STUDIES ON THE MECHANISMS OF ION AND FLUID SECRETION BY MALPIGHIAN TUBULES OF LOCUSTA MIGRATORIA L.

BY

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Declaration

I hereby declare that the work presented in this document is based on research carried out by me, and that no part has previously been submitted for a degree in this or any other university.

Statement of Copyright

Sarah L. Marshall

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November 1995

To my Mam and Dad

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ABSTRACT

Inhibitors of known transport processes, in conjunction with ion substitution were used in biochemical, physiological and microelectrode studies to investigate the mechanisms underlying ion and fluid secretion across the Malpighian tubules of *Locusta migratoria*.

Immunocytochemical localization and cell fractionation methods used in conjunction with biochemical analyses demonstrate the presence of Na^+/K^+-ATPase activity on the basal cell membranes and HCO_3^--stimulated and V-type ATPase activities on apical cell membranes. The biochemical properties of the HCO_3^--stimulated and V-type ATPase activities are compared and the results discussed.

Specific inhibitors of Na^+/K^+-ATPase and V-type ATPases inhibited fluid secretion whilst cAMP was found to stimulate fluid secretion. The continued secretion of K^+ when tubules were bathed in K^+-free saline was attributed to the presence of mineral concretions shown in ultrastructural studies to be distributed throughout the cytoplasm. The effects of ouabain, NEM and cAMP on the cationic composition of the "urine" were studied. In control saline, ouabain and NEM increased the level of Na^+ in the "urine" whereas cAMP caused an increase in the amount of K^+ secreted. In Rb^+-saline, ouabain caused "urine" levels of Na^+ to increase and levels of Rb^+ to fall whereas cAMP and NEM had no effect on the cationic composition of the "urine".

Ion substitution experiments used in conjunction with intracellular microelectrodes suggest the basal cell membrane contains an inward rectifying K^+ channel. Blocking the basal inward rectifier with Rb^+ drove the potential of this membrane towards the emf of the apical membrane potential. In the presence of Rb^+, bafilomycin A_1, a specific V-type ATPase inhibitor, reduced this potential suggesting the apical membrane potential is produced by a V-type ATPase.

The results are discussed and a hypothetical model is proposed to account for the mechanisms of ion and fluid movement across apical and basal cell surfaces.

GLOSSARY

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumen fraction V
Ca^{2+}-ATPase	Magnesium-dependent, calcium-stimulated adenosine triphosphatase
cAMP	Cyclic adenosine 3', 5'-monophosphate
DCCD	N, N'-dicyclohexylcarbodiimide
DDSA	Dodecenyl succinic anhydride
DDW	Distilled deionized water
DMP	2, 4, 6, tri [dimethylaminomethyl] phenol
DMSO	Methyl sulfoxide
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol bis (β-aminoethyl ether)-N, N'-tetraacetic acid
emf	Electromotive force
FITC	Fluorescein isothiocyanate
HEPES	4-(2-hydroxylethyl)-1-piperazine-ethanesulphonic acid
Imidazole	1, 3-diaza-2,4-cyclopentadiene
Ins-1,4,5-P_3	D-*myo*-inositol 1, 4, 5-trisphosphate
kDa	Kilo Daltons
K_i	Inhibition constant
MEM	Minimum essential medium
MOPS	4-Morpholinopropanesulphonic acid
mV	Millivolts
Na^+/K^+-ATPase	Magnesium-dependent, sodium-potassium-stimulated adenosine triphosphatase

NEM	N-ethylmaleimide
NP	Nitrophenol
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P_i	Inorganic phosphate
ppm	Parts per million
psi	Pounds per square inch
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean/Scanning electron microscope
SITS	4-acetamide-4'-isothiocyano-2 2'-di-sulphonic acid stilbene
TEMED	N, N, N', N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl)aminoethane
Triton X-100	t-Octylphenoxypolyethoxyethanol
Tween 20	Polyoxyethylenesorbitanmonolaureate
V_A	Apical membrane potential
V_B	Basal membrane potential
VDR	Voltage divider ratio
V-type ATPase	Vacuolar, magnesium-dependent, hydrogen-stimulated adenosine triphosphate

CHAPTER 1

INTRODUCTION

The first detailed studies on the mechanisms of ion and fluid transport in insect Malpighian tubules were carried out by Ramsay in the 1950's (Ramsay, 1953, 1954, 1956 and 1958). From these studies he 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 insect species (e.g. *Calliphora vicina* (was *C. erythrocephala*), Berridge, 1968, 1969; *Rhodnius prolixus*, Maddrell, 1969; *Locusta migratoria*, Anstee and Bell 1975, 1978, Anstee *et al.*, 1979; *Glossina austeni*, Gee, 1975; *Glossina morsitans*, Gee, 1976a, b). These studies confirmed that water movements were a consequence of ion movements (Maddrell, 1977) and that active transport of K^+ was the "prime mover" in the formation of "urine" in the majority of insect species studied with the exception of *Rhodnius prolixus* which is able to produce "urine" in the presence of Na^+ or K^+ (Maddrell, 1969) and *Glossina morsitans* in which Na^+ is the "prime mover" (Gee, 1976a, b).

Ramsay originally suggested that the active secretion of K^+ accompanied by an anion might create a high osmotic pressure in the tubule and so cause water to move passively inwards (Ramsay, 1953). However, further studies on *Carausius morosus* (was *Dixippus morosus*) showed secretion of a slightly hypo-osmotic "urine" (Ramsay, 1954). Other insects, e.g. *Dysdercus fasciatus*, also secrete a hypo-osmotic "urine" (Berridge, 1965), although the majority do secrete a "urine" which is 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).

Other workers (Taylor, 1971; Maddrell, 1971) have proposed and elaborated a "local osmosis" theory (Diamond, 1964) for insect Malpighian tubules. Taylor, (1971)

and Maddrell, (1971) suggested that the cytoplasm is marginally hypertonic to the bathing medium due to active solute transport and likewise the lumen is marginally hypertonic to the cytoplasm. Water would then move passively from the cytoplasm to the lumen. 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).

Curran, (1960) postulated a model stating that active solute transport taking place into an enclosed compartment could establish and maintain a large osmotic gradient. This model was developed further as the "standing gradient osmotic flow theory" (Diamond and Bossert, 1967, 1968) to explain iso-osmotic and hyperosmotic secretion by tissues. These workers noted that many fluid transporting epithelia possessed a complex system of channels at their surfaces (such as basal infoldings, lateral paracellular spaces, intracellular canaliculi and brush border microvilli) and proposed a theory for solute-linked water transport based upon the ultrastructure of the transporting tissues. This theory was applied to Malpighian tubule cells by Berridge and Oschman, (1969). Malpighian tubule cells have channels on both surfaces created by either basal infoldings or microvilli. Fluid at the entrance to the basal channels will be isotonic to the haemolymph but as it approaches the inner end of the channels active solute uptake into the cytoplasm will make it hypotonic and it will become increasingly hypotonic the further it progresses down the channel. The hypotonicity of the fluid in the channel, with respect to the cytoplasm, will cause a passive flow of water into the cell. At the apical surface, fluid which is isotonic to the cytoplasm will enter the base of the microvilli. Solute will be pumped from this fluid into the channels formed between adjacent microvilli and as at the basal surface a standing osmotic gradient will arise; the tip of the microvilli showing the greatest hypotonicity with respect to the channel. In this situation water will move from the area of hypotonicity (the microvilli) into the channels which are hypertonic. However, this theory was criticized by Taylor, (1971) and Maddrell, (1977) who argued that basal infoldings and apical microvilli of Malpighian tubules were too

short for standing solute gradients to develop along their lengths. Diamond and Bossert, (1967) originally proposed the standing-gradient osmotic flow model for structures such as gall bladder and kidney; apical microvilli of Malpighian tubule cells are considerably shorter than the corresponding channels in these tissues. Data obtained for ion concentrations along basal infolds and apical channels using electron-probe X-ray microanalysis was also incompatible to that predicted by the model (Gupta *et al.*, 1976, 1977). Based on mathematical calculations, Hill, (1975a) concluded that cell membranes would have to have abnormally high osmotic permeabilities in order to secrete isotonic fluid. However, O'Donnell *et al.*, (1982) discovered that the Malpighian tubules of *Rhodnius prolixus* were much more permeable than had previously been thought; thereby suggesting they may have high enough osmotic permeabilities to allow solute and water flows to be coupled by osmosis.

Electro-osmosis is another theory that has been proposed to explain the mechanism of ion and water movements across epithelia (Hill 1975b, 1977). This hypothesis was applied to insect Malpighian tubules by Maddrell, (1977), who suggested that apical electrogenic cation pumps produce an electrical potential difference across the membrane. The resulting electrochemical gradient would draw Cl^- out of the cell; it would cross the apical membrane where it would frictionally interact with water molecules drawing them out of the cell too. This theory depends on the maintenance of a favourable potential gradient across the apical membrane.

There are two main pathways for transepithelial ion movement; a transcellular route in which ions move through the cells crossing the basal and apical membranes, and a paracellular pathway in which ions travel through the lateral spaces between cells (Hanrahan, 1984). Epithelia are often characterized by the relative permeability of these two pathways. In tight epithelia, paracellular junctions have a high resistance, therefore, most transport occurs across the cells. Conversely, in leaky epithelia, movement occurs through the low resistance paracellular pathway (Lewis *et al.*, 1984).

There is some evidence of paracellular fluid flow in some insect epithelia (O'Donnell and Maddrell, 1983). Gupta *et al.*, (1978) and Gupta and Hall, (1979)

suggested paracellular fluid movement may be occurring in the salivary glands of *Calliphora vicina*. This has also been proposed for the rectal papillae of *Calliphora* (Gupta *et al.*, 1980), the salivary glands of *Periplaneta americana* (Gupta and Hall, 1983) and the rectal pads of *Periplaneta* (Wall *et al.*, 1970). Paracellular fluid flow would negate the necessity of the cell membranes to show a high osmotic permeability as described by Hill, (1975a). However, studies carried out by Maddrell, (1980) on the Malpighian tubules of *Rhodnius prolixus* produced results that suggested fluid transport did occur transcellularly. Large organic solutes such as xylose, sucrose and inulin are likely to cross the epithelium passively by paracellular routes. If fluid movement also occurs by this pathway then a correlation would be expected between the rate of fluid secretion and the movement of these solutes across the tubule wall; in fact when the rate of fluid secretion was increased seventy-fold by the application of 5-HT no increase in the rate of movement of the solutes was seen. Furthermore, if large molecules such as inulin can penetrate the paracellular junctions it would be difficult to imagine how osmotic gradients could be established required to draw water though the clefts at the observed rates (Maddrell, 1980). Additionally, the surface area of the tubule taken up by the paracellular clefts was only 0.034% of the total tubule surface area (Maddrell, 1980) this feature of limited areas of high permeability has been used to explain the low passive permeability of the tubules. The paracellular channels also have septate junctions running down their lengths (Maddrell, 1980; Phillips, 1981) which reduce the area available for paracellular permeation (Lord and Dibona, 1976; Filshie and Fowler, 1977).

Malpighian tubules display characteristics of both tight and leaky epithelia, as they can maintain a large potential difference across the cells, and also produce high rates of fluid secretion (Beyenbach, 1995). Williams and Beyenbach, (1984) classified the Malpighian tubules of *Aedes aegypti* as a moderately tight epithelium.

Once it was established that solute transport was the driving force for fluid transport attention was turned to how solute transport was carried out. Ramsay, (1953) investigated fluid secretion in eight insect species and discovered that the concentration

of K^+ was always higher in the "urine" than in the bathing medium. Furthermore, he also found that in the majority of cases this ion was moved against an electrical gradient. From these results he deduced that the transport of K^+ was an active process. Later studies, building on this work, confirmed that K^+ was the "prime mover" in fluid secretion in a number of insects (Berridge, 1968; Coast, 1969; Maddrell, 1969 and Maddrell and Klunsuwan, 1973), including *Locusta migratoria*, (Anstee *et al.*, 1979). From these studies it has been elucidated that K^+ must be actively transported from the haemolymph to the lumen, in doing so K^+ must cross the basal and apical membranes.

Many epithelial cells display a large basal K^+ conductance and, as will be discussed later, many cells also possess a Na^+/K^+-ATPase on their basal surface which pumps Na^+ out of the cell in exchange for K^+. The large basal K^+ conductance allows K^+ entering the cell through the Na^+/K^+-ATPase to return to the serosal side (Hanrahan *et al.*, 1986). This permeability to 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 if there is 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 Na^+, K^+, Ca^{2+} and Cl^- (Van Driessche and Zeiske, 1985).

"Urine" formation depends on the availability of anions to accompany the active transport of K^+ (Berridge, 1969) in order to maintain electroneutrality. Since the "urine" is rich in KCl, it has generally been accepted that Cl^- is the accompanying anion. Cl^- transport across the basal membrane is thought to be by secondary active transport, i.e. 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 Cl^- channels have been

detected in the Malpighian tubules of the 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). 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 Cl^- channels have been detected (Wright and Beyenbach, 1987; Wieczorek *et al.*, 1989). However, there is some suggestion that Cl^- transport may occur paracellularly in the ant (Dijkstra *et al.*, 1994a).

Berridge, (1967) and Berridge and Oschman, (1969) postulated that K^+ entered the cell across the basal surface via a coupled sodium-potassium pump. Skou, (1957) was the first to demonstrate a Mg^{2+}-dependent ATPase that was synergistically stimulated by Na^+ and K^+. Further studies revealed that the enzyme is ubiquitous in animal cells and established some of its major features - it is located in cell membranes and inhibited by ouabain (Skou, 1965 and Schuurmans Stekhoven and Bonting, 1981). The enzyme hydrolyses ATP to cause a vectorial movement of cations against their electrochemical gradients, moving Na^+ out of and K^+ into the cell (Skou, 1972) and so is thought to be responsible for most cells having a relatively high K^+ concentration but relatively low Na^+ concentration (Schuurmans Stekhoven and Bonting, 1981). Hydrolysis of ATP takes place on the inside of the membrane and results in the movement of 3 Na^+, per energy-rich bond hydrolysed, to the outside of the cell. In red blood cells and squid giant axon this is accompanied by the transfer of $2K^+$ to the inside of the cell (Schuurmans Stekhoven and Bonting, 1981).

Na^+/K^+-ATPase activity has been investigated in a number of insect tissues, e.g. brain (Cheng and Cutkomp, 1972) and compound eye, (Rivera, 1975) and is commonly demonstrated by inhibitor studies using ouabain - a specific inhibitor of the Na^+/K^+-ATPase (Skou, 1965). Preliminary studies did not demonstrate ouabain inhibition of fluid secretion by Malpighian tubules (Berridge, 1968; Maddrell, 1969, Pilcher, 1970 and Gee, 1976b). However, it has been found that ouabain sensitivity differs between enzyme systems of different tissues of the same animal and from the same tissues in

different animals (Skou, 1965). This difference in sensitivity has been attributed to differing rates of ouabain dissociation between tissues (Tobin *et al.*, 1972), however there is evidence to suggest that it cannot be solely due to this (Pitts *et al.*, 1977 and Wallick *et al.*, 1980).

Biochemical studies have shown Na^+/K^+-ATPase to exist in the Malpighian tubules and/or hindgut of *Locusta migratoria* Anstee and Bell, (1975, 1978); Peacock, (1976); Anstee *et al.*, (1979) and Fogg *et al.*, (1991). Other studies have provided evidence for the existence of Na^+/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). Injection studies (where ouabain is delivered, *in vivo*, into the haemolymph of an insect using a needle) have demonstrated inhibition of the normal processes of fluid secretion in *Glossina morsitans* (Gooding, 1975) and *Drosophila hydei* (Weber Von Grotthuss *et al.*, 1974; Atzbacher *et al.*, 1974). Anstee and Bowler, (1979) propose that conflicting results may be due to differences in experimental methodology when investigating ouabain sensitivity of tissues. An important experimental variable is temperature as it has been shown that the ouabain-sensitivity of the Malpighian tubules of *Locusta migratoria* is affected by changes in temperature (Donkin and Anstee, 1980).

In support of the proposal by Berridge and Oschman, (1969) that a basal Na^+/K^+-ATPase is responsible for K^+ entry into the cell, it has been shown that in tissues which display Na^+/K^+-ATPase activity this activity is associated predominantly with the basolateral membranes (Rodriguez and Edelman, 1979; Ernst *et al.*, 1980; Anstee and Bowler, 1984, Lechleitner and Phillips, 1988, Lebovitz *et al.*, 1989 and Fogg *et al.*, 1991).

Generally it has been accepted that there is an electrogenic cation pump on the apical membrane (Gee, 1976a; Berridge, 1967, 1968; Maddrell, 1971, 1977; Phillips, 1981 and O'Donnell and Maddrell, 1984). This apical pump is believed to be relatively non-specific in nature as although it mainly transports K^+, 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. Caesium and sodium could also support fluid secretion but at a lower rate. 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 Na^+ or K^+ (Maddrell, 1969). These results along with the fact that *Rhodnius* is able to secrete at the same rate in K^+-free solutions led Maddrell, (1969) to propose a mechanism whereby at low K^+ concentrations the pump mechanism could switch from pumping K^+ to 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 Na^+ and K^+ and why in the insect *Glossina morsitans*, Na^+ was the "prime mover". In the case of *Rhodnius prolixus* it was suggested that the apical pump had a higher affinity for Na^+, but the permeability of the basal membrane and the electrochemical gradient of the ions involved would determine the availability of Na^+ and K^+ for the apical pump. Therefore, the secretion of a Na^+-rich "urine" by the Malpighian tubules of *Glossina morsitans* (Gee, 1976a, b) and *G. austeni* (Gee, 1975) would result if the basal membrane had a higher permeability to Na^+ than K^+. However, if the basal membrane had a higher permeability to K^+ then K^+ would be the dominant cation in the cytoplasm and be transported via the apical pump into the lumen thereby producing a K^+-rich "urine" as in *Locusta migratoria* (Anstee *et al.*, 1979).

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, (1968) 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) and 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.

Investigations into the nature of the apical K^+ pump found ouabain had no effect on K^+ transport (Berridge *et al.*, 1976; Maddrell, 1977 and Harvey, 1980), therefore, apical K^+ transport was not believed to occur via Na^+/K^+-ATPase. However, Wolfersberger, (1979) and Wolfersberger *et al.*, (1982) used biochemical assays to demonstrate a K^+-modulated ATPase activity in the midgut of *Manduca sexta*. Similarly, Cioffi and Harvey, (1981), were able to detect a K^+ 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). Wieczorek, (1982) carried out biochemical assays on the proboscis of a fly and discovered a K^+-sensitive ATPase, this enzyme activity was further characterized by Wieczorek and Gnatzy,

(1985). Deaton, (1984) identified a K^+-stimulated activity in the microsomes of the midgut of *Manduca sexta* and Wieczorek *et al.*, (1986) also located a K^+-stimulated ATPase activity in midgut goblet cell apical membranes. As fluid secretion had been maintained in the presence of cations other than K^+, likewise ATPase activity could be stimulated by other cations. The characteristics of this enzyme differed to those of an H^+/K^+-ATPase (Wieczorek *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 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 on the apical surface of Malpighian tubules of *Locusta* (Fogg *et al.*, 1991). Wieczorek *et al.*, (1986) also discovered that the enzyme activity was not inhibited by ouabain, orthovanadate, azide or oligomycin but 150μM 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 portasomes 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.*, 1983a) 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.

Maddrell, (1971) noticed similarities in fluid production between the Malpighian tubules of *Calliphora erythrocephala* and *Carausius morosus*. Both insects used K^+ as the "prime mover". In K^+-free solutions secretion was very slow but inclusion of a small amount of K^+ had a marked stimulatory effect. In K^+-solutions, inclusion of a small amount of Na^+ increased the rate of fluid production but this could not be attributed to a Na^+/K^+ pump since ouabain had no effect on secretion. Therefore, to explain these characteristics Maddrell, (1971) proposed the following model (Fig. 1.1.) in which active transport is envisaged at both membranes.

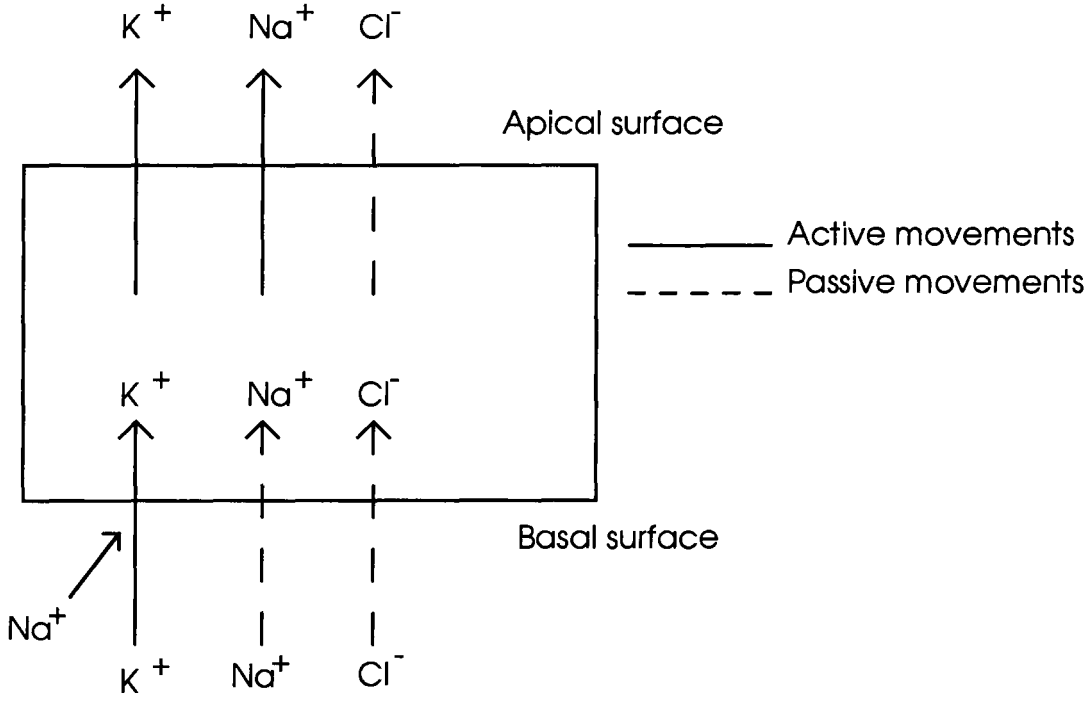


Fig. 1.1. Re-drawn from Maddrell, (1971).

In Fig. 1.1. an electrogenic K^+ pump on the basal surface was stimulated by Na^+, with Na^+ and Cl^- entering passively. At the apical surface Na^+ and K^+ were actively pumped but Cl^- followed passively.

However, a different model had to be constructed to account for fluid secretion across the Malpighian tubules of *Rhodnius prolixus* (see Fig. 1.2.). *Rhodnius prolixus*

was able to secrete just as fast in K^+-free solutions, but in K^+-solutions with no Na^+, secretion was greatly reduced. Inclusion of a small amount of Na^+ caused acceleration to almost normal rates. The ability to secrete in a K^+-free medium can be explained if the cell can transport Na^+ at a similar rate to K^+. However, the prime mover was still K^+; K^+ was still preferentially secreted to Na^+.

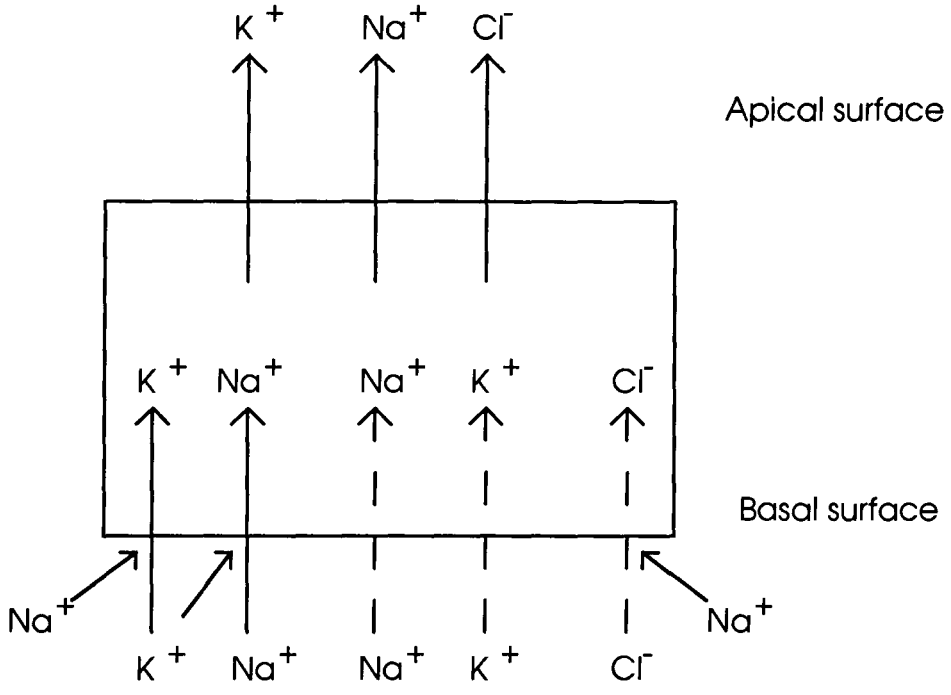


Fig. 1.2. Re-drawn from Maddrell, (1971).

The model shown in Fig. 1.2. was proposed to explain these features. It was suggested that a Na^+ pump must exist at the basal surface as Na^+ continued to be secreted at low extracellular levels of Na^+. There must be an apical Cl^- pump as a negative transwall potential existed and at low Cl^- concentrations the tubules still secreted at the maximum rate. In the absence of Na^+ the secretion of K^+ and Cl^- fell, this can be interpreted as a Na^+- stimulation of K^+ and Cl^- entry at the basal surface. Conclusions that can be drawn from this model are that in the absence of K^+, the apical K^+ pump must pump Na^+ or the apical Na^+ pumps must accelerate since the Na^+ output increases under these conditions.

Later Maddrell, (1977) suggested a revised model (shown in Fig. 1.3.) to explain fluid secretion by the Malpighian tubules of any insect. He accommodated for the differences in the cation acting as the "prime mover" (either K^+ or Na^+) by producing a single model that would embrace both ions. He suggested that a pump existed on the apical surface that had a higher affinity for Na^+ but that the ion which was transported would be the one present at the highest concentrations in the cytoplasm. Na^+, K^+ and Cl^- were all suggested to enter passively across the basal membrane with Cl^- also passively leaving the cell across the apical membrane.

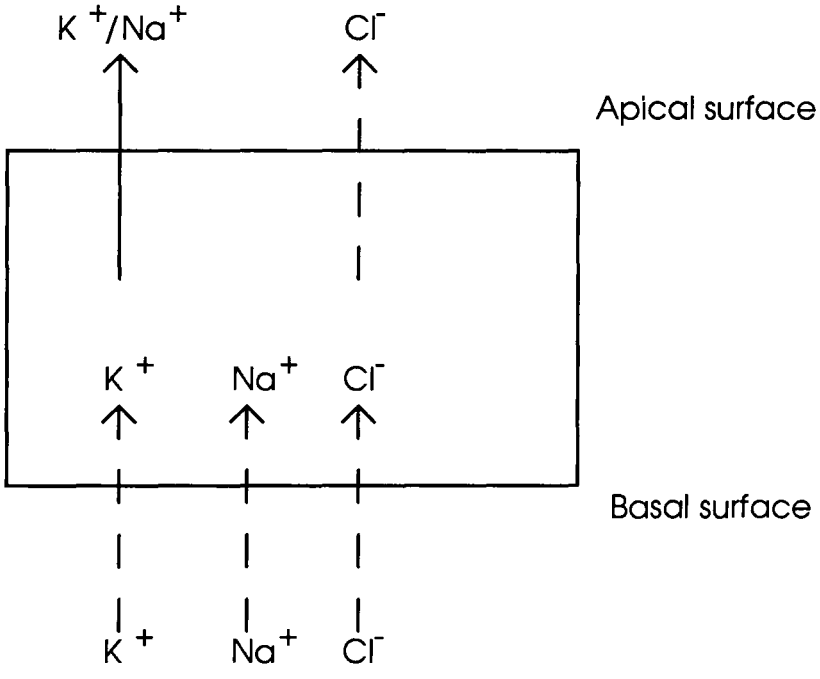


Fig. 1.3. Re-drawn from Maddrell, (1977).

The previous model (Fig. 1.3., Maddrell, 1977) was modified by Morgan and Mordue, (1983) to include an electroneutral mechanism on the basal surface of the Malpighian tubules of *Locusta migratoria* (Fig. 1.4.). Morgan and Mordue, (1983) measured electrochemical gradients across the Malpighian tubules of *Locusta migratoria*, and discovered that when tubules were stimulated with diuretic hormone or

cAMP, no change was seen in the basal membrane potential. Therefore, they concluded that an electroneutral entry mechanism must operate at this surface. Finding the basal membrane was permeable to K^+ it was suggested that K^+ entered the tubules passively. It was also suggested that Na^+ entered the cells passively due to a favourable concentration gradient. Cl^- entry was postulated to be active, due to the unfavourable concentration gradient for passive entry, but linked to the entry of Na^+ and/or K^+ to maintain electroneutrality. At the apical surface extrusion of both Na^+ and K^+ was proposed to take place via an active pump since considerable electrical and concentration gradients were measured opposing passive exit with Cl^- following passively down a large electrical gradient.

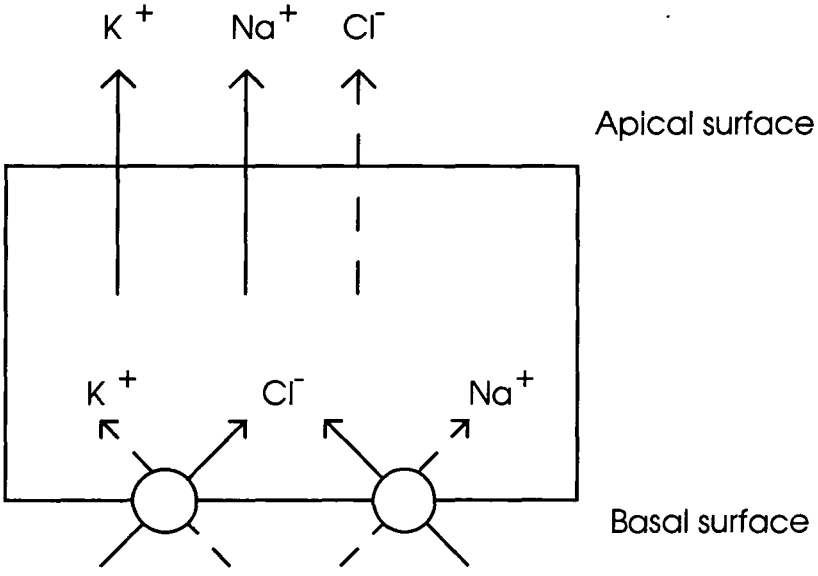


Fig. 1.4. Re-drawn from Morgan and Mordue, (1983).

