (final concentration 1mM) and incubations were carried out at 35°C for 30 min as described in the Methods.

Each point represents the mean  $\pm$  S.E.M.

**Figure 5.14**

Effect of KHCO<sub>3</sub>, (n=3).

**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** [KHCO<sub>3</sub>] mM.

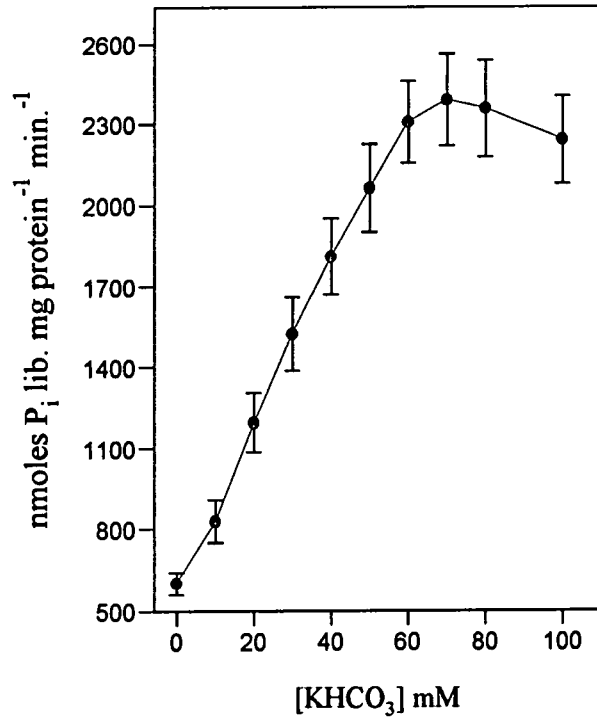
**Figure 5.15**

Effect of NaHCO<sub>3</sub>, (n=3).

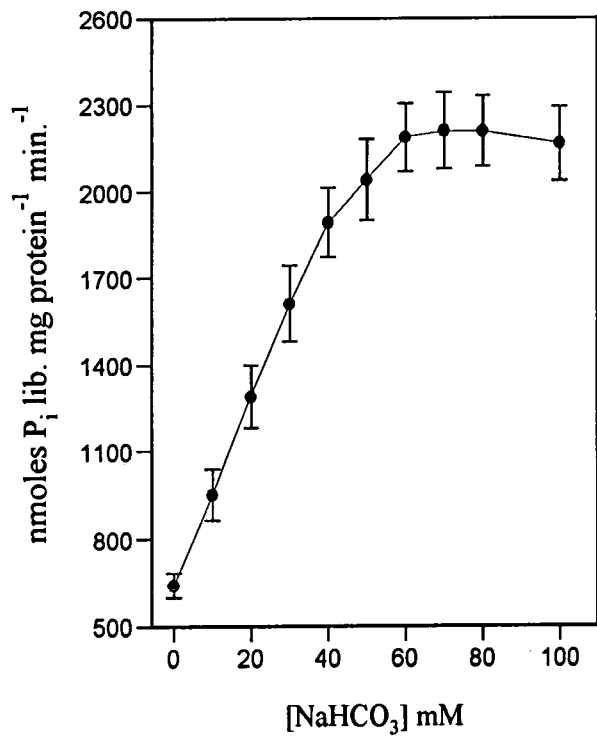
**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** [NaHCO<sub>3</sub>] mM.

**Fig. 5.14** Effect of  $\text{KHCO}_3$  on V-type ATPase activity



**Fig. 5.15** Effect of  $\text{NaHCO}_3$  on V-type ATPase activity



ATPase activity. Maximum stimulation was observed in the presence of 40mM ( $942.3 \pm 75.9$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ ).

#### **Effect of Potassium Fluoride (KF) on V-type ATPase activity:**

Figure 5.18 shows the relationship between V-type ATPase activity and different concentrations of KF (0 - 30mM). Inclusion of KF in the assay medium caused an increase in the activity of the enzyme. Maximum activity was observed in the presence of 20mM  $1006.3 \pm 30.4$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$  (see also Appendix 4, Table 4.19).

#### **Effect of KBr on V-type ATPase activity:**

Figure 5.19 shows the effect of different concentrations of KBr (0-60mM) on the V-type ATPase activity. An increase in the enzyme activity was observed with increasing KBr concentration up to 30mM. Maximal stimulation was recorded in the presence of 30mM KBr, when the activity was  $828.6 \pm 41.7$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$  (~120%) compared to the activity in the absence of KBr  $673.8 \pm 32.6$  nmoles  $P_i$  liberated  $\text{mg protein}^{-1} \text{ min}^{-1}$ . (for further see also Appendix 4, Table 4.20).

#### **Effect of $\text{KNO}_3$ on V-type ATPase activity:**

The effect of different concentrations of  $\text{KNO}_3$  (0-30mM) on the V-type ATPase activity was investigated. The results obtained from three experiments (as mean  $\pm$  SEM) are shown in Figure 5.20 and Appendix 4, Table 4.21. It can be seen that  $\text{KNO}_3$  inhibited ATPase activity; thus a concentration of 1mM  $\text{KNO}_3$  effected a 20% inhibition of activity, and in the presence of 30mM a significantly reduction 70% ( $p < 0.005$ ) was observed.

**Figures 5.16, 5.17, 5.18, 5.19, and 5.20**

Effect of varying concentrations of various salts on V-type ATPase activity in *Locusta migratoria* Malpighian tubules microsomal preparations.

The V-type ATPase activity recorded in assay medium: 1mM MgCl<sub>2</sub>, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN<sub>3</sub> and 0.1mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.5. Different concentrations of the salt were included in this medium and stimulation or inhibition recorded. Reactions were started by adding ATP (final concentration 1mM) and incubations were carried out at 35°C for 30 min as described in the Methods.

Each point represents the mean  $\pm$  S.E.M.

**Ordinate:** V-type ATPase activity expressed in nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

**Abscissa:** [Salt] mM.

**Figure 5.16**

Effect of KSO<sub>4</sub>, (n=3).

**Figure 5.17**

Effect of K Gluconate, (n=3).

**Figure 5.18**

Effect of KF, (n=3).

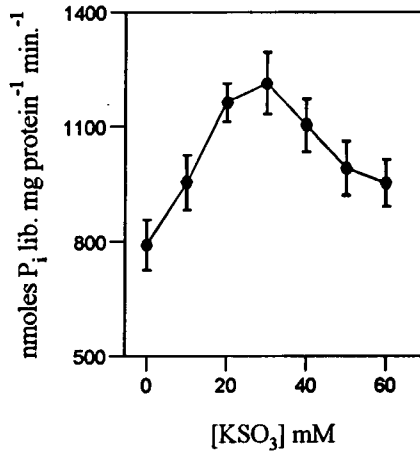
**Figure 5.19**

Effect of KBr, (n=3).

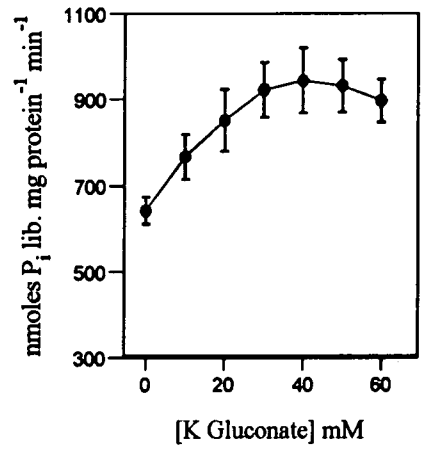
**Figure 5.20**

Effect of KNO<sub>3</sub>, (n=3).

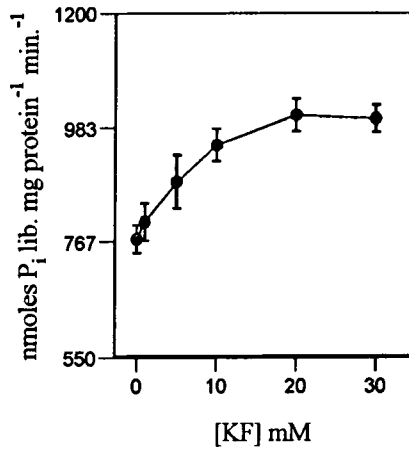
**Fig. 5.16** Effect of  $\text{KSO}_3$  on V-type ATPase activity



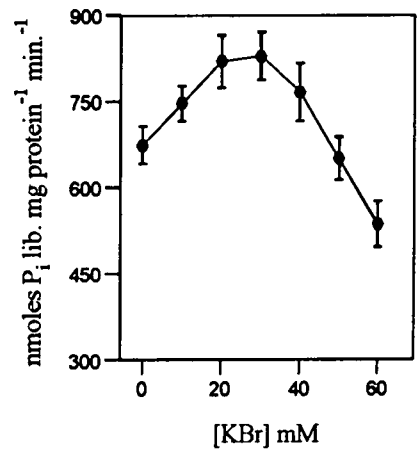
**Fig. 5.17** Effect of K Gluconate on V-type ATPase activity



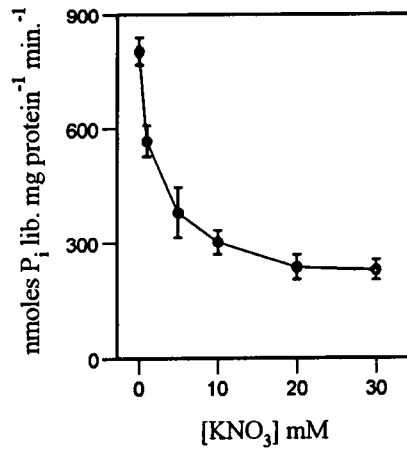
**Fig. 5.18** Effect of KF on V-type ATPase activity



**Fig. 5.19** Effect of KBr on V-type ATPase activity



**Fig. 5.20** Effect of  $\text{KNO}_3$  on V-type ATPase activity



Hegner and Anika (1975) for rumen epithelium and Gerencser and Lee (1985) for intestine of *Aplysia*. Ivashchenko *et al.*, (1975) found that 15-77% stimulation of ATPase activity using a different tissues of rat was produced by a concentration of  $\text{HCO}_3^-$  between 10 and 30mM. Ho and Chan (1981) obtained a slightly higher value of 30-40mM  $\text{HCO}_3^-$  for the gill tissue of eel, and Turbeck *et al.*, (1968) discovered the concentration which produced the greatest stimulation of ATPase activity in midgut of *Hyalophora cecropia* was 50mM  $\text{HCO}_3^-$ .

The results presented in this study showed different values of stimulation of  $\text{Mg}^{2+}$ -ATPase activity by 20mM  $\text{NaHCO}_3$  in basal membrane fraction ( $\text{P}_4$ ) which was 645.0 nmoles  $\text{P}_i$  lib. mg protein<sup>-1</sup> min<sup>-1</sup> and 979.6 nmoles  $\text{P}_i$  lib. mg protein<sup>-1</sup> min<sup>-1</sup> in apical membrane fraction ( $\text{P}_5$ ). These results indicate that there are possibly two different  $\text{Mg}^{2+}$ -dependent  $\text{HCO}_3^-$ -stimulated ATPases activities located on the basal (mitochondrial ATPase) and apical (microsomal ATPase) membrane cell. A similar stimulation of the enzyme activity was obtained by Marshall (1995). However, in a previous study by Fathpour (1980) the stimulation was larger being approximately 50%. Different degrees of stimulation by  $\text{HCO}_3^-$  have been recorded in different tissues; Suzuki (1978) discovered that mouse kidney microsomes were only stimulated by 10-15% when  $\text{HCO}_3^-$  was included in the reaction medium but the mitochondrial preparation from the same tissue was stimulated by almost 50%. Van Amelsvoort *et al.*, (1978) working on red blood cells found a stimulation of 27% was caused by  $\text{HCO}_3^-$ . A larger  $\text{HCO}_3^-$  stimulation was recorded from gastric mucosa (~54%) by Van Amelsvoort *et al.*, (1977) and Liang and Sacktor (1976) working on renal proximal tubule of rabbit recorded a 60% stimulation of activity by 50mM  $\text{HCO}_3^-$ .

In red blood cells, Duncan (1975) recorded a stimulation of 47% when 20mM  $\text{NaHCO}_3$  was substituted for  $\text{NaCl}$ .

It would appear that considerable non-mitochondrial  $\text{HCO}_3^-$ -stimulated ATPase activity is associated with the apical cell membrane-rich fraction of locust Malpighian tubules. Previous studies have also revealed anion-stimulated ATPase activity in membrane fractions from Malpighian tubules of *Locusta migratoria* (Fogg, 1990; Fogg *et al.*, 1991; Fathpour and Dahlman, 1994; Marshall, 1995), the recta of *Schistocerca gregaria* (Herrera *et al.*, 1978), larval dragonfly (Komnick *et al.*, 1980), the midgut and integument of *Manduca sexta* (Deaton, 1984), the midgut of *Hyalophora cecropia* (Turbeck *et al.*, 1968), and in various other animal tissues (reviewed by Gerencser and Lee, 1983). Linkage of such activity to active anion transport has been proposed (Komnick *et al.*, 1980; Bornancin *et al.*, 1980; Gerencser and Lee, 1983; 1985). One problem encountered in determining the subcellular site of anion-stimulated ATPase is mitochondrial contamination of plasma membrane fractions. In the present study, the distribution of anion-stimulated ATPase activity was not equivalent to that of the mitochondrial marker enzyme, SDH. This may be taken as support for the suggestion that such activity is not exclusively mitochondrial in origin.

$\text{Mg}^{2+}$ -dependent  $\text{HCO}_3^-$ -stimulated ATPase activity, which could be inhibited by  $\text{SCN}^-$  (Turbeck *et al.*, 1968; Wiebelhaus *et al.*, 1971; De Renzis and Bornancin, 1977; Van Amelsvoort, 1977), but not ouabain, was first discovered in microsomal preparations from frog gastric mucosa (Kasbekar *et al.*, 1965), and since has been reported in a variety of different animal species (Blum *et al.*, 1971; Wiebelhaus *et al.*, 1971; De Pont *et al.*, 1972; Sachs *et al.*, 1972a; Izutsu and Siegel, 1975; Suzuki, 1978). The enzyme has been implicated in  $\text{Cl}^-$  (Hanrahan and Phillips, 1983;

Lechleitner and Phillips, 1988) and  $\text{HCO}_3^-$  transport (Simon *et al.*, 1972a),  $\text{Cl}^-/\text{HCO}_3^-$  exchange (Herrera *et al.*, 1978),  $\text{H}^+/\text{HCO}_3^-$  transport (Simon *et al.*, 1972a) and  $\text{Na}^+/\text{H}^+$  exchange (Kinne-Saffran and Kinne, 1974; Liang and Sacktor, 1976).

Hanrahan and Phillips (1983) have suggested that active  $\text{Cl}^-$  transport is a primary transport process in locust rectum, and possibly involves an apical anion-stimulated ATPase. Indeed working on locust rectum (Lechleitner and Phillips, 1988) provided some evidence for an apical plasma membrane anion-stimulated ATPase, which may be responsible for active  $\text{Cl}^-$  transport. These workers found such ATPase activity to be stimulated to the greatest extent by sulphite, followed by  $\text{HCO}_3^-$  and finally  $\text{Cl}^-$ , and strongly inhibited by thiocyanate. Similarly, Anstee and Fathpour (1981) also found the enzyme in the Malpighian tubules of *Locusta migratoria* to be stimulated to the greatest extent by sulphite, followed by  $\text{HCO}_3^-$  and bromide (see also, Blum *et al.*, 1971; Sachs *et al.*, 1972b; Simon *et al.*, 1972a,b; Ebel and Lardy, 1975; Liang and Sacktor, 1976). However, these workers found  $\text{Cl}^-$  had no significant effect on enzyme activity, and nitrite inhibited ATP hydrolysis. The lack of specificity of ATPase for stimulation or inhibition by different anions led to the more appropriate description of anion-sensitive ATPase (Van Amelsvoort *et al.*, 1977). Fogg *et al.*, (1991) proposed that since the succinate dehydrogenase and  $\text{HCO}_3^-$ -ATPase showed a different distribution in the different membrane fractions of the Malpighian tubules of *Locusta migratoria*, then the  $\text{HCO}_3^-$ -ATPase activity could not be exclusively mitochondrial in origin.

It would appear that the Malpighian tubules of *Locusta migratoria* possess a  $\text{HCO}_3^-$ -stimulated ATPase in both the basolateral and apical cell membrane-rich fractions, with a higher specific activity associated with the former.  $\text{Cl}^-/\text{HCO}_3^-$

exchange at the basal surface may be responsible for  $\text{Cl}^-$  entry into the cell, with the  $\text{Na}^+/\text{K}^+$ -ATPase responsible for  $\text{K}^+$  entry. This would result in the transport of  $\text{K}^+$  and  $\text{Cl}^-$  into the cell and is potentially more adaptable than the  $\text{K}^+-\text{Cl}^-$  cotransporter, with  $\text{K}^+$  and  $\text{Cl}^-$  being transported independently, aided by intracellular pH. Because thiocyanate also inhibits alkali/acid secretion by pancreas and gastric mucosa and fluid secretion by the Malpighian tubules of *Locusta migratoria*, it has been suggested that the anion-sensitive ATPase may be involved in secretory processes (Blum *et al.*, 1971; Simon and Thomas, 1972; Fathpour and Dahlman, 1994). Another possible role for the  $\text{HCO}_3^-$ -stimulated ATPase which has been suggested is in active  $\text{Cl}^-$  transport across the apical membrane of locust rectum (Lechleitner and Phillips, 1988). However, since in the Malpighian tubules of *Locusta*,  $\text{Cl}^-$  transport is most likely to be passive, moving down a favourable electrical gradient, (Baldrick *et al.*, 1988) then an involvement in active  $\text{Cl}^-$  transport in this tissue seems unlikely.