A later model proposed by O'Donnell and Maddrell, (1984), suggested that Na^+ and Cl^- transport were also linked at the basal membrane in *Rhodnius prolixus*, this was a rational idea as electrochemical gradients favoured Na^+ but not Cl^- entry. The loop diuretics furosemide and bumetanide, known inhibitors of NaCl co-transport (Palfrey and Rao, 1983 and Palfrey and Greengard, 1981), had effects consistent with the presence of a NaCl co-transport mechanism in the Malpighian tubules. Furthermore, Cl^- absorption

by a variety of epithelia is the consequence of an electrically silent Na^+-coupled transport process. However, these workers also discovered evidence that some Cl^- entry could be linked to K^+ transport and therefore proposed that an electrically silent $Na^+/K^+/2\ Cl^-$ co-transporter existed at the basal membrane (see Fig. 1.5.). The $Na^+/K^+/2\ Cl^-$ co-transporter is also known to be affected by loop diuretics (Geck *et al.*, 1980).

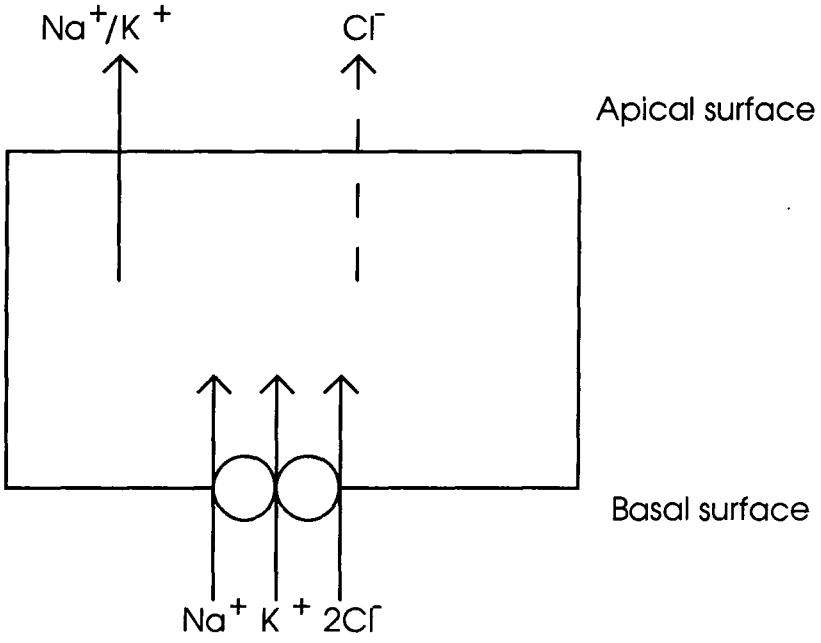


Fig. 1.5. Re-drawn from O'Donnell and Maddrell, (1984).

This model (Fig 1.5.) was further modified by Maddrell and Overton, (1988) when they discovered that ouabain stimulated Na^+ and fluid secretion in the Malpighian tubules of *Rhodnius prolixus*. Studies with ^3H labelled ouabain located binding sites on the basal membrane and so a model was proposed with a Na^+/K^+ pump situated on this surface (Fig. 1.6.). A Na^+/K^+-ATPase had already been identified in the Malpighian tubules of *Locusta migratoria* (Anstee and Bell, 1975; 1978; Anstee *et al.*, 1979).

Using this model (Fig. 1.6.) it was possible to explain the different cationic composition of the "urine" under different circumstances. The fluid secreted by non-stimulated tubules contained only low amounts of Na^+, as under these conditions much

of the Na^+ entering via the co-transporter was returned to the haemolymph via the Na^+/K^+-ATPase. During fast fluid secretion, when the tubules had been stimulated, the co-transporter and the apical pump were working at much greater rates than usual resulting in concentrations of Na^+ and K^+ in the "urine" being approximately equal (Maddrell, 1969). If ouabain was included in the bathing medium the Na^+/K^+-ATPase was inhibited, this prevented Na^+ being returned to the haemolymph, against the direction of fluid transport, and so secretion of Na^+ increased.

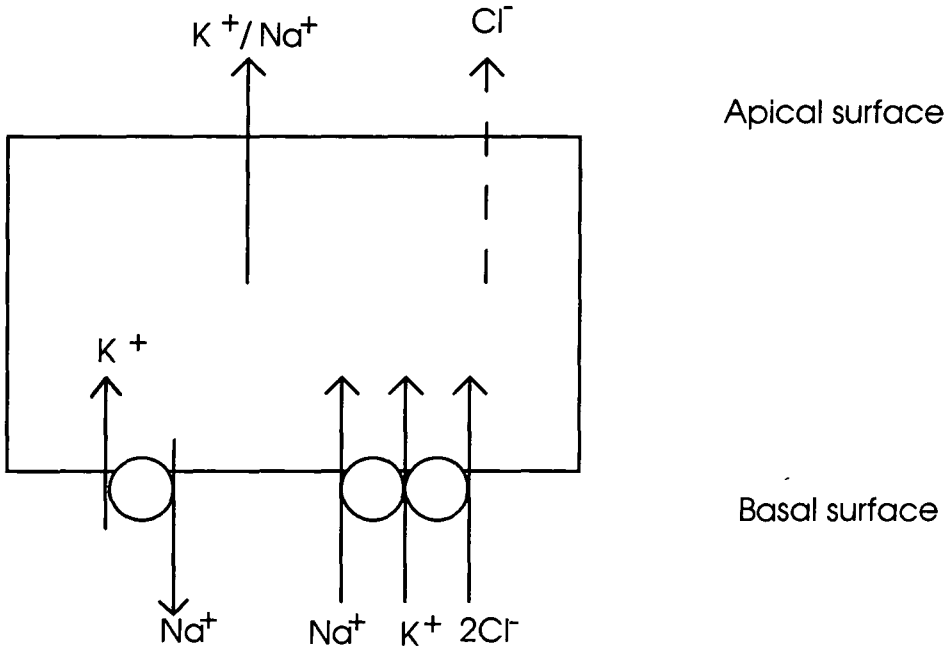


Fig. 1.6. Re-drawn from Maddrell and Overton, (1988).

Baldrick *et al.*, (1988) working on the Malpighian tubules of *Locusta migratoria* attributed the basal cell membrane potential to its permeability to K^+ and relative impermeability to Na^+ and Cl^-. They 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 was not as conclusive as for *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) and

there was some suggestion that Cl^- might also be co-transported with K^+ as Cl^- secretion still occurred in Na^+-free saline. As had been suggested for other insects, it was proposed that Na^+ and K^+ were actively transported across the apical membrane with Cl^- following passively.

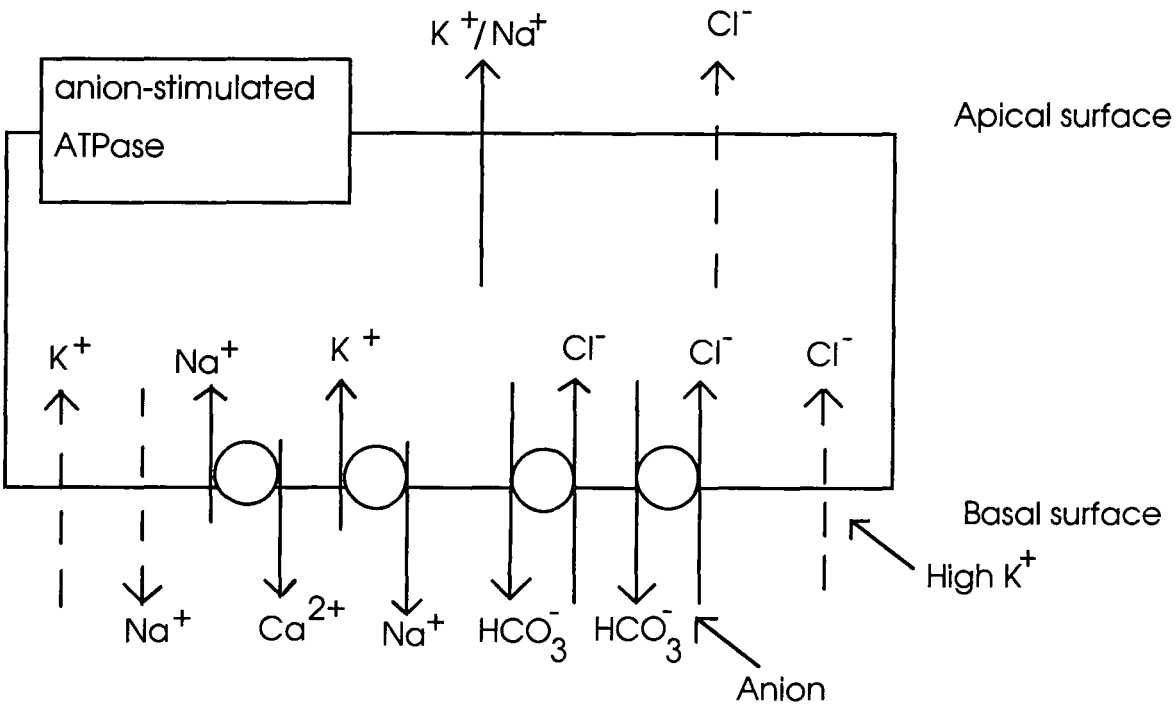


Fig. 1.7. Re-drawn from Fogg, (1990).

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 (see Fig. 1.7.).

Cl^- entry was also suggested to occur in exchange for HCO_3^- and by the activity of (Cl^-+HCO_3^-) - ATPase (Fogg, 1990). Previous ion substitution experiments using Cl^--free and HCO_3^--free saline containing acetazolamide gave similar results (Baldrick, 1987) suggesting that $Cl^-$and HCO_3^- transport were linked. Furosemide has been suggested to have a relatively non-specific effect (Palfrey *et al.*, 1980). It has been shown to effect Cl^-/HCO_3^- exchange (Chipperfield, 1986). Additionally Fogg, (1990) detected an anion-stimulated ATPase on the basal membrane and this enzyme has been related to Cl^-/HCO_3^- exchange (Herrera *et al.*, 1978) and active Cl^- transport (Lechleitner and Phillips, 1988). Finally Fogg, (1990) and Fogg *et al.*, (1991), 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.

Experiments conducted on the Malpighian tubules of *Aedes aegypti* by Hegarty *et al.*, (1991) did produce evidence for an electroneutral co-transporter, and they suggested a model based on one that was originally proposed by Phillips, (1981), (see Fig. 1.8.). Both cAMP and bumetanide caused an increase in the secretion of Na^+ and a decrease in the secretion of K^+. cAMP increases the permeability of the basal membrane to Na^+ (Sawyer and Beyenbach, 1985) hence Na^+ becomes the dominant cation in the cytoplasm and so is extruded by the "common cation pump".

Bumetanide blocks one of the routes of K^+ entry into the cell, therefore the level of intracellular K^+ would decrease causing Na^+ to become the dominant ion in the cytoplasm and so the one transported into the lumen by the cation pump. Because K^+ secretion was affected in the presence of bumetanide, a $Na^+/K^+/2Cl^-$ co-transporter was suggested rather than just a Na^+/Cl^- transporter. Active Cl^- entry was postulated at the basal surface as fluid secretion was inhibited by SITS. Na^+ channels were thought to exist at the basal surface as this membrane was found to have a high Na^+ conductance in

Aedes aegypti, (Sawyer and Beyenbach, 1985). Recently, Leyssens *et al.*, (1994) working on the tubules of *Formica polyctena* have suggested K^+ entry to be via K channels and active co-transporters, the relative contributions of which depending on $[K^+]_o$.

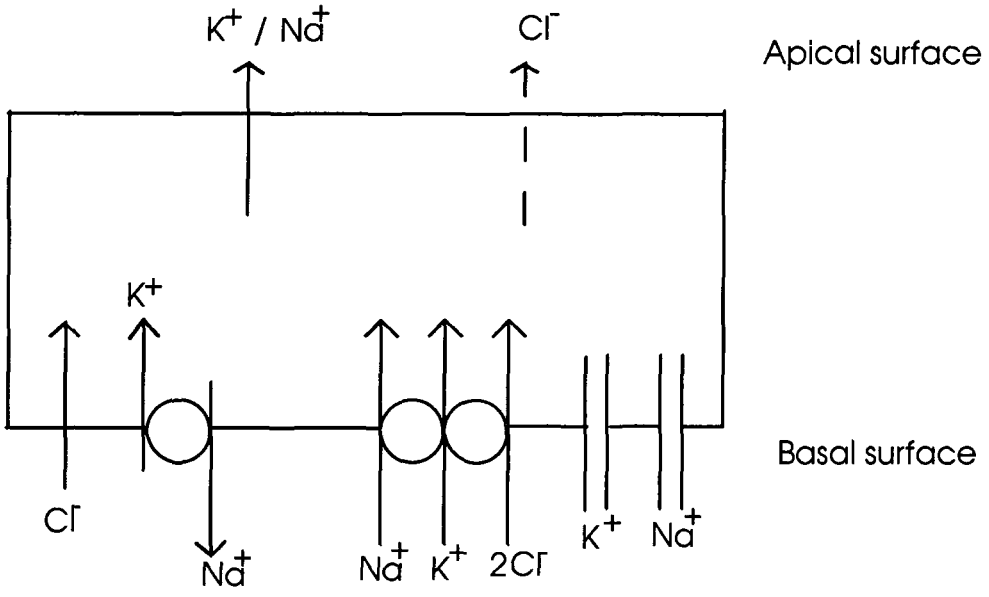


Fig. 1.8. Re-drawn from Phillips, (1981) and Hegarty *et al.*, (1991)

Proton ATPases can be divided into three classes, the F-type ATPases, the P-type (or phosphorylated) ATPases and the V-type (or vacuolar) ATPases (Mellman *et al.*, 1986). The F-type ATPases represent the class found on the inner mitochondrial, chloroplast and plasma membranes of prokaryote cells. An example of a phosphorylated proton ATPase is the proton pump found in the plasma membranes of yeast and *Neurospora crassa*. A proton ATPase of this type is found in mammals in the stomach epithelium; it is the H^+/K^+-ATPase which secretes acid into the stomach. The reaction cycle involves the formation of a phosphorylated intermediate which is inhibited by the transition state analogue sodium orthovanadate. It belongs to the same general class as Na^+/K^+-ATPase and Ca^{2+}-ATPase although it is not sensitive to ouabain. Then there are the V-type ATPases which differ from the other two classes by their inhibitor

specificities, their lack of coupling to counter ion transport and their intracellular distribution (Mellman *et al.*, 1986). The majority of membrane organelles are part of what is known as the vacuolar system, this consists of the exo- and endocytic pathways. Both the inward- and outward-directed pathways consist of a series of organelles through which material passes in transport vesicles in a sequential manner (Mellman *et al.*, 1986). Most of these organelles contain H^+-ATPases which produce an internal acidic environment. For other reviews on proton ATPases see Forgac, (1989) and Nelson, (1989).

A fundamental discovery was made by Schweikl *et al.*, (1989), using membrane bound ATPase prepared from the midgut of *Manduca sexta*, they were able to demonstrate 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 is a specific inhibitor of Vacuolar or V-type ATPases when used at appropriate concentrations; V-type ATPases are sensitive to much lower concentrations of NEM (1-2μM) than the phosphorylated or P-type ATPases (100μM-1mM) and the F-type ATPases characteristic of mitochondria, chloroplasts and bacteria are virtually resistant (Forgac, 1989). In the study by Schweikl *et al.*, (1989) 1μM 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.

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.

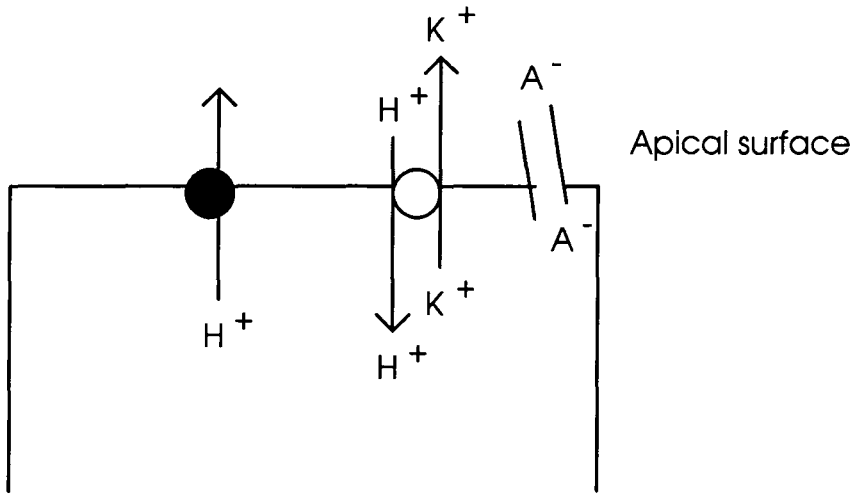


Fig. 1.9. Re-drawn from Wieczorek *et al.*, (1989).

Fig. 1.9. shows the model proposed by Wieczorek *et al.*, (1989) 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 more than $1H^+$ was exchanged for K^+ during an exchange cycle of the antiport. 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 and Grinstein and Wieczorek, 1994). Recent work by Azuma *et al.*, (1995) using the static head method has 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. The revised model resulting from these studies is shown in Fig. 1.10.

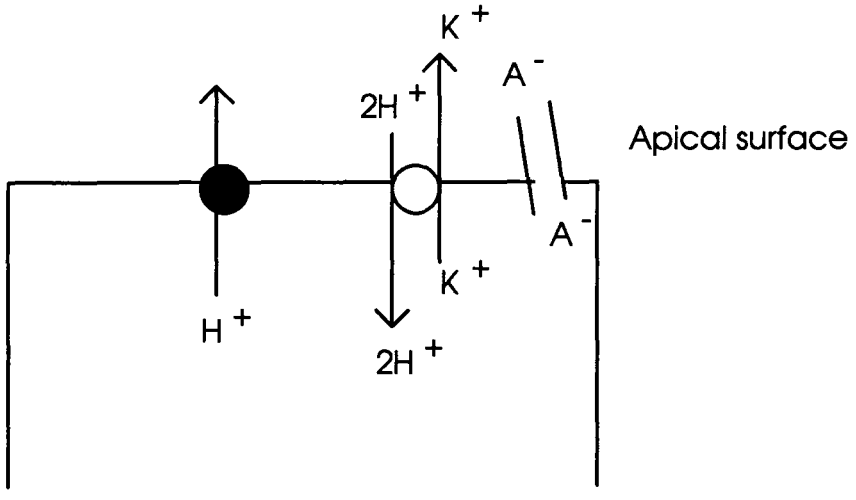


Fig. 1.10. From Wieczorek *et al.*, (1991); Azuma *et al.*, (1995).

Therefore there is strong evidence for the electrogenic nature of the antiporter in the midgut of *Manduca sexta*.

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 Fig. 1.11.). Weltens *et al.*, (1992) suggest a similar apical exit mechanism for K^+ in the Malpighian tubules of *Formica polyctena.*

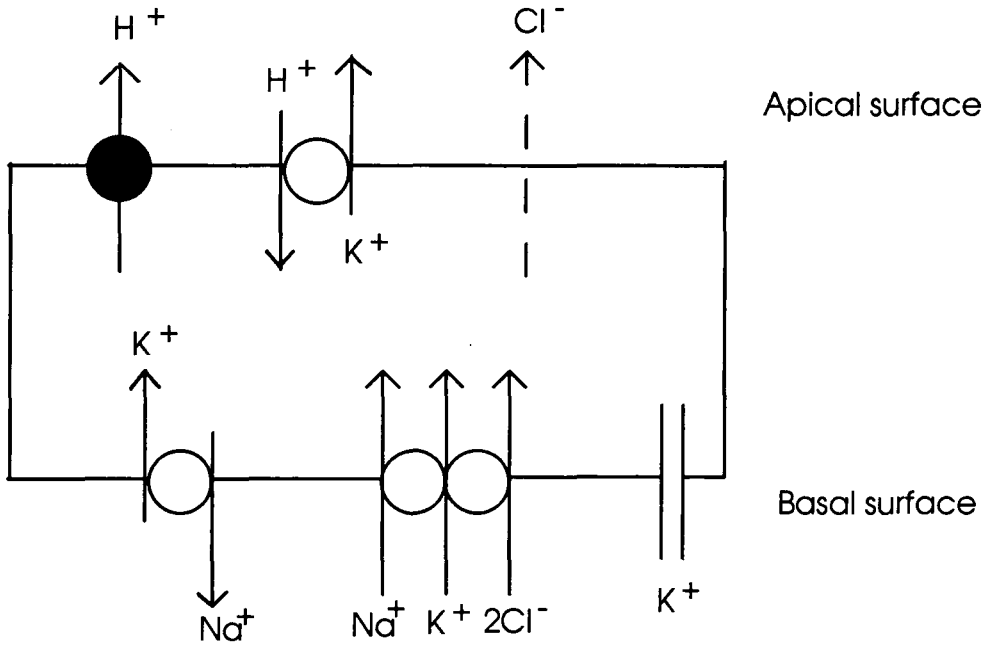


Fig. 1.11. Re-drawn from Bertram *et al.*, (1991).

Zeiske, (1992) proposed a model to explain fluid secretion by Malpighian tubules of *Manduca sexta.* He suggested that insects with low haemolymph K^+ possessed both the antiport and a Na^+/K^+-ATPase on their basal membrane, but insects with rich haemolymph K^+ possessed K^+ channels in the basal membrane which provided the main entry mechanism for K^+, a Na^+/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 (see also Leyssens *et al.*, 1992; Nicolson, 1993).

Zhang *et al.*, (1994) working with *Formica polyctena* measured cellular and luminal pH of the 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).

An anion-stimulated ATPase has been reported in the Malpighian tubules of *Locusta migratoria* (Anstee and Fathpour, 1979, 1981; Fathpour and Anstee, 1981; Fogg *et al.*, 1991). Anion-stimulated ATPases have been found in many other tissues, e.g. pancreas, salivary gland, gill and erythrocytes (see Schuurmans Stekhoven and Bonting, 1981) 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 and 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* (Fogg *et al.*, 1991).

The anion-stimulated ATPase has been suggested to take part in the active transport of anions (Komnick, 1978, Gerencser and Lee, 1985 and Lechleitner and Phillips, 1988) and has also been linked to ion/water transport mainly because

thiocyanate, an inhibitor of the anion-stimulated ATPase, also inhibits secretion by some epithelia (Simon and Thomas, 1972 and Blum *et al.*, 1971).

The function of cyclic adenosine -3',-5' monophosphate (cAMP) as a secondary messenger mediating the action of many hormones has been established in many vertebrate hormone systems (Robison *et al.*, 1968). A similar role has been suggested in some insect hormone systems (Wyatt, 1972). 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, 1976); *Cenocorixa blaisdelli* (Cooper *et al.*, 1988); *Glossina morsitans*, (Gee, 1976a); *Locusta migratoria* (Anstee *et al.*, 1980; Morgan and Mordue, 1981); *Schistocerca gregaria* (Maddrell and Klunsuwan, 1973; James *et al.*, 1993); *Papilio demodocus* (Nicolson and Millar, 1983); *Onymacris plana* (Nicolson and Hanrahan, 1986); *Cenocorixa bifida* (Szibbo and Scudder, 1979); *Aedes aegypti* (Petzel and Stanley-Samuelson, 1992) and *Acheta domesticus* (Kim and Spring, 1992), led to the proposal that it was mediating the action of diuretic hormone in these organs. Although there are some cases where cAMP had no effect on the rate of fluid secretion, for example, *Musca domestica* (Dalton and Windmill, 1980). This theory 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; Lehmberg *et al.*, 1993). cAMP also increases 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).

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 and Mordue (1984) produced both direct and indirect evidence for the presence of two diuretic peptides. Diuretic activity was found 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_3 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 calcium. Evidence from other studies points to a link between inositol trisphosphate and intracellular calcium release (Berridge, 1983; Streb *et al.*, 1983; Berridge and Irvine, 1984). Recently, it has been discovered that nitric oxide 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 Ca^{2+} signalling system (Dow *et al.*, 1994a).

The aim of the project described in this thesis was to elucidate the mechanisms involved in ion and fluid transport in the Malpighian tubules of *Locusta migratoria*; specifically, the pathways for the movement of monovalent cations across the apical and basal cell membranes. The apical transporting pathway of insect secretory epithelia has been the focus of much research recently. 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. However, the involvement of a V-type ATPase and antiporter in fluid secretion by the Malpighian tubules of *Locusta migratoria* has not been established. Therefore, the main aim of this study was to investigate if a V-type ATPase was responsible for

cation transport in the Malpighian tubules of *Locusta*. 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, therefore one of the aims of this project was to investigate this suggestion experimentally.

Intracellular microelectrode recording methods, immunocytochemical localization techniques and biochemical and fluid secretion studies using cAMP or inhibitors of known transport processes would be used to examine the transport mechanisms at the basal and apical surfaces of Malpighian tubules of *Locusta migratoria*. The information provided would allow a hypothetical model for ion and fluid secretion to be proposed.

CHAPTER 2

MATERIALS AND METHODS

Maintenance of insects

A stock population of *Locusta migratoria* L., was reared in gregarious phase in an insectary at 28 ± 0.5°C, 60 ± 5% relative humidity and a photoperiod of 12 hr light : 12 hr dark. Animals were kept in gregarious phase by rearing them at sufficiently high population density (Joly and Joly, 1953). Air was extracted at constant rate and replaced by fresh air using a fan-driven ventilator (Xpelair) and air was circulated by three electric fans. Humidity was maintained by three humidifiers (Lumatic, Humidifier Group, Bromley, Kent, England). Locusts were reared in glass-fronted cages (41 cm x 41 cm x 60 cm, supplied by Philip Harris Biological Ltd., Oldmixon, Weston-super-Mare, Avon, England). Each cage was illuminated with a single 25 Watt bulb, resulting in cage temperatures varying 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 and water.

Manduca sexta larvae (4th instar) were a gift from Prof. S.E. Reynolds, University of Bath.

Glassware

Pyrex glassware was used throughout. Prior to use it was cleaned by soaking overnight in a 2% solution of Teepol laboratory detergent followed by several rinses in hot and cold tap water and final rinsing in DDW (twice). 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 a gift from I.C.I. Dyestuffs division. Primary monoclonal antibodies (clones #90-7, 221-67, 230-3 and 224-3), 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. Mouse monoclonal antibody IgG α 5 raised against the α-subunit of the avian sodium pump was generously donated by Prof. D.M. Fambrough, The Johns Hopkins University, Baltimore, Maryland, USA. Bafilomycin A_1 was purchased from Prof. Dr. K. Altendorf, Universität Onsabrück, Germany.

Solutions

The composition of control saline solution (Anstee *et al.*, 1979; 1986) and other experimental salines which were used in ion substitution experiments are shown in Table 2.1.

Bafilomycin A_1 was purchased in a form which was not 100% pure although it did not contain any other active compounds (K. Altendorf, personal communication). The actual concentration was estimated photometrically (Bowman *et al.*, 1988) using an absorbance maxima of 245nm.

In experiments involving bafilomycin A_1 it was necessary to dissolve it in DMSO before adding it to the appropriate saline. The final concentration of DMSO in the experimental salines was <1% and in all studies using this chemical, the same concentration of solvent was included in the controls.

Table 2.1. Composition of experimental salines (concentrations in mM)

Salt	Control	Rb^+-saline	K^+-free saline	High $[K^+]$, Na^+-free
NaCl	100	100	108.6	-
KCl	8.6	-	-	108.6
$CaCl_2$	2.0	2.0	2.0	2.0
$MgCl_2$	8.5	8.5	8.5	8.5
NaH_2PO_4	4.0	4.0	4.0	-
$NaHCO_3$	4.0	4.0	4.0	-
NaOH	11.0	11.0	11.0	-
Glucose	34.0	34.0	34.0	34.0
HEPES	25.0	25.0	25.0	25.0
RbCl		8.6		-
KH_2PO_4				4.0
$KHCO_3$				4.0
KOH				11.0

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 Fischer and Yates (1963). Values where $P<0.05$ were taken as significant. Data are presented as mean ± SEM unless otherwise stated.

Rate of fluid secretion

In vitro measurements of the rate of fluid secretion were carried out following the method of Maddrell and Klunsuwan (1973) modified later by Anstee and Bell, (1975) and Donkin and Anstee, (1980). Locusts were killed by twisting the head to break the arthroidal membrane. Then the tip of the abdomen was cut off which allowed the gut of the animal, with the head attached, to be drawn out of the body cavity. The gut of the locust bearing the Malpighian tubules was placed in a hollow groove in a perspex dish which contained control saline, the head rested on the lip of the dish. The whole

preparation was covered in water-saturated liquid paraffin, tubules were drawn out of the saline bath and looped around pegs. Each tubule was punctured at a convenient point, using a fine tungsten needle, allowing the formation of a near-spherical droplet of "urine". The temperature was maintained at 30 ± 0.5°C by placing the perspex chamber on a thermostatically controlled hotplate. Up to ten Malpighian tubules could be set up in this way and fluid secretion monitored.

The rate of fluid secretion was determined by measuring the rate of change in diameter of each secreted droplet of "urine", as described elsewhere (Ramsay, 1954; Maddrell, 1969). After an initial 15 min equilibration period, the secretion rate was determined by measuring the diameter of the secreted droplet at 5 min intervals over a period of 25 min to give rate 1. The saline bathing the tubules was then replaced by a fresh solution of either the same (control) or modified (experimental) composition and after a further equilibration period of 20 min, the new secretion rate (rate 2) was determined over a second 25 min period. The effect of treatment was determined by comparing rate 1 to rate 2. In this way, each tubule served as its own control and variation between individual tubules was accounted for.

The radius of the droplet was measured using an eyepiece graticule and the volume of the droplet was determined by assuming it to be a sphere according to the formula $^4/_3\pi r^3$ (where r is the radius of the droplet). The rate of fluid secretion was expressed in nl/min.

Collection of secreted "urine"

Malpighian tubules were set up as described for measurements of rates of fluid secretion. After an initial equilibration period of 15 min the "urine" droplet was removed and discarded. The "urine" secreted over the next 60 min was dislodged from each tubule using a fine glass rod and pooled on a clean glass coverslip under the liquid paraffin. The pooled droplet of "urine" was then taken up in a clean glass pipette, transferred to a clean plastic petri dish and 1µl or 0.5µl volumes collected in microcap

capillaries. The samples were then diluted to 10ml with DDW in a plastic universal tube.

Determination of Na^+, K^+ and Rb^+ concentrations in the "urine"

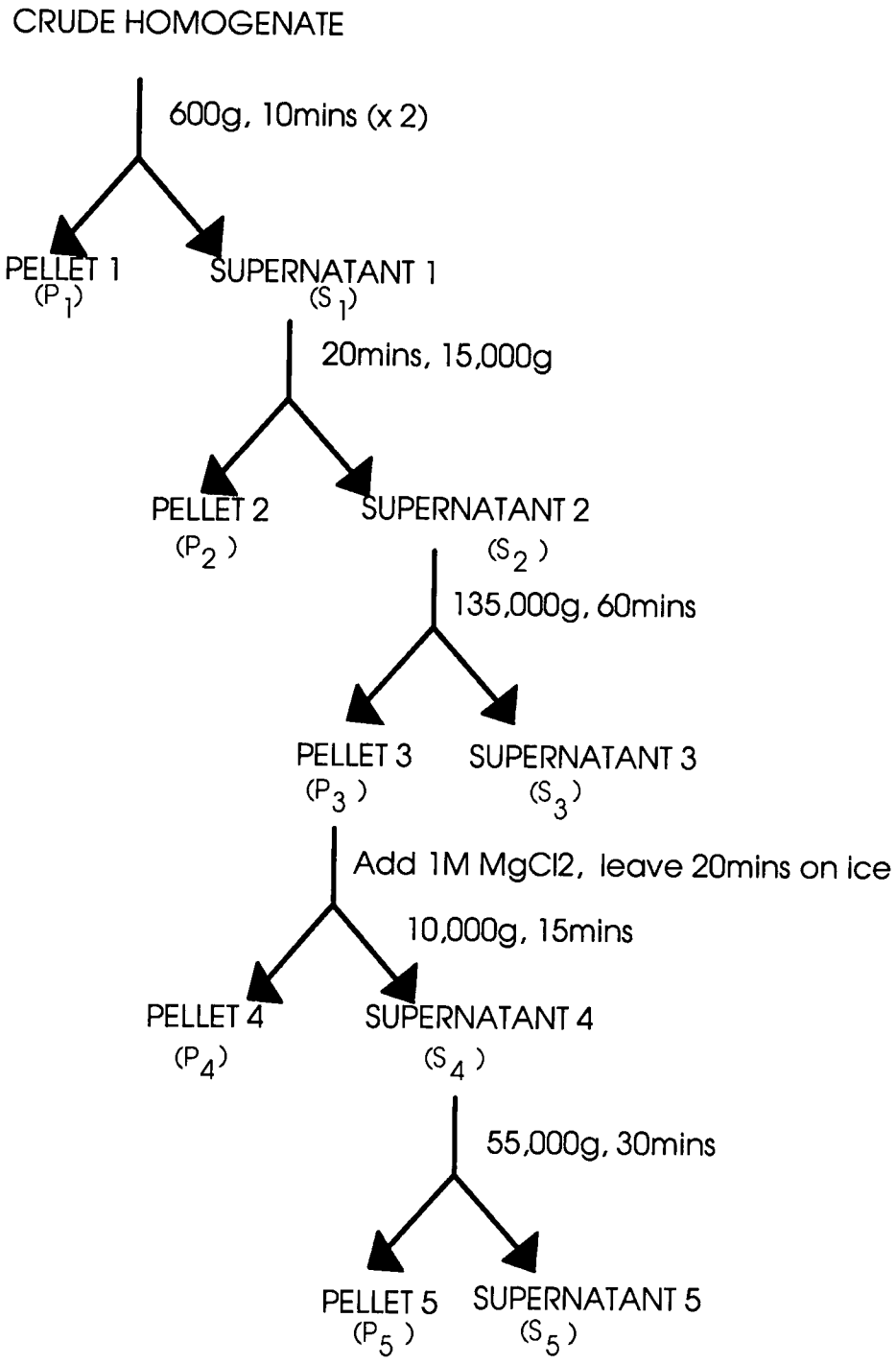
The Na^+, K^+ and Rb^+ concentrations in the secreted fluid were determined by atomic absorption (for Na^+ and K^+) and atomic emission spectrophotometry (for Rb^+) using a Perkin-Elmer 5000 spectrophotometer. Emission and absorbance readings were related to standard calibration curves constructed by serial dilution of NaOH, KOH and RbCl stock solutions (see Appendix, Figs. A.1., A.2. and A.3.).

Cell fractionation

The technique used for cell fractionation was a modification of that reported by Rodriguez and Edelman (1979) and Fogg *et al.*, (1991) for isolation of the apical and basolateral membranes of toad bladder cells and Malpighian tubule cells of *Locusta*, respectively. A flow diagram summarizing this method is shown in Fig 2.1.

The Malpighian tubules were quickly dissected out as described previously in ice-cold homogenization medium (5mM imidazole-HCl buffer, pH 7.5, containing 250mM sucrose) and then placed in a homogenization tube which contained an appropriate amount of ice-cold buffer (tubules from approximately 20 locusts to 10ml buffer). Homogenization was carried out as described by Anstee and Fathpour, (1979) and the resulting homogenate centrifuged at 600 x g for 10 min using an MSE Coolspin centrifuge. This pellet was then resuspended and the process repeated. All subsequent centrifugations were carried out using a Fisons MSE "Prepspin 50" Ultracentrifuge, fitted with an 8 x 50 ml, fixed angle rotor head. The supernatant (S_1) obtained from the initial centrifugation was centrifuged at 15,000 x g for 20 min. This resulted in the production of a mitochondria-rich pellet (P_2). The supernatant (S_2) produced by this centrifugation was then centrifuged at 135,000 x g for 60 min and the pellet obtained (P_3) resuspended and rehomogenized in 10ml of homogenization medium with 1M

Fig. 2.1. Schematic representation of the centrifugation steps taken to produce apical and basal plasma membranes of Malpighian tubules



P_1 - crude membrane pellet
P_2 - mitochondria-rich pellet
P_4 - basal membrane-rich pellet
P_5 - apical membrane-rich pellet

$MgCl_2$ added to give a final concentration of 10mM $MgCl_2$. This was then left to stand on ice for 20 min. The resuspended P3 was then centrifuged at 10,000 x g for 15 min. The resulting supernatant (S4) was then centrifuged at 55,000 x g for 30 min. The pellet (P5) 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 DDW. All steps were carried out at 4°C.

ATPase assays

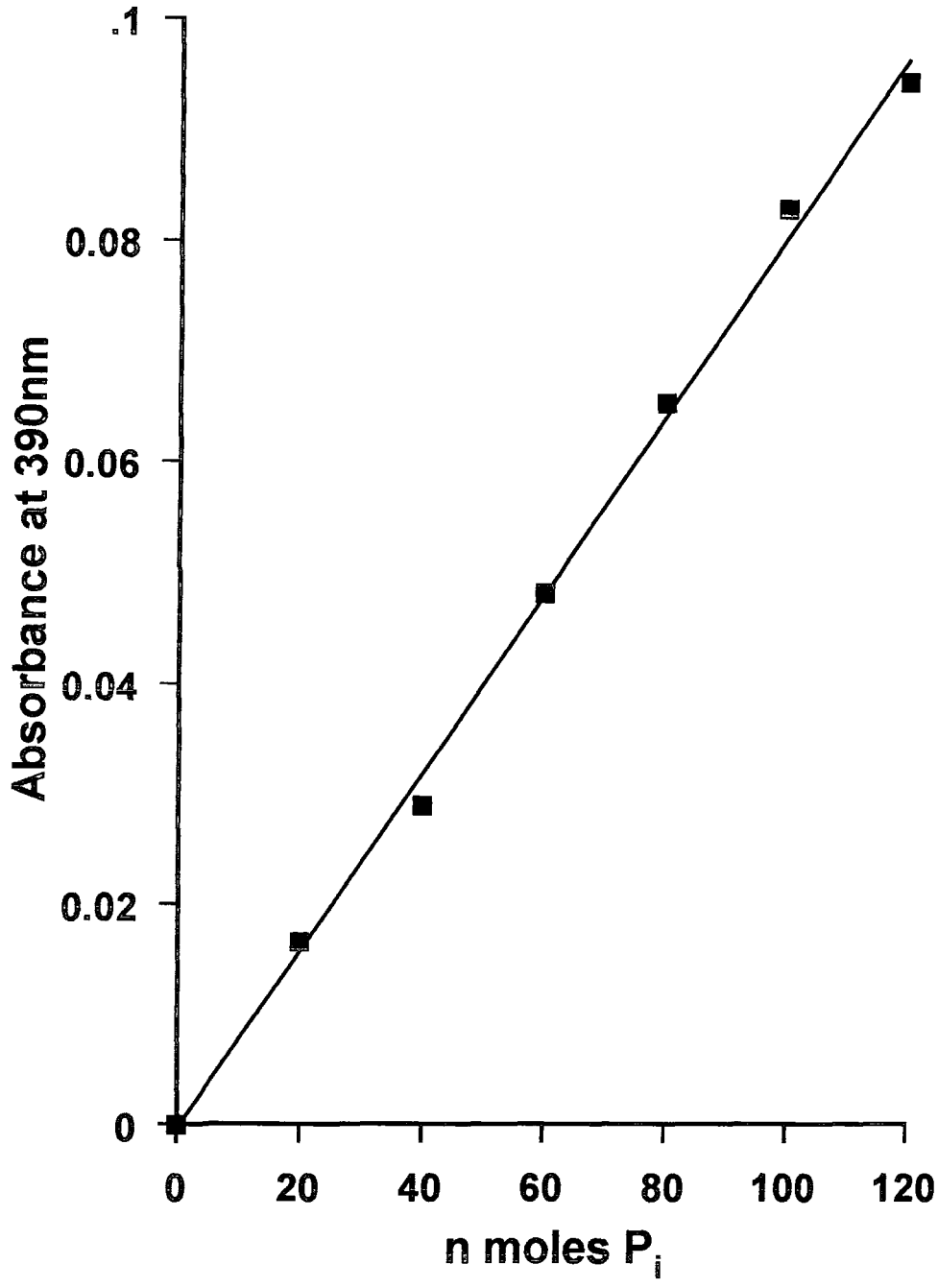
Incubation media which consisted of 100µl of the appropriate ionic medium and 50µl of membrane homogenate were thermoequilibriated for 15 min in Eppendorf tubes in a water bath. Reactions were started by adding 50µl of 12mM ATP (Tris salt) and incubations were carried out at 35°C for 30 min. Reactions were stopped by the addition of 400µl of a 1:1 mixture of 1% Lubrol and 1% ammonium molybdate in 0.9M sulphuric acid (Atkinson *et al.*, 1973). At the end of this time tubes were left to stand at room temperature for 10 min to allow the yellow colour of the reaction product to develop. Controls were run to determine the extent of non-enzymatic hydrolysis of ATP.

Determination of inorganic phosphate

Inorganic phosphate was determined using the method of Atkinson *et al.*, (1973). After centrifugation at 13,000 rpm for 5 min in a Heraeus Biofuge 13, at 4°C, to spin down any protein which might have precipitated, the amount of inorganic phosphate released (indicated by a yellow colour) was determined by reading at 390nm in a Beckman Du 7500 spectrophotometer. To convert the absorbance readings to amount of inorganic phosphate a standard calibration curve relating amount of inorganic phosphate to absorbance was produced. A stock solution of 0.6mM Na_2HPO_4, was serially diluted to give a concentration range of 0-0.6mM (Fig 2.2.). To 200µl of each concentration 400µl of a Lubrol/acid molybdate solution (1 volume of 1% Lubrol in

Fig. 2.2.

Standard calibration curve for the determination of inorganic phosphate



deionized water : 1 volume of 1% ammonium molybdate in 0.9M sulphuric acid) was added. Each mixture was left to stand for 10 min at room temperature and the absorbance read at 390nm.

Determination of HCO_3^--stimulated ATPase activity

Appropriate reaction media were thermoequilibriated for 15 min as described previously. The reaction media had the following final concentration of ions, unless otherwise stated :

i) 4mM $MgCl_2$ in 20mM imidazole buffer, pH 7.5,
ii) (i) plus 20mM NaCl,
iii) (i) plus 20mM $NaHCO_3$.

Liberation of P_i was measured as described previously. HCO_3^--stimulation of ATPase activity was calculated as the difference in P_i liberated in reaction medium (iii), containing $MgCl_2$ and $NaHCO_3$, and that released in medium (ii), containing $MgCl_2$ and NaCl. Controls were run where ATP was not added until the reaction had been stopped 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_i liberated /mg protein/ min.

Determination of Na^+/K^+-ATPase activity

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

i) 4mM $MgCl_2$ in 20mM imidazole buffer, pH 7.2,

ii) (i) plus 100mM NaCl and 20mM KCl,

iii) (ii) plus 1mM ouabain.

The amount of P_i released from ATP hydrolysis was measured as described previously. Na^+/K^+-ATPase activity was determined as the difference in P_i liberated in reaction medium (ii), containing Mg^{2+}, Na^+ and K^+, and that released in medium (iii), containing Mg^{2+}, Na^+ and K^+ 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_i liberated /mg protein/ min.

Determination of azide- and orthovanadate-insensitive ATPase activity

Incubations were carried out as described previously (see ATPase assays). The reaction medium (Schweikl *et al.*, 1989) had the following final concentration of ions (mM) :

$MgCl_2$ 1, KCl 20, NaN_3 0.5, Na_3VO_4 0.1, 0.1mM EGTA and 0.3mg BSA/ml in 50mM Tris-MOPS buffer, pH 7.5.

Activity was expressed in nmoles P_i liberated /mg protein/ min. Controls were run where ATP was not added until the reaction had been stopped to determine the extent of non-enzymatic hydrolysis.

Determination of succinate dehydrogenase activity

The method used was similar to that described by King (1967). Cuvettes containing reaction media were thermoequilibriated at 35 ± 0.1°C for 15 min. The reaction media included 0.75 ml of 0.2M phosphate buffer (pH 7.8), 0.1ml of 0.6M sodium succinate, 0.15ml of 1% BSA, 0.05ml of 0.03M potassium ferricyanide (freshly prepared) and 0.25ml distilled water. Reactions were started by the addition of 0.25ml

membrane homogenate. Control tubes were run in parallel in which 0.25ml deionized water replaced the 0.25ml membrane homogenate. Changes in absorbance at 420nm were measured at known intervals over a 20 min period using a Unicam SP1800 spectrophotometer. As the rate of decrease in light absorbance at 420nm, due to ferricyanide reduction by succinate, is used as a measure of enzyme activity. The observed changes in absorbance were converted to units of mM succinate oxidized, as described by King (1967), i.e., 0.485 x change in absorbance at 420nm. The specific activity was expressed as nmoles succinate oxidized/mg protein/min.

Determination of alkaline phosphatase activity

The method of Bowers and McComb (1966) was used with slight modification. Reaction media were thermoequilibriated in cuvettes at 35 ± 0.1°C for 15 min. The reaction media consisted of 1ml of buffered substrate (1mM $MgCl_2$ and 25mM p-nitrophenyl phosphate in Sigma buffer 221, pH 10.3) and 0.5 ml DDW. Reactions were started by adding 0.5ml membrane homogenate. Controls were run in parallel in which 0.5ml DDW water replaced the membrane homogenate.

Changes in absorbance at 420nm were measured at known intervals over a 20 min period using a Unicam SP1800 spectrophotometer. Changes in absorbancy were converted to nmoles p-nitrophenol (NP) liberated by reference to a standard p-nitrophenol curve (Fig. 2.3.) produced by serial dilution of a 2mM stock solution.

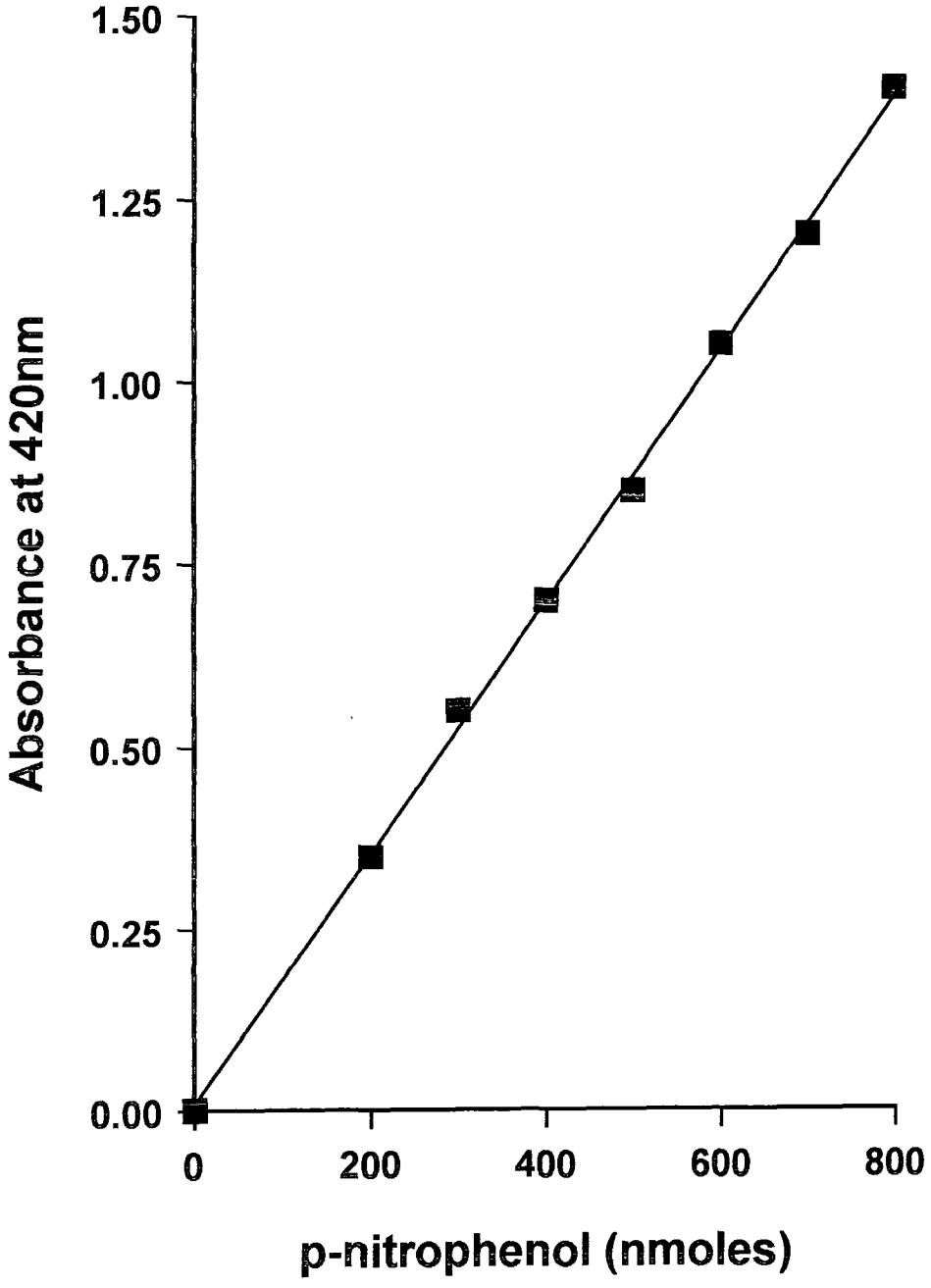
Determination of protein content

Protein determinations were made using the Coomassie brilliant blue binding method of Bradford (1976), using bovine serum albumin fraction V (BSA) as standard. The dye reagent used was Bio-Rad protein assay dye reagent concentrate.

Protein determinations were carried out by placing 5-50µl of the sample in a cuvette to which 2ml of protein reagent was added. The solutions were mixed and then

Fig. 2.3.

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



left for 10 min at room temperature before reading their absorbances at 595nm using an Ultraspec 4050 spectrophotometer.

Protein content was determined by reference to a freshly made standard BSA curve relating protein amount (0-50μg) to absorbance of standard solutions. Fig 2.4. shows a typical example of a standard calibration curve.

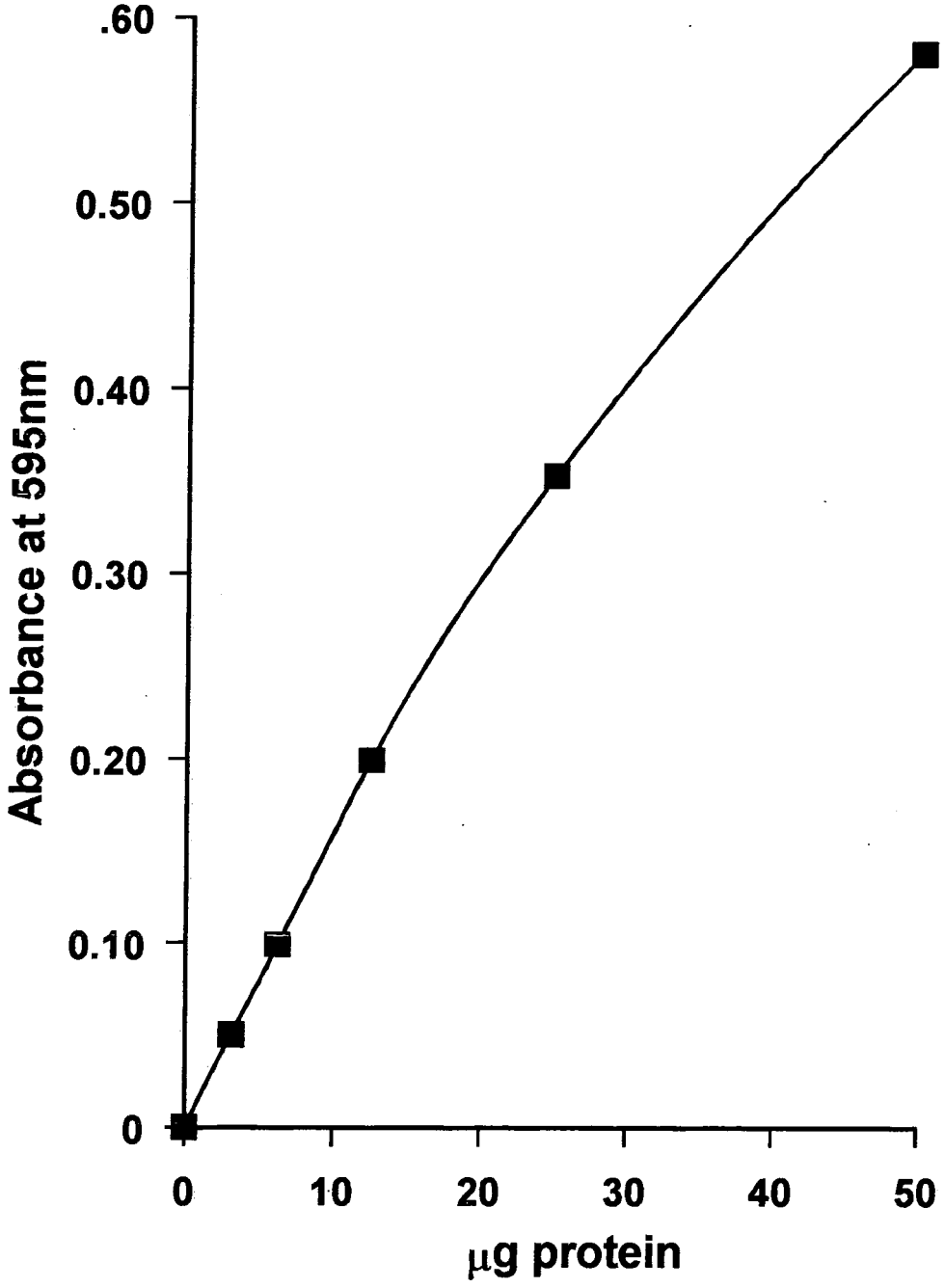
Cell Culture

First instar locusts were killed as described earlier. The Malpighian tubules were dissected out in sterile-filtered saline, rinsed and then incubated at 25°C in a sterile solution of 0.5% dispase for 30 min. At the end of this time tubules were washed in fresh saline three times and then triturated gently. The tubules were then transferred to a droplet of insect cell culture medium on a Matrigel dish. These were ordinary sterile tissue culture dishes coated with a thin layer of Matrigel, which had been diluted with MEM (1ml Matrigel : 3ml MEM). Matrigel (Becton-Dickinson) is a solubilized basement membrane preparation which has been shown to be biologically active and to stimulate the growth and differentiation of certain cells (Kleinman *et al.*, 1986). A coverslip was then placed on top of the tubules and pressed down firmly to encourage attachment to the Matrigel, 2ml of culture medium was then added. The culture medium used was insect medium IPL-41 (Sigma). To 10ml of this medium 0.5ml of penicillin/streptomycin and 1ml of foetal calf serum were added and mixed thoroughly. 2ml of the mixture was added to each culture dish.

The dish was then transferred to a CO_2 incubator at 37°C. The culture medium was replaced every 2-3 days and the condition of the cells was monitored under the microscope. Photographs were taken using a Zeiss Axiovert 135 (for transmitted light and incident-light fluorescence).

Fig. 2.4.

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



Determination of viability of cells

A dish containing cultured cells which were 21 days old was chosen and 2ml of filtered trypan blue stain (0.5%) was added to the culture medium. The coverslip was eased up gently to allow the stain to reach the cells. This a convenient technique to assess the number of living cells in a culture. As the surface membrane of living cells is able to selectively exclude certain substances but dead cells will take it up (Paul, 1975). Photographs were taken using a Zeiss microscope.

Preparation of Malpighian Tubules for Transmission Electron Microscopy

Locusts were killed and Malpighian tubules dissected out in ice-cold Karnovsky's fixative (see below) as described previously. Tubules were prepared for electron microscopy by fixation in Karnovsky's fixative (Karnovsky, 1965), which comprised :-

<u>Solution A</u>	
Paraformaldehyde	2g
Distilled water	40ml
NaOH	drops added until the paraformaldehyde dissolves (heat applied too).
<u>Solution B</u>	
25% Gluteraldehyde	10ml
0.2M Cacodylate buffer, pH 7.3	50ml

Solutions A and B were mixed in a 1:1 ratio just before use. 0.25M sucrose was also included in the fixative. Material was fixed for 1-1.5 hours on ice.

After fixing, material was post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.5) for 1 hr, on ice. Following post-fixation, the tubules were

treated to the following procedure :-

i) Dehydration through a series of graded alcohols ;

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

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

(c) 15 min in absolute alcohol, with 3 changes,

ii) 30 min in absolute alcohol /propylene oxide in 1:1 ratio, with 3 changes,

iii) 30 min in propylene oxide, with 3 changes,

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

v) 30 min in Araldite alone at 45°C,

vi) embedded in fresh Araldite alone for polymerisation at 45°C overnight,

vii) preparation moved from 45°C to 60°C for a further 24 hr.

Silver or gold sections were cut on a Reichart NK microtome using glass knives. Sections were expanded using chloroform and collected on Formvar coated copper grids. The sections were then stained with uranyl acetate (1% solution in 70% alcohol) and lead citrate (Reynolds, 1963) before examination in a Phillips EM 400T.

Preparation of pellets for Transmission Electron Microscopy

Pellets obtained from the membrane separations (see cell fractionation) were prepared for electron microscopy as described above. Pellets (P_2, P_3, P_4 and P_5) were obtained they were resuspended (individually) in Karnovsky's fixative and placed in

Eppendorf tubes. They were then spun down, at a low speed spin, to form a pellet in a Heraeus Biofuge 13, at 4°C, and left on ice for 1.5 hr. For post-fixation, 1% buffered osmium tetroxide was pipetted onto the pellet in the Eppendorf tube and left to penetrate the material for 1 hr, on ice. The pellet was then dehydrated and embedded according to the procedure described above, the substances being applied to the surface of the pellet.

Scanning Electron Microscopy of single Malpighian tubule cells

The procedure followed was based upon that described by Satmary and Bradley, (1984). Single cells were prepared using the method described previously (see cell culture). Incubation in dispase lasted for up to 1 hour to facilitate separation of individual cells from the tubules. The cells were taken up as a suspension in a syringe, this was inserted into the top of a plastic filter holder and by exerting slight pressure the suspension was put through a 25mm diameter millepore filter, pore size 0.45μM Then the cells were washed several times with saline. The cells were fixed on the filter for 1 hour, on ice, in $^{1}/_{2}$-strength Karnovsky fixative prepared as described for transmission electron microscopy but diluted with sodium cacodylate buffer (0.25M sucrose was still included in the fixative). The cells were then dehydrated through an ethanol series as described previously. Once in 100% ethanol the filter could be transferred to a E300 jumbo critical point dryer (Polaron). Once in the dryer the transfer liquid (ethanol) is replaced by liquid CO_2. Hot water is then passed through the water jacket surrounding the dryer raising the temperature of the drying chamber to 32°C. This causes a pressure rise from about 800 p.s.i. to about 1,150 p.s.i. At this point the liquid/gas meniscus disappears and so the chamber contains only dry gas. The gas is then released from the chamber to leave dry tissue. The filters were then attached to scanning electron microscope (SEM) stubs using double sided sellotape. Specimens were coated with gold palladium using an SEM coating unit E5100 (Polaron instruments limited) and viewed with a JSM-IC848 scanning electron microscope.