$\text{Na}^+$  transport across the apical membrane could be mediated by an anion-stimulated ATPase in the form of electrogenic  $\text{Na}^+/\text{H}^+$  exchange. It may be possible that this exchange process preferentially accepts  $\text{K}^+$  rather than  $\text{Na}^+$ , due to intracellular levels of  $\text{K}^+$  being relatively high compared to  $\text{Na}^+$ . However, Fogg (1990) suggested that the effects of  $\text{SCN}^-$  on  $V_A$ , would be most difficult to explain on the basis of anion-stimulated ATPase being the apical cation pump. It may be that these systems help regulate intracellular pH, whilst other transport processes, such as the electrogenic ( $\text{Na}^+/\text{K}^+$ )-ATPase and apical cation pump serve as the major ion transporters.

**Studies of the biochemical properties of the apical membrane-rich V-type ATPase activity prepared from Malpighian tubules of *Locusta migratoria*:**

The effect of treating the tubules with NEM, an inhibitor of V-type ATPases, was also studied. NEM is specific for sulphhydryl groups and inhibits V-type ATPases between the concentration range of 1-2 $\mu$ M, P-type ATPases between 0.1-1mM and F-type ATPases are essentially unaffected (Forgac, 1989). In this study the concentration needed to produce a significant inhibition of fluid secretion was 100 $\mu$ M. At this concentration it was possible that effects on P-type ATPases were occurring, although 100 $\mu$ M is at the lower end of the sensitivity range for P-type ATPases. However, the availability of the NEM at the site of inhibition may be reduced, as it is found to inhibit membrane preparations at lower concentrations, it is then conceivable that NEM was inhibiting an enzyme activity at concentrations less than 100 $\mu$ M, which is below the concentration level that inhibits other classes of ATPases. Previous studies on the effect of NEM on the rate of fluid secretion by Malpighian tubules have produced variable results (Marshall, 1995 and the present study Chapter 3). Weltens *et al.*, (1992), working on the tubules of *Formica polyctena* found that a concentration of 500 $\mu$ M reduced the rate of fluid secretion to 7%. However Al-Ahmadi (1993) looking at the tubules of *Spodoptera littoralis* discovered that 50% inhibition of fluid secretion occurred with 1 $\mu$ M whilst complete inhibition occurred at 100 $\mu$ M. Bertram *et al.*, (1991) observed significant effects on the rate of fluid secretion by the Malpighian tubules of *Drosophila hydei* at concentrations greater than 10 $\mu$ M, with complete inhibition occurring at 1mM NEM. In a related study Dijkstra *et al.*, (1994b) found

that 500 $\mu$ M NEM applied to the solution bathing the Malpighian tubules of *Formica* caused a drop in the short circuit current to 21% of the control value.

A NEM-bafilomycin A<sub>1</sub> sensitive (V-type) ATPase was partially purified from the apical membrane-rich fractions of Malpighian tubules of *Locusta migratoria*. In the present study the properties of this enzyme were investigated. The preliminary results indicated that the ATPase activity was insensitive to both azide and orthovanadate which inhibit P-ATPases and F-ATPases respectively (Forgac, 1989; Nelson, 1992). However, the enzyme activity was extremely sensitive to bafilomycin A<sub>1</sub> (95% inhibition at 10 nM), the activity was also sensitive to NEM (~85% inhibition at 10  $\mu$ M). These inhibitor characteristics are similar to those reported in Malpighian tubules of *Locusta migratoria* (Marshall, 1995), other insect secretory tissues (Wieczorek *et al.*, 1989; 1991; Bertram *et al.*, 1991; Weltens *et al.*, 1992; Bertram and Wessing, 1994), and in various tissues (Bowman *et al.*, 1988; Mattsson *et al.*, 1991). V-type ATPase activity was located in the midgut of *Manduca sexta* (Schweickl *et al.*, 1989) by similar procedures. These latter investigators prepared apical membrane-rich fractions from goblet cells and identified an ATPase activity which was azide and orthovanadate insensitive but sensitive to NEM (it was inhibited by ~50% at a concentration of approximately 1 $\mu$ M). In another related study Minami *et al.*, (1991) obtained significant inhibition of a midgut ATPase activity isolated from the brush border of *Bombyx mori* using 100 $\mu$ M NEM.

V-type ATPases are extremely sensitive to NEM and bafilomycin A<sub>1</sub>. NEM has been used in various studies to determine the presence of V-type ATPases in microsomal fractions (see review by Al-Awqati, 1986) by their high sensitivity to the inhibitor ( $I_{50}$  < 100 $\mu$ M). This is below the value stated by Forgac (1989) needed to

inhibit P-type ATPases. In the present study concentrations of NEM up to 1mM were not found to inhibit  $\text{Na}^+/\text{K}^+$ -ATPase activity. NEM is an alkylating agent and is relatively selective for sulphhydryl groups (Forgac, 1989) however it is possible that it could have general toxic effects (Weltens *et al.*, 1992). Bafilomycin  $\text{A}_1$ , a macrolide antibiotic, has been shown to be an extremely potent inhibitor of V-type ATPases (Bowman *et al.*, 1988). F-type ATPases were not affected by this substance. P-type ATPases were moderately sensitive, but could be distinguished from the V-type ATPases by their relative sensitivity to the inhibitor. Mattsson *et al.*, (1991) also reported that bafilomycin  $\text{A}_1$  was  $10^4$  times less potent on P-type than V-type ATPases. Another advantage of using bafilomycin  $\text{A}_1$  to investigate the V-type ATPase activity is that it has no general toxic effects, this was reported by Yoshimori *et al.*, (1991) who studied its effects on lysosomes of living cultured cells.

Nitrate ( $\text{NO}_3^-$ ) is a well known inhibitor of anion ATPases. Anstee and Fathpour (1981) working on the Malpighian tubules of *Locusta migratoria*, discovered that 20mM  $\text{NaNO}_3$  caused the anion-stimulated activity to fall to 71.7% and 65.8% of control values in the presence of 20mM  $\text{NaCl}$  and 20mM  $\text{NaHCO}_3$ , respectively. In fact, nitrate inhibited the majority of the stimulation due to bicarbonate. Other workers have observed nitrate inhibition of anion-stimulated ATPases; Van Amelsvoort *et al.*, (1977) discovered that nitrate caused the anion-stimulated ATPase activity of rabbit gastric mucosa to fall to 65% of the value recorded in the presence of  $\text{Cl}^-$ . Ebel and Lardy (1975) reported that 20mM nitrate caused 50% inhibition of ATP hydrolysis in the presence of 20mM  $\text{HCO}_3^-$ , in rat liver mitochondrial ATPase. Also Izutsu and Siegel (1975) working on rat liver found that in the presence of 25mM nitrate ATPase activity became approximately half what it was in the presence of the incubation

solution only. Nitrate is also a known inhibitor of V-type ATPases. O'Neill *et al.*, (1983), and Bennett and Spanswick (1983) demonstrated a nitrate sensitive  $H^+$ -ATPase from corn roots, this activity was also stimulated by anions but inhibited by DCCD. The vacuolar ATPase of *Saccharomyces carlsbergensis* was sensitive to nitrate and Bowman (1983) comparing the sensitivity of the V-type ATPase of *N. Crassa* to mitochondrial and plasma membrane ATPases, found only the V-type activity was sensitive to nitrate.

The biochemical experiments in the present study were carried out to assess the properties of V-type ATPase activity. In this study the optimal pH required for maximal V-type ATPase activity was pH 7.5. It had previously been determined in mammalian kidney brush border as 7.4 (Ait-Mohamed *et al.*, 1986; Wang and Gluck, 1990). In contrast the optimum pH for V-type ATPase from midgut of *Manduca sexta* was recorded at pH 8.3 (Wieczorek *et al.*, 1986).

The present study located a  $K^+$ -stimulated ATPase activity to the apical membrane of the Malpighian tubules of *Locusta*, maximum stimulation occurring at 30mM KCl. The ATPase was also stimulated by various other salts, the maximum stimulation was achieved at (concentration in mM) 70NaHCO<sub>3</sub>, 70KHCO<sub>3</sub>, 40K gluconate, 30KBr, 30KSO<sub>4</sub>, 50Tris, 20KF, 50choline Cl, 40NaCl, 40RbCl, and 40LiCl. The effect of the same concentration (30mM) of these various salts on ATPase activity was investigated. The results are shown in Table, 5.1. Bicarbonate salts were a potent stimulator of activity. The Na salt causing  $152.4 \pm 8.4\%$  and the K salt  $142.4 \pm 18.4\%$ . Chloride salts also caused increase in activity in the following ascending order RbCl, LiCl, choline Cl, NaCl, KCl and Tris-HCl. Comparing potassium salts, KNO<sub>3</sub> caused inhibition of activity but the other salts tested all raised activity in the following

ascending order KBr, KF, KSO<sub>4</sub>, K gluconate, KCl and KHCO<sub>3</sub>. The chloride and bicarbonate salts of potassium caused a significantly larger increase in activity when compared to other tested. This suggests the activity was sensitive to these anions.

**Table 5.1. Influence of various salts on V-type ATPase activity**

Salt (30 mM)	Relative specific activity of V-type ATPase (Mean $\pm$ SEM)	<i>n</i>	<i>p</i>
No salt*	100		
KCl	202.3 $\pm$ 17.1	5	<0.01
NaCl	191.0 $\pm$ 17.5	3	<0.01
Choline Cl	171.3 $\pm$ 20.3	3	<0.02
RbCl	134.7 $\pm$ 11.5	3	<0.05
LiCl	150.1 $\pm$ 9.8	3	<0.05
KHCO <sub>3</sub>	242.4 $\pm$ 18.4	3	<0.001
NaHCO <sub>3</sub>	252.4 $\pm$ 8.4	3	<0.001
Tris-HCl	241.5 $\pm$ 20.1	3	<0.001
KBr	125.9 $\pm$ 2.5	3	n.s.
K Gluconate	140.4 $\pm$ 14.0	3	<0.05
KSO <sub>4</sub>	138.3 $\pm$ 8.2	3	<0.05
KF	130.3 $\pm$ 11.6	3	<0.05
KNO <sub>3</sub>	29.2 $\pm$ 2.1	3	<0.0001

\* (No salt) Unstimulated ATPase activity obtained in assay medium: 1mM MgCl<sub>2</sub>, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN<sub>3</sub> and 0.1mM Na<sub>3</sub>VO<sub>4</sub>, pH 7.5. The specific activity recorded in this assay medium was depended on each salt experiment (see appendix 5).

*p* values obtained by comparison of activity in the presence and absence of salt by Student's *t*-test.

Similar results are reported by Marshall (1995). However, Fogg *et al.*, (1991) did not find an ATPase on the apical membrane of the tubules of *Locusta* that was stimulated by K<sup>+</sup>. The finding of Fogg *et al.*, (1991) agreed with that of Keynes (1973) who could not demonstrate a K<sup>+</sup>-stimulated ATPase in the midgut of *Cecropia*.

However, other related studies on insect tissues have been able to locate a  $K^+$ -stimulated ATPase.

Wolfersberger (1979) demonstrated the existence of a  $K^+$ -modulated ATPase in the midgut of *Manduca sexta*. Later Wieczorek (1982) reported an ATPase activity in the proboscis of a fly that was insensitive to azide and stimulated by  $K^+$ , this enzyme activity was further characterized by Wieczorek and Gnatzy (1985) who found the ATPase activity could also be stimulated by  $Na^+$  and  $Rb^+$ . Deaton (1984) working on the midgut of *Manduca sexta* was also able to locate a  $K^+$ -stimulated activity, the optimal activity was produced with 25mM  $K^+$ ; optimum pH was 7.5. Wieczorek *et al.*, (1986) working on the same tissue found maximum stimulation at 30mM KCl and ~pH 8. In agreement with the present study they also showed that the enzyme activity could be stimulated by various anions, e.g. KBr, KF and  $KHCO_3$  but was inhibited by  $KNO_3$ . The  $K^+$  stimulation was confirmed in a later study (Schweickl *et al.*, 1989) as well as the demonstration that the ATPase was stimulated by different anions and cations but still inhibited by nitrate.

One explanation for the active transport of  $K^+$  across epithelia such as Malpighian tubules, midgut and salivary glands, was that it was achieved by a  $K^+$ -ATPase (Harvey *et al.*, 1983b) which was located in portosomes on the apical membrane. This ATPase activity was later referred to as an alkali metal ion pump due to its relatively unspecific nature (Wieczorek *et al.*, 1986). However, now it is generally accepted that it is a V-type ATPase activity which is responsible for  $K^+$  transport, this would be stimulated by  $K^+$ . The biochemical properties of the enzyme activity reported in this chapter in conjunction with ultrastructural and immunocytochemical evidence confirm that a V-type ATPase exists on the apical

surface of the Malpighian tubules of *Locusta*. It is proposed that in common with other insect secretory epithelia in which a V-type ATPase is present, that this enzyme, in parallel with an antiporter, is responsible for the active transport of  $K^+$  across the apical membrane which in turn is the driving force for fluid secretion (Wessing and Zierold, 1996).

The V-type ATPase was stimulated by a variety of different anions and cations;  $HCO_3^-$  was found to be the most potent cationic activator of ATPase activity and the only anions which inhibited activity were nitrate and thiocyanate. The anion stimulation was inhibited by NEM, a typical inhibitor of V-type ATPase activity and thiocyanate a typical anion-stimulated ATPase inhibitor. These findings suggest that the  $HCO_3^-$ -stimulated activity could represent, in part, an anion-sensitive V-type ATPase, this proposal was tentatively suggested by Fogg *et al.*, (1991).

The data in Table 5.1 suggest that the V-type ATPase is non-specifically stimulated by a variety of monovalent cations. The fact that the enzyme is activated by both choline and Tris suggest that this may be an ionic activation effect and so may not be directly related to cation transport. However, it is more likely that the enzyme responds to stimulation with  $HCO_3^-$  and inhibition by  $NO_3^-$  and so is affected by specific anions.

In the present study, comparison of  $P_5$  V-type ATPase and  $HCO_3^-$ -stimulated ATPase activities revealed that the anion-stimulated ATPase exhibited some characteristics which were relatively similar to V-type ATPase activity. Like V-type ATPase activity the apical rich-membrane fraction  $HCO_3^-$ -stimulated ATPase activity was shown to be insensitive to azide and orthovanadate. On other hand, the anion -

stimulated ATPase does exhibit some different characteristics which emerge when the sensitivity of the anion-stimulated ATPase to bafilomycin A<sub>1</sub> and NEM is compared to that of the V-type ATPase. The V-type ATPase activity is inhibited by nanomolar concentrations of bafilomycin A<sub>1</sub>, however, HCO<sub>3</sub><sup>-</sup>-stimulated ATPase is only substantially inhibited at higher concentrations of 10 and 100μM (Marshall, 1995). At these concentrations it is possible that P-type ATPases are affected therefore the enzyme activity cannot be confirmed as a V-type ATPase on the basis of its sensitivity to this inhibitor. Likewise NEM only causes a noticeable reduction of the HCO<sub>3</sub><sup>-</sup>-stimulated ATPase activity at values of 100μM and 1mM. At these levels P-type ATPases may also be inhibited. A possible explanation for some of these differences in sensitivity could be due to the difference in the assay media. When investigating V-type ATPase activity azide and orthovanadate were included in the assay medium and these inhibitors would eliminate most ATPase activity associated with P- or F-type ATPases. However these inhibitors were not included in the assay medium when HCO<sub>3</sub><sup>-</sup>-stimulated ATPase was monitored hence it is possible that there would be a persistent amount of Mg<sup>2+</sup>-ATPase activity present in the preparation which would not be inhibited by bafilomycin A<sub>1</sub> and NEM.

## CHAPTER 6

### **Partial purification and immunocytochemical localization studies on the apical and basal membrane-rich fractions molecular biology technique.**

#### INTRODUCTION

The V-type ATPase proton pump most commonly functions in the acidification of cytoplasmic vesicles and vacuoles and it is present in most eukaryotic cells (Nelson, 1992). First identified in organelles, it is also localized in the plasma membrane of vertebrate transporting epithelia, such as frog skin (Harvey, 1992), in osteoclasts (Chatterjee *et al.*, 1992) and in kidney (Brown *et al.*, 1992). The V-type ATPase is arranged in two structural sectors, a peripheral or  $V_1$  sector and an integral or  $V_o$  sector. The peripheral sector is composed of multiple subunits and, among others, it contains the catalytic subunit A and the regulatory subunit B, both of which possess ATP binding sites. Three molecules of both A and B are thought to be present in  $V_1$  in a single ATPase complex (Nelson, 1992; for reviews of V-type ATPases, see Harvey and Nelson, 1992; Dow 1994). The  $V_o$  sector is mainly composed of the putative proton channel formed by six copies of a protein known as subunit c or 16 kDa proteolipid, with molecular masses ranging from 15 to 18 kDa depending on the organism (Meagher *et al.*, 1990; Mandel *et al.*, 1988; Hanada *et al.*, 1991; Hasebe *et al.*, 1992; Dow *et al.*, 1992; Pietrantonio and Gill, 1993; 1995)

Immunocytochemical localization of V-type ATPase has been demonstrated in various tissues (Gluck and Caldwell, 1987; Pouyet *et al.*, 1992; Sasaki *et al.*, 1994; Wang and Gluck, 1990; Brown *et al.*, 1992; Fok *et al.*, 1993; Sekiguchi *et al.*, 1992; Bastani, 1995). Brown *et al.*, (1988a,b; 1992) prepared monoclonal and polyclonal

antibodies against the isolated kidney V-type ATPase, allowing its location in the kidney to be established by immunocytochemistry. These experiments confirmed that the kidney V-type ATPase resides in the plasma membrane of the renal tubular epithelial cells in the same segments of the nephron in which proton pumps had been identified in physiological studies (Gluck and Al-Awqati, 1984 and for reviews see Forgac, 1989; Gluck and Nelson, 1992).