Electrophysiology

Locusts were killed as described previously, Malpighian tubules were dissected out in control saline, washed and placed in fresh control saline. In order to record electrical potentials a perspex perfusion chamber was designed (Fig 2.5.) based on the "gap" system of Berridge and Prince (1972a). A length of Malpighian tubule (about 2cm) was pinned out in experimental bath A, the ends being held under flaps of Sylgard, which coated the bottom of the bath to a depth of approximately 4mm. Microelectrode recordings took place in bath A which was superfused by the experimental saline via a glass inlet tube B. The tubule was positioned over a raised Sylgard block (D) in bath A to aid microelectrode penetration. Saline superfusing the experimental chamber arrived from one of a number of perfusion bottles. These bottles were surrounded by a water jacket to allow the contained saline to be thermoequilibriated at 30°C and each bottle was connected to a perspex manifold (Fig 2.6.) via plastic tubing. A clip on the tubing coming from the base of each bottle was opened to allow superfusion of the appropriate saline. Experiments where amaranth dye was placed into one of the bottles and then allowed to circulate through the system showed no observable mixing with other solutions at the manifold junctions. Liquid was drawn away from the system via a glass outlet tube C, positioned over bath A, by a peristaltic pump (Watson Marlow 502S) and passed to a waste bottle.

The tubing used was either P.V.C. (Gallenkamp) or silicon (Watson Marlow). The temperature of the perspex experimental chamber was maintained at 30 ± 0.1°C by placing it on the surface of a water-heated metal bath (provided by a Gallenkamp Haake DI water bath). Liquid paraffin placed in the metal bath ensured thermal contact between the bath and experimental chamber. A fibre optic light (Ealing Beck Ltd., Watford, Herts., England), placed at one side of the chamber, provided illumination without heating the preparation.

Fig. 2.5. Experimental chamber used for microelectrode recordings

Drawing of the perspex experimental chamber. Experimental bath (A) was superfused and recordings of the basal membrane potential occurred here. B was the inlet tube and C the outlet tube for the solutions superfusing the experimental chamber. The tubule was pinned over (D), a Sylgard block. E shows the position of the agar bridge, which ran from a pot containing 3M KCl to the experimental chamber (A).

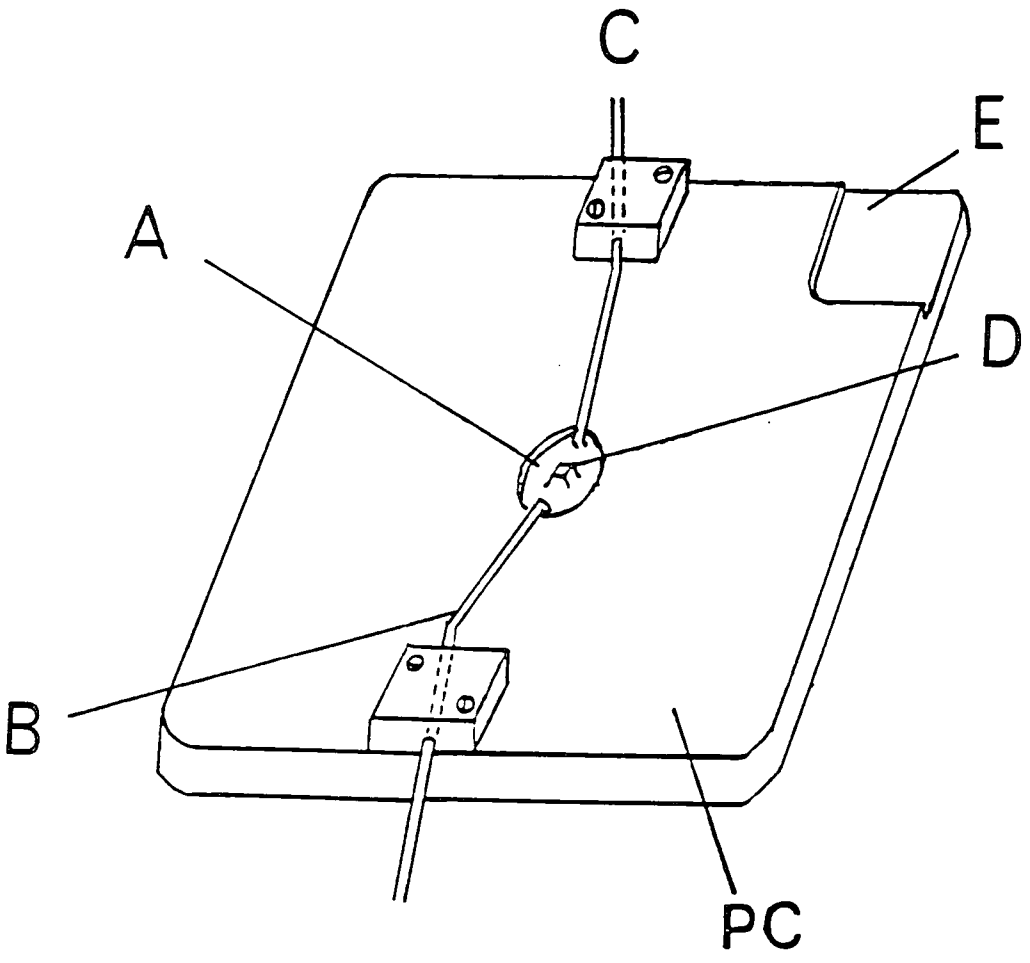
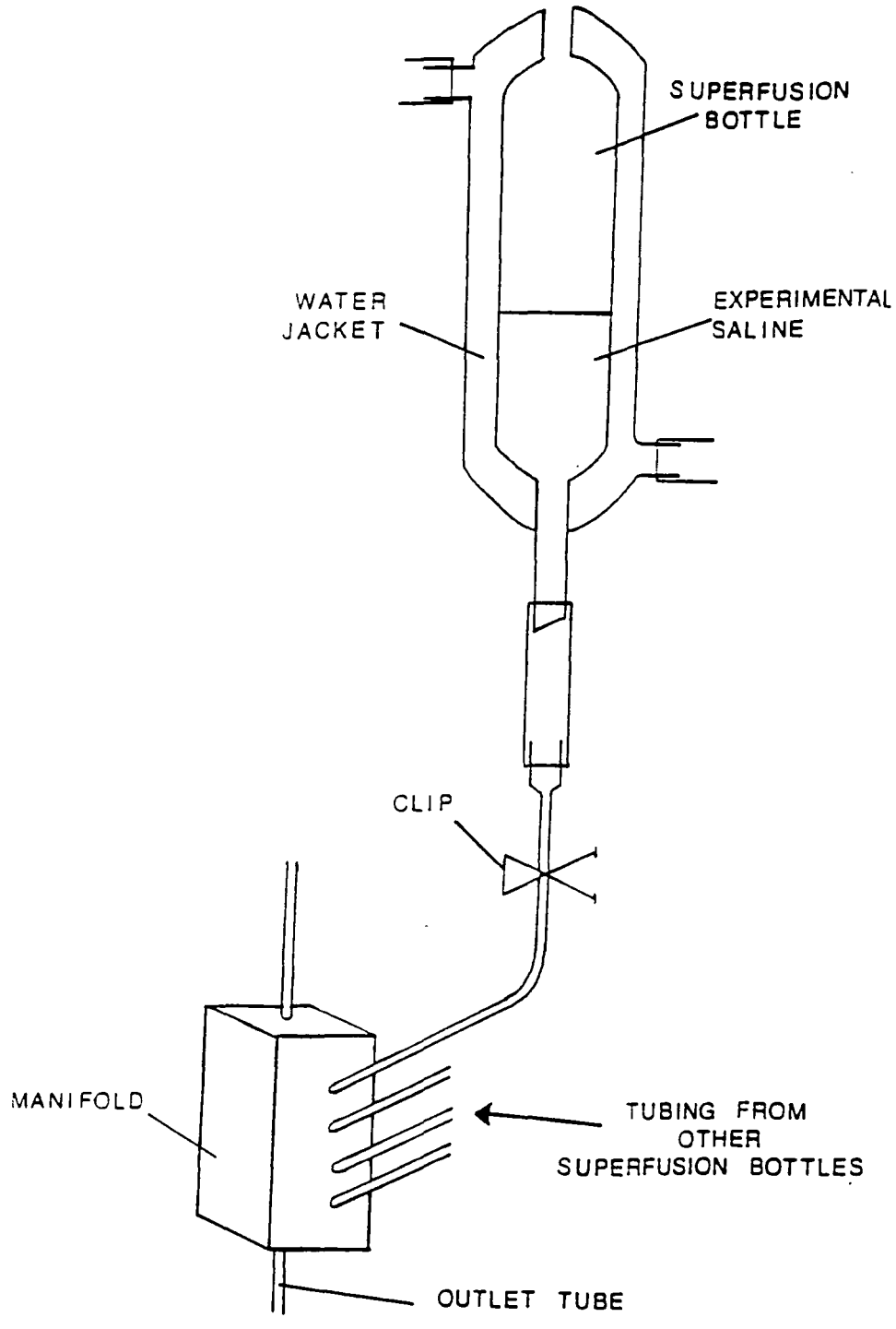


Fig. 2.6. Superfusion bottle used to supply saline to the experimental chamber

Drawing of one of the superfusion bottles which supplied the experimental chamber. The jacketing is shown which served to maintain the temperature of the contained solution at a constant, specific temperature. The manifold received inputs from all the bottles but had only one outlet to the experimental chamber.

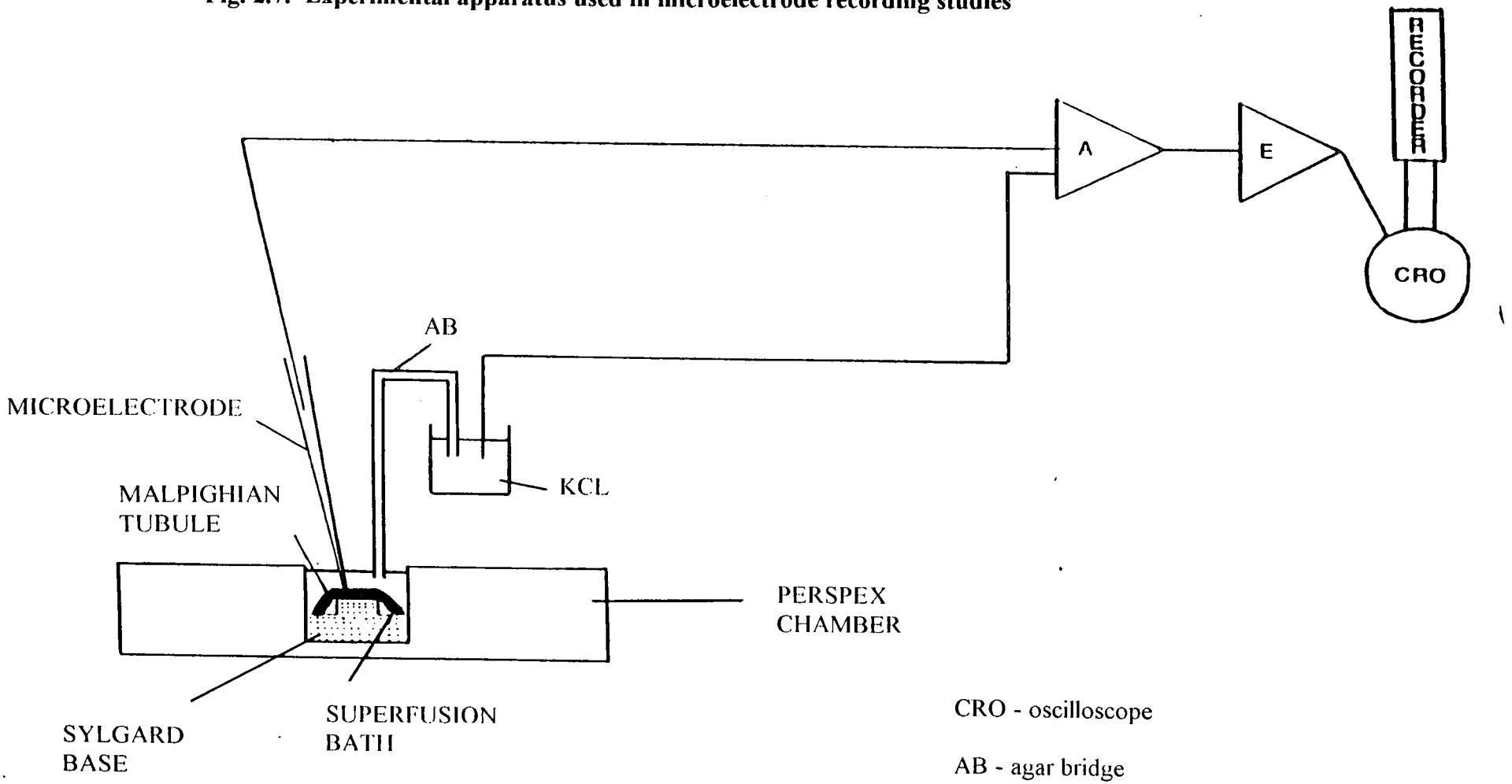


Electrical Recording

In order to record electrical potentials an experimental set-up was designed, based on that of Berridge and Prince (1972a). Fig 2.7. shows that the basal potential was measured by connecting bath A (the experimental chamber) to an Ag/AgCl electrode in 3M KCl via glass 3M KCl/4% agar bridges (AB). The Ag/AgCl electrode was connected to an oscilloscope (CRO) (Telequipment Type D1010) and a pen recorder (Servogor 460 Metrawatt, Nurnburg, Germany) via two components of a high input resistance field effect amplifier (E) based on the design of Colburn and Schwartz, (1972). The system was zeroed at the beginning of each experiment by placing the microelectrode in bath A. Deviations away from the baseline were due to junction potentials between KCl and Ag/AgCl electrodes or old agar bridges (Barry and Diamond, 1970). This problem was solved by scraping the silver wires and leaving them in a strong salt solution overnight or by using a sintered pellet. Membrane potentials were measured using single barrelled microelectrodes made from 1mm bore diameter thin walled glass capillary tubing with an inner filament (GC 200F-10, Clarke Electromedical, Reading, England). These were pulled on a single-barrelled vertical microelectrode puller (Harvard) and back-filled with 3M potassium acetate using a syringe. A thin, chloride-coated silver wire was inserted into the bottom of the microelectrode and connected to the input stage (A) of the high resistance field-effect amplifier (E) the output of which was displayed on the oscilloscope (CRO) and recorded on the Servogor chart recorder.

Microelectrodes were positioned in the perfusate near the Malpighian tubule using a micromanipulator (Prior, England). Microelectrode tips were ~1μm in diameter, enabling easy cell penetration and reducing cell damage. The resistances of the microelectrodes were measured using a constant current generator system incorporated in the amplifier. A 1nA current was passed down a microelectrode resulting in a voltage deflection on the oscilloscope. Using Ohm's law (V=IR) a value for microelectrode resistance was calculated. Resistances were measured in the superfusing fluid and were

Fig. 2.7. Experimental apparatus used in microelectrode recording studies



CRO - oscilloscope

AB - agar bridge

A, E - components of the high resistance amplifier

at least 20MΩ rising to 50MΩ.

Before an experiment, the microelectrode was equilibrated in the superfusate and the resistance noted. The microelectrode was then manoeuvred above the tubule using the fine advance on the micromanipulator, the microelectrode was held at an angle of about 60° in the micromanipulator with respect to the tubule. Gentle tapping of the baseplate to which the micromanipulator was attached caused rapid penetration of a cell, shown by the sudden appearance of a resting potential on both the oscilloscope and the pen recorder.

A successful microelectrode penetration resulted in the following observations:

i) The change in potential from the baseline was sudden,

ii) The intracellular voltage remained relatively constant after impalement,

iii) The voltage returned to the original baseline, or close to it , when the microelectrode was removed from the tubule cell.

Setting up and penetration of the tubules was viewed under a Zeiss microscope. All experiments were carried out in a Faraday cage to prevent electrical interference and a vibration-damped bench was used. After a successful penetration experiments involving solution changes could be carried out. Tip resistances were checked after experimentation for blockages. The experimental chamber was washed thoroughly between experiments. All potentials were measured in millivolts (mV).

Gel electrophoresis

SDS-PAGE

The method followed was essentially that described by Laemmli (1970), for discontinuous gels. Samples (enriched membrane fractions from Malpighian tubules of *Locusta* and a crude homogenate of midgut from *Manduca sexta*) were dissolved in sample buffer containing 0.0625M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 10% (w/v)

SDS, 5% (v/v) 2-mercaptoethanol and 1% (w/v) bromophenol blue. These samples were then heated for 5 min at 100°C in a water bath before being loaded on to a 12.5% (w/v) polyacrylamide slab gel via a 3.9% (w/v) stacking gel. Approximately equal amounts of protein (~15μg) were loaded in each lane. The gels were run at a constant voltage (40mV) using a mini PROTEAN II electrophoresis system (Bio-Rad) with the power supply from a PowerPac 300 (Bio-Rad). 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. Gels were destained in a solution containing 40% methanol and 10% acetic acid.

Western blotting

Proteins were blotted from acrylamide gels to nitrocellulose using the semi-dry blotting method of Kyhse-Andersen (1984). Transfer was carried out on a Bio-Rad Trans blot, semi-dry transfer cell. 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 all of which had been equilibrated in transfer buffer (48mM Tris, 39mM glycine, 0.0375g/l (w/v) SDS and 20% methanol (Analar) pH 9.2; Bjerrum and Schafer-Nielsen, 1986) for 15 min. Each mini-gel was transferred at 25V and 220mA (5.5mA/cm^2) for 30 min using a power pack (Flowgen).

After blotting, the nitrocellulose membrane was removed and transferred immediately into blocking solution (PBS, 5% Marvel and 1% Tween-20) for 1 hour at room temperature. The blot was then incubated with the primary antibody in anti-sera buffer (PBS, 5% Marvel and 0.1% Tween-20) overnight at 4°C shaking. Best results were obtained using 1:4000 dilution. 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. The nitrocellulose was then washed 3 x 5 min in anti-sera buffer before incubation with the secondary antibody for 2 hr at room temperature, 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 2 x 5 min and 1 x 15 min, shaking. Finally the membrane was rinsed in water for 2 x 5 min. The membrane was then kept in water.

ECL Detection

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^2 membrane.

ii) The excess water was drained from the membrane.

iii) Detection reagent was poured onto the membrane (protein side up) and left for 1 min.

iv) The membrane was drained as before and then placed between acetate sheets in a cassette.

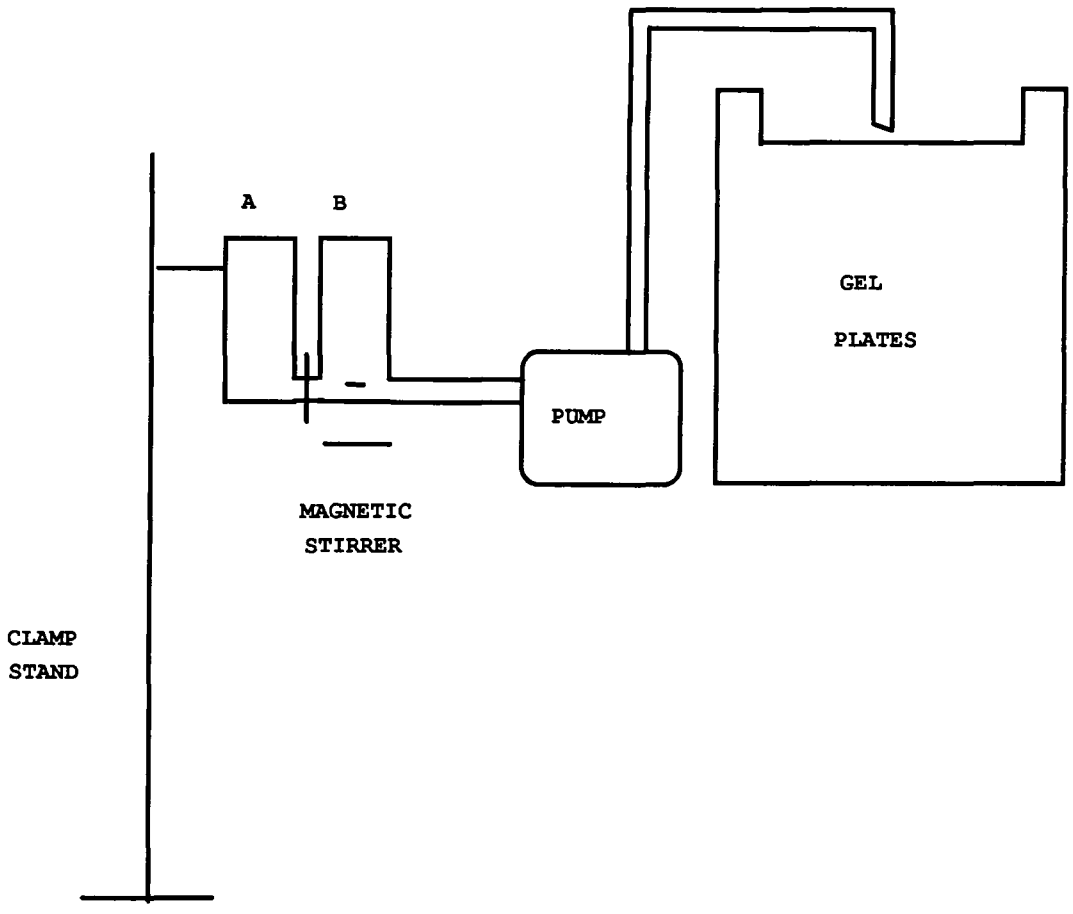
v) The membrane was exposed to film overnight.

Native gradient polyacrylamide gels

The typical apparatus needed to produce concentration gradient gels is shown in Fig 2.8. Chambers A and B contained acrylamide mixtures corresponding to the lowest and highest concentrations in the gel and were made up as shown in Table 2.2. The highest acrylamide concentration mixture contained 15% and the lowest 3% acrylamide respectively. TEMED and ammonium persulphate were added just before the gel was poured, after the gels have been degassed, to prevent premature polymerization. The 3% mixture was added to chamber A and the clip of the connecting tube was opened in order to fill it and then closed. The 15% mixture was then added to chamber B. An equal

Fig 2.8.

Apparatus for the production of gradient polyacrylamide gels.



A was the reservoir of the gradient maker and B was the mixing chamber which contained a magnetic flea. B was suspended above a magnetic stirrer by a clamp. A clip isolated the two chambers (A and B). Tubing which connected the gradient maker to the peristaltic pump and glass gel plates was silicon tubing (1.6mM, inner diameter). The end of this tubing was cut at a 45° angle and held at the centre of the rear gel plate with the cut edge facing this plate.

volume of each concentration was added to each chamber, 3.8ml of each mixture was used as this produced the correct final volume for the resolving gel. The magnetic stirrer was turned on, the clip undone and the peristaltic pump (Watson-Marlow, 505S) turned on at a rate of 55rpm. Immediately after the gel was poured it was overlaid with water. After polymerization, the water was removed and a stacking gel was poured (Table 2.2.).

Table 2.2. Gel mixtures for a 3-15% gradient gel and a stacking gel

Reagent	15%	3%	Stacking
DDW	3.5 ml	12.7 ml	6.18 ml
1.5M Tris, pH 8.8	5 ml	5 ml	-
10% Triton	0.2 ml	0.2 ml	0.2 ml
30% Acrylamide	10 ml	2 ml	1 ml
Glycerol	1.2 ml	-	-
10% Ammonium persulphate	75 µl	100 µl	100µl
TEMED	10 µl	15 µl	20 µl
0.5M Tris, pH 6.8	-	-	2.5 ml

The following procedure was based on the method described by Schweikl *et al.*, (1989) with minor modifications. The enriched apical-membrane pellet (P_5) was produced as described previously. This was resuspended in the following buffer: 10mM Tris-HCl, pH 7.5, 0.32mM EDTA and 5% glycerol. Then Triton X-100 was added to produce a final concentration of 1%. This mixture was then put on ice and stirred for 10 min before being centrifuged at 100,000 x g for 1 hour, using a 12 x 10 rotor, in a Beckman L-70 Ultracentrifuge. The solubilized enzyme was then mixed with sample buffer in a 1:1 ratio. The sample buffer consisted of 0.0625M Tris-HCl, pH 6.8, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol (modified from Laemmli, 1970). 10µl of each sample was loaded onto the gel. The gel was then run at 20mA constant current for 4-5 hours at 4°C.

The incubation and localization procedure followed that described by Schweikl *et al.*, (1989). The incubation medium: 5mM $MgSO_4$ and 100mM Tris-HCl (final concentrations), pH 7.5 was thermoequilibriated for 15 min at 35°C, in a waterbath. The

gel was added and thermoequilibriated for 15 min before 5mM (final concentration) ATP or β-glycerophosphate was added. The gel was left for 1 hour. At the end of this time it was washed in DDW and then 2.5mM of Pb (III) acetate in 80mM Tris malate, pH 7 was added and left for 30 min at room temperature. The gel was then thoroughly washed in DDW and 2% ammonium sulphide was added until the bands appeared on the gel when it was rinsed again in DDW.

Immunofluorescence microscopy

The method used was according to Klein *et al.*, (1991) with minor changes. All steps were carried out at approximately 4°C. Malpighian tubules from adult *Locusta* and midgut tissue from 4th instar *Manduca* larvae were dissected out in cold PLP fixative (2% paraformaldehyde, 0.075% lysine, 0.01M sodium periodate in 0.1M sodium phosphate buffer, pH 7.3. (McLean and Nakane, 1974) and then left in fixative for 2h, on ice. They were then washed in phosphate buffer, 3 x 5 min, and transferred via 10% sucrose in phosphate buffer for 4 x 15 min into 30% sucrose in phosphate buffer overnight. The tissue was embedded in Tissue Tek embedding medium for frozen tissue sections and then frozen in liquid nitrogen. Sections were cut in a cryostat at -20°C and collected on slides coated with 0.01% aqueous poly-L-lysine.

For immunofluorescence staining, sections were taken through the following steps, in which each step lasted for a 5 min period:

i) 0.1M sodium phosphate buffer, pH 7.5, 0.5M NaCl and 0.02% NaN_3 (PBSN) with 0.01% Tween 20,

ii) PBSN with 50mM NH_4Cl,

iii) PBSN,

iv) PBSN with 0.5% gelatine (blocking solution).

The sections were then incubated in the primary antibody diluted in blocking solution overnight at 4°C in a moist chamber. The concentration of primary antibody used was either:

i) 3/1000, for monoclonal antibodies to the V-type ATPase of *Manduca* midgut,

or

ii) 50μg protein/ml, for monoclonal antibody to the α-subunit of avian Na^+/K^+-ATPase.

For controls the primary antibody was replaced with PBSN. Sections were then washed 3 x in PBSN and placed in FITC-conjugated sheep anti-mouse antibody (Sigma, Cat. No. F-6257) diluted, 1:40, for 1 hr at room temperature in the dark. The sections were then washed 3 x in PBSN and mounted in Eukitt.

CHAPTER 3

Studies on ion and fluid transport across the Malpighian tubules of *Locusta migratoria*

RESULTS

Malpighian tubule structure

The structure of the Malpighian tubules of *Locusta migratoria* has been described in detail in previous studies by Bell, (1977); Bell and Anstee, (1977) and Donkin, (1981). An electronmicrograph of a transverse section through a Malpighian tubule is shown in Plates 3.1A and 3.1B confirming that the tubules were made up of two cell types, the primary (type I) cells which represent approximately 90% of the total tubule cell number (Baldrick, 1987) and stellate (type II) cells which made up the remainder. Primary cells are thought to mainly be concerned with ion and water movements (Maddrell, 1971) and so in common with other secretory epithelia are characterized by amplifications of both the basal and apical cell membranes; such as in insect salivary gland (Oschman and Berridge, 1971), mammalian gall bladder (Tormey and Diamond, 1967) and mammalian kidney distal tubule (Berridge and Oschman, 1972). Charnley, (1982) suggested that the type II cells could be mucocytes. Recently a novel secretory "granular" cell has been identified in the initial segment of the Malpighian tubules of *Locusta* (Prado *et al.*, 1992). These cells contain large numbers of cytoplasmic granules concentrated in the apical region as well as abundant rough endoplasmic reticulum and Golgi bodies. Prado *et al.*, (1992) state that the granules are not the same as those previously reported by Sohal, (1974) in *Musca domestica.* The granules in *Musca domestica* being less abundant, more electron dense and only spherical in shape. A role in secretion of digestive enzymes is suggested for these "granular" cells (Prado *et al.*, 1992).

Studying the fine structure of the primary (type I) cells it was found that the basal cell membrane infoldings formed long, narrow extracellular channels which extended approximately one quarter to one third of the way into the cytoplasm; mitochondria were associated with them. The apical infoldings are known as microvilli and these projected into the tubule lumen and often had mitochondria running along their length. The central region of the cells lying between the projections of the two surfaces was seen to contain profiles of circular nuclei, mitochondria and other organelles (Plate 3.1A and 3.1B). A variety of vacuolar structures, ranging from clear vacuoles to lamellated concretions and dense bodies were found in this region and are shown in Plates 3.2A-3.3B. Homogeneous electron dense circular bodies and also other bodies which appeared to be made up of concentric circles of different electron density, which were named lamellated concretions (Donkin, 1981), were distributed throughout the cytoplasm, as shown in Plate 3.2A. Multivesicular bodies, which consisted of membrane bound structures which were filled with smaller membrane bound bodies or vesicles were also a common occurrence in the cytoplasm and are shown in Plate 3.2B. Vacuoles were also located throughout the cytoplasm, often they had an electron dense ring around their outside an example of this is shown in Plate 3.2C. A wide range of such structures can be found together in a small area the cytoplasm. Plate 3.3A shows a vacuole, multivesicular body, dense body and lamellated concretions of different sizes. There are also two darker concretions in which concentric layers can just be made out. Different sizes of the concretions observed and to some extent the different banding patterns could be explained by the plane of sectioning. The vacuoles which were found in the cytoplasm were sometimes "empty" (Plate 3.2C) or contained varying amounts of electron dense material as shown in Plate 3.3B

Fluid secretion studies

Table 3.1. shows that under experimental conditions when Rb^+ replaced K^+ in the bathing medium for the measurement of rate 2, the rate of fluid production recorded

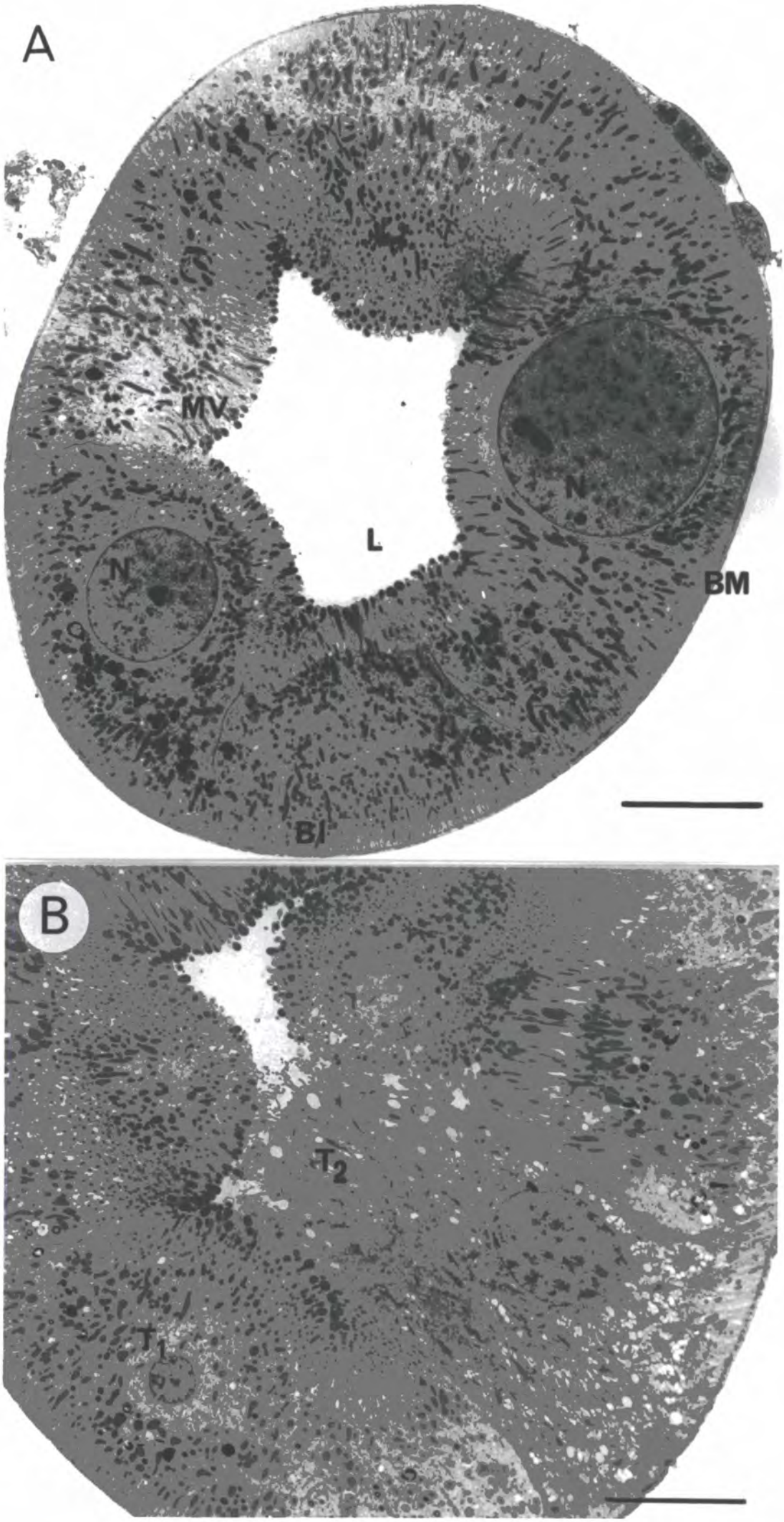

A
MV
L
N
N
BM
BI
B
T2
T1

Plate 3.1A

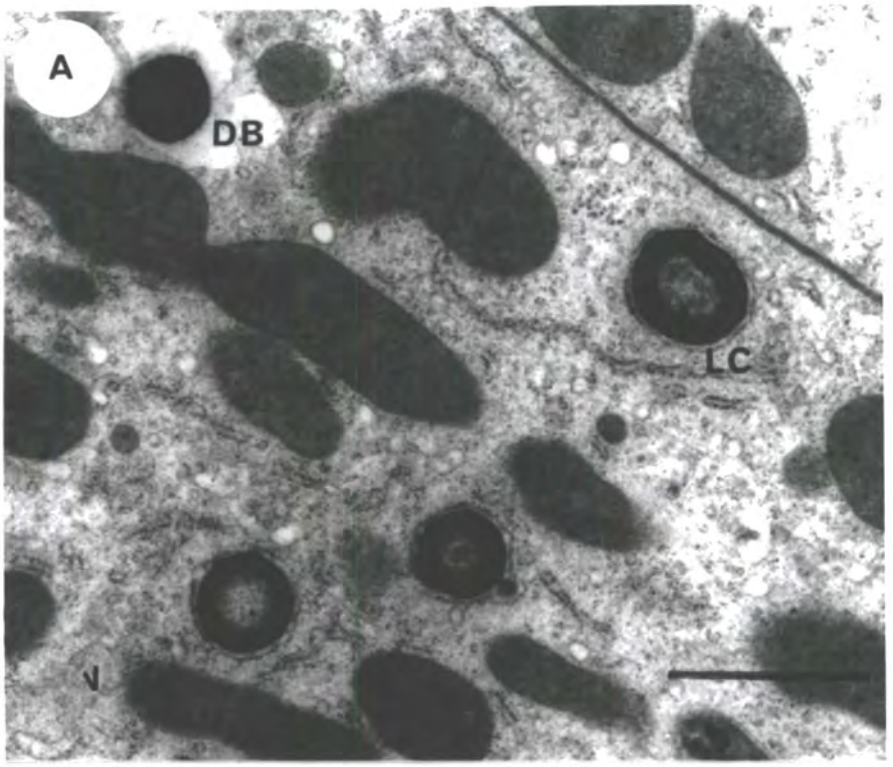
A low power electronmicrograph of a transverse section through a Malpighian tubule of *Locusta migratoria.* The tubule cells were characterized by both basal infoldings (BI) and microvilli (MV) which projected into the lumen (L) from the apical surface. The tubule was one cell thick and large circular nuclei (N) were seen in some of the cells. Mitochondria were present throughout the cytoplasm but were especially concentrated at the basal and apical surfaces. The tubule was completely surrounded by a basement membrane (BM).

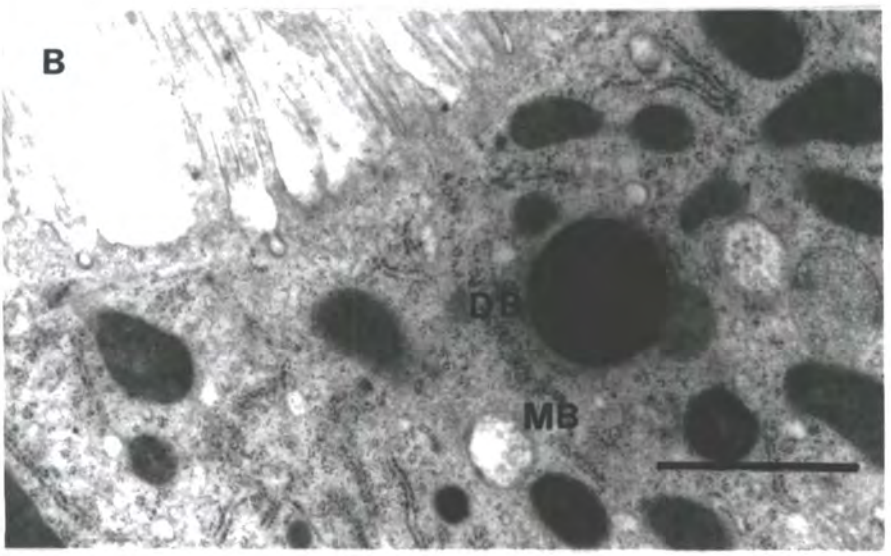
Scale : 10μm

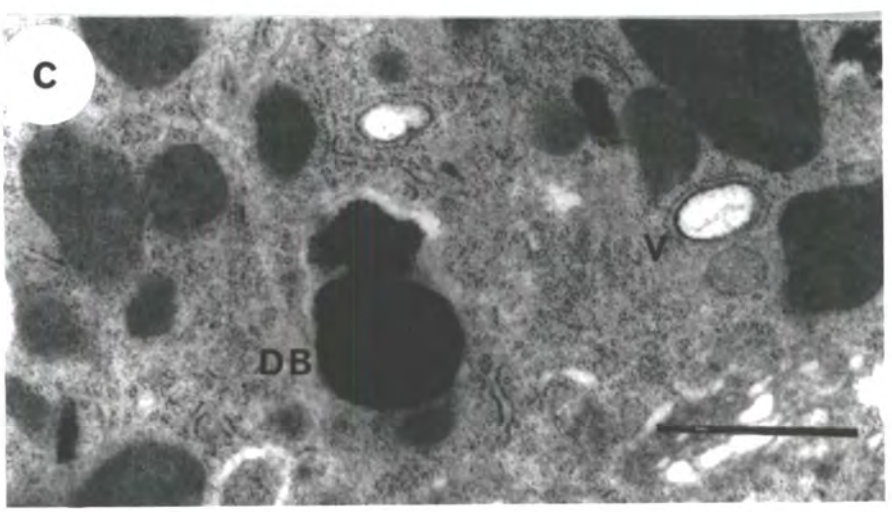
Plate 3.1B

A transverse section through a Malpighian tubule of *Locusta migratoria* showing the presence of two cell types. The type 1 (T_1) or primary cells and the type 2 (T_2) or secondary cells. The cytoplasm of the type 2 cell contained numerous vacuoles towards the apical surface.

Scale : 10μm


A
DB
LC
V


B
DB
MB


C
V
DB

Plates 3.2A-3.3B

Electronmicrographs of transverse sections through primary cells of the Malpighian tubules of *Locusta migratoria* illustrating the various vacuolar structures observed.

Plate 3.2A

Transverse section through a Malpighian tubule cell showing a dense body (DB) and lamellated concretions (LC), which were made up of concentric layers of electron dense material.

Scale : 1μm

Plate 3.2B

The cytoplasm of the Malpighian tubule cell also contained multi-vesicular bodies (MB), which were circular membrane bound structures that contained a large number of smaller circular membrane bound structures. A dense body (DB) was also present.

Scale : 1μm

Plate 3.2C

In the cytoplasm a vacuole (V) could be seen with an electron-dense perphery. A dense body (DB) was also present.

Scale : 1μm

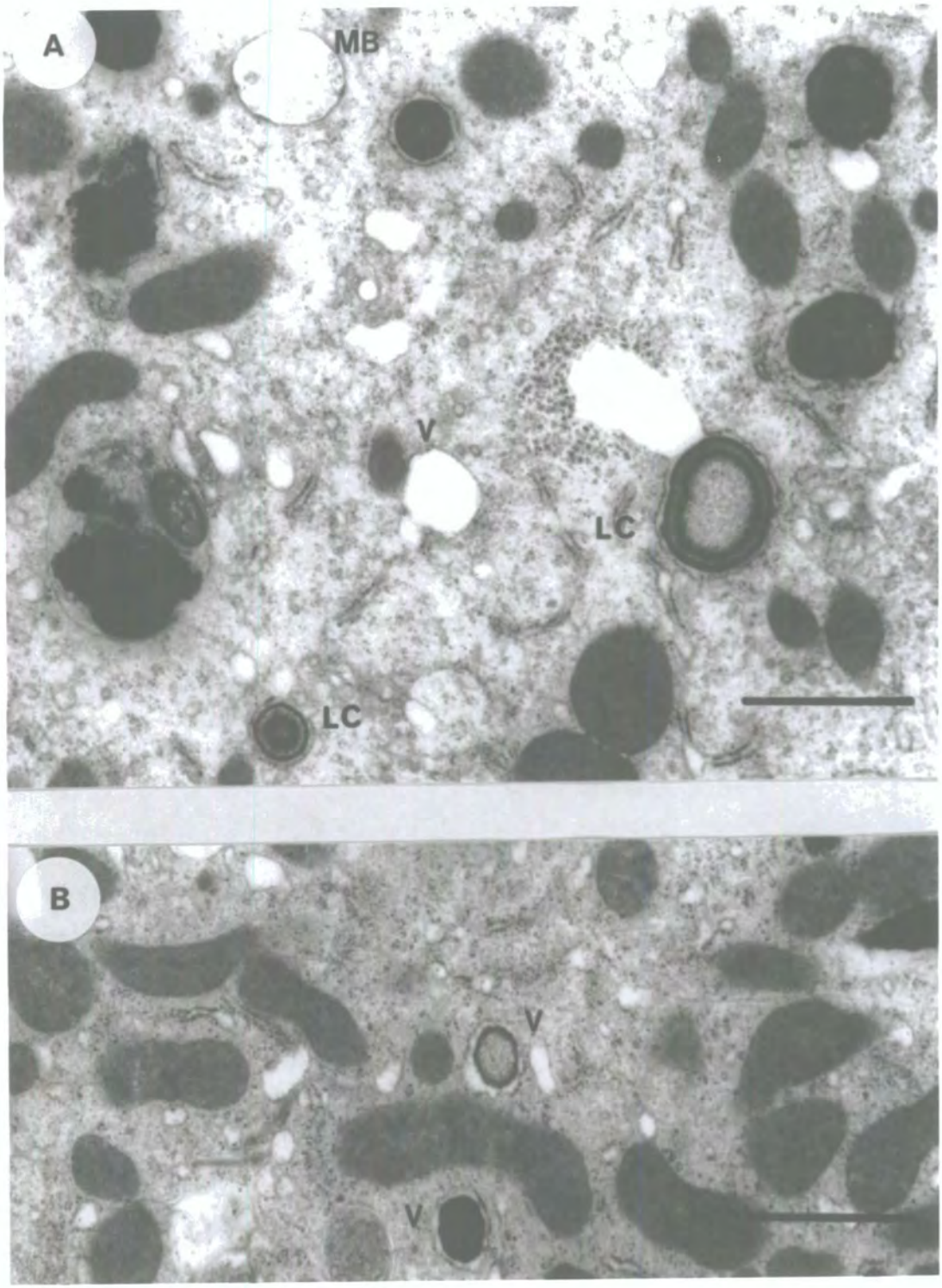

A
MB
V
LC
LC
B
V
V

Plate 3.3A

This plate shows the variety of the structures that were found throughout the cytoplasm of the tubule cells including a clear vacuole (V), multi-vesicular body (MB) and a number of lamellated concretions (LC). Note the different banding patterns found in the lamellated concretions.

Scale : 1μm

Plate 3.3B

Electronmicrograph showing the detailed structure of vacuoles (V) which existed in the cytoplasm which were found to contain varying amounts of electron-dense material. The most dense region being the periphery.

Scale : 1μm

was ~51% of that measured for rate 1 in control saline. In contrast, under control conditions (both rates being measured in control saline) rate 2 was ~92.0% of the rate measured during the initial period. When the normalized data were compared using Student's *t*-test the experimental treatment was found to significantly decrease the rate of fluid secretion ($P<0.001$).

Table 3.1. Effect of replacement of 8.6mM K^+ in the bathing medium with 8.6mM Rb^+ on Malpighian tubule fluid secretion *in vitro*

Treatment	n	Mean rate of fluid secretion in nl/min ± SEM		Rate 2 normalized against Rate 1	P
		Rate 1	Rate 2		
Control	40	2.21 ± 0.45	2.04 ± 0.39	91.96 ± 1.96	
Experimental	38	2.82 ± 0.44	1.25 ± 0.17	50.70 ± 4.2	<0.001

P values were obtained by comparing normalized values with Student's *t*-test.

Effect of 1mM ouabain in control saline on the rate of fluid secretion

The mean rate of fluid secretion was 1.33 ± 0.35nl/min (n=10), when the tubules were bathed in control saline over the initial period of measurement. When 1mM ouabain was included in the bathing medium the rate of fluid secretion fell to 0.52 ± 0.13nl/min (n=10), representing a fall of approximately 44%. In contrast when comparing this to the control situation when both rate 1 and rate 2 were measured in "normal" saline little difference was seen between the initial (2.01 ± 0.28nl/min) and final (1.77 ± 0.31nl/min) measurement periods (n=23). The rate in the final period being measured as ~90% of the initial rate. Analyzing the normalized values for control and experimental data with Student's *t*-test revealed a significant difference ($P<0.05$) between the two rates, therefore, inclusion of 1mM ouabain in the bathing solution did cause a

significant decrease in the rate of fluid secretion. Results are summarized in Table 3.2. below

Table 3.2. Effect of 1mM ouabain and 1mM cAMP on the rate of fluid secretion

Treatment	n	Mean rate of fluid secretion in nl/min ± SEM		Rate 2 normalized against Rate 1	P
		Rate 1	Rate 2		
Control	23	2.01 ± 0.28	1.77 ± 0.31	90.1 ± 8.1	
1mM ouabain	10	1.33 ± 0.35	0.52 ± 0.13	56.2 ± 10.5	P<0.05
1mM cAMP	8	1.63 ± 0.32	1.84 ± 0.31	123.1 ± 12.5	P<0.05

For controls both rate 1 and rate 2 were measured in control saline. For experimentals, rate 2 was measured in saline containing either 1mM ouabain or 1mM cAMP.

Effect of 1mM cAMP in control saline on the rate of fluid secretion

The mean rate of fluid secretion measured over an initial period (rate 1), from tubules bathed in control saline, was 1.63 ± 0.32nl/min (n=8). The inclusion of 1mM cAMP in the bathing saline increased the rate of fluid secretion to 1.84 ± 0.31nl/min (rate 2), representing a mean increase of approximately 23%. No such increase was observed under control conditions (see Table 3.2.). Comparison of normalized values by Student's *t*-test showed that inclusion of 1mM cAMP in the bathing medium produced a significant ($P<0.05$) increase in the rate of fluid secretion.

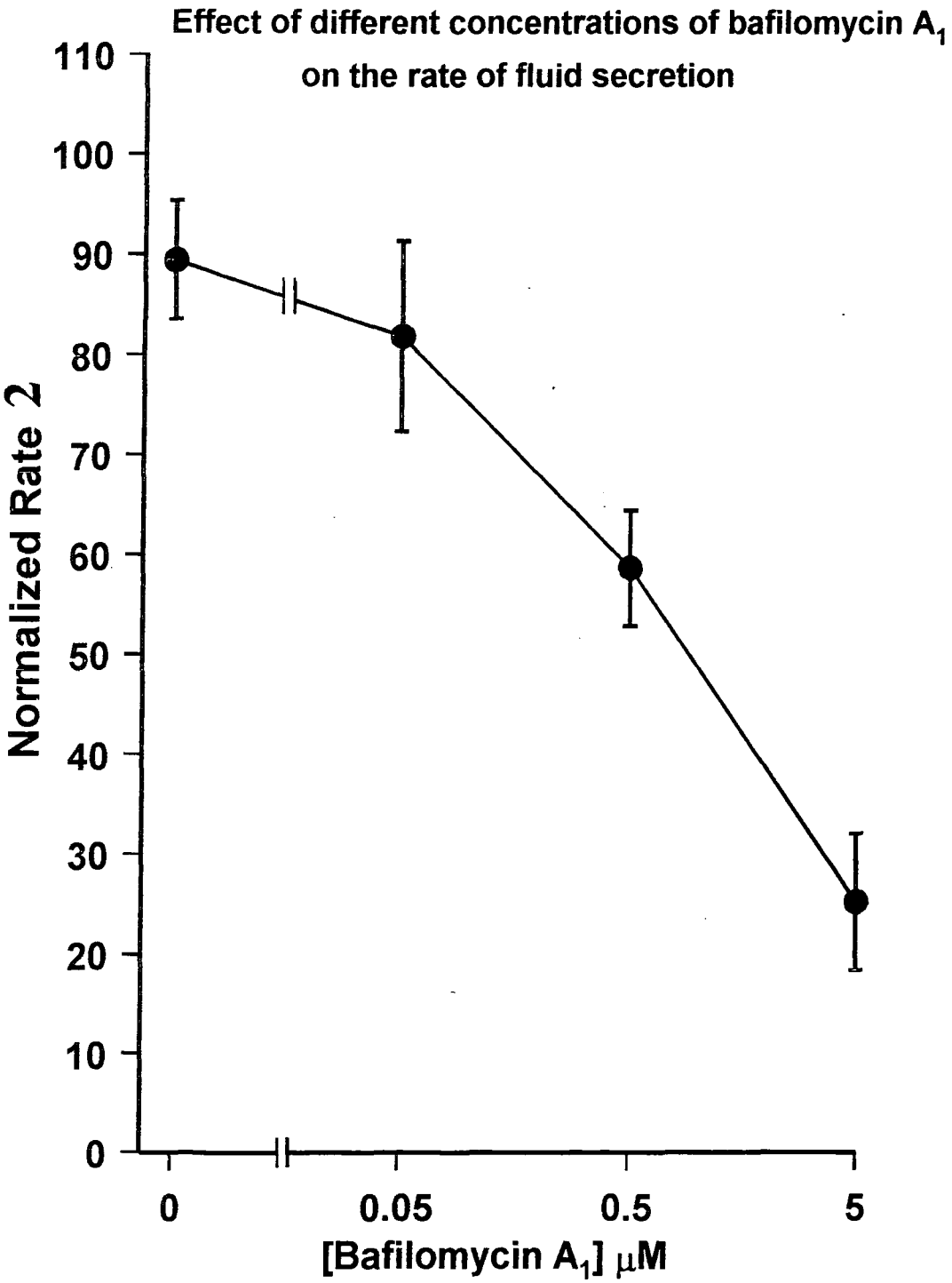
Effect of bafilomycin A_1 in control saline on the rate of fluid secretion

Effect of various concentrations of bafilomycin A_1, ranging from 5 to 0.05μM, on the rate of fluid secretion were investigated and the results are shown in Fig. 3.1. It can be seen that very low concentrations of bafilomycin A_1 (5μM and 0.5μM) had a marked inhibitory effect on the rate of fluid secretion.

Fig. 3.1. Effect of different concentrations of bafilomycin A_1 on the rate of fluid secretion.

The rate of fluid secretion was measured over two time periods. For contols both measurements occurred in control saline. To determine the effect of bafilomycin A_1 on fluid secretion the first measurement of rate was recorded in control saline over a period of 25 min whilst the second measurement of rate of fluid secretion was carried out over a period of 25 min in saline containing bafilomycin A_1. This second rate was then normalized with respect to that recorded over the first time period in control saline.

Fig. 3.1.



Control value was measured in the presence of DMSO (<1%)

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

Table 3.3. Effect of bafilomycin A_1 on the rate of fluid secretion

Treatment	Rate 1 nl/min ± SEM	Rate 2 nl/min ± SEM	Rate 2 normalized against Rate 1	n	P
Control	2.04 ± 0.39	1.95 ± 0.46	89.4 ± 5.9	10	
0.05μM baf A_1	0.99 ± 0.24	0.89 ± 0.3	81.8 ± 9.5	9	n.s.
0.5μM baf A_1	0.77 ± 0.13	0.44 ± 0.1	58.6 ± 5.8	12	<0.01
5μM baf A_1	1.96 ± 0.42	0.61 ± 0.33	27.3 ± 7.1	12	<0.001

For controls both rate 1 and rate 2 were measured in control saline. For experimentals, rate 2 was measured in saline containing bafilomycin A_1.
P values were obtained by comparing normalized values by Student's *t*-test.
n.s. - not significant (P>0.05).

Comparison between normalized control and experimental values, using Student's *t*-test, established that including 5μM bafilomycin A_1 in the bathing saline caused a significant inhibition of the rate of fluid secretion (P<0.001); the mean rate decreasing by approximately 73%. Similarly, 0.5μM bafilomycin A_1 effected a significant decrease in the rate of fluid secretion (P<0.01), reducing the rate by approximately 41%. However, at 0.05μM concentration, bafilomycin A_1 did not significantly affect the rate of fluid secretion. In controls, where DMSO (<1%) was included in the bathing saline when measuring rate 2, rate 2 was ~90% of rate 1. Results are summarized in Table 3.3.

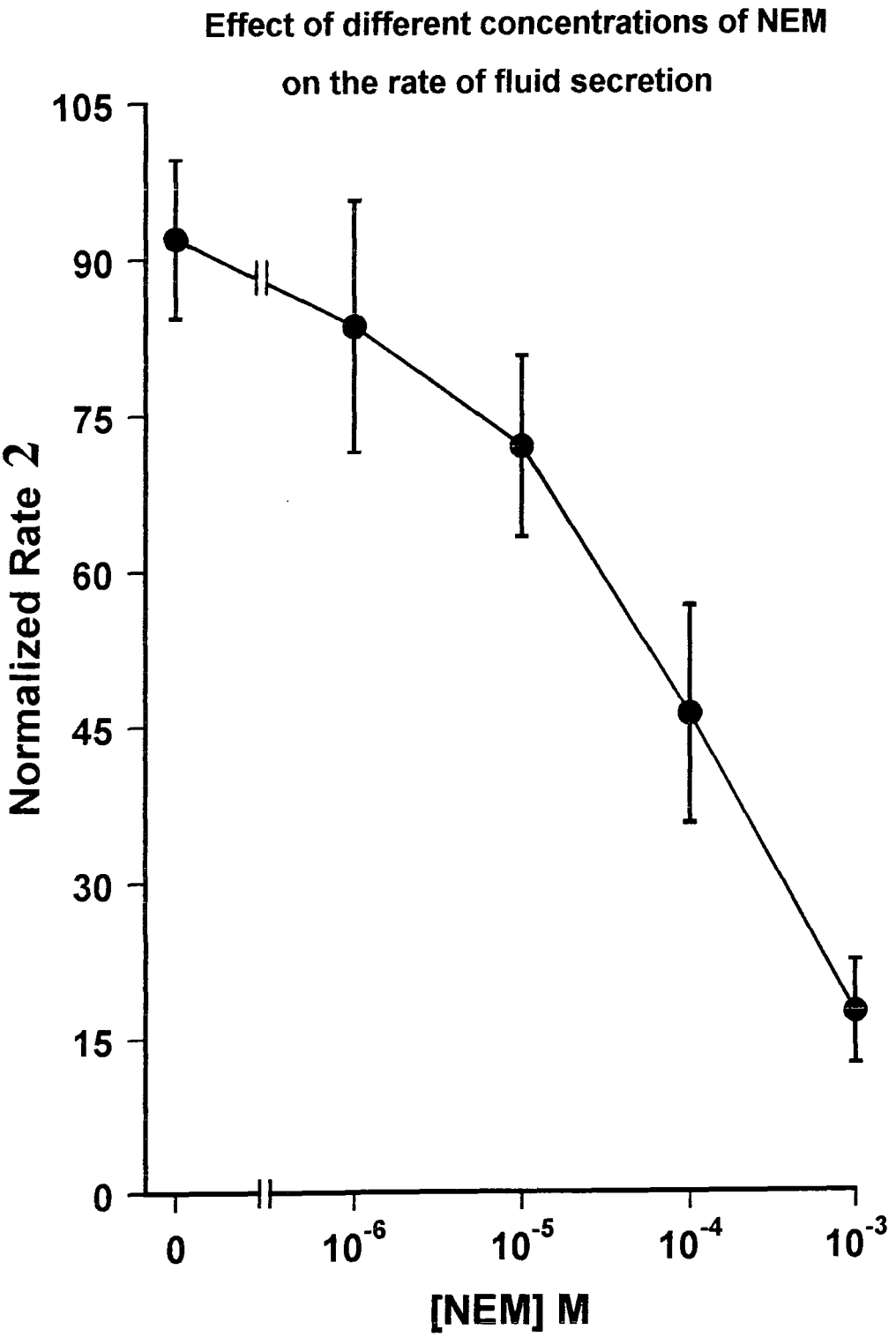
Effect of NEM in control saline on the rate of fluid secretion

Fig. 3.2. and Table 3.4. show the effects of NEM on the rate of fluid secretion at concentrations ranging from 10^{-6}M to 10^{-3}M. Inclusion of NEM in the bathing saline for the measurement of rate 2 caused inhibition of fluid secretion in a dose dependent manner. Comparing normalized control and experimental values by Student's *t*-test indicates that 10^{-4} and 10^{-3}M NEM effected significant inhibition of the rate of fluid secretion (P<0.001), by ~54% and ~83% respectively.

Fig. 3.2. Effect of different concentrations of N-ethylmaleimide (NEM) on the rate of fluid secretion.

The rate of fluid secretion was measured over two time periods. For contols both measurements occurred in control saline. To determine the effect of NEM on fluid secretion the first measurement of rate was recorded in control saline over a period of 25 min whilst the second measurement of rate of fluid secretion was carried out over a period of 25 min in saline containing NEM. This second rate was then normalized with respect to that recorded over the first time period in control saline.

Fig. 3.2.



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

Table 3.4. Effects of NEM on rates of fluid secretion

Treatment	Rate 1 nl/min ± SEM	Rate 2 nl/min ± SEM	Rate 2 normalized against Rate 1	n	P
Control	3.1 ± 0.37	2.93 ± 0.47	92.0 ± 7.6	21	
10^{-6}M NEM	2.69 ± 0.31	1.9 ± 0.29	83.5 ± 12.1	31	n.s.
10^{-5}M NEM	1.75 ± 0.18	1.21 ± 0.19	71.9 ± 8.7	28	n.s.
10^{-4}M NEM	2.79 ± 0.64	1.28 ± 0.35	46.1 ± 10.5	19	<0.001
10^{-3}M NEM	2.35 ± 0.41	0.37 ± 0.1	17.4 ± 5.0	18	<0.001

For controls both rate 1 and rate 2 were measured in control saline. For experimentals, rate 2 was measured in saline containing NEM.
P values were obtained by comparing normalized values with Student's *t*-test.

Effect of 100μM NEM in Rb^+-saline on the rate of fluid secretion

The control rate of fluid secretion was recorded when the tubules were bathed in Rb^+-saline (see Table 2.1, in the Methods section) for measurement of both rate 1 and rate 2. In experimentals, rate 1 was measured in Rb^+-saline with rate 2 being measured in Rb^+-saline containing 100μM NEM.

Table 3.5. Effect of 100μM NEM in Rb^+-saline on fluid secretion *in vitro*

Treatment	n	Mean rate of fluid secretion in nl/min ± SEM		Rate 2 normalized against Rate 1	P
		Rate 1	Rate 2		
Rb^+-Control	22	1.95 ± 0.28	1.43 ± 0.24	76.2 ± 5.9	
Experimental	20	2.80 ± 0.56	1.24 ± 0.27	50.92 ± 7.3	<0.01

For controls both rate 1 and rate 2 were measured in control saline. For experimentals, rate 2 was measured in saline containing 100μM NEM.
P values were obtained by comparing normalized values by Student's *t*-test.