In insect tissue purification of the V-type ATPase from the plasma membrane of *Manduca sexta* midgut offered the possibility to produce polyclonal and monoclonal antibodies against this enzyme (Russell *et al.*, 1992). V-type ATPase was immunocytochemically detectable in the apical membrane of Malpighian tubules and other insect epithelia (Russell *et al.*, 1992; Schweikl *et al.*, 1989; Wieczorek *et al.*, 1989; 1990; Klein *et al.*, 1991; Klein, 1992; Just and Walz, 1994; Pietrantonio and Gill, 1993; 1995). Furthermore, in a comparative immunological study (Klein and Zimmermann, 1991) demonstrated a V-type ATPase in Malpighian tubules of *M. sexta* and sensillae of *Antheraea pernyi*. Crude homogenate fractions from these tissues were tested in immunoblots after SDS-PAGE with the immune serum directed to the enzyme. The polyclonal antibodies were found to cross react with protein bands corresponding in size to the main subunits of the midgut V-type ATPase (Schweikl, *et al.*, 1989). Furthermore, immune sera directed against the whole enzyme or single subunits of xenic V-type ATPases from plant tonoplast and bovine chromaffin granules cross reacted in immunoblots with corresponding subunits of the midgut V-type ATPase (Russell, *et al.*, 1992). These results suggested that a V-type ATPase may be a common constituent of insect plasma membranes bearing the  $K^+$  pump, as an

alternative to the classical concept of plasma membrane energizing in animal cells by the  $\text{Na}^+/\text{K}^+$ -ATPase (Wieczorek, *et al.*, 1989; 1991).

When monoclonal antibodies directed against defined subunits of the midgut V-type ATPase were used to probe microscopical sections of the midgut epithelium (Klein *et al.*, 1991; Klein, 1992; Russell, *et al.*, 1992), they demonstrated the localization of the V-type ATPase in agreement with the biochemical findings (Wieczorek, *et al.*, 1986; Schweikl, *et al.*, 1989). As analysed by light and electron microscopy, clear labeling of the antibodies was found in the folding of the goblet cell apical membrane (Klein *et al.*, 1991; Klein, 1992; Russell, *et al.*, 1992).

Cytochemical localization studies on other insect epithelia such as the salivary glands of *Periplaneta americana* (Just and Walz, 1994) and ovarian follicles of the *M. sexta* (Janssen *et al.*, 1995) also demonstrated the presence of V-type ATPase. Apical enriched membrane fractions produced from the Malpighian tubules of *Locusta* contained a protein (~28 kDa) which was recognized by antibody 230-3 specific for a subunit of *Manduca* midgut V-type ATPase (Marshall, 1995). The apical localization of plasma membrane V-type ATPase in Malpighian tubules of *Locusta migratoria* (Marshall, 1995) was also indicated in fluorescence immunocytochemical studies using monoclonal antibody 230-3 specific for a subunit of *Manduca* midgut V-type ATPase. Other insect ion-transporting epithelia were found to contain a V-type ATPase on their apical membrane when they were probed immunocytochemically. Malpighian tubules of *Manduca sexta* were labelled in fluorescence immunocytochemistry at their brush border by the monoclonal antibodies to the *Manduca sexta* midgut V-type ATPase as well as by the polyclonal antibodies to the plant tonoplast V-type ATPase (Klein *et al.*, 1991; Klein, 1992; Russell, *et al.*, 1992). V-type ATPase in antennal sensilla apical

membrane of the saturniid moth *Antheraea pernyi* was examined with monoclonal antibodies to the *Manduca sexta* midgut V-type ATPase by light and electron microscopy (Klein, 1992; Klein and Zimmermann, 1991).

In the present study immunocytochemical using microgradient native and SDS electrophoresis and immunogold localization techniques were used to confirm the conclusion from the biochemical study (chapter 5) that the V-type ATPase activity was located on the apical membrane of *Locusta migratoria*.

MATERIALS AND METHODS

**Gel Electrophoresis:**

**Native microgradient polyacrylamide gels:**

**Preparation of linear microgradient polyacrylamide gels:**

The method was performed according to Schweikl *et al.*, (1989) and Hames (1990), with slight modifications. The apparatus producing linear microgradient slab gels is shown in Figure (6.1). In this study two acrylamide mixtures, 15% and 3% concentrations, were prepared according to the details in (Table 6.1).

**Table 6.1 Gel mixtures for a 3-15% native microgradient separating gel and 3% stacking gel**

Stock reagents	3%*	15%*	Stacking gel 3%
Acrylamide-bisacrylamide (30:0.8)	2	10	2
1.5 M Tris-HCl (pH 8.8)	5	5	-
0.5 M Tris-HCl (pH 6.8)	-	-	5
10% Triton 100-X	0.2	0.2	0.2
Deionized water	12.7	2.7	12.36
Glycerol	-	2	-
10% ammonium persulphate	0.1	0.05	0.1
TEMED	0.02	0.01	0.02

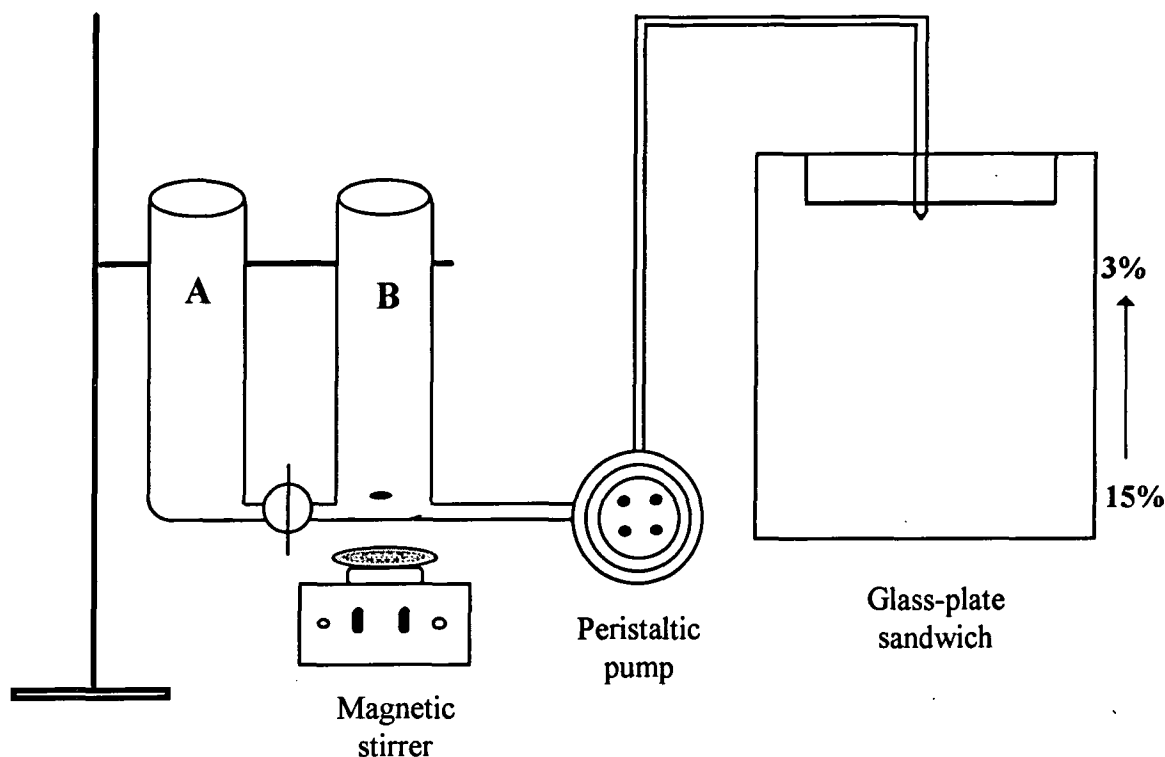
\*The columns represent volumes (ml) of the various reagents required to make 20 ml of gel mixture.

Ammonium persulphate and TEMED were add after degassing to initiate polymerization just before pouring the gel.

The protocol was as follows:

- 1) The gel mixtures were degassed to prevent premature polymerization.
- 2) TEMED and ammonium persulphate were added just before the gel was poured.

**Figure 6.1** Apparatus for the formation of microgradient polyacrylamide slab gels.



A and B refer to the reservoir and mixing chambers of the gradient maker, respectively. B was suspended about 1.5 cm above the magnetic stirrer by a clamp. Chamber B connected to A by a tunnel controlled by a clip. The two chambers A and B must be of exactly equal cross-section and are joined by an inter-connecting tunnel controlled by a two-way tap. The outlet from the gradient maker is connected via fine-bore Silicon tubing (1.6mm, inner diameter) to a peristaltic pump and then to the glass-plate sandwich which has been previously assembled. The silicon tubing is cut at an angle of 45° and the cut edge taped in position facing the rear (un-notched) plate at the centre of the assembly. According to this procedure, the most concentrated acrylamide solution enters the glass-plate sandwich first and runs down the inside of the un-notched glass plate to reach the gel bottom. As the level of acrylamide mixture rises in the sandwich, the acrylamide concentration steadily decreases.

The exact composition of the acrylamide mixtures used for gradient gel preparation will depend on the desired concentration range of the gradient and the buffer system used.

- 3) The low concentration mixture was added to reservoir A (Figure 6.1) and the connecting tube between the chambers of the gradient maker opened to fill it and then closed. Any gel solution which flowed into mixing chamber B was returned to reservoir A.
- 4) An equal volume of each mixture was now added to each chamber, 3.8 ml of each volume was calculated to correspond to half the volume of the final resolving gel. Chamber B can be mixed using a magnetic stirrer in which case care must be taken, to prevent heat from the magnetic stirrer causing premature polymerization. To avoid this, the gradient maker was supported (using a clamp) about 1.5 cm above the stirrer.
- 5) The chambers of the gradient maker were connected and the peristaltic pump and stirrer turned on, the clip undone and the peristaltic pump (Watson-Marlow, 505S) turned on the rate of the gel mixture into the glass-plate sandwich was about 3.0 ml/min (35 rpm).
- 6) Immediately after gel was poured it was overlaid by dipping the outlet tubing into a flask containing a buffer the same composition as in the gel mixture (7.5ml of 1.5M Tris-HCl pH, 8.8, 0.3ml of 10% Triton 100-X, 22.2ml of deionized water) and the flow rate reduced to 0.5ml/min (15 rpm) (Hames 1990).
- 7) After polymerization of the slab gel (30 min), the overlay was removed by tilting the gel and a stacking gel (Table 6.1) was poured.
- 8) The comb was immediately inserted into the stacking gel mixture, being careful to avoid trapping any air bubbles beneath it.
- 9) The assembly was left undisturbed whilst the stacking gel polymerized (30 min.).

- 10) After polymerization, the comb was carefully removed to expose the sample wells, which were rinsed-out and then filled with reservoir buffer. Any divisions between wells that had become displaced during comb removal were straightened with a syringe needle, care being taken to avoid damage.

### **Sample preparation and electrophoresis:**

The following procedure was based on the method described by Schweikl *et al.*, (1989) with minor modifications. The enriched apical-membrane pellet (P<sub>5</sub>) was produced as described previously. This was resuspended in the following buffer: 10mM Tris-HCl, pH 7.5, 0.32mM EDTA and 5% glycerol containing 1% Triton X-100. This mixture was then put on ice and stirred for 10 min before being centrifuged at 100,000xg for 1 hour, using a 12 x 10 rotor, in a Beckman L-70 Ultracentrifuge. The supernatant containing the solubilized enzyme was then mixed in a 1:1 ratio with sample buffer consisting of 125mM Tris-HCl, pH 6.8, 20% (v/v) glycerol and 0.02% (w/v) tracking dye, bromophenol blue (modified from Laemmli, 1970).

“Running” buffer (0.025M Tris-HCl, pH 8.3, 0.192M glycine) was added to the lower chamber and the same buffer with 0.1% Triton X-100 to the upper chamber (Dewald *et al.*, 1974). The gel surface was washed by directing a gentle stream of running buffer into each sample well using a Pasteur pipette. Before loading the sample the gel was pre-electrophoresed for a period of time (30-40 min), to remove traces of chemicals such as residual ammonium persulphate or acrylic acid which might reduce the biological activity of proteins (Hames, 1990).

10 $\mu$ l volumes of samples were carefully loaded into the wells using a microsyringe. The tip of the sample applicator was held only 1-2 mm above the gel surface to minimize sample mixing with the running buffer during loading. The unused wells were filled with an equivalent volume of blank sample buffer. The gel was run at 20 mA constant current, using a mini electrophoresis system (Anachem, MVI-DC) with power supply from Anachem, (Power Pac, PSU-400/200), for about 6-7 h at 4°C or until the tracking dye which was bromophenol blue reached the lower end of the gel.

#### **Detection of ATPase and alkaline phosphatase Activity in gels:**

The incubation and localization procedure followed that described by Schweickl *et al.*, (1989), and Gabriel (1974). The incubation medium: 5mM MgSO<sub>4</sub> and 100mM Tris-HCl (final concentrations), pH 7.5, was thermoequilibrated for 15 min at 35°C, in a waterbath. The gel was added and thermoequilibrated for 15 min before 5mM (final concentration) ATP (ATPase) or  $\beta$ -glycerophosphate (alkaline phosphatase) was added as substrate. The gel was left for 1 hour. At the end of this time it was washed in deionized distilled water and then 2.5mM of lead acetate in 80mM Tris maleate, pH 8.0, was added. Incubation was continued for a further 30min at room temperature, gels were then thoroughly washed in DDW. Finally, the gels were placed in aqueous 2% ammonium sulphide for at least 2 min and rinsed for 1 hour in deionized distilled water with frequent changes. Dark brown precipitate represented the site of ATPase/alkaline phosphatase activity.

#### **Microgradient SDS-PAGE:**

The buffer system (discontinuous gels) for the microgradient SDS PAGE (3-15%) is shown in Table (6.2) the methods used was performed according to

Laemmli (1970), and the method was essentially the same as described previously in native microgradient PAGE. Samples (enriched-membrane fractions from Malpighian tubules of *Locusta*) were dissolved in sample buffer containing 62.5mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue. These were heated for 5 min at 100°C in a water bath, then allowed to cool at room temperature. To remove any insoluble material, the samples were centrifuged at 15,000g for 10min at 4°C. Approximately equal amounts of protein (~15µg) were loaded onto a 3-15% microgradient gel in each lane. The gels were run at a constant voltage, 50mV for 30min, then at 150mV for 3-4hours, at room temperature, using a mini-electrophoresis system (Anacham, MVI-DC) and an Anachem PowerPac PSU-400/200. Gels were fixed and stained for protein using 0.25% (w/v) Coomassie Blue in 40% (v/v) methanol and 10% (v/v) acetic acid. Destaining was effected in a solution containing 40% methanol and 10% acetic acid.

**Table 6.2 Gel mixtures for a 3-15% SDS microgradient separating gel and 3.9% stacking gel**

Stock reagents	3%*	15%*	Stacking gel 3.9%
Acrylamide-bisacrylamide (30:0.8)	2	10	2.6
1.5 M Tris-HCl (pH 8.8)	5	5	-
0.5 M Tris-HCl (pH 6.8)	-	-	5
10% SDS	0.2	0.2	0.2
Deionized water	12.7	2.7	12.1
Glycerol	-	2	-
10% ammonium persulphate	0.1	0.05	0.1
TEMED	0.02	0.01	0.02

\* The columns represent volumes (ml) of the various reagents required to make 20 ml of gel mixture.

Ammonium persulphate and TEMED were added after degassing to initiate polymerization just before pouring the gel.

**Western blots of microgradient PAGE:**

Proteins were blotted from acrylamide gels to nitrocellulose using the semi-dry blotting method of Kyhse-Andersen (1984). Each transfer unit (containing one gel) comprised a sandwich arrangement of a sheet of 3MM paper; a sheet of nitrocellulose; the gel to be blotted and another sheet of 3MM paper. Transfer was carried out on a Bio-Rad Trans-blot, semi-dry transfer cell using the following procedure:

- 1) The nitrocellulose membrane and pieces of 3MM paper were cut to the size of the gel and pre-soaked in transfer buffer (48mM Tris, pH 9.2, 39mM glycine, 0.0375g/l (w/v) SDS and 20% methanol) for 15min (Bjerrum and Schafer-Nielsen, 1986).
- 2) The corner of the gel was cut off to mark its orientation and then the gel was equilibrated in transfer buffer for 15min.
- 3) Soaked nitrocellulose membrane was placed onto 3MM paper, and then the gel was placed on top followed by a layer of 3MM paper.
- 4) Each mini-gel was transferred at 25V and 220mA ( $5.5\text{mA}/\text{cm}^2$ ) for 1hour using a power pack (Anachem, PSU-400/200).
- 5) After blotting, the nitrocellulose membrane was removed and transferred immediately into blocking solution (PBS, 5% Marvel and 1% Tween-20) for 1hour at room temperature.
- 6) The blot was then incubated with the primary antibody (230-3, raised against to ~28kDa subunit of *Manduca sexta* midgut V-type ATPase) in anti-sera buffer (PBS, 5%

skimmed milk and 0.1% Tween-20) overnight, at 4°C, with shaking. Best results were obtained using 1:4000 dilution.

- 7) The primary monoclonal antibodies, directed to the V-ATPase from insect plasma membrane (midgut of *Manduca sexta* larvae) were kindly supplied by Prof. Dr. Ulla Klein, Laboratory Wieczorek, Zoological Institute of the University of Munich.
- 8) The nitrocellulose membrane was then washed 3x5min in anti-sera buffer before incubation with the secondary antibody for 2hour, at room temperature, with shaking. The secondary antibody used was a goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (from Bio-Rad Cat. No. 170-6516) at 1:5000 dilution. At the end of this time the membrane was washed in PBS/ 0.1% Tween-20 for 2x5min and 1x15min, with shaking.
- 9) Finally, the membrane was rinsed in distilled water for 2x5min. The membrane was then kept in distilled water.

#### **ECL detection of V-type ATPase:**

ECL or enhanced chemiluminescence is a non-radioactive, light emitting method for the detection of immobilized antigens conjugated directly or indirectly with horseradish peroxidase labelled antibodies.

These steps were carried out quickly in the dark:

- i) Equal volumes of detection reagents 1 and 2 (Amersham's ECL detection system) were mixed together, such that the total volume was 0.125ml/cm<sup>2</sup> membrane.
- ii) The excess water was drained from the membrane.
- iii) Detection reagent was poured onto the membrane (protein side up) and left for 1min.

- iv) The membrane was drained as before and then placed between acetate sheets in a cassette.
- v) The membrane was exposed to film for 5min.

**Immunogold electron microscopy:**

The method used was according to Klein *et al.*, (1991) and Just and Walz (1994). *Locusta* Malpighian tubules were dissected out in cold PLP fixative (2% paraformaldehyde, 0.075M lysine, 0.01M sodium periodate in 0.1M sodium phosphate buffer, pH 7.3) and then fixed for 3hours at 4°C. They were then washed in 0.1M sodium phosphate buffer, 3 x 5 min. Samples were then dehydrated in a graded ethanol series (12.5, 25, 50, 70%) 3x10min each. Samples were infiltrated with a 2:1 mixture of LR white : 70% ethanol for 1.5hour, then transferred to LR white only for 1h and then to fresh LR white, overnight, on a rotamix at room temperature. In the morning, the LR white was changed once more and then the samples were embedded in LR white in gelatine capsules. Capsules were polymerized at 50°C for 24hour. Thin sections were cut on an ultramicrotome and collected on uncoated nickel grids.

The following protocol for immunolabelling thin sections was followed (all steps were carried out in a moist chamber at room temperature and all solutions were sterile filtered before contact with the sections ):

I) Grids were pre-incubated on 25µl droplets of the following, in sequence:

- 1) PBS (150mM NaCl in 10mM phosphate buffer pH, 7.4)
- 2) 50mM NH<sub>4</sub>Cl in PBS.
- 3) 0.01% Tween 20 in PBS.

4) PBS.

5) Blocking buffer (0.1% gelatine, 1% bovine serum albumin (BSA) in PBS.  
Each step lasting 10min.

II) The grids were transferred to a 25µl droplet of primary antibodies diluted 1/500 in the 0.1% BSA/PBS buffer, pH 7.4, for 48hour at 4°C in a moist chamber.

The primary antibodies used were:

1) Monoclonal antibody 230-3 raised against to 28kDa subunit of the V-type ATPase of *Manduca sexta* midgut.

2) Monoclonal antibody IgG α-5 to the α-subunit of avian Na<sup>+</sup>/K<sup>+</sup>-ATPase

III) Grids were then washed to remove unbound primary antibodies by placing them on a 50µl droplet of blocking buffer for 5 min x 5.

IV) Grids were transferred to a 25µl droplet of gold conjugate (15nm gold-conjugated goat anti-mouse IgG antibody; Bioclin immunogold reagent - GAM15) diluted 1:20 in PBS buffer, pH 8.2, for 1 hour.

V) Unbound gold conjugate was removed by placing grids on 50µl droplets of PBS and distilled water for 2 min x 5.

VI) Sections were left to dry before staining in 2% aqueous uranyl acetate for 10min.

Controls consisted of either omitting the primary antibodies and replacing them with buffer or using one of the other antibodies which did not recognize the locust V-type ATPase or Na<sup>+</sup>/K<sup>+</sup>-ATPase.

**RESULTS**

**Section I**

**Detection of apical membrane-rich fraction enzyme activities:**

Following successful native microgradient electrophoretic separation, the gel assays were carried out as quickly thereafter as possible. The gels were incubated in reaction media with appropriate substrate as described previously.

The results obtained are shown in Plate (6.1). When the gels were incubated in media containing ATP as substrate, two enzyme activity bands were revealed (Plate 6.1, lane A). However, in the presence of  $\beta$ -glycerophosphate as substrate, only one band was observed (Plate 6.1, lane F). This band represents alkaline phosphatase activity and was identical in position to the lower band shown in Plate 6.1, lane A, suggesting that the activity corresponding to this latter band hydrolysed ATP and  $\beta$ -glycerophosphate as substrates.

An investigation into the effect of various inhibitors on the enzyme activities located on the gels was carried out by incubation of the gels in reaction media as previously described. Plate 6.1, lanes B and C, shows that only the upper band was sensitive to bafilomycin A<sub>1</sub> and NEM, specific inhibitors of V-type ATPase. However, the ATPase activity presenting in the upper band (Plate 6.1, lanes D and E) was not inhibited by azide or orthovanadate respectively.

**Plate 6.1**

Native microgradient PAGE of the apical membrane ATPase activity from Malpighian tubules of *Locusta migratoria*.

**A:** The gel was stained for ATPase activity by incubation for 1 hour at 35 °C in substrate solution containing 5 mM ATP, 5 mM MgSO<sub>4</sub>, 100 mM Tris-HCl, pH 7.5. Then the gel was washed in dionize water and incubated in a solution containing 2.5 mM lead acetate in 80 mM Tris-malate, pH 7, for 30 min. To visualize the white lead salt precipitates, gel was incubated with 2% ammonium sulphide which turned precipitates dark brown.

**B:** In the presence of 5 μM Bafilomycin A<sub>1</sub>.

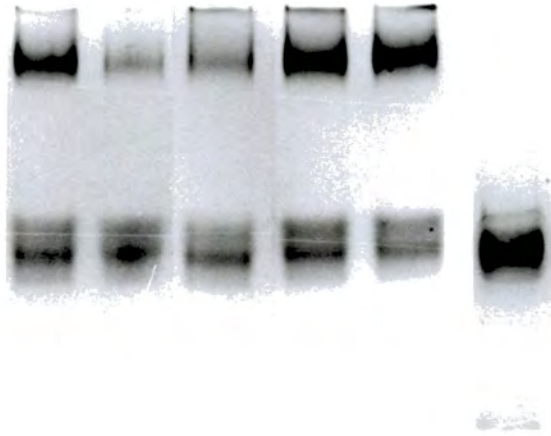
**C:** In the presence of 10 mM NEM.

**D:** In the presence of 100 mM sodium azide (NaN<sub>3</sub>).

**E:** In the presence of 1 mM orthovanadate (Na<sub>3</sub>VO<sub>4</sub>).

**F:** Exactly the same procedure was followed as described above for Plate 6.1, A apart from the fact that the reaction medium contained 5 mM β-glycerophosphate as the substrate instead of ATP.

A B C D E F



**Western blotting of native microgradient PAGE:**

Using an identical gel the enzyme activities were transferred to a nitrocellulose membrane and incubated with monoclonal antibody 230-3 to the V-type ATPase of *M. sexta*. The results are shown in Plate 6.2. It can be seen that the results are consistent with the detection of enzyme activity assays referred to above. The antibody recognized the upper band (Plate 6.2, lane B), indicating that this band represented a V-type ATPase present in Malpighian tubules of *Locusta migratoria*.

**Specificity of V-type ATPase to apical membrane:**

The different enriched-membrane fractions, (mitochondrial P<sub>2</sub>, basal P<sub>4</sub> and apical P<sub>5</sub>) were prepared as described previously, and separated by microgradient SDS-PAGE (Plate 6.3, lanes A, B and C, respectively). The observed proteins were then transferred to a nitrocellulose membrane and probed with antibody 230-3 against the 28kDa subunit of *Manduca sexta* midgut V-type ATPase. Plate 6.4, lane A, represents a western blot of lane C and shows that the antibody immunoreacted to the 28kDa subunit of the apical rich-membrane fraction (P<sub>5</sub>). No reactivity was observed with any subunit in the other membrane fractions. These results show that V-type ATPase was only present in the apical membrane fraction of Malpighian tubule cells of *Locusta migratoria*.

**Plate 6.2**

Two microgradient gels prepared as those as described previously for Plate 6.1, A.

Lane A: The gel was stained for ATPase activity using lead capture technique.

Lane B: The gel was probed with antibody 230-3, to the 28kDa subunit of the midgut V-type ATPase of *Manduca sexta*. ECL was used for detection.

A



B



**Plate 6.3**

Two microgradient gels were prepared in exactly the same way and run simultaneously using the same equipment. One was stained with Coomassie blue (Lanes B, C and D) and one used for Western blotting (Plate 6.4 Lane A).

Stained microgradient SDS-PAGE gel. Lanes A, B, D and E represented different protein samples:

Lane A: Enriched mitochondrial membrane fraction.

Lane B: Enriched basal membrane fraction.

Lane C: Enriched apical membrane fraction.

Lane D: Molecular weight markers.

**Plate 6.4**

Lane A: Western blot of a microgradient gel identical to the one shown above. The blot was probed with a monoclonal antibody 230-3, to the 28kDa subunit of the midgut V-type ATPase of *Manduca sexta*. The antibody recognized a protein band of similar molecular weight present only in the apical-enriched membrane fraction of the Malpighian tubules of *Locusta migratoria*.

Plate 6.3

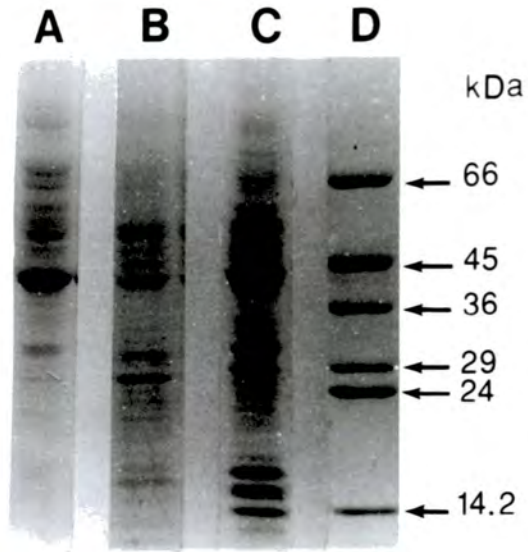
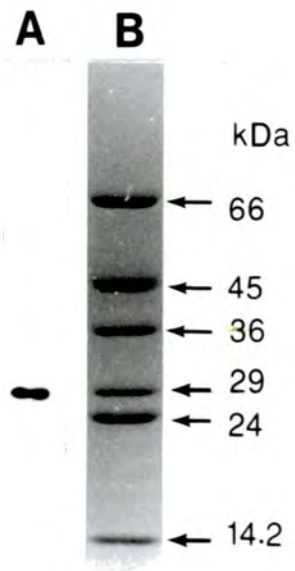


Plate 6.4



## Section II

### **Immunogold localization of V-type ATPase:**

Monoclonal antibody 230-3 raised against the V-type ATPase from the midgut of *Manduca sexta* was used to investigate the presence of a V-type ATPase in *Locusta* Malpighian tubules.

Thin sections of Malpighian tubules probed with the antibody showed specific binding to the apical region of the cross-sections (Plates 6.5 A and B). This apical region was the site of the microvilli where the electron-dense 10-nm particles were situated (Klein *et al.*, 1991; Klein, 1992; Marshall, 1995). No staining was observed in any other region of Malpighian tubule cell (Plates 6.5A and B). When antibody 230-3 was replaced by non-immune serum in control experiments, no specific labeling could be detected in any part of the cell (Plate 6.5C).

### **Immunogold localization of Na<sup>+</sup>/K<sup>+</sup> ATPase:**

Monoclonal antibody IgG  $\alpha$  5 raised against the catalytic subunit of the chicken sodium pump (Takeyasu *et al.*, 1988) was used to map the distribution of the Na<sup>+</sup>/K<sup>+</sup> - ATPase in the cells of Malpighian tubules of *Locusta migratoria*. Plates (6.6A and B) illustrate the results demonstrated by electron microscopy. Cross-sections through the Malpighian tubules show that the gold labeling was confined to the basal region of the cell. No specific binding was detected at the apical surface (Plate 6.6C).

**Plates 6.5A and B**

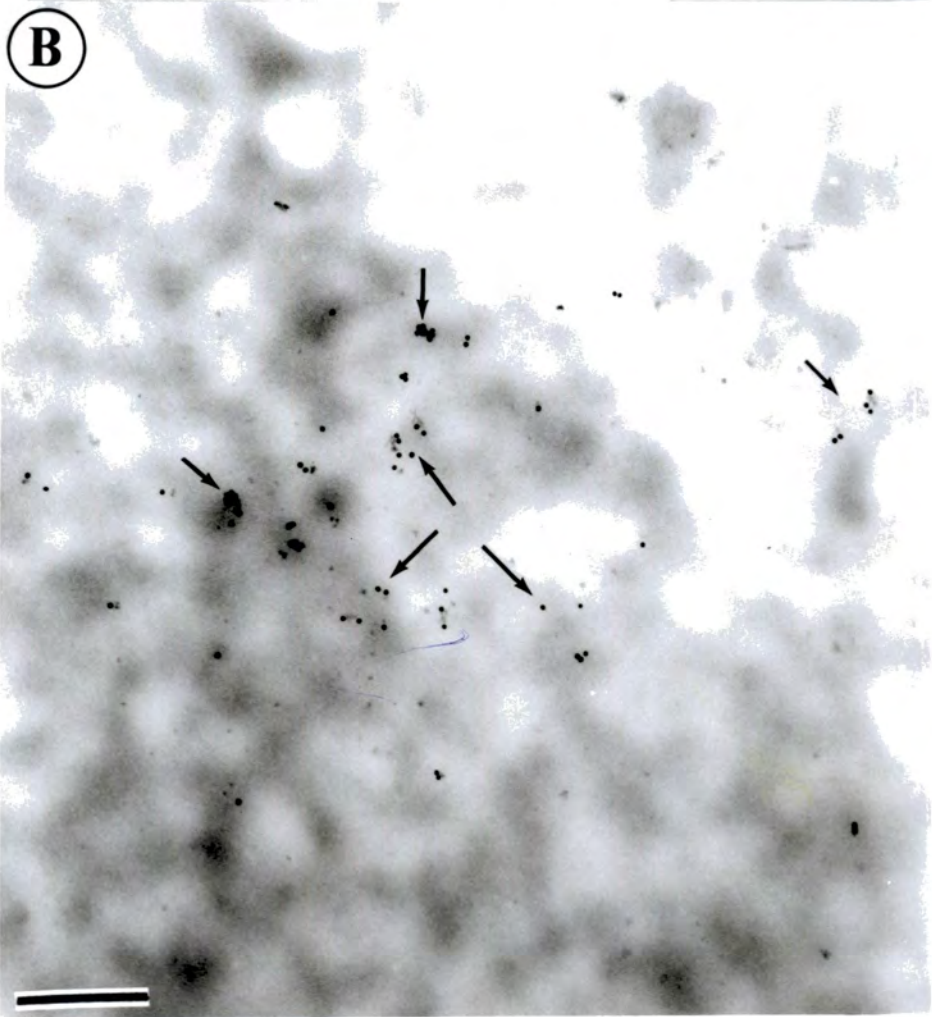
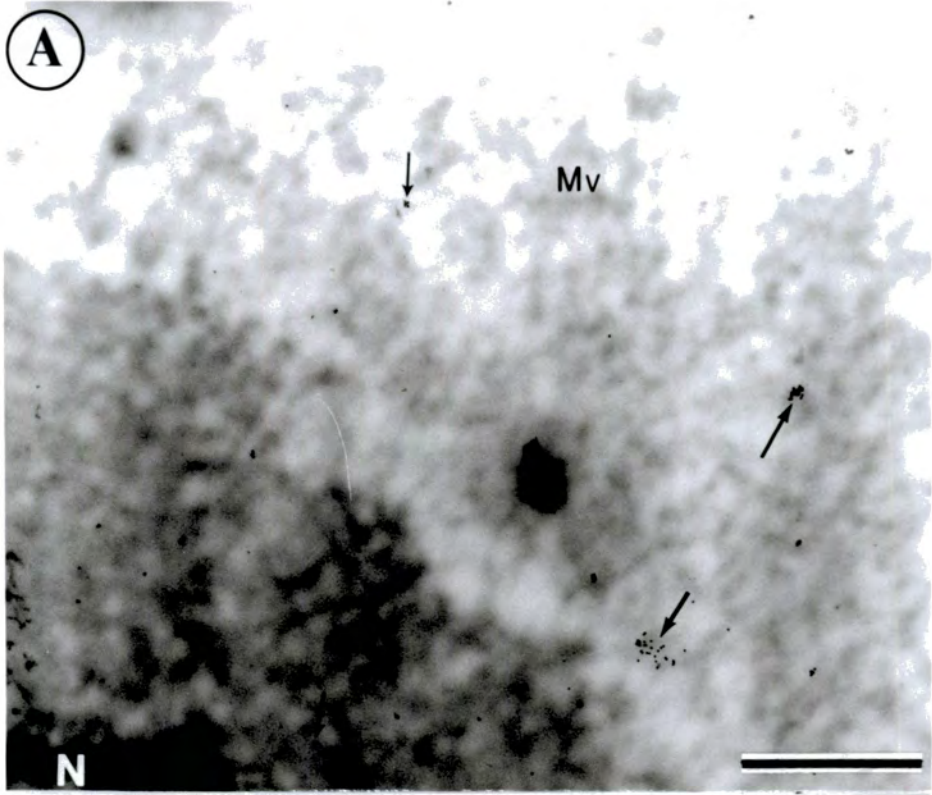
A: Electron micrographs showing immunogold localization of V-type ATPase in Malpighian tubules of *Locusta migratoria*. The sections were labelled with primary monoclonal antibody 230-3, to midgut V-type ATPase of *Manduca sexta*. The secondary antibody, GAM-IgG, was coupled to 15-nm colloidal gold. The microvilli (MV) of the Malpighian tubules cell are intensely stained by 230-3 (small arrows).