Table 3.5. shows that inclusion of 100μM NEM in Rb^+-saline bathing the tubules caused a significant decrease ($P<0.01$) in the rate of fluid secretion, when comparing control and experimental normalized values using Student's *t*-test. The control treatment produced a rate in the final period (rate 2) which was ~76% of the rate during the initial period (rate 1); with the experimental treatment, however, rate 2 was recorded as only ~51% of rate 1.

Pre-incubation times and the cation composition of the "urine"

The "urine" was collected and analysed as described previously (see Chapter 2). Tubules were pre-incubated for varying times, in either control or Rb^+-saline solution. Original saline was then replaced with fresh saline of the same composition and "urine" collected over the following 60 min period. Table 3.6. shows the monovalent cation composition of the secreted fluid collected over the 60 min period. Values were compared using Student's *t*-test.

Table 3.6. Effect of pre-incubation in control and Rb^+-saline solutions on cation secretion by Malpighian tubules *in vitro*.

Pre-incubation time (min)	Na^+ (mM)	K^+ (mM)	Rb^+ (mM)	n
Control saline				
5	80.5 ± 25.3	110.6 ± 22.2	0	11
15	65.4 ± 22.4	110.7 ± 10.4	0	11
30	55.8 ± 19.8	123.5 ± 26.3	0	10
45	62.9 ± 14.1	111.6 ± 7.5	0	12
Rb^+-saline				
5	53.4 ± 16.1	115.9 ± 9.6	15.6 ± 1.8	11
15	58.2 ± 16.7	87.8 ± 17	17.0 ± 2.6	11
30	71.4 ± 9.3	83.2 ± 11.5	15.9 ± 1.6	11
45	65.4 ± 17.6	61.9 ± 17.0	11.7 ± 1.5	10

Tubules were incubated in control or Rb^+-saline for 5, 15, 30 or 45 min and the "urine" produced over the subsequent 60 min, with the tubule bathed in the same saline, was collected and analysed by atomic absorption or emission spectrophotometry.

In the case of control saline (8.6mM K^+, 119mM Na^+) Table 3.6. shows that the length of the pre-incubation period did not significantly affect the ionic composition of the secreted fluid, which varied between 55.8 ± 19.8 and 80.5 ± 25.3mM for Na^+ and between 110.7 ± 10.4 and 123.5 ± 26.3mM for K^+. After pre-incubation in Rb^+-saline the [Na^+] in the secreted fluid did not vary significantly with pre-incubation time. There was also no significant difference from the concentration secreted in control saline. In contrast, the [K^+] in the secreted fluid became progressively less with increased pre-incubation time. However, it was only the levels produced by the 5min (115.9 ± 9.6mM) and 45min (61.9 ± 16.9mM) pre-incubations that were significantly different ($P<0.02$). Comparing the concentrations of K^+ present in control and Rb^+-saline significant differences ($P<0.02$) were found between the 45min pre-incubation period in Rb^+-saline and the 45min pre-incubation time in control saline. Rb^+ ions were now present in the secreted fluid with levels ranging from 11.7 ± 1.5 to 17.0 ± 2.6mM, these concentrations were not significantly different.

Table 3.7. shows that the secreted fluid from tubules incubated in control saline contains 115.1 ± 3.8mM K^+ and 37.5 ± 2.3mM Na^+. Incubation in saline containing 1mM ouabain had negligible effect on [K^+], the level of Na^+ increased to 54.3 ± 3.7mM and this increase was significant ($P<0.01$). Incubation in 100µM NEM caused a large rise in [Na^+] ($P<0.001$) and an insignificant increase in [K^+]. Incubation in 1mM cAMP had no effect on the Na^+ content of the secreted fluid as compared to control saline but the K^+ content increased significantly to 135.1 ± 6.6mM ($P<0.02$).

Incubation in Rb^+-saline did not significantly affect the Na^+ and K^+ composition of the secreted fluid. However, the "urine" now contained Rb^+ (24.1 ± 2.0mM) as well as these two cations. Incubation of the tubules in Rb^+-saline with 1mM ouabain did affect the Na^+ and Rb^+ content of the "urine". The concentration of both ions altered significantly, Na^+ rose from 36.4 ± 4.6 to 61.9 ± 3.4mM ($P<0.001$) and Rb^+ fell from 24.1 ± 2.0 to 13.0 ± 3.3 mM ($P<0.02$). K^+ levels were not affected.

Effect of ouabain, NEM or cAMP on cation levels in control or Rb^+-saline

Table 3.7. Ionic composition of fluid secreted by Malpighian tubules incubated in control or Rb^+-saline containing ouabain, NEM or cAMP.

Treatment	Na^+ (mM)	K^+ (mM)	Rb^+ (mM)	n
Control saline				
Control	37.5 ± 2.3	115.1 ± 3.8	-	8
Ouabain (1mM)	54.3 ± 3.7	106.3 ± 3.2	-	6
NEM (100µM)	65.7 ± 4.3	119.8 ± 6.9	-	12
cAMP (1mM)	43.6 ± 3.5	135.1 ± 6.6	-	10
Rb^+ saline				
Control	36.4 ± 4.6	109.0± 6.1	24.1 ± 2.0	7
Ouabain (1mM)	61.9 ± 3.4	98.0 ± 4.9	13.0 ± 3.3	7
NEM (100µM)	50.6 ± 4.6	102.4 ± 6.4	21.3 ± 5.5	15
cAMP (1mM)	39.6 ± 7.9	117.3 ± 8.1	31.2 ± 3.1	7

Tubules were pre-incubated in the appropriate saline, containing 100µM NEM, 1mM ouabain, or 1mM cAMP for 15min. The tubules were then incubated with fresh saline of the same composition and the "urine" produced over the subsequent 60min collected and analysed.

In contrast to results obtained from tubules bathed in control saline, the inclusion of 100µM NEM in Rb^+-saline did not affect the Na^+, K^+ or Rb^+ content of the secreted fluid. Similarly, the inclusion of 1 mM cAMP in Rb^+-saline did not significantly affect levels of any of these cations. Comparing the effect of the inhibitors or cAMP on the composition of the fluid between the two salines, in the case of NEM there was a significant effect, $[Na^+]$ rose in control but not Rb^+-saline ($P<0.05$). Also including cAMP in control saline produced an increase in $[K^+]$, this was not the case in Rb^+-saline.

Na^+/K^+-ATPase activity in the presence of Rb^+

Na^+/K^+(or Rb^+)-ATPase activity was measured (as described in the Methods section) in the presence of different concentrations of K^+ or Rb^+, ranging from 0 to 40mM, for three independent preparations. The effects of replacing K^+ in the ionic medium with Rb^+ are shown in Fig. 3.3. Na^+ concentration was kept constant at 100mM. There was a significant difference in the enzyme activity recorded when Rb^+ was substituted for K^+ in the ionic medium; the activity in the presence of Rb being approximately 80% of that obtained with K^+ ($P<0.001$, paired *t*-test).

Table 3.8. Effect of monovalent cations on Mg^{2+}-ATPase activity.

Mg^{2+}-ATPase activity of the basal membrane-enriched fraction in the presence of various combinations of the monovalent cations K^+, Na^+ and Rb^+.

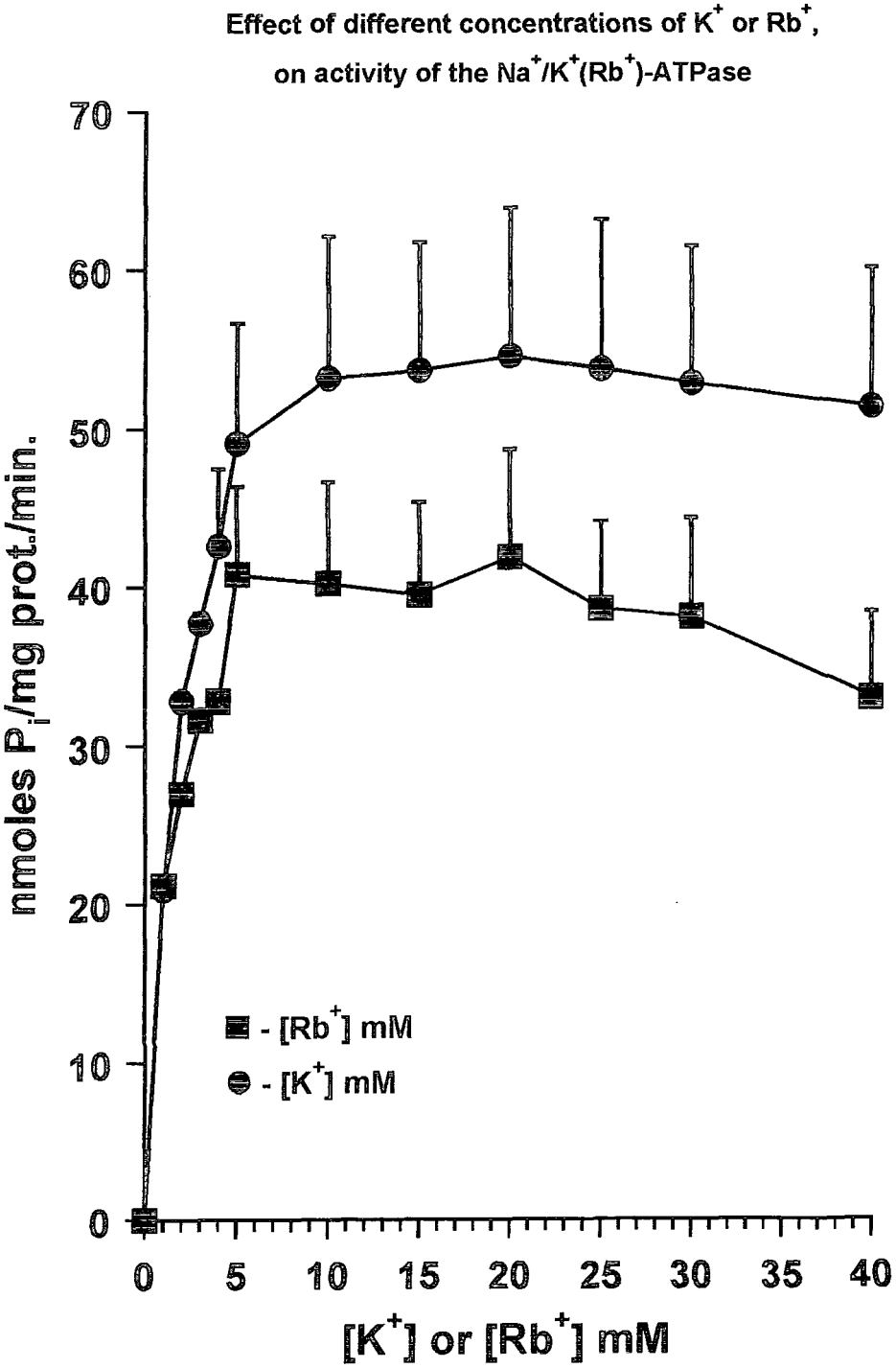
Ionic medium (final concentrations)	nmoles P_i liberated/ mg. Prot./ min.
4mM Mg^{2+}	8.69
4mM Mg^{2+}+20mM K^+	12.98
4mM Mg^{2+}+100mM Na^+	7.18
4mM Mg^{2+}+20mM Rb^+	14.7
4mM Mg^{2+}+20mM K^++100mM Na^+	73.1
4mM Mg^{2+}+20mM Rb^++100mM Na^+	55.3
4mM Mg^{2+}+20mM K^++100mM Na^++1mM ouabain	6.95
4mM Mg^{2+}+20mM Rb^++100mM Na^++1mM ouabain	5.68

Table 3.8. shows a typical example of the Mg^{2+}-ATPase activity produced in the presence of various combinations of cations. It can be seen that the ATPase activity recorded in the presence of Mg^{2+} alone was stimulated slightly in the presence of 20mM K^+ or 20mM Rb^+, but no stimulation was observed by Na^+ alone. However, synergistic stimulation of activity was observed in the presence of K^+(or Rb^+) and Na^+ together,

Fig. 3.3. Effect of different concentrations of K^+ or Rb^+, on activity of the Na^+/K^+ (Rb^+)-ATPase.

The activity of the $Na^+/K^+(Rb^+)$-stimulated Mg^{2+}-ATPase present in the basal membrane-enriched fraction produced from the Malpighian tubules of *Locusta* was measured in the presence of different concentrations of K^+ or Rb^+. Na^+ concentration was kept at 100mM.

Fig. 3.3.



Each point represents the mean ± S.E.M.,
calculated from 3 independent preparations

i.e., the activity in the presence of the two cations was far greater than the sum of the activities recorded in the presence of both ions separately. In addition this synergistic stimulation of the Mg^{2+}-ATPase activity (whether by $Na^+ + Rb^+$ or $Na^+ + K^+$) was inhibited by 1mM ouabain.

DISCUSSION

In this study the effects of known inhibitors of ion transporting ATPases were investigated on the Malpighian tubules of *Locusta migratoria*. Bafilomycin A_1 was found to inhibit fluid secretion most dramatically; 0.5μM and 5μM concentrations effecting ~41% and ~73% inhibition respectively. Bafilomycin A_1 is the most specific inhibitor of V-type ATPases known at present (Bowman *et al.*, 1988). Half-maximal inhibition of purified or membrane bound V-type ATPase from *Neurospora crassa*, chromaffin granules and plant vacuoles was produced by 0.02-2.6nM bafilomycin A_1, whereas P-type ATPases were inhibited at micromolar concentrations, for example, Na^+/K^+-ATPase of ox brain required approximately 25μM bafilomycin A_1 to effect 50% inhibition (Bowman *et al.*, 1988; Bertram *et al.*, 1991). Likewise Moriyama and Nelson, (1989) reported that bafilomycin A_1 inhibited ATP dependent H^+-transport activities in various animal endomembrane systems as well as purified H^+-ATPase from chromaffin granules at nanomolar concentrations. In contrast, vanadate-sensitive ATPase from chromaffin granules and mitochondrial F_oF_1 ATPase were not inhibited at concentrations up to 1μM although at higher concentrations partial inhibition of F_oF_1 ATPase did occur. The concentrations (0.5μM-5μM) which produced a significant inhibition of fluid secretion in the present study were higher than those reported to produce specific inhibition of V-type ATPases, but, were lower than those that affect P- and F-type ATPases. Similar results to this study have been reported by Bertram *et al.*, (1991). They found concentrations of bafilomycin A_1 above 0.1μM reduced fluid secretion by the Malpighian tubules of *Drosophila hydei* and at a concentration of 10μM fluid secretion was stopped completely. Weltens *et al.*, (1992) demonstrated that fluid secretion was reduced by 55% in the presence of 5μM bafilomycin A_1 in the tubules of *Formica polyctena*. In a study to investigate the effect of bafilomycin A_1 on the Malpighian tubules of *Aedes aegypti* Pannabecker and Beyenbach, (1993) discovered that fluid secretion was inhibited by 81% with a K_i of 5.8μM. Also bafilomycin A_1 eradicated fluid secretion by the tubules of *Drosophila hydei* at a concentration of 50μM

(Dow *et al.*, 1994b) and in a study by Dijkstra *et al.*, (1994b) bafilomycin A_1 was found to half-maximally inhibit fluid secretion by the Malpighian tubules of *Formica* at a concentration of 10^{-5}mol/l when it was applied to the luminal side. Therefore, in these other studies, the concentration of bafilomycin A_1 required to inhibit fluid secretion was also higher than that stated by Bowman *et al.*, (1988) and Moriyama and Nelson, (1989). Furthermore, Dijkstra *et al.*, (1994b), state that in general membrane-bound ATPases are normally less sensitive to bafilomycin A_1 by a factor of 10-100 than the purified form. In the present study, a higher concentration of bafilomycin A_1 was needed to produce significant inhibition of fluid secretion when compared to the concentration needed to significantly inhibit enriched enzyme preparations (see following Chapter). This suggests that the intracellular concentration of the inhibitor may be lower than that present in the medium bathing the tubules. This could be due to (1) the inhibitor not being able to cross the tubule wall very successfully and/or (2) the inhibitor being compartmentalized in the cells and/or (3) the inhibitor being detoxified by the cells. Any one of these conditions would result in the amount of inhibitor reaching the target enzyme being reduced.

The effect of treating the tubules with NEM, another inhibitor of V-type ATPases, was also studied. NEM is specific for sulphydryl groups and inhibits V-type ATPases between the concentration range of 1-2μM, 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 100μM. At this concentration it was possible that effects on P-type ATPases were occurring, although 100μM is at the lower end of the sensitivity range for P-type ATPases. However, as mentioned above the availability of the NEM at the site of inhibition may be reduced, as it too is found to inhibit membrane preparations at lower concentrations, it is then conceivable that NEM was inhibiting an enzyme activity at concentrations less than 100μM, 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. Weltens *et al.*, (1992), working on the tubules of

Formica polyctena found that a concentration of 500μM 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 1μM whilst complete inhibition occurred at 100μM. Bertram *et al.*, (1991) observed significant effects on the rate of fluid secretion by the Malpighian tubules of *Drosophila hydei* at concentrations greater than 10μM, with complete inhibition occurring at 1mM NEM. In a related study Dijkstra *et al.*, (1994b) found that 500μM 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.

1mM ouabain (a specific inhibitor of the Na^+/K^+-ATPase, Skou, 1969) significantly ($P<0.05$) inhibited fluid secretion by Malpighian tubules of *Locusta* by ~44% in the present study. Therefore, Na^+/K^+-ATPase is involved in fluid secretion by Malpighian tubules of *Locusta*. There is a wealth of evidence for the existence of Na^+/K^+-ATPase in the Malpighian tubules of *Locusta*, (see Anstee and Bell, 1975; Anstee *et al.*, 1979; Anstee *et al.*, 1980 and Donkin and Anstee, 1980). A similar inhibition of fluid transport was reported by Atzbacher *et al.*, (1974) in the tubules of *Drosophila hydei*. There is also evidence for the presence of Na^+/K^+-ATPase in other tissues such as the rectum of locust (Goh and Phillips, 1978) and the Malpighian tubules of the pill millipede (Farquharson, 1974). These studies support the view that a Na^+/K^+-ATPase activity is involved in fluid secretion. However, evidence from other insect epithelia suggests that this is not the case. Jungreis and Vaughan, (1977) showed the ouabain insensitivity of midgut epithelium in three Lepidoptera and criticized previous work which supported the presence of a Na^+/K^+-ATPase in insect ion transporting epithelia. These criticisms were later shown to be inaccurate (see Anstee and Bowler, 1979). However, further work carried out on the midgut of *Manduca sexta* (Harvey *et al.*, 1983a) confirmed the ouabain insensitivity of the tissue. Indeed Zeiske, (1992) states that Na^+/K^+-ATPase is not involved in ion transport in the midgut of phytophagous caterpillars. Early work often did not detect ouabain sensitivity in Malpighian tubules (Berridge, 1968; Maddrell, 1969: Gee, 1976b) but recent evidence for the presence of

Na^+/K^+-ATPase in the Malpighian tubules of insects has increased. Al-Ahmadi, (1993) found that the tubules of *Spodoptera littoralis* ceased to secrete as efficiently in the presence of ouabain; Maddrell and Overton, (1988) discovered evidence for the existence of Na^+/K^+-ATPase in the Malpighian tubules of *Rhodnius prolixus* whilst Bertram *et al.*, (1991) showed that 100μM ouabain caused the rate of fluid secretion by the tubules of *Drosophila hydei* to fall by approximately 50%. Therefore, there seems to be a major difference in the ion transport processes responsible for fluid secretion in these two tissues.

Evidence in support of Na^+/K^+-ATPase involvement in tubule function is also provided by ouabain inhibition of ATPase activity. Furthermore, microsomal Mg^{2+}-dependent ATPase activity was synergistically stimulated by the presence of Na^+ and K^+. Both of these features are major characteristics of Na^+/K^+-ATPase activity (Skou, 1965). It was also possible to synergistically stimulate the microsomal Mg^{2+}-dependent ATPase activity in the presence of Rb^+ and Na^+, although levels only reached ~80% of the stimulation achieved with K^+. A similar result was obtained by Warden *et al.*, (1989) working on rabbit collecting duct.

1mM cAMP caused a significant increase in the rate of fluid secretion by the Malpighian tubules of *Locusta migratoria*. Similar results have been found by Maddrell *et al.*, (1971) in the tubules of *Rhodnius* and *Carausius*; Maddrell and Klunsuwan, (1973) in the tubules of *Schistocerca gregaria*; Petzel *et al.*, (1987) working on the tubules of *Aedes aegypti* and Coast *et al.*, (1991) investigating the effect of cAMP on fluid secretion by the Malpighian tubules of *Acheta domesticus*. From studies like these where exogenously applied cAMP was found to increase the rate of fluid secretion it was concluded that cAMP performs as an intracellular second messenger in Malpighian tubules where it responds to diuretic peptides (see also Nicolson, 1976 and Anstee *et al.*, 1980). It has also been discovered that diuretic peptides or tissue extracts containing diuretic activity increase intracellular levels of cAMP in the tubules (Proux *et al.*, 1987;

Fogg *et al.*, 1990; Coast *et al.*, 1991; Coast *et al.*, 1992; Audsley *et al.*, 1993 and Coast and Kay, 1994).

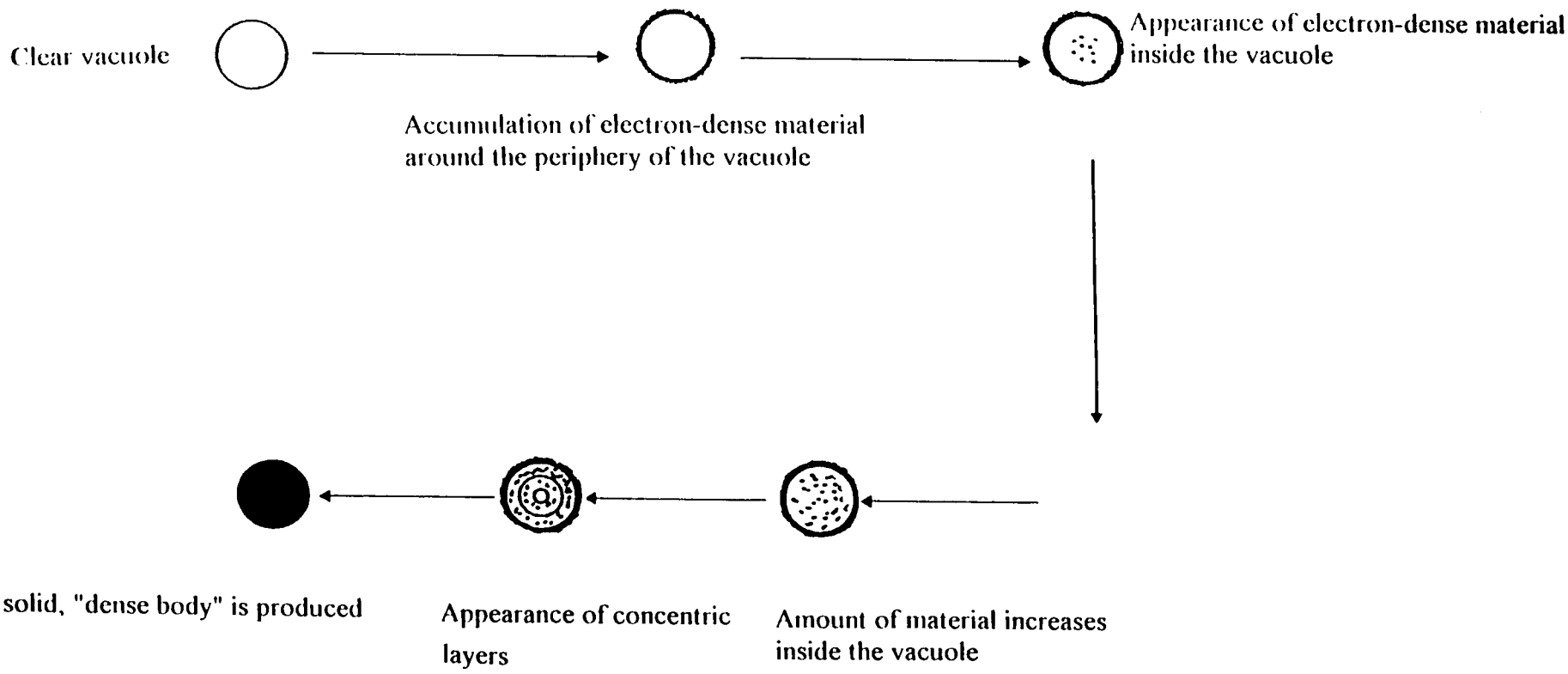
It has been suggested that fluid secretion by the Malpighian tubules of *Locusta* is controlled by two diuretic peptides, located in the corpora cardiaca which exert an effect at different receptor sites and act through different intracellular pathways one of which involves cAMP (Morgan and Mordue, 1985). Kay *et al.*, (1991) demonstrated that a diuretic peptide (*Locusta*-DP) 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. Therefore it seems feasible that *Locusta*-DP could represent the diuretic peptide which acted via cAMP reported by Morgan and Mordue, (1985).

The cytoplasm of the Malpighian tubule cells contains a variety of inclusions ranging from vacuoles and multi-vesicular bodies to lamellated concretions and dense bodies (Plates 3.2A-3.3B). It has been speculated that these bodies represent different stages in concretion formation (Sohal *et al.*, 1976; Donkin, 1981, Al-Ahmadi, 1993) as the variation in the amount of electron dense material might suggest a progressive build up. For example, in Plate 3.3A a clear vacuole can be seen, however, in Plate 3.2C the vacuole seems to have an electron-dense shell around it. Vacuoles are also seen where an accumulation of electron-dense material seems to be occurring inside them (Plate 3.3B). Also the appearance of the lamellated concretions suggests a gradual deposition of layers. A schematic diagram of a possible method of concretion formation is shown in Fig. 3.4. This proposed theory for the development of concretions is speculative as the evidence is derived from static electronmicrographs.

Previous studies have described cytoplasmic granules under a variety of different names such as urospherites (Berkaloff, 1960), concentric inclusions and mineralized spheres (Wigglesworth and Salpeter, 1962), mineral concretions (Gouranton, 1968) and excretory globules (Mello and Bozzo, 1969). These inclusions have been known to occur in insect Malpighian tubules for many years. In 1931, Wigglesworth described the

Fig. 3.4.

A schematic diagram showing a possible method for the formation of concretions in the Malpighian tubules of *Locusta migratoria*



cytoplasm of the upper segment Malpighian tubule cells in *Rhodnius* to be full of spherical granules. Later Wigglesworth, (1972) distinguished between the granules present in the cytoplasm and the excretory granules in the lumen which are composed largely of uric acid. He reported that the cytoplasmic granules were composed of minerals and, in 1977, Sohal *et al.*, suggested that the universal term "mineral" concretion be used to describe the mineral-containing structures regardless of appearance.

Advances in techniques such as X-ray microanalysis have allowed the chemical composition of the concretions to be elucidated. The concretions in the Malpighian tubules of *Musca domestica* contain P, S, Cl, K, Cu, Fe, Ca and Zn to varying degrees (Sohal *et al.*, 1976, 1977). Concretions in the tubules of *Musca autumnalis* are primarily inorganic, Ca, Mg and P contributing 98% of the inorganic element but Cl, Mg, K, Si, Zn, Cu and Mn were also detected (Grodowitz *et al.*, 1987). Wessing *et al.*, (1992) report two types of concretion in *Drosophila* Malpighian tubules, one rich in Ca, Mg and P, the other rich in K. S, Cl and Na were also detected. Three types of "dark body" have been reported in the tubule cells of *Locusta*, one rich in Ca and P and two rich in K (Pivovarova *et al.*, 1994a). These "dark bodies" are so called as they appear electron dense in cyrosections when viewed under the EM. The "dark bodies" were 0.1-0.5μm across and were distributed throughout the cytoplasm and some of them were located in vacuoles. Concretions in *Locusta* were also found throughout the cytoplasm, sometimes being located in vacuoles. They were found in various sizes going up to approximately 1 μm in diameter. Wessing *et al.*, (1988) report that electron dark vacuoles seen via cryofixation, in *Drosophila* Malpighian tubules, are the same as the dark vacuoles observed in conventional electron micrographs after osmium fixation. Later, it was proposed that in freeze dried cyrosections the type II concretions, which are rich in K, appear as electron dark particles (Wessing *et al.*, 1992 and Wessing and Zierold, 1993). A similar observation has been made in *Locusta* (Pivovarova *et al.*, 1994a).

There have been different theories put forward for the role of the concretions. In some cases, as in *Musca autumnalis*, they act as mineral storage sites for puparial cuticle

mineralization (Grodowitz *et al.*, 1987). It has been suggested that they may accumulate substances from the haemolymph, transport them across the cell and extrude them into the lumen (Wessing and Eichelberg, 1975). Sohal *et al.*, (1976), believed that the concretions might act as deposit sites for the storage of minerals or even a deposit excretion site. Kapoor, (1994) proposed that concretions existed as a storage site for material from the haemolymph. Wessing *et al.*, (1992) suggested that concretions provided an economical way to excrete large amounts of ions in only a small amount of water. Humbert, (1978) proposed that in *Collembola* the temporary accumulation of concretions may be involved in ionic maintenance of the intracellular environment. Brown, (1982) suggested that, in general, concretions of a concentric nature made up of different elements were important in storage-excretion processes. The consistent presence of these concretions not only in Malpighian tubules but in organs of other invertebrates especially with digestive, storage and excretory functions fuels speculation that they are critical in the excretory process. In insects, mineral concretions are also found in *Collembola* in midgut (Humbert, 1978) and in the midgut wall of cercopid larvae and adults (Gouranton, 1968) and larval *Drosophila melanogaster* (Tapp and Hockaday, 1977).

It is known that some types of concretions can alter their mineral make up. K^+ in the dark bodies of *Locusta* was interchangeable for Rb^+ (Pivovarova *et al.*, 1993; 1994a). Wessing *et al.*, (1992) showed that type II concretions which occur in the tubule cells of *Drosophila* and are rich in K^+ can be altered by the composition of luminal fluid. It was also shown that concretions in *Drosophila* can change their metal composition by feeding on a metal-enriched diet (Wessing and Zierold, 1992) in fact these investigators suggested that the concretions were like ion exchangers. Thinking of the concretions as ion exchangers helps interpret some of the results found in the present study.