*Scale* = 2 $\mu$ m.

B: Higher magnification of part of the microvillar border.

*Scale* = 0.5 $\mu$ m.

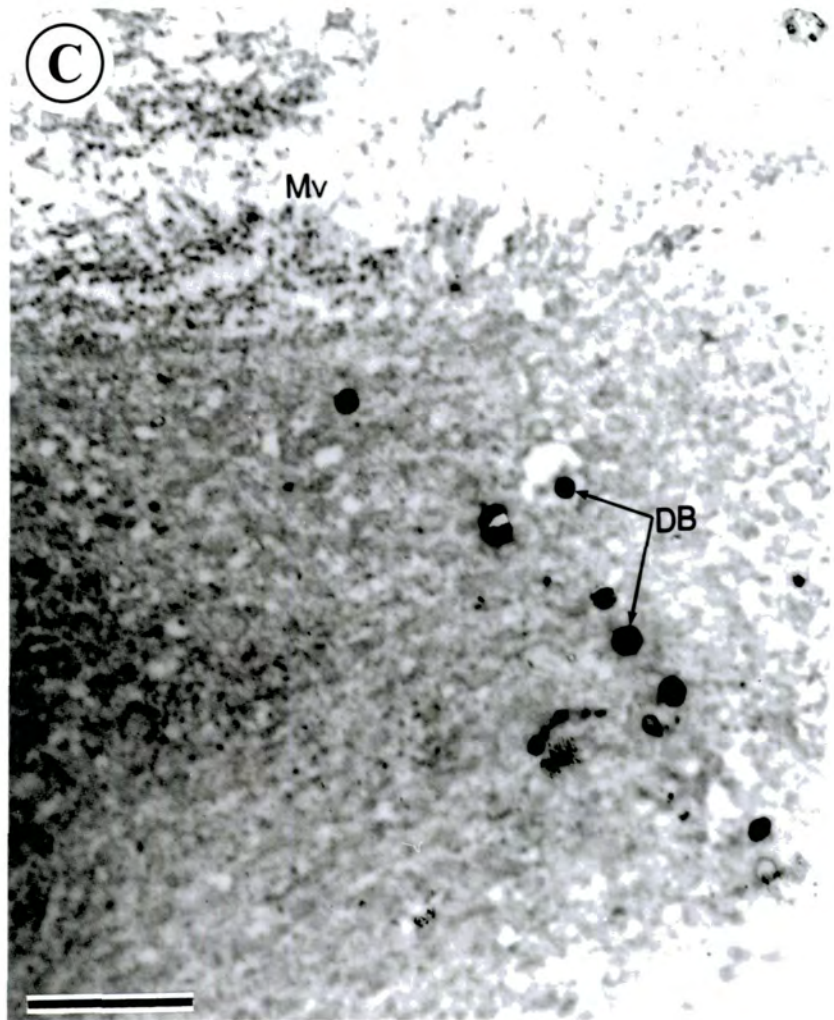
N: Nucleus.



**Plate 6.5C**

Electron micrograph showing the control of immunogold localization of V-type ATPase. The primary monoclonal antibody 230-3 was replaced with buffer contained (0.1% BSA/PBS buffer, pH, 7.4) and the secondary antibody, GAM-IgG. Note that there is no specific staining at apical region of the Malpighian tubules cell. A dense bodies (BD) were present.

*Scale* = 10 $\mu$ m.



**Plate 6.6A and B**

**A:** Electron micrographs showing immunogold localization of  $\text{Na}^+/\text{K}^+$ -ATPase in Malpighian tubules of *Locusta migratoria*. The sections were labelled with primary monoclonal antibody IgG  $\alpha$  5 to  $\alpha$ -subunit of avian  $\text{Na}^+/\text{K}^+$ -ATPase. The secondary antibody, GAM-IgG, was coupled to 15-nm colloidal gold. Note that the immunoreactivity is found on the basal part of Malpighian tubules cell (small arrows).

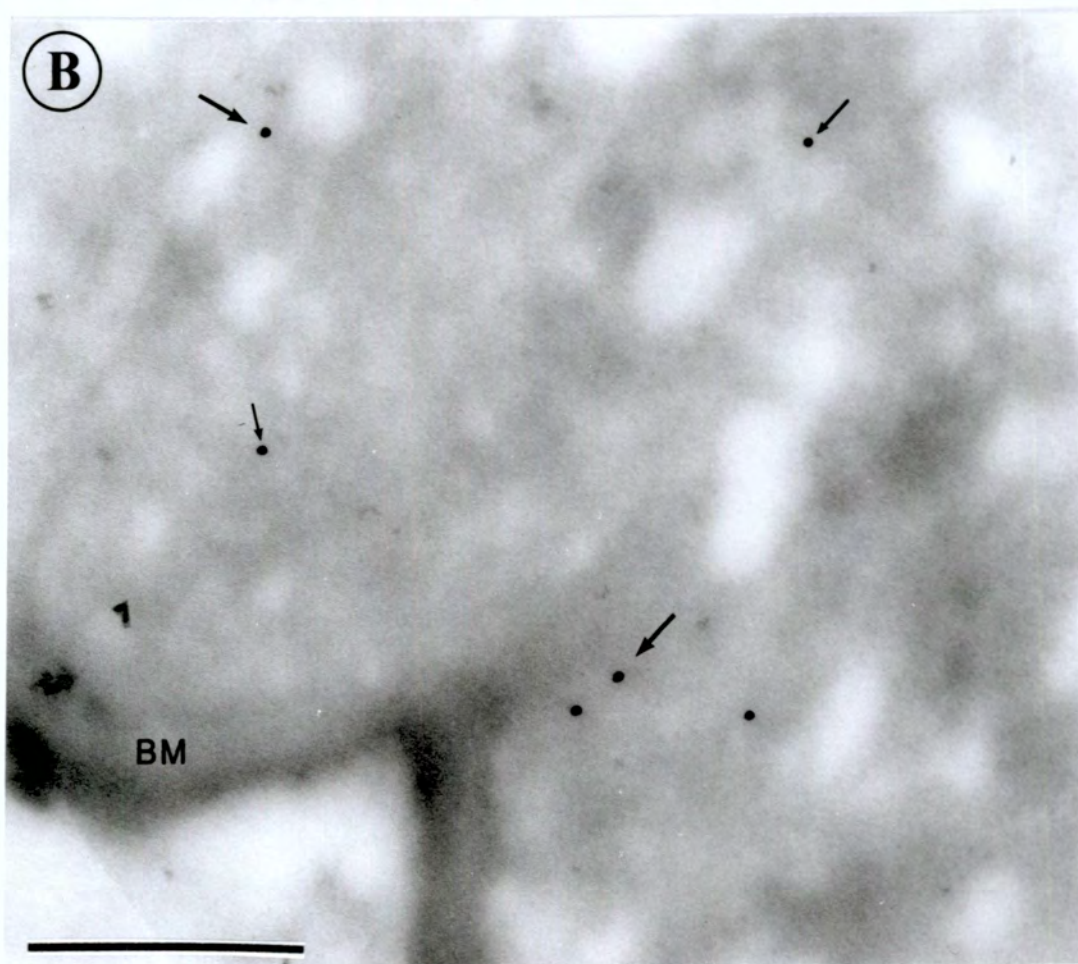
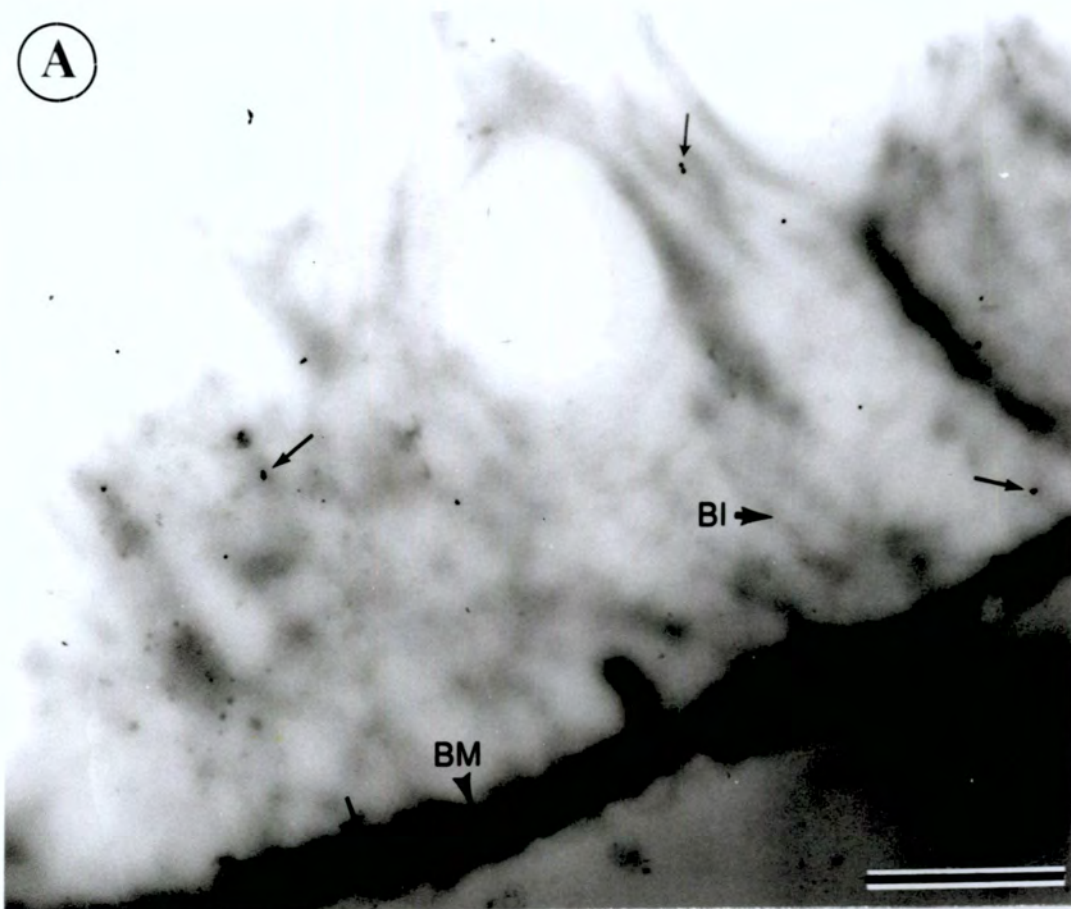
*Scale* = 1  $\mu\text{m}$ .

**B:** Higher magnification of part of the basal region.

*Scale* = 0.5  $\mu\text{m}$ .

BM: Basal membrane.

BI: Basal infoldings.



**Plate 6.6C**

Electron micrograph showing the control of immunogold localization of Na<sup>+</sup>/K<sup>+</sup>-ATPase. The primary monoclonal antibody was replaced with buffer contained (0.1% BSA/PBS buffer, pH, 7.4) and the secondary antibody, GAM-IgG. Note that there is no specific staining at basal region of the Malpighian tubules cell.

*Scale* = 0.5μm.

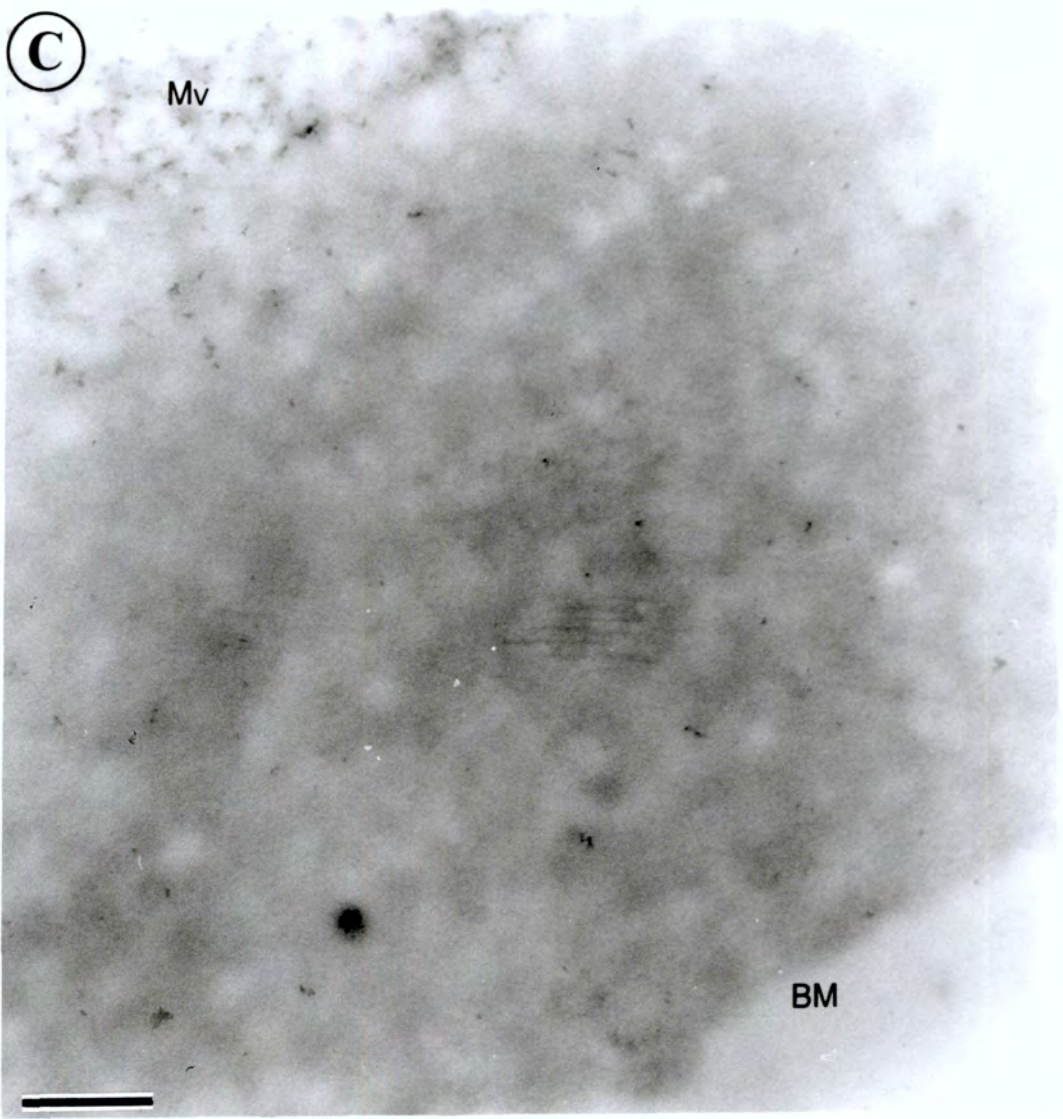
BM: Basal membrane.

Mv: Microvilli.

©

Mv

BM



DISCUSSION

In the present study the apical membrane-enriched fraction was subjected to native microgradient gel electrophoresis. Gels were incubated with two different substrates. When the substrate used was  $\beta$ -glycerophosphate only one band resulted; this band could be attributed to alkaline phosphatase activity. However, when the gel was incubated with ATP, as a substrate, two bands became visible. The upper band of ATPase activity was inhibited by the specific V-type ATPase inhibitors (bafilomycin A<sub>1</sub> and NEM) but was insensitive to azide or orthovanadate suggesting that this band represented sites of V-type ATPase activity. These findings are consistent with the clear biochemical evidence (see chapter 5) which demonstrated the presence of V-type ATPase activity in the apical membrane-enriched pellets prepared from Malpighian tubules of *Locusta migratoria*. This activity was associated with the fraction containing mainly apical plasma membranes and inhibited by bafilomycin A<sub>1</sub> and NEM, whilst, it was insensitive to azide or orthovanadate. Similar results have been reported by Schweickl, *et al.*, (1989) when NEM inhibited two bands of ATPase activities in the native microgradient PAGE preparations of *Manduca sexta* midgut.

Further support for a V-type ATPase being located on the apical membrane was provided by probing a Western blot of apical membrane ATPases with V-type ATPase monoclonal antibody. A Western blot of the native microgradient gel, was probed with monoclonal antibody 230-3 (raised against 28kDa subunit midgut V-type ATPase of *Manduca sexta*), recognized a protein present in the apical membrane fraction corresponding to the band exhibiting V-type ATPase activity as revealed by lead capture studies. Therefore, it can be concluded that the 28kDa subunit of midgut

V-type ATPase of *Manduca sexta* has a molecular weight similar to that present in the apical membrane V-type ATPase of Malpighian tubules of *Locusta migratoria*. The localization of this molecule on the apical membrane of the Malpighian tubules has previously been confirmed by fluorescent labelling immunocytochemistry in *Manduca sexta* midgut (Russell *et al.*, 1992); Malpighian tubules of *Formica polyctena* (Garayoa *et al.*, 1995); *Heliothis virescens* midgut and Malpighian tubules (Pietrantonio and Gill, 1995). Just and Walz (1994) using fluorescence labeling localized an H<sup>+</sup>-pump to 10nm-particles on the cytoplasmic surface of microvilli in the salivary glands of the cockroach *Periplaneta americana*.

In Malpighian tubules of *Locusta migratoria*, Marshall (1995) produced fluorescent labeling of V-type ATPase at the apical surface. V-type ATPases are known to have high homology since polyclonal antibodies raised against subunits of plant and mammalian V-type ATPases recognized subunits of *Manduca sexta* midgut ATPase (Russell *et al.*, 1992). Monoclonal antibodies which had been raised to different subunits of the V-type ATPase present in *Manduca sexta* midgut were used to probe different membrane fractions of the Malpighian tubules of *Locusta migratoria*, (Marshall 1995). This worker found that antibody 230-3 raised to the 28kDa subunit of the V-type ATPase from the midgut of *Manduca sexta*, recognized the same subunit in the Malpighian tubules of *Locusta*, but, antibodies 221-67, 224-3 and 90-7 raised to the 67, both 56 and 20, and 28kDa subunits of the V-type ATPase in the midgut of *Manduca sexta*, respectively, did not cross react with the V-type ATPase from *Locusta* Malpighian tubules. This lack of recognition could be due to the epitope (the antigenic region of the *Manduca sexta* protein subunit) not being conserved between the corresponding subunits of the V-type ATPase in the Malpighian tubules of *Locusta*.

The 28kDa subunit is commonly acknowledged as part of the V-type ATPase (Graf *et al.*, 1994a), hence, localization of this subunit on the apical surface of the Malpighian tubules of *Locusta* supports biochemical, structural and physiological evidence that a V-type ATPase activity is present in the tubules. Zheng *et al.*, (1992) found that the 28kDa subunit of *Manduca sexta* midgut contained a nucleotide binding site, however, this could be a non-catalytic binding site as has been shown previously (Moriyama and Nelson, 1987). Graf *et al.*, (1994a) suggested that this subunit may be involved in membrane targeting. Recent discovery of a novel 14kDa subunit in *Manduca sexta* midgut (Graf *et al.*, 1994b) led to speculation that this was a unique part of plasma membrane V-type ATPase and not endomembrane V-type ATPase so perhaps this is how the enzyme is targeted.

More recent work by Sumner *et al.*, (1995) demonstrated that V-type ATPase activity could be regulated by the dissociation of the peripheral  $V_1$  subunits from the ATPase, ( $V_1$  comprising of at least subunits A, B and E) as it was found that during a larval/larval moult, the midgut of *Manduca sexta* displayed reduced  $K^+$  transport, which corresponded to the loss of the  $V_1$  subunits from the polypeptide. Recently, Lepier *et al.*, (1996) provided an evidence that the tobacco hornworm V-ATPase contains a novel 13-kDa subunit which as a member of the peripheral  $V_1$  complex. Since the 14-kDa subunit first cloned from *Manduca sexta* (Graf *et al.*, 1994b) has already been designated as subunit F (Nelson *et al.*, 1994), the 13-kDa subunit has been suggested as subunit G (Lepier *et al.*, 1996). Other study (Pietrantonio and Gill, 1995) showed for the first time the immunolocalization of the  $V_o$  sector component or the 17 kDa subunit c in the midgut goblet cell apical membrane and in the Malpighian tubule apical membrane of lepidopteran larvae. These labeling patterns in tissue

sections of *Heliothis virescens* and *Manduca sexta* obtained with the anti-peptide antibody and with the anti-B subunit antiserum are similar to those shown with monoclonal antibodies against the midgut V-type ATPase V<sub>1</sub> sector in *Manduca sexta* midgut and Malpighian tubules by Klein *et al.*, (1991).

*Manduca sexta* goblet cell plasma membrane V-type ATPase is multi-subunit enzyme and similar to that reported from plant, fungi and other animal species. Other laboratories have reported, from studies using native gels, a holoenzyme size of 600-900 kDa, and denaturing SDS-PAGE analysis revealed that the V-ATPase composed of at least ten polypeptides of subunits of 67, 57, 40, 43, 28, 20, 17, 16, 14 and 13 kDa. In addition, there appear to be glycoproteins of 40 and 20 kDa. (Wieczorek, 1992; Dow, 1994; Graf *et al.*, 1992; 1994a,b; Pannabecker, 1995; Pietrantonio and Gill, 1995; Lepier *et al.*, 1996). It has been reported by Forgac (1989), that all V-type ATPases share at least three common subunits; 70kDa, 60kDa and 17kDa. Subunits A, B and E, which represent the 70kDa 60kDa and 28kDa polypeptides, respectively, have been cloned and sequenced from *Manduca sexta* midgut and these all show a high sequence homology to subunits from other sources (Graf *et al.*, 1992; 1994a,b; Novak *et al.*, 1992; Pietrantonio and Gill, 1995; Lepier *et al.*, 1996). The function of the different subunits is still being investigated but it has been proposed that subunit A, which has been shown to contain ATP and NEM binding sites, is the catalytic subunit and subunit B has a regulatory function (Nelson, 1989). The V-type ATPase present in *Manduca sexta* midgut also contains a proteolipid subunit of 16kDa, Dow *et al.*, (1992), cloned and sequenced this subunit from the tubules and midgut of *Manduca sexta* and found a high sequence homology to other 16kDa subunits. It is believed that

six of these subunits constitute the proton pore (Dow *et al.*, 1992; Nelson 1989; Forgac, 1989).

In the present study, electron microscopy immunogold labeling with monoclonal antibody 230-3 (specific for the 28kDa subunit of midgut V-type ATPase of *Manduca sexta*), localized V-type ATPase to the microvilli of Malpighian tubules of *Locusta migratoria*, providing strong evidence that a V-type ATPase is localized at the apical plasma membrane. However, the fixation protocol resulted in relatively poor ultrastructural preservation of the tissue with only sparse and patchy immunogold labeling. Other studies have been produced a similar result for example, a V-type ATPase has been localized in *Manduca sexta* midgut (Klein *et al.*, 1991; Klein *et al.*, 1992; Russell *et al.*, 1992); in sensilla epithelia (Klein and Zimmermann, 1991) and in salivary glands of the cockroach, *Periplaneta americana* (Just and Walz, 1994). Studies on vertebrate, (Brown *et al.*, 1987) using immunogold labeling were also able to localize an H<sup>+</sup>-pump to particles studding toad urinary bladder.

The ultrastructure of the apical surface of the Malpighian tubules as revealed by electron microscopy, identified structures resembling portosomes on the cytoplasmic membrane of the microvilli. These particles stud the membranes of many ion transporting epithelia. They were first reported by Gupta and Berridge (1966) in rectal papillae of blowfly and since have been found in salivary gland of *Calliphora* (Berridge and Oschman, 1972); larval Malpighian tubules of *Aedes taeniorhynchus* (Bradley *et al.*, 1982); sensilla of the fly *Protophormia terraenovae* (Wieczorek, 1982) and the midgut of *Manduca sexta* (Klein *et al.*, 1991).

The ATPase of the inner mitochondrial membrane is located in spheres called  $F_1$  particles which are anchored to the membrane by  $F_0$  units (responsible for proton conduction). The main function of this class of ATPases is to couple the downhill movement of protons to ATP synthesis (Forgac, 1989). The similarity in the structure of the  $F_1$  particles of mitochondria and the portosomes of ion translocating epithelia lead to the suggestion that they were involved in  $K^+$  transport (Anderson and Harvey, 1966) and subsequently the site of a V-type ATPase (Klein *et al.*, 1991). Since then studies have been carried out on the molecular structure of F-type and V-type ATPases (Forgac, 1989; Nelson, 1989) and these show that both ATPases are multi-subunit complexes with similar molecular masses. Support for the argument that these portosomes are the site of ion transport comes from evidence that particles of a similar appearance have been discovered on the cytoplasmic side of membranes known to have a V-type ATPase. These include fungal vacuoles: Bowman *et al.*, (1989) examined the vacuolar membranes of *Neurospora crassa* and found ball and stalk structures similar in size but different in shape to the  $F_1F_0$  particles. Treatment with nitrate removed the ball and stalk structures from the vacuolar membranes but had no effect on mitochondrial membranes. A similar study was carried out by Morre *et al.*, (1991) on plant tonoplasts (vacuolar membrane) of soybean and the same results were found. Stadler and Tsukita (1984) investigating the synaptic vesicles of guinea pig brain showed that the vesicles contained an ATPase which was inhibited by DCCD. They also showed that the vesicles, which had "knob-like" protrusions on their surface, could be acidified.

In the present study, when monoclonal antibody IgG  $\alpha$  5 used in immunogold electron microscopy showed a specific binding to an epitope only on the basal

membrane of Malpighian tubules. Monoclonal antibody IgG  $\alpha$  5 has been raised against the catalytic  $\alpha$ -subunit of the chicken sodium pump (Takeyasu *et al.*, 1988; 1989; Lemas *et al.*, 1992) where it recognizes all three isoforms ( $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ). The antibody cross-reacts with the  $\alpha$ -subunits of  $\text{Na}^+/\text{K}^+$ -ATPases of other invertebrate epithelia (*Drosophila melanogaster*, Lebovitz *et al.*, 1989; *Apis mellifera*, Baumann and Takeyasu 1993; *Calliphora erythrocephala*, Baumann *et al.*, 1993; *Periplaneta americana*, Just and Walz, 1994). In *Drosophila*, only one gene for the  $\text{Na}^+/\text{K}^+$ -ATPase has been found and IgG  $\alpha$ -5 binds specifically to the product of this gene (Lebovitz *et al.*, 1989). The results in the present study confirm the membrane separation techniques in conjunction with electron microscopy studies (see chapter 4) which demonstrated the presence of sodium pump at the basal membrane of Malpighian tubules of *Locusta migratoria*.  $\text{Na}^+/\text{K}^+$ -ATPase activity has previously been reported in this tissue (Anstee and Bell, 1975; 1978; Anstee and Bowler, 1984; Anstee *et al.*, 1986). Cytochemical localization studies revealed that this enzyme associated with the basal cell membrane of Malpighian tubules of *Locusta migratoria* (Fogg *et al.*, 1992; Kalule-Sabiti, 1985). Indeed, localization of  $\text{Na}^+/\text{K}^+$ -ATPase has been reported on basolateral and basal cell membrane infoldings in several secreting epithelia, e.g., (avian salt gland, Ernst, 1972; alimentary tract of *Locusta migratoria*, Peacock, 1976; rectum of *Aeshna cyanea* larvae, Komnick and Achenbach, 1979).

In conclusion, the present study emphasizes the evidence produced by earlier studies which reported that monoclonal antibody 230-3, raised to the V-type ATPase from *Manduca sexta* midgut, was found to cross react with a protein only present in apical enriched membrane preparations of Malpighian tubules of *Locusta*. The apical

localization of the V-type ATPase was confirmed using immunocytochemical and immunogold electron microscopy.

## CHAPTER 7

### GENERAL DISCUSSION

A number of researchers have produced evidence which supports V-type ATPase activity being involved in ion and fluid transport across insect Malpighian tubules, e.g. in *Drosophila hydei*, (Bertram *et al.*, 1991); *Drosophila melanogaster*, (Dow *et al.*, 1994b); *Formica polyctena*, (Weltens *et al.*, 1992; Dijkstra *et al.*, 1994b) and *Manduca sexta*, (Klein *et al.*, 1991; Klein, 1992; Zeiske, 1992). However, the present study demonstrated biochemically V-type ATPase like activity in apical membrane-enriched fractions of Malpighian tubules. Other researchers have biochemically characterized V-type ATPase activity in other epithelia (Wieczorek *et al.*, 1986; Schweikl *et al.*, 1989), as well as by other experimental approaches (Just and Walz, 1994; Schirmanns and Zeiske, 1994). Wieczorek *et al.*, (1989) proposed that the V-type ATPase is involved in the mechanisms of ion and fluid secretion in transporting epithelia, by forming a component of the apical membrane electrogenic cation pump. It is suggested that the V-type ATPase produces a  $H^+$  gradient which energizes an apical antiporter which is responsible for the extrusion of  $K^+$  (Wieczorek *et al.*, 1989; 1991). The present study has found that *Locusta migratoria* secretes a  $K^+$ -rich "urine".  $K^+$  is known to be actively transported across the tubule cells, the transport from cell to lumen being by an electrogenic  $K^+$  pump. It is tempting to suggest that, on the basis of the evidence presented here, that the V-type ATPase is involved in a similar capacity in the Malpighian tubules of *Locusta migratoria*, i.e. energizing apical  $K^+$  extrusion. The presence of a V-type ATPase in Malpighian tubules replaces the idea of a common cation pump, unique to insect tissues, with a universal mechanism for energization (Wieczorek *et al.*, 1989; Dow, 1994).

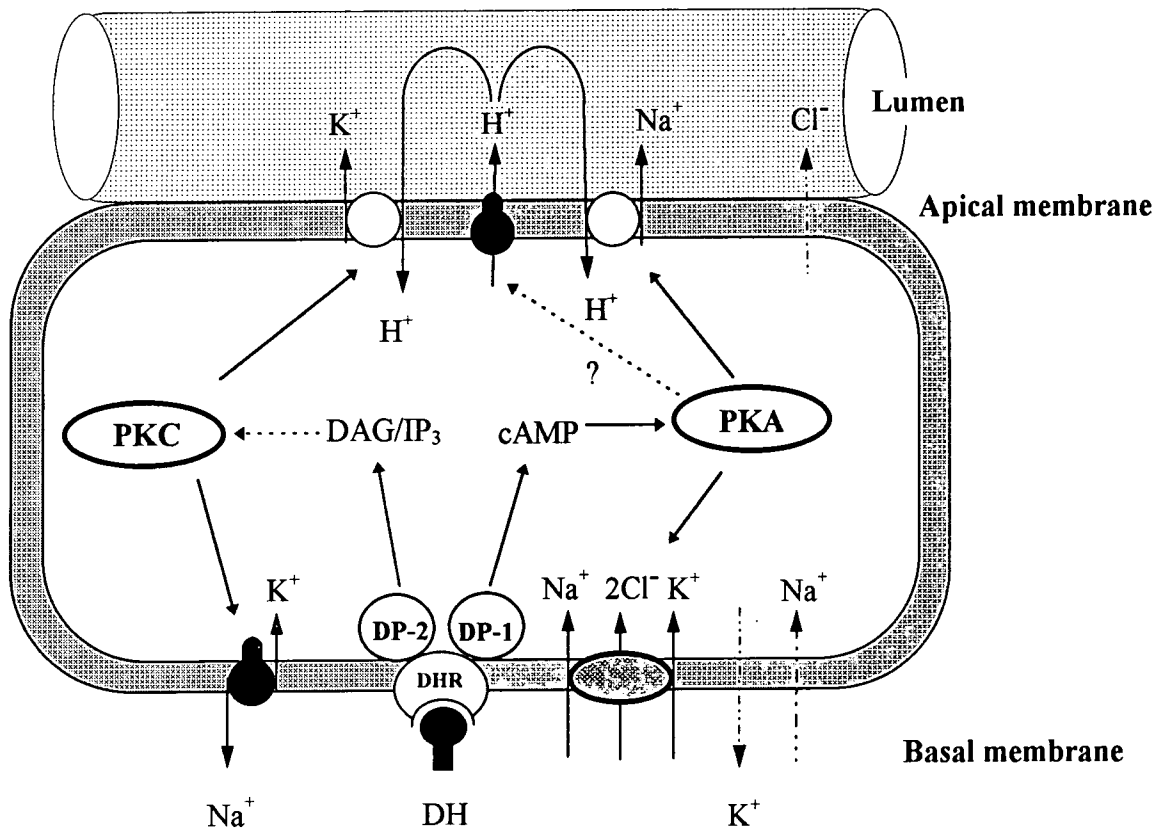
The present study has produced evidence (Chapter 5) for the existence of both an  $\text{HCO}_3^-$ -stimulated ATPase and V-type ATPase activities on the apical membrane. The V-type ATPase activity displayed some characteristic features of the  $\text{HCO}_3^-$ -stimulated ATPase; it was stimulated by a number of different anions and was inhibited by thiocyanate and nitrate. However, although these features suggest that the two enzymes represent the same entity, the  $\text{HCO}_3^-$ -stimulated ATPase was not sensitive to NEM and bafilomycin  $\text{A}_1$  at levels that V-type ATPase activity was affected. Nevertheless, it is possible that a component of the  $\text{HCO}_3^-$ -stimulated ATPase preparation is bafilomycin  $\text{A}_1$ -sensitive at concentrations specific for V-type ATPases and therefore can be ascribed to V-type ATPase activity. Fogg *et al.*, (1991) proposed that the active extrusion of  $\text{K}^+$  across the apical membrane of the Malpighian tubules of *Locusta migratoria* could be carried out by an anion-stimulated V-type ATPase situated on this membrane. However, that proposal was speculative.