In the present study (Table 3.5.) a large amount of K^+ (61.9 ± 16.9mM) was still being secreted by the Malpighian tubule cells of *Locusta* even after they had been pre-incubated in Rb^+- (K^+-free) saline for 45min (Pivovarova *et al.*, 1994a). This is a

significantly lower amount than that found in the secreted fluid after 45 min incubation in K^+-saline (111.6 ± 7.5mM), $P<0.02$ and than the amount (115.9 ± 9.6mM) secreted by the tubule cells after 5 min pre-incubation in Rb^+- (K^+-free) saline, $P<0.02$. This result is consistent with that found by Anstee *et al.*, (1979), Fathpour, (1980) and Bell, (1977); who recorded K^+ still being secreted (at levels of approximately 40mM) when tubules were bathed in K^+-free saline. Therefore, although the amount of K^+ being secreted fell with respect to the length of time it had been in Rb^+- (K^+-free) saline, as would be expected as no K^+ was entering the cell, a substantial amount of K^+ was still being secreted. Furthermore, as shown by X-ray microanalysis (Pivovarova *et al.*, 1994a) the cell still contained approximately 10% of the K^+ found in control cytoplasm (after 45 min in K^+-free saline).

The question arises as to where this K^+ could be coming from. The major proteins in the basal lamina are type IV collagen and laminins but, there are also proteoglycans (Fransson, 1987) which are able to bind cations, Gupta, (1989) suggested that this substance may be able to trap K^+ leaving the cell and recycle it back in via the Na^+/K^+-ATPase. As from previous studies it had been shown that the basement membrane of *Calliphora* salivary gland was able to sequester K^+ and exclude Na^+ and Cl^- (Gupta and Hall, 1979) this had also been found for the Malpighian tubules of *Rhodnius* (Gupta *et al.*, 1977). However, when Pivovarova *et al.*, (1994a) measured the K^+ concentration in the basement membrane after it had been incubated in Rb^+- (K^+-free) saline for 45 min they found it below the detection limit (<4mM). These glycosaminoglycans/proteoglycans are not only found in the basement membrane, they also occur in storage granules (Fransson, 1987). In rabbit ileum the presence of K^+ at the apical surface, lateral interspaces and basal laminae when there is 0mM K^+ in the bathing medium (Gupta, 1989) is attributed to the presence of mucoid matrices in these regions. In *Locusta*, the K^+ content of some of the mineral concretions is higher than the surrounding cytoplasm (Pivovarova *et al.*, 1994a), it is possible, therefore, that the dark bodies or mineral concretions found in these cells are acting as intracellular stores. Wessing and Eichelberg, (1975), Hevert, (1975) and Wessing *et al.*, (1992) have

reported the presence of glycosaminoglycans and proteoglycans in the concretions found in *Drosophila,* these two substances are negatively charged and so can attract and hold positively charged ions. It is tempting to speculate that K^+ is stored in the concretions of *Locusta* by binding to these insoluble mucoids.

There is a gradient of K^+ across the cells of *Locusta* increasing from the basal to apical surface (Pivovarova *et al.*, 1993; 1994a). A gradient of K^+ also exists across the cells of *Drosophila hydei* (Wessing and Zierold, 1993) along with a gradient of Cl^-. It is possible Donnan effects cause these gradients but it is very unusual to have these gradients when unbound ions are highly mobile and diffusion pathways would not be very large. Another question which needs to be addressed is why does Na^+ not adopt the same gradient? Wessing and Zierold, (1993) raise the possibility that the negatively-charged proteoglycans or glycosaminoglycans are able to attract K^+ more strongly than Na^+ and that the binding of the positively charged K^+ ions then attracts Cl^- and Oschman, (1978) reported that heparin sulphates, which make up a large proportion of mucoid matrices, have a predisposition to bind K^+ rather than Na^+. Furthermore, hyaluronic acid which is a component of proteoglycans or glycosaminoglycans (Alberts *et al.*, 1989) is important for the attraction of water which is needed for the transport of ions across the cell. Hence, Wessing and Zierold, (1993) suggest that potassium increases in concentration across the cell due to binding to the "dark bodies". It is a possibility that the same thing is occurring in *Locusta.* Further information can be derived from these studies where Rb^+ replaces K^+ to clarify what is occurring at the apical and basal cell surfaces.

When K^+ levels in the cytoplasm had fallen to approximately 10% of control levels a significant amount of the cations being secreted in the "urine" were still K^+. This suggests that the ion pump at the apical surface must have a high affinity for K^+. At the same time the amount of Na^+ being secreted remained fairly constant even though through X-ray microanalysis we know its intracellular content rose considerably when tubules had been incubated in Rb^+-saline. This suggests that if there is a common exit pump for K^+ and Na^+ that the pump has a much greater affinity for K^+ or perhaps Na^+

leaves by a different apical mechanism. This contradicts other evidence that the apical pump is unspecific, in fact, Maddrell, (1977) named it a common cation pump and proposed that it had a higher affinity for Na^+.

The levels of Rb^+ secreted were low and relatively constant. The rate of fluid secretion decreased when Rb^+ replaced K^+ in the medium bathing the tubules (Table 3.1.). Warden *et al.*, (1989) reported that Rb^+ did not fully substitute for K^+ in rabbit cortical collecting duct, causing a fall in the rate of secretion, due to in part a reduction in the apical membrane conductance and also the fact that Rb^+ does not support Na^+/K^+-ATPase activity as well as K^+, in fact it falls to 80%, approximately the same reduction was found in this study (Fig. 3.3.). Could this alone explain the inability of Rb^+ to be present at the same intracellular levels as K^+ in control cells? Other possible entry mechanisms for K^+ at the basal surface include the Na^+-K^+-$2Cl^-$ co-transporter and Owen and Prastein (1985) report that Rb^+ can substitute quantitatively for K^+ in human fibroblasts. However, a study on rat renal cortex by Beck *et al.*, (1988) showed that Rb^+ was not handled in the same way as K^+. Rb^+ was not secreted efficiently. Another study by Zehran, (1980) using radioactive tracers to measure flux across the midgut of *Hyalophora cecropia* larvae found Rb^+ only carried 50-60% of the expected current. Another possible entry mechanism for Rb^+ would be through K^+ channels. The basal membrane is highly permeable to K^+ (Baldrick, 1987; Baldrick *et al.*, 1988; Fogg, 1990), and as will be discussed in greater detail in Chapter 5, the basal membrane potential is close to the K^+ equilibrium potential (E_K). At E_K there is no net movement of K^+. Therefore, if Rb^+ can substitute for K^+ then the system will remain at equilibrium and there will be limited Rb^+ entry. Conversely, if Rb^+ cannot substitute for K^+ a loss of K^+ would be expected across the basal membrane indeed Gallacher *et al.*, (1984) found that some K^+-selective channels in epithelial cells can distinguish between K^+ and Rb^+ allowing only limited Rb^+ entry. However, Rb^+ is known to block certain K^+ channels (Hille, 1992) hence if these channels exist in the basal membrane the exit of K^+ and the entry of Rb^+ may be prevented. Because Rb^+ had become the major cation in the cytoplasm suggests that Rb^+ had the greatest difficulty in substituting for K^+ in ion

transport processes at the apical surface, this in turn leads to the conclusion that the pump on the apical surface is very specific for K^+. This result also suggests that it does not compete with Na^+ effectively for exit mechanisms. However, it appears that K^+ was lost from the cytoplasm more quickly than Rb^+ could be taken up as Rb^+ levels never equalled the level of K^+ in the control cells (Pivovarova *et al.*, 1994a, b) and this could be explained by poor Rb^+ entry at the basal surface.

To help interpret results using Rb^+ substitution and to elucidate further the processes occurring at both surfaces further experiments into cation secretion were carried out using the substances ouabain, NEM and cAMP. Pivovarova *et al.*, (1994b) discovered that a K^+ gradient was maintained within the cell even when the cell had been treated with ouabain, NEM or cAMP, all of which effect the intracellular level of ions. These agents affect the intracellular levels of ions by affecting processes occurring at the basal (entry) or apical (exit) surfaces. Ouabain inhibits the Na^+/K^+-ATPase in Malpighian tubules of *Locusta* (Anstee and Bell, 1975; Atzbacher *et al.*, 1987; present study). NEM at certain concentrations is a specific inhibitor of V-type ATPase enzymes (Forgac, 1989) and cAMP is reported to stimulate cation transport at the apical surface. (Berridge and Prince, 1972a, 1972b; Fogg *et al*, 1989, 1990). If the K^+ gradient was due to processes occurring at the two surfaces it would be expected to alter in accordance with the change in entry/exit of ions produced by the inhibitor/agonist. As it does not it suggests that the gradient is due to things within the cells, possibly as mentioned before, the dark bodies or concretions.

When 1mM ouabain was included in control saline, intracellular levels of Na^+ increased and levels of K^+ decreased in all regions of the cell (Pivovarova *et al.*, 1994b). In the secreted fluid there was a rise in $[Na^+]$ but $[K^+]$ was not significantly different. Ouabain is a specific inhibitor of the Na^+/K^+-ATPase (Skou, 1969) and this enzyme is present in membrane fractions of *Locusta* Malpighian tubules (Anstee and Bell, 1975, 1978; Fogg *et al.*, 1991). The Na^+/K^+-ATPase pumps K^+ into the cell and Na^+ out, so inhibition of the pump by ouabain would lead to a rise in $[Na^+]_i$ and a fall in $[K^+]_i$ and this is what was recorded using X-ray microanalysis. In the secreted fluid the level of

Na^+ rose significantly (as more was present in the cytoplasm) from 37.5 ± 2.3mM to 54.3 ± 3.7mM ($P<0.01$). But the level of K^+ being secreted from the cell did not fall significantly. This result is consistent with data produced by Bertram *et al.*, (1991) as in Malpighian tubules of *Drosophila* and for other insects K^+ is able to enter passively through basal membrane pathways (Bradley, 1989) the main function of the Na^+/K^+-ATPase in these cases is to maintain Na^+ at a low intracellular level. However, Anstee *et al.*, (1986) reported that the Na^+/K^+-ATPase activity in Malpighian tubules of *Locusta migratoria* was sufficient to account for significant K^+ entry, implying a major role in ion and fluid secretion for this enzyme, which has also been suggested for other insect Malpighian tubules by Maddrell and Overton, (1988). Perhaps the dark bodies (some of which are rich in K^+, Pivovarova *et al.*, 1994a) were responsible for maintaining the level of K^+ in the secreted fluid. However, ouabain does cause a significant ($P<0.05$) decrease in the rate of fluid secretion, therefore, the total loss of Na^+ and K^+ did decrease.

NEM which has been reported as a specific inhibitor of V-type ATPases, at low concentrations (Forgac, 1989), caused an increase in $[K^+]_i$ in all regions of the cell except the basal infoldings but $[Na^+]_i$ was not significantly different (Pivovarova *et al.*, 1994b). The secreted fluid showed an significant increase in $[Na^+]$ (from 37.5 ± 2.3mM to 65.7 ± 4.3mM, $P<0.001$) and an insignificant rise in $[K^+]$. The increase in $[K^+]_i$ in all regions of the cell excepting the basal infolds and the subsequent increase in the K^+ gradient across the cell (Pivovarova *et al.*, 1994b) suggests that K^+ extrusion at the apical surface is reduced, that NEM is causing this reduction supports the theory that a V-type ATPase present on the luminal surface is responsible for this process (see reviews by Nicolson, 1993; Beyenbach, 1995 and Pannabecker, 1995). Although 100µM NEM reduced fluid secretion significantly ($P<0.001$), so the total number of K^+ and Na^+ leaving the cell decreased, the cells did not stop secreting fluid. The V-type ATPase was still functioning only at a reduced level so the presence of the same concentration of K^+ in the secreted fluid was not surprising, what was, was the increased concentration of Na^+ being extruded. That intracellular levels of Na^+ remained the same suggests that

the secretion of Na^+ may not be dependent on the V-type ATPase, possibly Na^+ has a different exit mechanism to K^+ and when V-type ATPase is inhibited the Na^+ extrusion mechanism becomes the most dominant. Although Bertram *et al.*, (1991) working on the Malpighian tubules of *Drosophila hydei* found NEM had no effect on the concentrations of Na^+ and K^+ secreted in the "urine".

1mM cAMP was applied to the tubule cells and caused an increase in $[Na^+]_i$ and a decrease in $[K^+]_i$ (Pivovarova, personal communication). In the "urine" there was a significant increase in $[K^+]$ (from 115.1 ± 3.8mM to 135.1 ± 6.6mM, $P<0.02$) but $[Na^+]$ was not significantly different. cAMP stimulates the apical extrusion mechanism for K^+ (Fogg *et al.*, 1989, 1990), hence there was a decrease in $[K^+]_i$ and an increase in $[K^+]$ in the "urine". There was also an increase in $[Na^+]_i$ this could be caused if cAMP inhibited Na^+/K^+-ATPase, but there is no evidence for this and it would be improbable as it would cause a decrease in $[K^+]_i$ when the cell is under demand to produce more "urine". It is also possible that it could stimulate the co-transporter as $[Cl^-]_i$ was found to increase too (Pivovarova, personnel communication), this would also ensure an increased uptake of K^+ to supply the increased demands at the apical surface. Evidence that cAMP influences the co-transporter has been provided by Dijkstra *et al.*, (1995); Audsley *et al.*, (1993) and Hegarty *et al.*, (1991). Due to the increased rate of fluid secretion caused by cAMP, in the present study secretion increased by approximately 23% in the presence of 1mM cAMP ($P<0.05$), more Na^+ will be leaving the cell even though it is appearing not to.

The same treatments were carried out in Rb^+-saline and were compared to the Rb^+ controls. Again, a high concentration of K^+ was still present in the "urine" even though the tubule was bathed in K^+-free saline. When 1mM ouabain was included in Rb^+-saline only $[K^+]_i$ was not significantly different to the levels recorded in Rb^+-saline alone. $[Na^+]_i$ increased and $[Rb^+]_i$ decreased (Pivovarova *et al.*, 1994b). In the "urine" $[Na^+]$ increased significantly to 61.9 ± 3.4mM from 36.4 ± 4.6mM ($P<0.001$) and $[Rb^+]$ decreased from 24.1 ± 2.0mM to 13.0 ± 3.3mM ($P<0.02$) but K^+ was present in the same amounts. Ouabain inhibits the Na^+/K^+-ATPase which is responsible for pumping

K^+ into the cell and Na^+ out, therefore, if no K^+ is present in the bathing solution the effect of the inhibitor on this pump will have no effect on $[K^+]_i$ meaning the same levels will be secreted as were found in Rb^+-saline. $[Rb^+]_i$ did decrease as this ion can substitute for K^+ in the Na^+/K^+-ATPase (at levels of 80% efficiency) and so Rb^+ entry was affected by ouabain inhibition, $[Na^+]_i$ levels increased for the converse reasons. Secreted levels of Na^+ increased as more was present in the cell and levels of Rb^+ decreased as less was available.

When 100μM NEM was included in Rb^+-saline both $[Na^+]_i$ and $[K^+]_i$ increased and $[Rb^+]_i$ levels in the apical cytoplasm and microvilli decreased (Pivovarova *et al.*, 1994b). $[K^+]_i$ increased as the rate of fluid secretion decreased by approximately 50% ($P<0.01$), see Table 3.4., due to inhibition of the V-type ATPase combined with the reduction in secretion which is seen when Rb^+ replaces K^+ in the bathing medium, as K^+ was not leaving the cell as efficiently intracellular levels rose. Fluid secretion was still able to take place only at reduced levels so the concentration of K^+ secreted in the "urine" remained the same . $[Na^+]_i$ increased due, in part, to the reduction in the activity of the Na^+/K^+-ATPase seen in Rb^+-saline, however, it did not increase in the secreted fluid as it did when NEM was applied in control saline perhaps Rb^+ can compete with Na^+ for an exit site (which is different to the K^+ exit mechanism) as its levels did remain the same in the secreted fluid.

cAMP in Rb^+-saline did not cause a significant increase in $[Na^+]_i$, the co-transporter on the basal surface was probably not working efficiently as Rb^+ had replaced K^+ in the bathing medium. $[Rb^+]_i$ levels decreased; it was possible that some was exchanged for K^+ in the dark bodies as $[K^+]_i$ did increase (Pivovarova, personal communication) and it could not be entering from the bathing medium. The concentration of Na^+ in the secreted fluid did not change as levels of $[Na^+]_i$ had not. Levels of K^+ in the secreted fluid did not increase as they did in control saline possibly because Rb^+ was competing for the same apical site.

An increase in the intracellular concentration of K^+ was also seen when NEM was applied in Rb^+-saline. Pivovarova *et al.*, (1994b) suggest that the dark bodies are

releasing K^+, this, in combination with the reduced extrusion of K^+ at the apical surface due to the action of NEM would account for the observed results.

In Rb^+-saline the "urine" contained less K^+ when compared to control saline for all treatments (not significant) so it could be that $Rb^+$and K^+ compete for the apical site, but the pump has a much higher affinity for K^+. The fact that fluid secretion in Rb^+-saline is inhibited by NEM supports the theory that Rb^+ can leave by the apical pump. But, if this pump is inhibited Rb^+ is able to exit via another mechanism (as shown by the results obtained when NEM is included in Rb^+-saline).

In summary, the mineral concretions found in the Malpighian tubule cells, appear to have an important role in secretion. It is possible that they can maintain levels of intracellular K^+ when the cell is subjected to K^+-free or K^+ poor conditions. This suggests that in *Locusta* secretion cannot proceed normally without K^+. The extrusion mechanisms seem very insensitive to changes in intracellular ion composition which indicates that the pump(s) are relatively specific and that fluid secretion is not a very flexible process.

CHAPTER 4

Studies on the enzyme activities located in the Malpighian tubules of *Locusta migratoria*

RESULTS

The use of marker enzyme assays to identify membrane fractions produced from the Malpighian tubules of *Locusta migratoria*

Using a modified version of the technique for cell fractionation produced by Rodriguez and Edelman, (1979) an attempt was made to prepare enriched basal and apical cell membranes from the Malpighian tubules of *Locusta migratoria*. Biochemical assays were carried out to determine the Na^+/K^+-ATPase, alkaline phosphatase and succinate dehydrogenase activities within each pellet produced during the separation procedure (see Fig. 2.1.). These enzymes acted as markers for basal, apical and mitochondrial membranes, respectively, and hence provided a means of identifying which membranes were present in each pellet.

Previous studies have demonstrated Na^+/K^+-ATPase activity in microsomal preparations from tubules of *Locusta* (Anstee and Bell, 1975; 1978; Fogg, 1990; Fogg *et al.*, 1991). The present study found the major activity was present in pellet 4 (P_4), but substantial activity was also found in pellet 3 (P_3) (Fig. 4.1.). Alkaline phosphatase has previously been shown by cytochemical localization studies to be confined to the apical surface of Malpighian tubules of *Locusta* (Fogg, 1990). In the present study the vast majority of alkaline phosphatase activity was associated with pellet 5 (P_5) (Fig.4.2.). The data presented in Figs. 4.1. and 4.2. shows that pellet 3 (P_3) was successfully separated to produce enriched apical (P_5) and basal membrane (P_4) fractions.

Succinate dehydrogenase activity is used as a mitochondrial marker, the present study revealed a minimal amount of mitochondrial contamination in pellet 5 (P_5) (Fig. 4.3.) a larger amount being associated with P_4.

Fig. 4.1.

Measurement of Na^+/K^+-ATPase activity in various membrane fractions produced by differential centrifugation from the Malpighian tubules of *Locusta migratoria*. The results from three independent preparations are shown.

Fig. 4.2.

Measurement of alkaline phosphatase activity in various membrane fractions produced by differential centrifugation from the Malpighian tubules of *Locusta migratoria*. The results from three independent preparations are shown.

Fig. 4.3.

Measurement of succinate dehydrogenase activity in various membrane fractions produced by differential centrifugation from the Malpighian tubules of *Locusta migratoria*. The results from three independent preparations are shown.

For Figs 4.1.-4.3.

C = Crude Malpighian tubule homogenate

P1 = Pellet produced after 600 g spin for 10 min.

P2 = Pellet produced after 15,000g spin for 20 min (mitochondrial pellet).

P3 = Pellet produced after 135,000g spin for 60 min.

P4 =Pellet produced after 10,000g spin for 15 min (basolateral pellet).

P5 =Pellet produced after 55,000g spin for 30 min (apical pellet).

Fig. 4.1.

Measurement of Na^+/K^+-ATPase activity in various membrane fractions produced by differential centrifugation from the Malpighian tubules of *Locusta migratoria*. The results from three independent preparations are shown.

Fig. 4.2.

Measurement of alkaline phosphatase activity in various membrane fractions produced by differential centrifugation from the Malpighian tubules of *Locusta migratoria*. The results from three independent preparations are shown.

Fig. 4.3.

Measurement of succinate dehydrogenase activity in various membrane fractions produced by differential centrifugation from the Malpighian tubules of *Locusta migratoria*. The results from three independent preparations are shown.

For Figs 4.1.-4.3.

The different membrane fractions described below were produced by differential centrifugation of a crude homogenate of the Malpighian tubules of *Locusta*. This centrifugation process is detailed in Fig. 2.1. in the Materials and Methods.

C = Crude Malpighian tubule homogenate

P1 = Pellet produced after 600 g spin for 10 min.

P2 = Pellet produced after 15,000g spin for 20 min (mitochondrial pellet).

P3 = Pellet produced after 135,000g spin for 60 min.

P4 =Pellet produced after 10,000g spin for 15 min (basolateral pellet).

P5 =Pellet produced after 55,000g spin for 30 min (apical pellet).

FIG 4·1

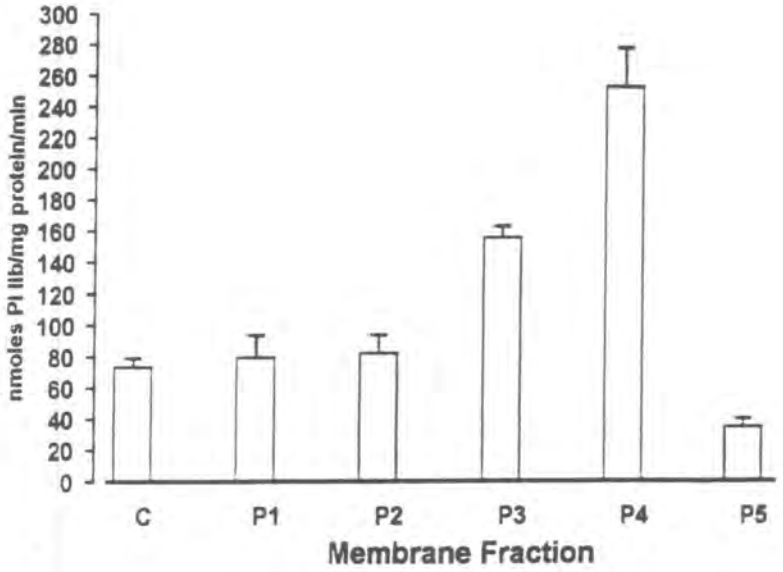


FIG 4·2

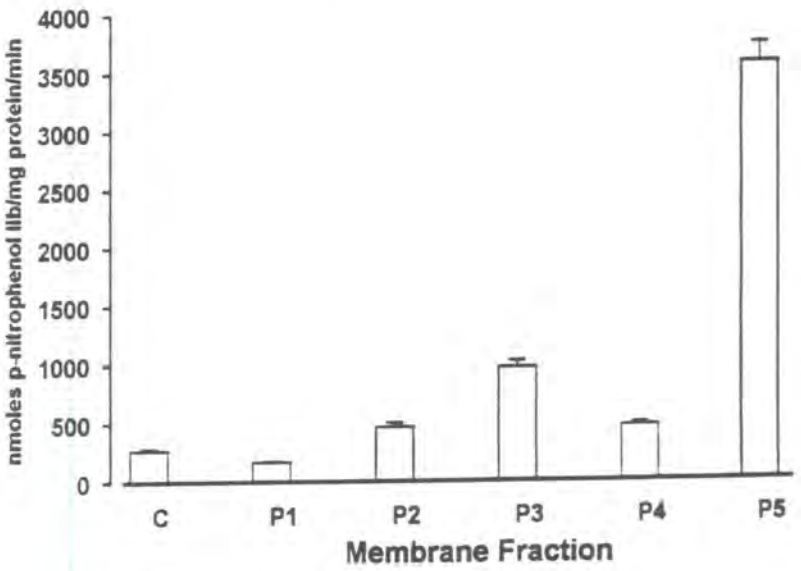
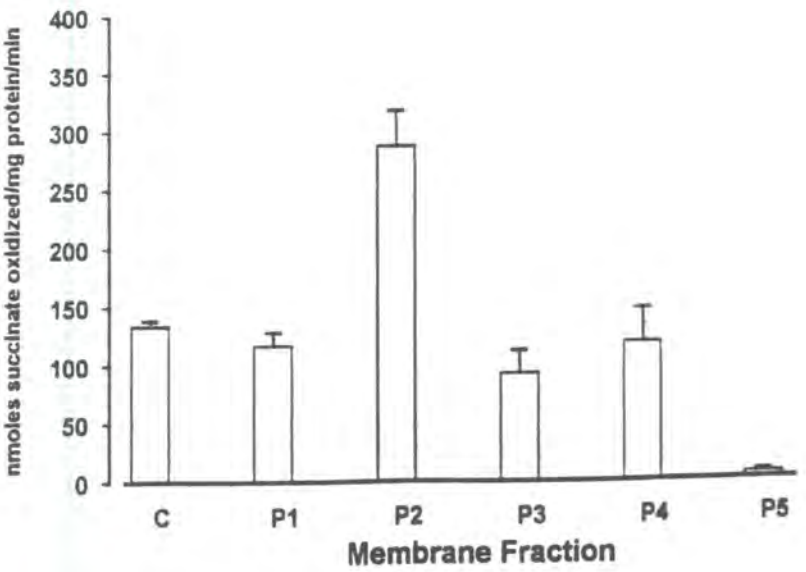


FIG 4·3



Biochemical studies on enzyme activities found in the apical membrane of *Locusta migratoria*

Effect of HCO_3^- and Cl^- concentration on Mg^{2+}-dependent ATPase activity

Mg^{2+}-dependent ATPase activity was assayed over a range of NaCl and $NaHCO_3$ concentrations. Fig. 4.4. shows the results obtained from a typical example and it can be seen that inclusion of $NaHCO_3$ in the reaction medium caused a stimulation of the Mg^{2+}-dependent ATPase activity; maximum stimulation being achieved with 20mM $NaHCO_3$. When NaCl was included in the reaction medium the activity of the Mg^{2+}-dependent ATPase was increased slightly at lower concentrations (5-15mM NaCl) but then fell returning towards to the basic Mg^{2+}-dependent ATPase activity level.

Results obtained from three preparations (Appendix, Table A.1.) gave mean activity in the presence of 2mM Mg^{2+} alone to be 231.4 ± 25.2 nmoles P_i liberated/mg prot./min. In the presence of 2mM Mg^{2+} and 20mM NaCl it was 236.7 ± 25.4 nmoles P_i liberated/mg prot./min. However, in the presence of 2mM Mg^{2+} and 20mM $NaHCO_3$ it rose to 295.0 ± 32.7 nmoles P_i liberated/mg prot./min representing a significant increase ($P<0.05$) in the normalized activity of 27.5 ± 2.0 compared to the activity recorded in the presence of 2mM Mg^{2+} alone. Additionally, a significant increase ($P<0.05$) in the normalized activity of 24.6 ± 1.1 was produced when compared to that recorded in the presence of 2mM Mg^{2+} and 20mM NaCl.

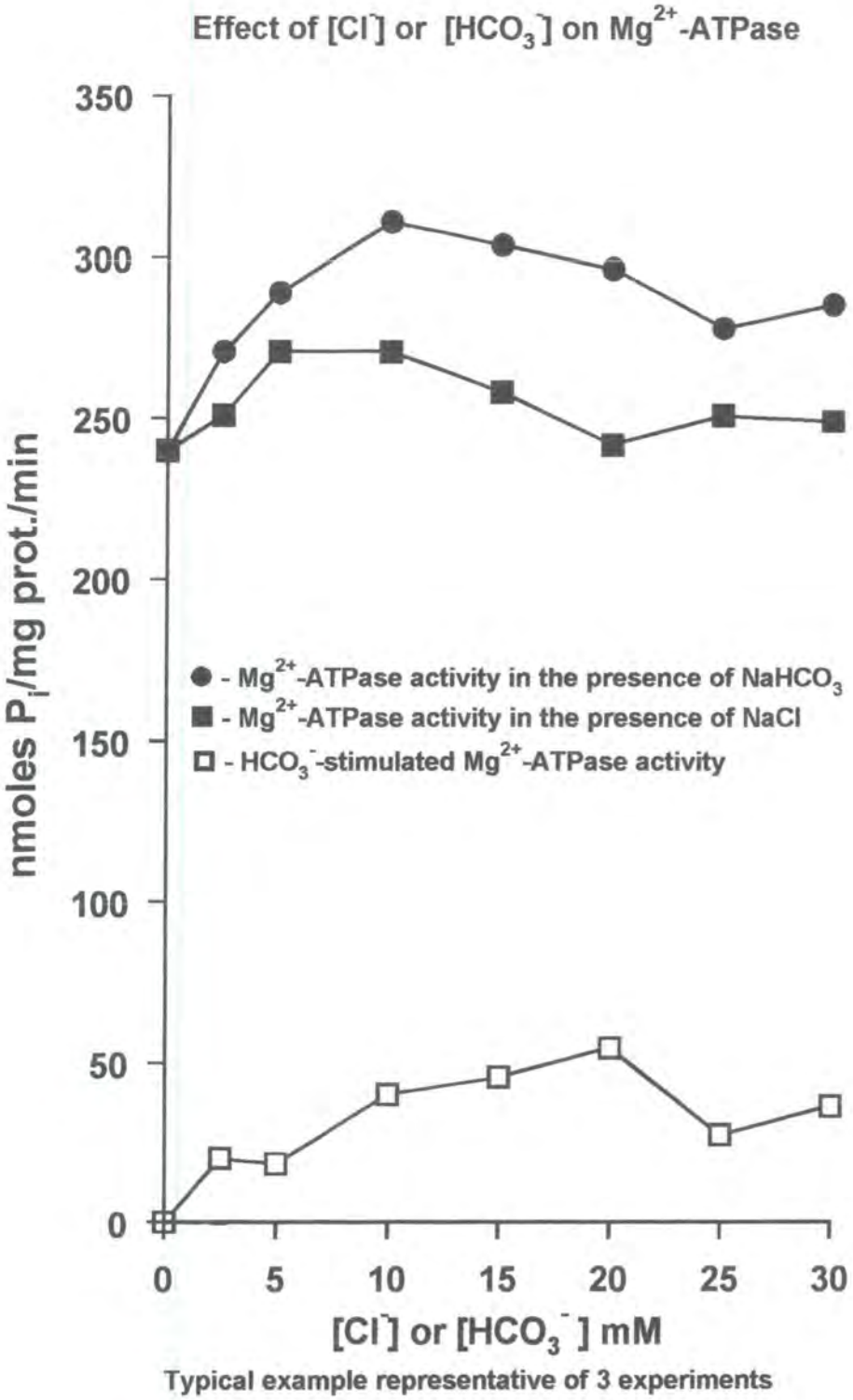
Effect of pH on Mg^{2+}-dependent ATPase activity

Reaction media having a pH ranging from 7 to 9 were created using a 30mM bis-tris propane buffer system. The desired pH was produced by adding HCl to the reaction medium. The results from a typical experiment are shown in Fig. 4.5., for results from three independent preparations see Appendix, Table A.2. The pH optimum (7.5) was the same in all three conditions. Although, unsurprisingly, the greatest activity was recorded

Fig. 4.4. Effect of $[Cl^-]$ or $[HCO_3^-]$ on Mg^{2+}-ATPase.