Basal  $\text{Na}^+/\text{K}^+$ -ATPase activity was demonstrated using immunogold techniques and membrane separation methods in conjunction with biochemical studies. Ouabain inhibition of fluid secretion, accompanied by increased and decreased intracellular levels of  $\text{Na}^+$  and  $\text{K}^+$  respectively (Pivovarova *et al.*, 1994b) and increased levels of  $\text{Na}^+$  in the "urine" (Marshall, 1995) indicated a role for this enzyme in fluid secretion. Other related studies support the existence of a  $\text{Na}^+/\text{K}^+$ -ATPase in the Malpighian tubules of *Locusta migratoria*, (Anstee and Bell, 1975; 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986; Fogg, 1990; Fogg *et al.*, 1991). Ouabain-binding studies on this tissue revealed substantial  $\text{K}^+$  transport could be carried out by the  $\text{Na}^+/\text{K}^+$ -ATPase (Anstee *et al.*, 1986). Hence it is proposed that the basal  $\text{Na}^+/\text{K}^+$ -ATPase is responsible or at least in part, for  $\text{K}^+$  entry into the cell, in exchange for  $\text{Na}^+$ , thus, maintaining the  $\text{Na}^+$

gradient. This function for the  $\text{Na}^+/\text{K}^+$ -ATPase had previously been suggested for the Malpighian tubules of *Locusta migratoria* by Fogg (1990) and Baldrick *et al.*, (1988) and also for the tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). The  $\text{Na}^+$  gradient established by the  $\text{Na}^+/\text{K}^+$ -ATPase can be used to drive the basal co-transporter.

The information obtained in the present study (Chapter 3 and Chapter 5) was used to construct a hypothetical model which seeks to explain the mechanisms of ion and fluid movements across the Malpighian tubules of *Locusta migratoria*. Figure 7.1 represents this model which is based upon that information generated in the present study and in previous studies carried out in this laboratory and elsewhere. It is suggested that the basal membrane possesses a classical  $\text{Na}^+/\text{K}^+$ -ATPase (Baldrick *et al.*, 1988) and a furosemide-sensitive ( $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ ) co-transporter, in addition to hormone receptors and anomalous rectified  $\text{K}^+$  channels (Hyde *et al.*, 1997). The apical membrane is considered to possess a V-type ATPase (Beyenbach, 1995) that creates a proton gradient that drives a  $\text{H}^+/\text{Me}^+$  co-transporter responsible for the secretion of  $\text{K}^+$  and  $\text{Na}^+$  into the lumen (O'Donnell *et al.*, 1996). The fact that the protein kinase C inhibitors, chelerythrine and staurosporine, the protein kinase A inhibitor, Rp-cAMP, and the protein phosphatase 1 and 2A, okadaic acid, inhibit fluid secretion suggests that protein kinases and protein phosphatases, change the phosphorylated status of transport proteins at the basal or apical surfaces or both and are therefore important in the regulation/control of tubule function.

A number of workers (Prince and Berridge, 1972; Berridge and Prince, 1972a,b; Berridge *et al.*, 1975b; Maddrell, 1980) have proposed models for the endocrine control of ion translocation by epithelial cells in insects in which cAMP acts as intracellular second messenger. Fogg *et al.*, (1989; 1990) found that depolarization of  $V_A$  was



**Figure 7.1**

A schematic diagram of the model proposed to explain anion and cation transport across the cells of the Malpighian tubules of *Locusta migratoria* and their endocrine control. In this model, the basal membrane faces the bathing medium, whilst the apical membrane faces the lumen.

In this model, it is proposed that a basal ( $\text{Na}^+/\text{K}^+$ )-ATPase maintains the cellular gradients for  $\text{Na}^+$  (O'Donnell and Maddrell, 1984; Baldrick *et al.*, 1988). It is proposed that passive exit of  $\text{K}^+$ , along its concentration gradient, and passive entry of  $\text{Na}^+$ , along its concentration and electrical gradient, occurs across the basal membrane. It would appear that passive  $\text{Cl}^-$  entry, along its concentration and electrical gradients, only occurs on elevation of external  $[\text{K}^+]$  (O'Grady *et al.*, 1987).  $\text{K}^+$  entry across the basal membrane seems to be by the action of the ( $\text{Na}^+/\text{K}^+$ )-ATPase (Anstee *et al.*, 1986).  $\text{Cl}^-$  and  $\text{K}^+$  may enter via the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter in a process driven by the  $\text{Na}^+$  gradient.  $\text{Cl}^-$  entry may be by  $\text{Cl}^-/\text{HCO}_3^-$  exchange and by the action of a ( $\text{Cl}^-/\text{HCO}_3^-$ )-ATPase both aided by increases in intracellular pH (Hanrahan and Phillips, 1983).

At the apical surface  $\text{K}^+$  and  $\text{Na}^+$  exit is postulated to occur via separate antiporters which are energized by a V-type ATPase (Wieczorek *et al.*, 1989; 1991; Marshall, 1995). The exit of  $\text{Cl}^-$  occurs passively down a favourable electrical gradient (Morgan and Mordue, 1983; Baldrick *et al.*, 1988).

It is suggested that diuretic hormone (DH), appears to interact with a basal membrane receptors DP-1 and DP-2, resulting in the elevation in levels of the intracellular second messengers, cAMP (Fogg *et al.*, 1989) and DAG/ $\text{IP}_3$ , respectively. cAMP, in turn, stimulates PKA which acts via the apical  $\text{Na}^+$  electrogenic cation pump and also may stimulate the basal  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  co-transporter (Hegarty *et al.*, 1991). PKC acts via the apical  $\text{K}^+/\text{H}^+$  antiporter and may also be involved in the mechanism of  $\text{Na}^+/\text{K}^+$ -ATPase regulation at the basal membrane (Bertorello *et al.*, 1991). These suggestions are consistent with the V-type ATPase requiring activation via PKA and PKC to maintain the  $\text{H}^+$  gradient.

effected by CC extract in control saline, but not by cAMP. Similarly, in the present study, CC extract effected changes in the  $[K^+]$  and  $[Na^+]$  in "urine" secreted, whilst cAMP did not. These observations suggested that diuretic factors in CC extract may exert a control epithelial  $Na^+$  and  $K^+$  transport in Malpighian tubules of *Locusta migratoria*. In addition, it would be consistent with findings of previous studies (Fogg *et al.*, 1989; 1990) to suggest that the different responses to CC extract, and cAMP indicated that cAMP alone cannot mediate the full effects of DH in Malpighian tubules of *Locusta migratoria*. Fogg *et al.*, (1990) suggested that some component of CC extract, in addition to controlling cation movements, is modifying  $Cl^-$  movement across both the basal and apical membranes and increased anion conductance may be independent of cAMP.

Fluid secretion by *Locusta* Malpighian tubules is controlled by two diuretic peptides (DP-1 and DP-2), derived from the corpora cardiaca which act at different receptor sites (Morgan and Mordue, 1987). DP-1 acts at a receptor which stimulates the adenylate cyclase/cAMP signaling pathway (Morgan and Mordue, 1985 and Coast *et al.*, 1993). DP-2 is suggested to act via the phosphoinositide/ $Ca^{2+}$  signaling pathway (Figure 7.1), Fogg *et al.*, (1990) have demonstrated that  $IP_3$  levels are also raised in tubule cells stimulated by CC extract. Additionally, Kay *et al.*, (1991) have demonstrated that a diuretic peptide (*Locusta*-DP) isolated from locust heads was also present in the corpora cardiaca and discovered that a synthetic analogue of this peptide stimulated cAMP production. More recently Coast *et al.*, (1993) have also shown that *Locusta*-DP increases the rate of fluid secretion by the tubules of *Locusta migratoria* and increases the levels of cAMP

The various effects of CC extract, cAMP alone and in the presence of PKC and PKA inhibitors on the apical surface can be best explained if  $Na^+$  and  $K^+$  exit the cell via different antiporters. The data presented are consistent with PKA activation of a  $Na^+/H^+$

antiporter and PKC activation of a  $K^+/H^+$  antiporter. The ability of CC extract to stimulate fluid production in the presence of PKC but not PKA inhibitors again emphasizes that at least two arms of the signalling pathway are essential. These data are consistent with the V-type ATPase requiring activation via PKA to maintain the  $H^+$  gradient without which  $Na^+$  and  $K^+$  extrusion via the co-transporter(s), which depend on that gradient (Zeiske, 1992) will slow progressively.

Little is known of the regulation of the function of the apical V-type ATPase or the putative apical  $H^+/Me^+$  counter-transporter but this too may involve protein phosphorylation. Indeed, Weinman and Shenolikar (1986), and Weinman *et al.*, (1988) have demonstrated that the renal tubule apical membrane  $Na^+/H^+$  exchanger is stimulated by PKC. The use of inhibitors of both PKC and PKA activity could provide useful further insight into the control of fluid secretion by Malpighian tubule cells.

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# APPENDICES

## APPENDIX (1)

### Appendix 1.1

#### Determination of IC<sub>50</sub> and 95% confidence limits:

##### Notation:

$y_i \dots y_n$  denotes available probits,

$x_i \dots x_n$  denotes corresponding effect of inhibitors.

##### Calculate:

$$\bar{x} = (\sum x)/n \quad \text{and} \quad \bar{y} = (\sum y)/n$$

$$S_{xx} = (\sum x^2) - \frac{(\sum x)^2}{n} \quad S_{yy} = (\sum y^2) - \frac{(\sum y)^2}{n} \quad S_{xy} = (\sum xy) - \frac{(\sum x)(\sum y)}{n}$$

##### Procedure:

$$y = a + b(x - \bar{x}),$$

estimated intercept =  $a = \bar{y}$ ,

$$\text{estimated slope} = b = \frac{S_{xy}}{S_{xx}}.$$

$$\text{Median effective inhibitor (PI}_{50}) = m = \bar{x} + \frac{5 - a}{b},$$

95% confidence limits set by  $m \pm \text{s.e. (mean)} \times t$ , where  $t$  is the tabulated student  $t$  value (95%) with  $n-2$  degree of freedom.

$$\text{s.e. of } m = \sqrt{\frac{s^2}{b^2} \left[ \frac{1}{n} + \left( \frac{M - x}{S_{xx}} \right)^2 \right]},$$

$$\text{where } s^2 = \frac{1}{n-2} \left[ S_{yy} - \left( \frac{S_{xy}}{S_{xx}} \right)^2 \right].$$

## APPENDIX (2)

**Table (2.1)**

Effect of different concentrations of chelerythrine on the fluid secretion by Malpighian tubules of *Locusta*.

	<b>Chelerythrine [<math>\mu</math>M]</b>	<b>Rate of fluid secretion nl/min <math>\pm</math> SEM</b>	<b>R2/R1x100 <math>\pm</math> SEM</b>	<b>p</b>	<b>n</b>
Rate 1	0.0	4.6 $\pm$ 0.3	95.4 $\pm$ 0.9	n.s.	30
Rate 2	0.0	4.3 $\pm$ 0.3			
Rate 1	0.0	3.9 $\pm$ 1.0	81.9 $\pm$ 3.5	<0.005	12
Rate 2	0.07	2.9 $\pm$ 0.6			
Rate 1	0.0	3.5 $\pm$ 0.4	72.3 $\pm$ 3.4	<0.001	11
Rate 2	0.1	2.2 $\pm$ 0.3			
Rate 1	0.0	4.8 $\pm$ 0.6	63.5 $\pm$ 3.1	<0.0001	17
Rate 2	1.0	2.9 $\pm$ 0.3			
Rate 1	0.0	4.2 $\pm$ 0.5	44.0 $\pm$ 4.6	<0.0001	7
Rate 2	10	1.6 $\pm$ 0.2			
Rate 1	0.0	4.8 $\pm$ 1.0	30.1 $\pm$ 3.7	<0.0001	7
Rate 2	20	1.1 $\pm$ 0.3			
Rate 1	0.0	4.5 $\pm$ 1.0	20.9 $\pm$ 1.8	<0.0001	6
Rate 2	30	0.6 $\pm$ 0.1			
Rate 1	0.0	4.4 $\pm$ 0.7	16.0 $\pm$ 2.2	<0.0001	7
Rate 2	60	0.7 $\pm$ 0.2			

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

**Table (2.2)**

Effect of different concentrations of Rp-cAMP on the fluid secretion by Malpighian tubules of *Locusta*.

	<b>Rp-cAMP [<math>\mu</math>M]</b>	<b>Rate of fluid secretion nl/min <math>\pm</math> SEM</b>	<b>R2/R1x100 <math>\pm</math> SEM</b>	<b>p</b>	<b>n</b>
Rate 1	0.0	4.9 $\pm$ 0.5	96.6 $\pm$ 2.9	n.s.	19
Rate 2	0.0	4.6 $\pm$ 0.5			
Rate 1	0.0	4.4 $\pm$ 0.4	85.4 $\pm$ 5.6	n.s.	13
Rate 2	1.0	3.8 $\pm$ 0.4			
Rate 1	0.0	4.0 $\pm$ 0.6	74.0 $\pm$ 5.3	<0.001	11
Rate 2	10	2.8 $\pm$ 0.5			
Rate 1	0.0	4.3 $\pm$ 0.5	65.2 $\pm$ 3.8	<0.0001	20
Rate 2	100	2.7 $\pm$ 0.3			
Rate 1	0.0	4.5 $\pm$ 0.5	57.1 $\pm$ 4.0	<0.0001	14
Rate 2	500	2.5 $\pm$ 0.5			
Rate 1	0.0	5.0 $\pm$ 0.8	38.0 $\pm$ 4.9	<0.0001	13
Rate 2	1000	1.8 $\pm$ 0.3			

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

**Table (2.3)**

Effect of different concentrations of NEM on the fluid secretion by Malpighian tubules of *Locusta*.

	NEM [ $\mu$ M]	Rate of fluid secretion nl/min $\pm$ SEM	R2/R1x100 $\pm$ SEM	p	n
Rate 1	0.0	4.2 $\pm$ 0.3	90.5 $\pm$ 1.8	n.s.	42
Rate 2	0.0	3.8 $\pm$ 0.3			
Rate 1	0.0	2.2 $\pm$ 0.3	70.8 $\pm$ 5.6	<0.005	10
Rate 2	0.1	1.5 $\pm$ 0.2			
Rate 1	0.0	4.9 $\pm$ 0.6	59.3 $\pm$ 6.0	<0.0001	8
Rate 2	1.0	2.9 $\pm$ 0.4			
Rate 1	0.0	3.7 $\pm$ 0.5	49.0 $\pm$ 6.2	<0.0001	15
Rate 2	10	1.8 $\pm$ 0.3			
Rate 1	0.0	7.5 $\pm$ 1.8	36.4 $\pm$ 5.0	<0.0001	9
Rate 2	100	2.3 $\pm$ 0.4			
Rate 1	0.0	3.5 $\pm$ 0.5	8.3 $\pm$ 1.0	<0.0001	10
Rate 2	1000	0.28 $\pm$ 0.1			

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

**Table (2.4)**

Effect of different concentrations of okadaic acid on the fluid secretion by Malpighian tubules of *Locusta migratoria*.

	Okadaic acid [nM]	Rate of fluid secretion nl/min $\pm$ SEM	R2/R1x100 $\pm$ SEM	p	n
Rate 1	0.0	4.6 $\pm$ 0.5	93.8 $\pm$ 1.6	n.s.	18
Rate 2	0.0	4.3 $\pm$ 0.5			
Rate 1	0.0	4.5 $\pm$ 0.6	87.2 $\pm$ 3.2	n.s.	9
Rate 2	0.01	3.96 $\pm$ 0.6			
Rate 1	0.0	5.1 $\pm$ 0.7	84.6 $\pm$ 4.3	n.s.	9
Rate 2	0.1	4.4 $\pm$ 0.6			
Rate 1	0.0	5.3 $\pm$ 0.6	79.2 $\pm$ 2.9	<0.01	10
Rate 2	0.5	4.2 $\pm$ 0.5			
Rate 1	0.0	4.5 $\pm$ 0.9	75.2 $\pm$ 2.7	<0.005	9
Rate 2	1.0	3.2 $\pm$ 0.5			
Rate 1	0.0	5.3 $\pm$ 0.7	70.0 $\pm$ 3.4	<0.002	9
Rate 2	5.0	3.7 $\pm$ 0.5			
Rate 1	0.0	4.0 $\pm$ 0.3	65.3 $\pm$ 2.6	<0.0001	7
Rate 2	10	2.6 $\pm$ 0.2			
Rate 1	0.0	4.4 $\pm$ 0.9	56.3 $\pm$ 4.6	<0.0001	9
Rate 2	50	2.7 $\pm$ 0.7			
Rate 1	0.0	3.8 $\pm$ 0.7	48.0 $\pm$ 3.5	<0.0001	13
Rate 2	100	1.9 $\pm$ 0.5			
Rate 1	0.0	5.7 $\pm$ 0.9	42.0 $\pm$ 4.6	<0.0001	8
Rate 2	500	2.3 $\pm$ 0.4			
Rate 1	0.0	3.2 $\pm$ 0.3	30.5 $\pm$ 2.7	<0.0001	11
Rate 2	1000	1.0 $\pm$ 0.1			

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

**Table (2.5)**

Effect of 1 $\mu$ M chelerythrine on ratio of Na<sup>+</sup>/K<sup>+</sup> concentrations in the secreted fluid by Malpighian tubules of *Locusta migratoria*.

Incubation media	n	Na <sup>+</sup> /K <sup>+</sup> Ratio (Mean $\pm$ S.E.M)	p
Rate1 normal	21	0.34 $\pm$ 0.01	
Rate2 normal	21	0.34 $\pm$ 0.01	n.s.
Rate1 normal	15	0.34 $\pm$ 0.01	
Rate2 + Ch.	15	0.40 $\pm$ 0.01	<0.0001
Rate1 normal	15	0.31 $\pm$ 0.01	
Rate2 + CC	15	0.54 $\pm$ 0.01	<0.0001
Rate1 + Ch.	15	0.38 $\pm$ 0.01	
Rate2 + Ch. + CC	15	0.48 $\pm$ 0.01	<0.0001
Rate1 normal	15	0.33 $\pm$ 0.01	
Rate2 + cAMP	15	0.31 $\pm$ 0.02	n.s.
Rate1 + Ch.	14	0.40 $\pm$ 0.01	
Rate2 + Ch. + cAMP	14	0.43 $\pm$ 0.01	n.s.

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

**Table (2.6)**

Effect of 1 $\mu$ M staurosporine on ratio of Na<sup>+</sup>/K<sup>+</sup> concentrations in the secreted fluid by Malpighian tubules of *Locusta migratoria*.

Incubation media	n	Na <sup>+</sup> /K <sup>+</sup> Ratio (Mean $\pm$ S.E.M)	p
Rate1 normal	21	0.34 $\pm$ 0.01	
Rate2 normal	21	0.34 $\pm$ 0.01	n.s.
Rate1 normal	13	0.33 $\pm$ 0.01	
Rate2 + St.	13	0.38 $\pm$ 0.01	<0.0001
Rate1 normal	15	0.31 $\pm$ 0.01	
Rate2 + CC	15	0.54 $\pm$ 0.01	<0.0001
Rate1 + St.	15	0.38 $\pm$ 0.01	
Rate2 + St. + CC	15	0.47 $\pm$ 0.01	<0.0001
Rate1 normal	15	0.33 $\pm$ 0.01	
Rate2 + cAMP	15	0.31 $\pm$ 0.02	n.s.
Rate1 + St.	19	0.36 $\pm$ 0.01	
Rate2 + St. + cAMP	19	0.37 $\pm$ 0.01	n.s.

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

**Table (2.7)**

Effect of 0.1mM Rp-cAMP on ratio of Na<sup>+</sup>/K<sup>+</sup> concentrations in the secreted fluid by Malpighian tubules of *Locusta migratoria*.

<b>Incubation media</b>	<b>n</b>	<b>Na<sup>+</sup>/K<sup>+</sup> Ratio (Mean ± S.E.M)</b>	<b>p</b>
Rate1 normal	21	0.34 ± 0.01	
Rate2 normal	21	0.34 ± 0.01	n.s.
Rate1 normal	15	0.30 ± 0.01	
Rate2 + Rp.	15	0.19 ± 0.01	<0.0001
Rate1 normal	15	0.31 ± 0.01	
Rate2 + CC	15	0.54 ± 0.01	<0.0001
Rate1 + Rp.	12	0.19 ± 0.01	
Rate2 + Rp. + CC	12	0.28 ± 0.01	<0.0001
Rate1 normal	15	0.33 ± 0.01	
Rate2 + cAMP	15	0.31 ± 0.02	n.s.
Rate1 + Rp.	11	0.19 ± 0.01	
Rate2 + Rp. + cAMP	11	0.28 ± 0.02	<0.0001

p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

### APPENDIX (3)

**Table (3.1)**

ATPases activity of various membrane fractions from Malpighian tubules of *Locusta migratoria*.

Membrane fractions	Mg <sup>2+</sup> -ATPase activity	Total-ATPase activity	Na <sup>+</sup> /K <sup>+</sup> -ATPase activity
Crude	163.4 ± 08.0	230.6 ± 9.3	66.8 ± 4.6
P1	196.7 ± 12.2	262.8 ± 15.6	55.4 ± 9.0
P2	279.4 ± 21.2	346.0 ± 26.6	71.4 ± 6.9
P3	440.7 ± 27.9	572.6 ± 33.2	131.9 ± 11.2
P4	446.5 ± 11.7	781.1 ± 15.4	331.9 ± 21.9
P5	597.4 ± 38.1	629.9 ± 45.7	26.7 ± 5.6
S1	192.3 ± 8.6	250.4 ± 12.7	65.1 ± 7.7
S2	169.6 ± 8.7	206.4 ± 10.5	48.2 ± 5.5
S3	21.8 ± 1.8	22.9 ± 1.9	1.1 ± 0.5
S5	37.5 ± 6.2	38.5 ± 7.2	2.4 ± 0.6

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (3.2)**

Purity of alkaline phosphatase and succinate dehydrogenase enzymes from the various membrane fractions obtained from Malpighian tubules of *Locusta migratoria*.

Membrane fractions	Alkaline phosphatase activity *	Succinate dehydrogenase activity §
Crude	325.4 ± 31.0	123.0 ± 6.0
P1	181.6 ± 12.1	115.4 ± 12.3
P2	466.3 ± 19.4	287.2 ± 29.6
P3	1017.7 ± 38.5	103.2 ± 15.0
P4	2530.1 ± 45.5	133.1 ± 23.4
P5	3063.3 ± 145.5	10.0 ± 1.5
S1	467.4 ± 30.7	113.8 ± 13.2
S2	402.4 ± 93.2	69.4 ± 15.3
S3	36.4 ± 4.1	16.5 ± 2.9
S5	182.0 ± 10.8	0.0

(\*) Activity is expressed as nmoles p-nitrophenol liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

(§) Activity is expressed as nmoles succinate oxidized mg protein<sup>-1</sup> min<sup>-1</sup>.

Each value represents the average for 3 experiments ± S.E.M.

## APPENDIX (4)

**Table (4.1)**

ATPase activity in the basal and apical membrane fractions from Malpighian tubules of *Locusta migratoria*.

Membrane fractions	Mg <sup>2+</sup> -dependent ATPase activity		stimulation due to the presence of NaHCO <sub>3</sub> instead of NaCl.
	+20mM NaCl	+20mM NaHCO <sub>3</sub>	
P4	435.8 ± 11.6	645.0 ± 38.9	178.3 ± 25.5
P5	650.3 ± 33.6	979.6 ± 43.7	282.8 ± 29.7

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 5 experiments ± S.E.M.

**Table (4.2)**

Effect of various inhibitors on ATPases activity of the apical membrane pellet (P<sub>4</sub>)

Inhibitors	Mg <sup>2+</sup> -dependent ATPases activity		stimulation due to the presence of NaHCO <sub>3</sub> instead of NaCl
	+20mM NaCl	+20mM NaHCO <sub>3</sub>	
Control	451.3±15.1	631.4±20.2	187.7±21.6
NaSCN	49.7±4.8	103.6±8.6	54.0±5.3
NEM	424.2±11.0	635.5±29.6	196.7±23.5
Baf.	431.8±18.1	631.7±33.6	191.3±25.4
Azide	216.8±15.9	301.2±13.8	150.6±22.0

Inhibitors concentration:

10mM NaSCN, 1mM NEM, Baf. = 1μM Bafilomycin A<sub>1</sub>, 1mM Azide.

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

Each value represents the average for 3 experiments ± S.E.M.

**Table (4.3)**Effect of various inhibitors on ATPases activity of the apical membrane pellet (P<sub>5</sub>)

Inhibitors	Mg <sup>2+</sup> -dependent ATPases activity		stimulation due to the presence of NaHCO <sub>3</sub> instead of NaCl
	+20mM NaCl	+20mM NaHCO <sub>3</sub>	
Control	719.6±35.2	1019.3±41.9	299.7±24.1
NaSCN	116.9±10.4	156.6±26.5	52.2±10.6
NEM	672.1±35.5	703.7±45.3	145.6±27.3
Baf.	689.8±43.8	743.3±53.0	176.8±35.5
Azide	528.9±36.3	735.7±27.3	300.6±19.1

Inhibitors concentration:

10mM NaSCN, 1mM NEM, Baf. = 1μM Bafilomycin A<sub>1</sub>, 1mM Azide.Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

Each value represents the average for 3 experiments ± S.E.M.

**Table (4.4)**

Effect of pH concentrations on V-type ATPase activity

pH	V-type ATPase activity
6.0	418.1 ± 29.1
6.5	912.0 ± 75.6
7.0	1773.3 ± 78.8
7.5	2153.4 ± 100.4
8.0	2076.8 ± 86.8
8.5	1659.3 ± 79.2
9.0	960.8 ± 79.6
10.0	29.6 ± 9.31

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.

Each value represents the average for 5 experiments ± S.E.M.

**Table (4.5)**

Effect of ATP concentrations on V-type ATPase activity

[ATP] mM	V-type ATPase activity
0.0	0.0
0.01	156.0 ± 25.4
0.05	320.7 ± 17.2
0.1	663.6 ± 38.3
0.5	1319.6 ± 48.6
1.0	1895.5 ± 84.8
2.0	2318.1 ± 76.7
3.0	2469.7 ± 50.2
4.0	2488.8 ± 55.1
5.0	2485.0 ± 48.3
6.0	2477.0 ± 44.2

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 5 experiments ± S.E.M.

**Table (4.6)**Effect of MgCl<sub>2</sub> on V-type ATPase activity

[MgCl <sub>2</sub> ] mM	Specific activity
0.0	0.0
0.01	175.5 ± 17.7
0.1	701.0 ± 66.5
0.5	1240.7 ± 64.5
1.0	1757.3 ± 91.91
2.0	2169.4 ± 83.5
3.0	2296.3 ± 50.0
4.0	2339.5 ± 77.9
5.0	2366.5 ± 72.0
6.0	2328.5 ± 92.7

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 5 experiments ± S.E.M.

**Table (4.7)**

Effect of NEM on V-type ATPase activity

[NEM] $\mu\text{M}$	Specific activity	% Activity remaining
Control	1945.5 $\pm$ 75.5	100.0
0.1	1874.1 $\pm$ 72.5	96.41 $\pm$ 1.1
0.5	1719.8 $\pm$ 117.5	89.91 $\pm$ 2.0
1.0	1013.9 $\pm$ 106.2	59.1 $\pm$ 4.2
5.0	373.4 $\pm$ 55.4	32.0 $\pm$ 2.4
10	172.5 $\pm$ 32.7	14.3 $\pm$ 1.3
50	97.1 $\pm$ 32.0	6.8 $\pm$ 0.7
100	17.6 $\pm$ 3.0	1.7 $\pm$ 0.1
1000	0.0	0.0

Activity is expressed as nmoles  $\text{P}_i$  liberated  $\text{mg protein}^{-1} \text{min}^{-1}$ .Each value represents the average for 5 experiments  $\pm$  S.E.M.**Table (3.8)**

Effect of Bafilomycin on V-type ATPase activity

[Bafilomycin] $\text{nM}$	Specific activity	% Activity remaining
Control	1640.8 $\pm$ 74.0	100.0
0.1	1267.2 $\pm$ 64.2	77.3 $\pm$ 1.3
0.5	852.3 $\pm$ 61.9	51.9 $\pm$ 2.9
1.0	491.6 $\pm$ 76.5	31.4 $\pm$ 2.1
5.0	167.2 $\pm$ 14.4	10.2 $\pm$ 0.6
10	58.9 $\pm$ 12.3	3.6 $\pm$ 0.6
50	40.6 $\pm$ 11.8	2.3 $\pm$ 0.6
100	15.6 $\pm$ 2.40	1.0 $\pm$ 0.1
500	0.0	0.0

Activity is expressed as nmoles  $\text{P}_i$  liberated  $\text{mg protein}^{-1} \text{min}^{-1}$ .Each value represents the average for 5 experiments  $\pm$  S.E.M.**Table (3.9)**

Effect of KCl on V-type ATPase activity

[KCl] $\text{mM}$	Specific activity
0.0	824.0 $\pm$ 23.4
1.0	942.3 $\pm$ 28.0
5.0	1058.7 $\pm$ 21.6
10.0	1201.8 $\pm$ 26.9
20.0	1399.2 $\pm$ 76.7
30.0	1594.5 $\pm$ 114.1
40.0	1501.8 $\pm$ 52.1
60.0	1158.4 $\pm$ 35.7

Activity is expressed as nmoles  $\text{P}_i$  liberated  $\text{mg protein}^{-1} \text{min}^{-1}$ .Each value represents the average for 3 experiments  $\pm$  S.E.M.

**Table (4.10)**

Effect of NaCl on V-type ATPase activity

[NaCl] mM	Specific activity
0.0	777.8 ± 60.1
10.0	1085.0 ± 40.2
20.0	1300.7 ± 68.5
30.0	1461.1 ± 62.9
40.0	1510.4 ± 87.5
50.0	1500.6 ± 71.1
60.0	1451.5 ± 35.9

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.11)**

Effect of LiCl on V-type ATPase activity

[LiCl] mM	Specific activity
0.0	760.3 ± 35.6
10.0	964.7 ± 51.4
20.0	1130.1 ± 40.6
30.0	1165.2 ± 60.1
40.0	1140.5 ± 53.8
50.0	1120.3 ± 61.2
60.0	1098.7 ± 45.0

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.12)**

Effect of Choline Cl on V-type ATPase activity

[Choline Cl] mM	Specific activity
0.0	788.6 ± 64.2
10.0	1020.7 ± 80.9
20.0	1225.0 ± 72.3
30.0	1416.7 ± 55.3
40.0	1478.3 ± 85.7
50.0	1490.5 ± 70.6
60.0	1442.1 ± 49.3

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.13)**

Effect of Tris-HCl on V-type ATPase activity

[Tris] mM	Specific activity
5.0	791.6 ± 68.3
10.0	956.0 ± 70.4
20.0	1311.2 ± 80.2
30.0	1701.3 ± 92.9
40.0	1916.7 ± 110.2
50.0	2008.1 ± 123.6
60.0	2000.4 ± 130.1
70.0	1881.9 ± 121.2
80.0	1650.3 ± 119.7

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.14)**

Effect of RbCl on V-type ATPase activity

[RbCl] mM	Specific activity
0.0	721.3 ± 42.0
10.0	815.1 ± 51.9
20.0	925.6 ± 60.3
30.0	985.3 ± 70.4
40.0	1020.9 ± 30.8
50.0	1010.7 ± 55.8
60.0	985.3 ± 65.2

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.15)**Effect of  $\text{KHCO}_3$  on V-type ATPase activity

[ $\text{KHCO}_3$ ] mM	Specific activity
0.0	601.9 ± 40.2
10.0	829.1 ± 78.91
20.0	1195.8 ± 110.1
30.0	1521.1 ± 135.9
40.0	1810.7 ± 142.1
50.0	2063.1 ± 160.7
60.0	2305.6 ± 151.8
70.0	2388.9 ± 172.1
80.0	2357.1 ± 180.5
100.0	2240.0 ± 163.2

Activity is expressed as nmoles  $\text{P}_i$  liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.16)**Effect of  $\text{NaHCO}_3$  on V-type ATPase activity

[ $\text{NaHCO}_3$ ] mM	Specific activity
0.0	640.2 ± 41.2
10.0	950.3 ± 87.5
20.0	1289.1 ± 110.1
30.0	1611.3 ± 130.5
40.0	1892.6 ± 121.7
50.0	2040.9 ± 140.1
60.0	2187.6 ± 117.8
70.0	2210.3 ± 131.6
80.0	2208.7 ± 121.7
100.0	2166.3 ± 128.1

Activity is expressed as nmoles  $\text{P}_i$  liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.17)**Effect of  $\text{KSO}_4$  on V-type ATPase activity

[ $\text{KSO}_4$ ] mM	Specific activity
0.0	790.3 ± 65.7
10.0	953.8 ± 71.0
20.0	1160.2 ± 50.3
30.0	1210.9 ± 80.5
40.0	1101.4 ± 69.6
50.0	990.6 ± 70.3
60.0	951.1 ± 60.4

Activity is expressed as nmoles  $\text{P}_i$  liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.18)**

Effect of K Gluconate on V-type ATPase activity

[K Gluconate] mM	Specific activity
0.0	642.1 ± 31.2
10.0	765.9 ± 52.3
20.0	850.1 ± 71.4
30.0	921.1 ± 63.7
40.0	942.3 ± 75.9
50.0	931.0 ± 61.2
60.0	895.7 ± 50.1

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.19)**

Effect of KF on V-type ATPase activity

[KF] mM	Specific activity
0.0	772.2 ± 45.7
1.0	805.1 ± 75.3
5.0	880.7 ± 50.9
10.0	949.8 ± 70.1
20.0	1006.3 ± 30.4
30.0	1000.1 ± 25.3

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.20)**

Effect of KBr on V-type ATPase activity

[KBr] mM	Specific activity
0.00	673.8 ± 32.6
10.0	746.3 ± 30.2
20.0	819.6 ± 45.8
30.0	828.6 ± 41.7
40.0	765.8 ± 50.2
50.0	650.3 ± 37.1
60.0	535.7 ± 40.2

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

**Table (4.21)**Effect of KNO<sub>3</sub> on V-type ATPase activity

[KNO <sub>3</sub> ] mM	Specific activity
0.0	801.3 ± 35.8
1.0	565.9 ± 40.1
5.0	379.7 ± 65.8
10.0	301.8 ± 30.9
20.0	235.2 ± 32.1
30.0	228.1 ± 25.3

Activity is expressed as nmoles P<sub>i</sub> liberated mg protein<sup>-1</sup> min<sup>-1</sup>.  
Each value represents the average for 3 experiments ± S.E.M.