Stimulation of Mg^{2+}-ATPase activity present in the apical membrane-enriched fraction (Pellet 5, P_5) of Malpighian tubules from *Locusta* by Cl^- and HCO_3^- was investigated. The assay medium consisted of 4mM $MgCl_2$ in 20mM imidazole buffer, 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.

Fig. 4.4.

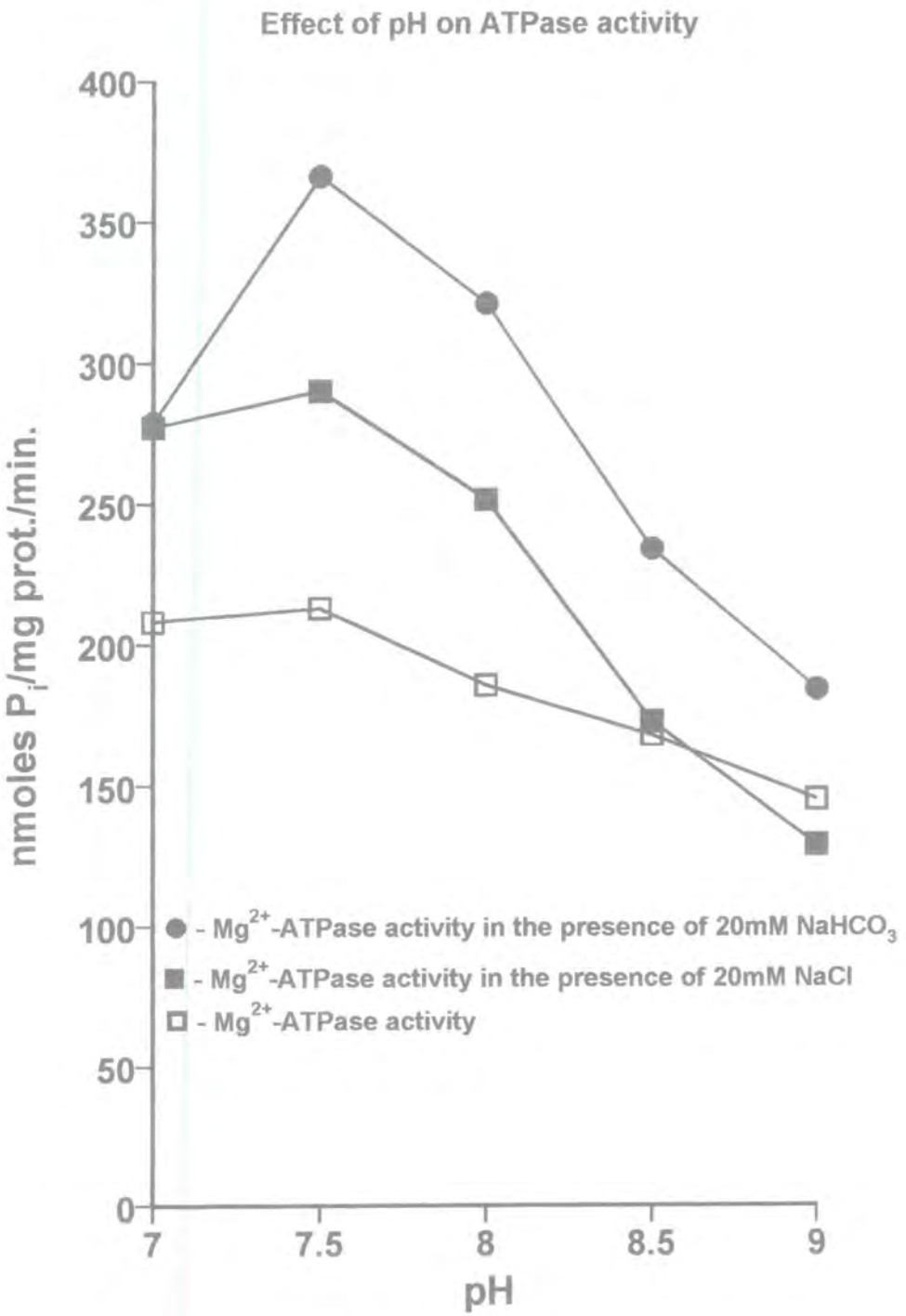


Typical example representative of 3 experiments

Fig. 4.5. Effect of pH on ATPase activity of the apical membrane-enriched fraction (Pellet 5, P_5) of Malpighian tubules from *Locusta*.

The effect of the pH on Mg^{2+}-ATPase activity and Mg^{2+}-ATPase activity in the presence of 20mM NaCl or 20mM $NaHCO_3$ was investigated. 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.

Fig. 4.5.



Typical example representative of 3 experiments

in the medium containing 2mM Mg^{2+} and 20mM $NaHCO_3$. In all three media activity fell sharply when the pH was increased above 7.5.

Effect of Mg^{2+} concentration on Mg^{2+}-dependent ATPase activity

Mg^{2+}-dependent HCO_3^--stimulated ATPase was assayed in reaction media in which the Mg^{2+} concentration varied from 0 to 10mM. Fig. 4.6. shows the relationship between ATPase activity and Mg^{2+} concentration (see also Appendix, Table A.3). With no Mg^{2+} present there was no activity. A marked increase in activity was observed with increasing Mg^{2+} concentration up to 2mM Mg^{2+} for Mg^{2+}-dependent HCO_3^--stimulated ATPase. Increasing the Mg^{2+} concentration above 2mM resulted in a sharp fall in activity which consequently plateaued out.

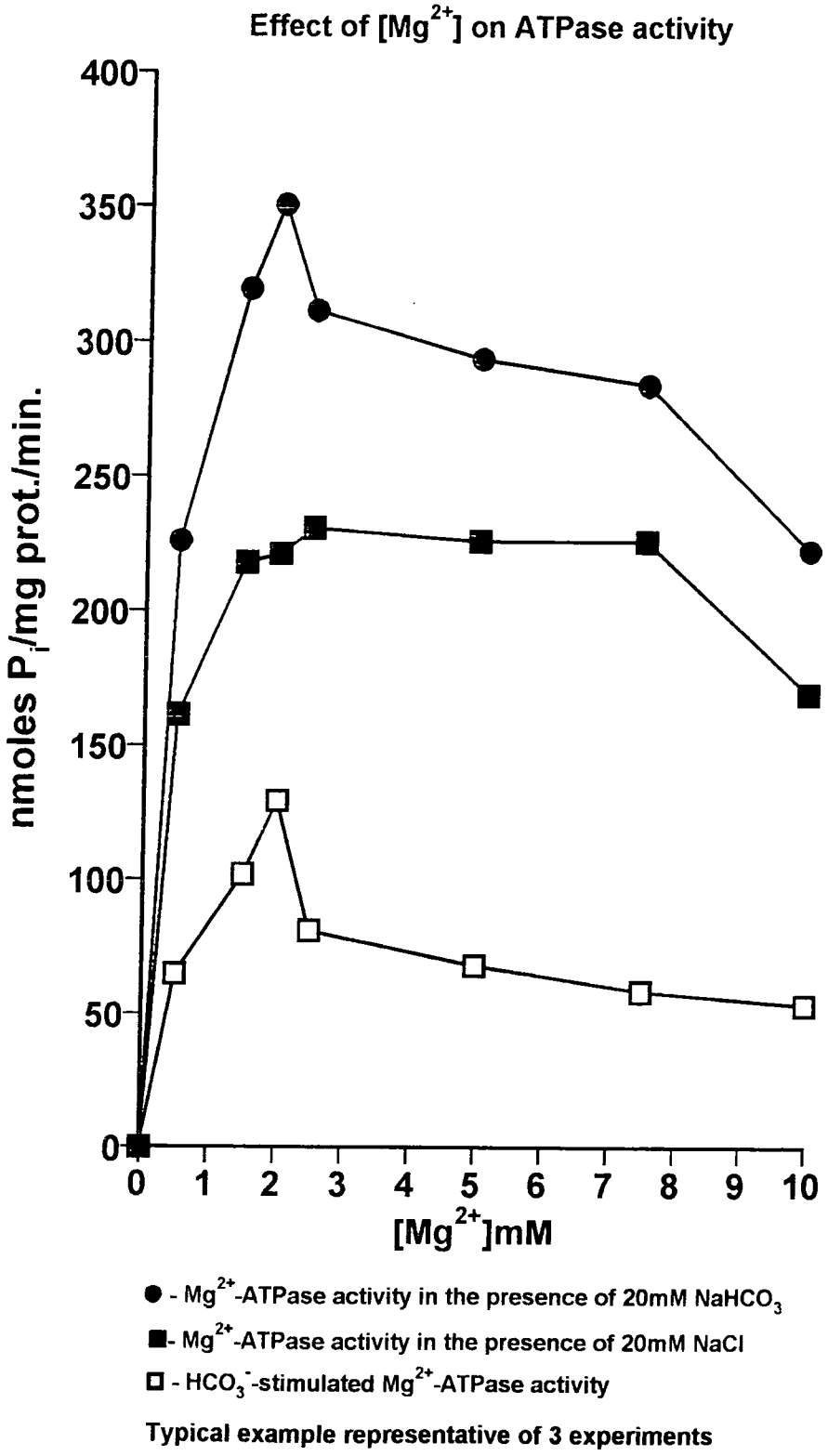
Effect of NEM on azide- and orthovanadate-insensitive ATPase activity (V-type ATPase)

The apical-enriched membrane fraction (P_5) was prepared as described previously and was assayed in reaction medium (1mM $MgCl_2$, 20mM KCl, 50mM Tris-MOPS, 0.1mM EGTA, 0.5mM NaN_3, 0.1mM Na_3VO_4 and 0.3mg/ml BSA, pH 7.5) containing concentrations of NEM ranging from 0 to 100μM. The relationship between ATPase activity and NEM concentration is shown in Fig. 4.7. It can be seen that enzyme activity was highly sensitive to NEM, significant ($P<0.05$) inhibition of approximately 50% being observed with a concentration of 1μM NEM. Higher concentrations of 10 and 100μM NEM significantly ($P<0.05$) reduced the ATPase activity by approximately 70 and 75% respectively but 0.1μM NEM had insignificant effect.

Fig. 4.6. Effect of $[Mg^{2+}]$ on ATPase activity apical membrane-enriched fraction (Pellet 5, P_5) of Malpighian tubules from *Locusta*.

The effect of $[Mg^{2+}]$ on ATPase activity was investigated in the presence of 20mM NaCl or 20mM $NaHCO_3$. 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.

Fig. 4.6.



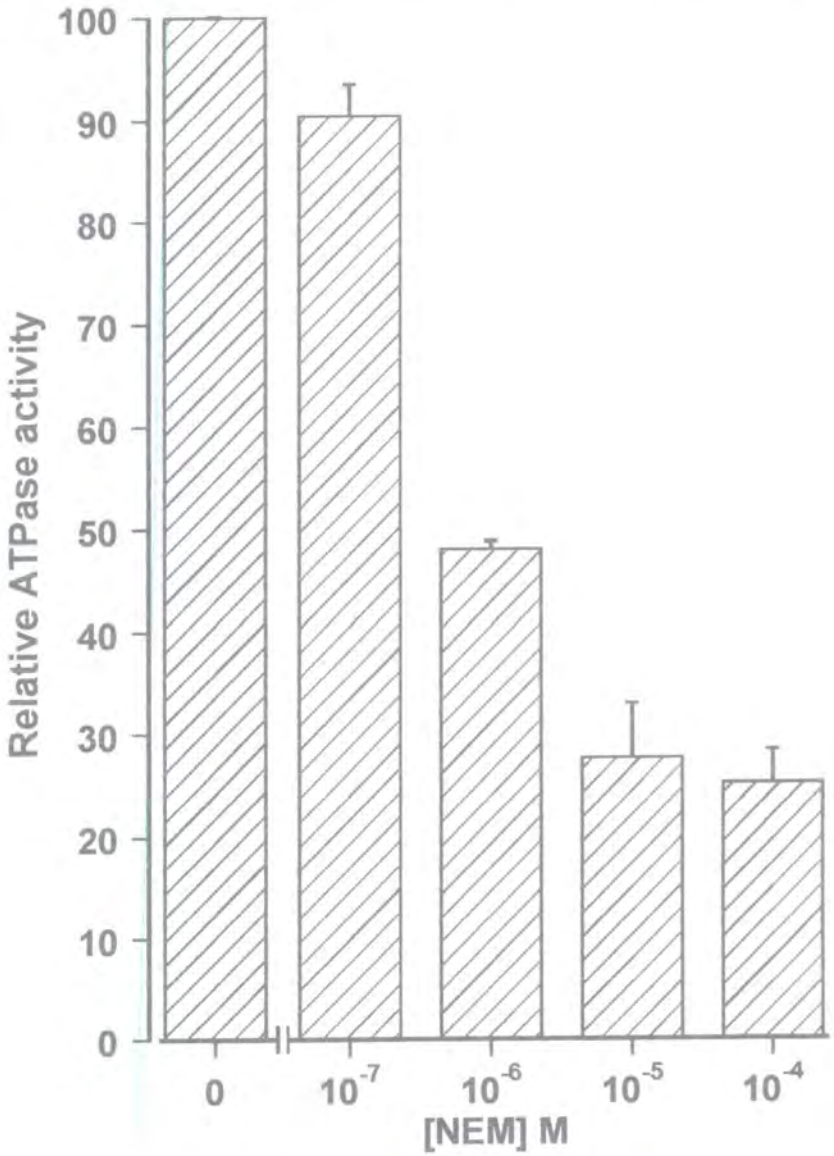
Typical example representative of 3 experiments

Fig. 4.7. Membrane-bound azide- and orthovanadate-insensitive ATPase activity in the presence of N-ethylmaleimide (NEM).

The azide- and orthovanadate-insensitive ATPase activity associated with the apical membrane-enriched fraction (P_5) of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of NEM. The reaction medium consisted of : 1mM $MgCl_2$, 20mM KCl, 50mM Tris-MOPS, 0.1mM EGTA, 0.5mM NaN_3, 0.1mM Na_3V0_4 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.

Fig. 4.7.

Membrane-bound azide- and orthovanadate-insensitive ATPase activity in the presence of NEM



Each bar represents the mean ± S.E.M.,
calculated from 3 independent preparations
Specific activity at 0: 266.5±44.7 nmoles P_i/mg prot/min.

Effect of bafilomycin A_1 on azide- and orthovanadate-insensitive ATPase activity (V-type ATPase)

The apical-enriched membrane fraction (P_5) and basal-enriched fraction (P_4) were prepared as described previously and assayed in a reaction medium (see above) containing concentrations of bafilomycin A_1 ranging from 0 to 50μM. Fig. 4.8. shows the relationship between ATPase activity and bafilomycin A_1 concentration. The enzyme activity of P_5 was extremely sensitive to bafilomycin A_1, all concentrations tested effecting significant ($P<0.05$) inhibition. At a concentration of only 5nM activity fell to approximately 20% of that recorded in the absence of bafilomycin A_1. The activity recorded fell as bafilomycin A_1 concentration increased, at 50μM activity fell to approximately 10% of the control value. In contrast the enzyme activity of P_4 was insensitive to bafilomycin A_1, even at the highest concentration used only 28-27% inhibition was observed. This was not a significant reduction.

Effect of NEM on HCO_3^--stimulated ATPase activity

The effect of different concentrations of NEM (0-1mM) on the Mg^{2+}-dependent HCO_3^--stimulated ATPase activity of pellet 5 (the apical membrane-enriched pellet) was investigated. Fig. 4.9 shows the mean results obtained from three independent preparations. The data was normalized with respect to the activity produced in the presence of 20mM NaCl. This value was 571.4 ± 203.0 nmoles P_i liberated/mg. protein/min. Fig 4.9. shows that HCO_3^--stimulated ATPase activity was noticeably inhibited in the presence of 0.1mM and 1mM NEM.

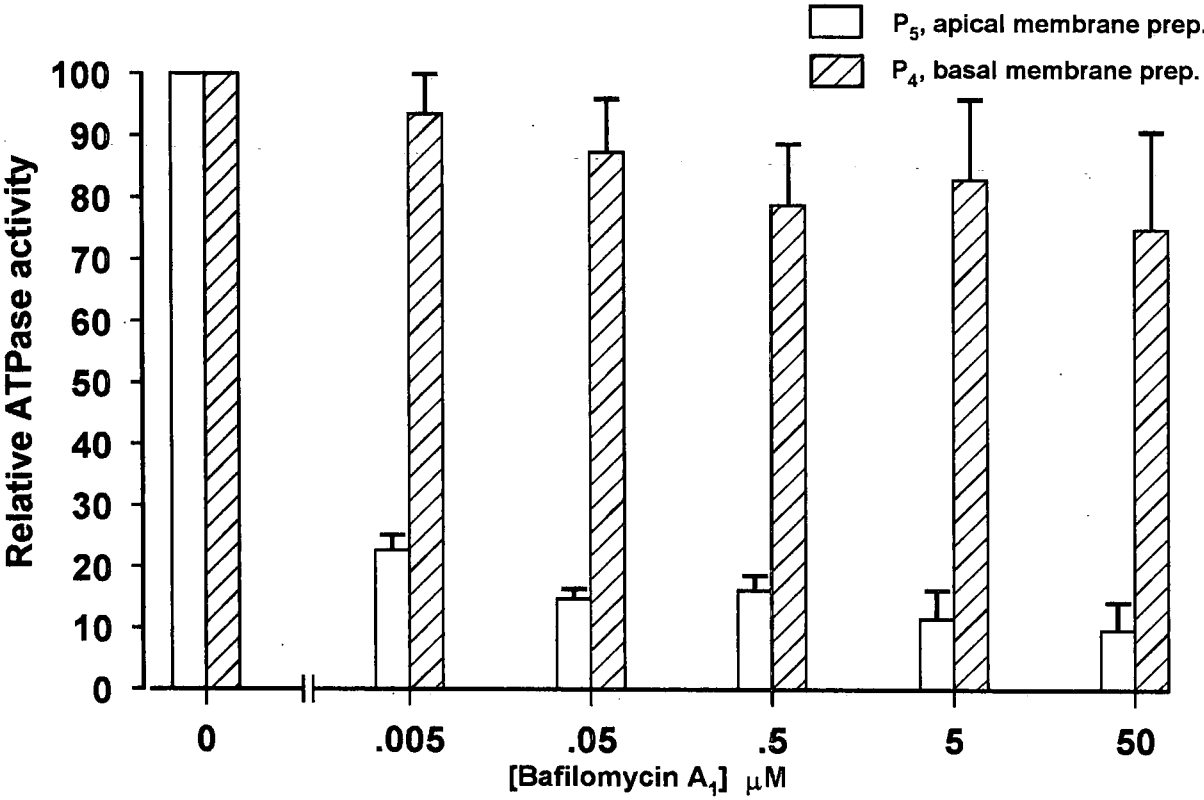
Effect of bafilomycin A_1 on HCO_3^--stimulated ATPase activity

The effect of different concentrations of bafilomycin A_1 (0-100μM) on the Mg^{2+}-dependent HCO_3^--stimulated ATPase activity of pellet 5 (apical membrane -

Fig. 4.8. Membrane-bound azide- and orthovanadate-insensitive ATPase activity of Pellet 4 (P_4) and Pellet 5 (P_5) in the presence of bafilomycin A_1.

The azide- and orthovanadate-insensitive ATPase activity associated with the basal (P_4) and apical (P_5) membrane-enriched fractions of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of bafilomycin A_1. The reaction medium consisted of : 1mM $MgCl_2$, 20mM KCl, 50mM Tris-MOPS, 0.1mM EGTA, 0.5mM NaN_3, 0.1mM Na_3V0_4 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.

Fig. 4.8. Membrane-bound azide- and orthovanadate-insensitive ATPase activity of P_4 and P_5 in the presence of bafilomycin A_1



The two bars at each concentration represent the mean ± S.E.M., calculated from 3 independent preparations

P_5 specific activity at 0: 1037.5±205.9 nmoles P_i/mg prot/min.

P_4 specific activity at 0: 218.0±29.0nmoles P_i/mg prot/min.

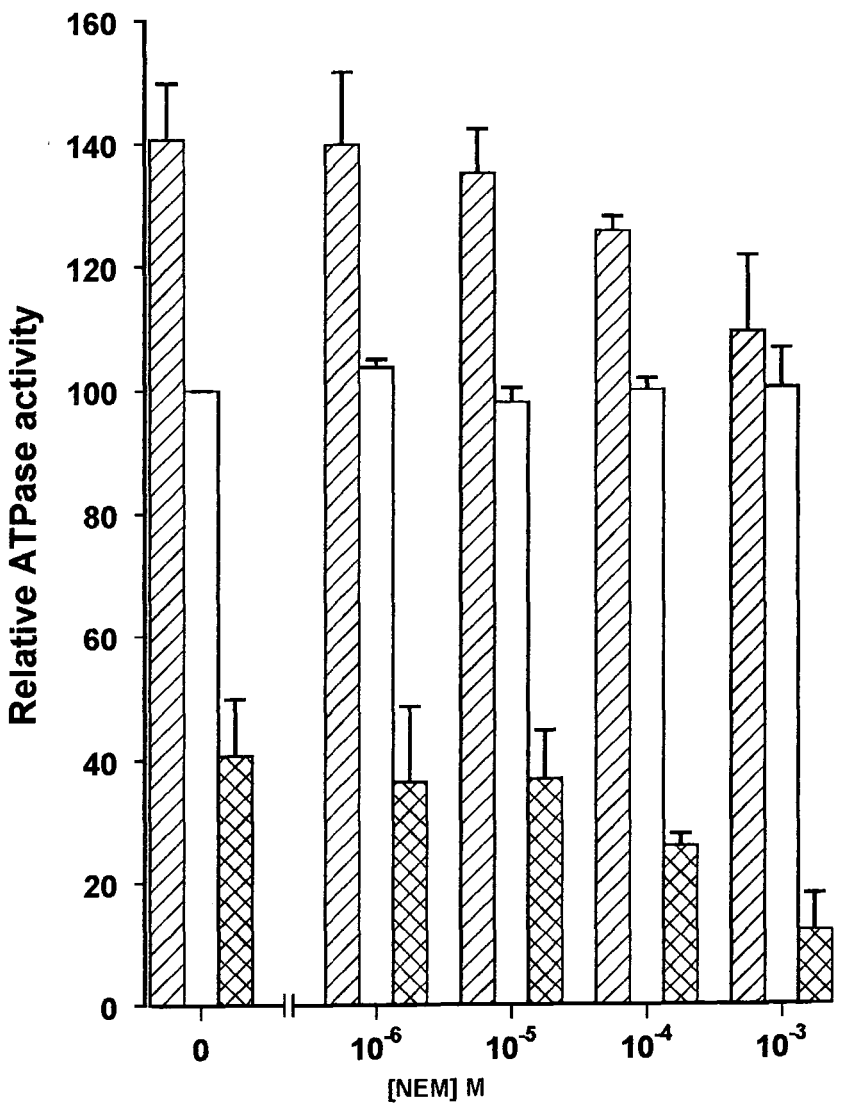
Fig. 4.9. HCO_3^--stimulated ATPase activity in the presence of NEM.

The HCO_3^--stimulated ATPase activity associated with the apical membrane-enriched fractions (P_5) of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of NEM. 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.

HCO_3^--stimulation of ATPase activity was measured as the difference in inorganic phosphate measured in the reaction medium containing 4mM $MgCl_2$, 20mM $NaHCO_3$ in 50mM HEPES buffer, pH 7.5 and that released in a reaction medium containing 4mM $MgCl_2$, 20mM NaCl in 50mM HEPES buffer, pH 7.5.

Fig. 4.9.

HCO_3^--stimulated ATPase activity in the presence of NEM



The three bars at each concentration represent the mean ± S.E.M., calculated from 3 independent preparations

enriched pellet) was investigated. Fig. 4.10. shows results obtained from the three experiments as mean ± S.E.M. The data was normalized with respect to the activity produced in the presence of 20mM NaCl. This value was 492.1 ± 91.8 nmoles P_i liberated/mg. protein/min. It can be seen that 100μM and 10μM bafilomycin A_1 both produced considerable reduction in the HCO_3^--stimulated ATPase activity.

Effect of NEM on Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity present in the basal membrane-enriched pellet (P_4) was assayed as described previously but different concentrations of NEM were included in the reaction medium. NEM did not inhibit the Na^+/K^+-ATPase activity at any of the concentrations used (see Fig. 4.11.).

Effect of K^+ on azide- and orthovanadate-insensitive ATPase activity (V-type ATPase) in the Malpighian tubules of *Locusta migratoria*

ATPase assays were carried out as described previously. The assay medium, based on that by Schweikl *et al.*, (1989), consisted of 1mM $MgCl_2$, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN_3 and 0.1mM Na_3VO_4, pH 7.5. The enzyme was solubilized using 0.05% Triton X-100. Different concentrations of KCl were assayed in order to find the maximum stimulation and the results are shown in Table 4.1.

It can be seen that inclusion of K^+ in the assay medium caused an increase in the activity of the enzyme. There is was an increase of ~20% when 1mM K^+ was included in the assay medium and this increased gradually until it reached a peak at 30mM K^+, when the stimulation was approximately 127%.

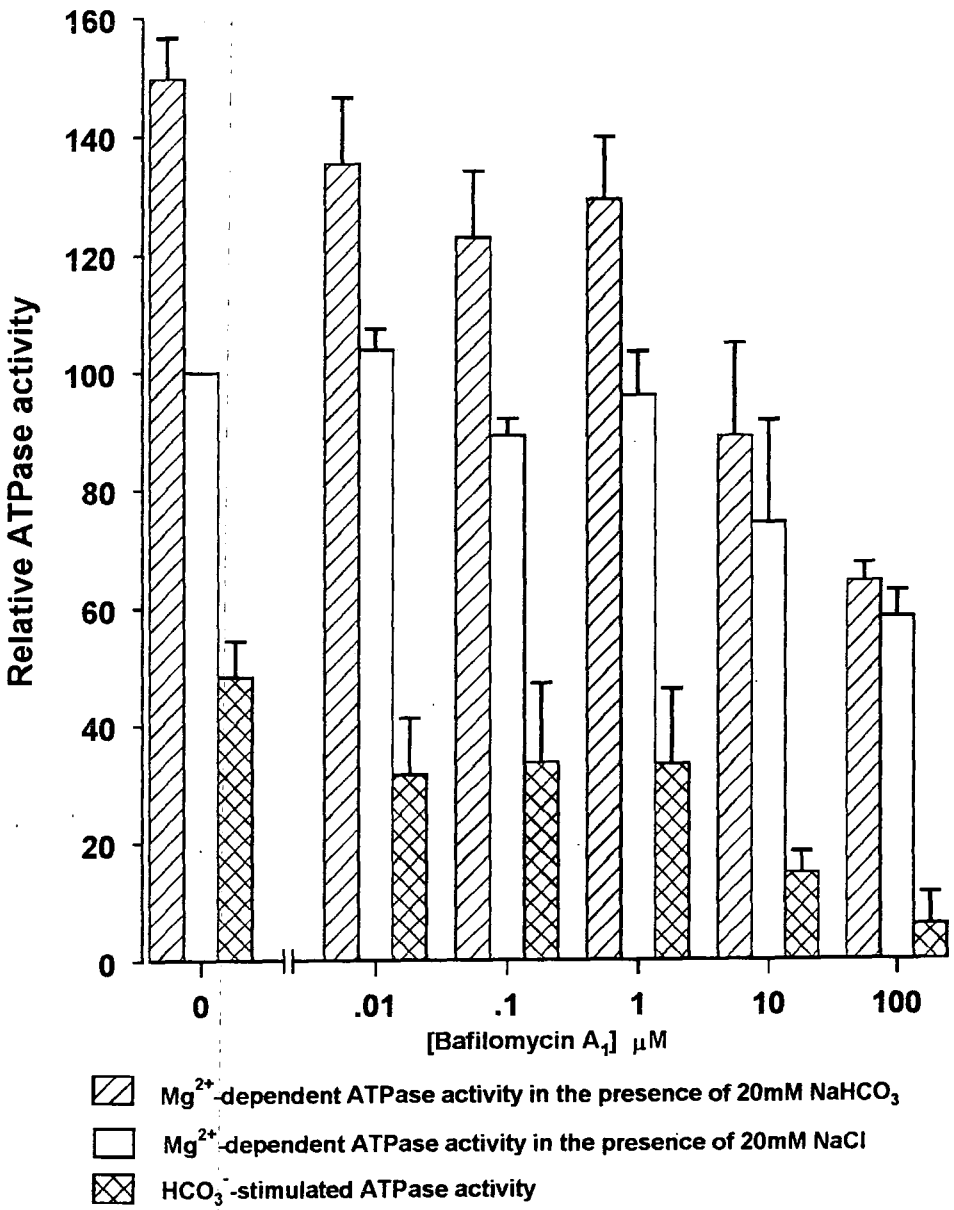
Fig. 4.10. HCO_3^--stimulated ATPase activity in the presence of bafilomycin A_1.

The HCO_3^--stimulated ATPase activity associated with the apical membrane-enriched fractions (P_5) of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of bafilomycin A_1. 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.

HCO_3^--stimulation of ATPase activity was measured as the difference in inorganic phosphate measured in the reaction medium containing 4mM $MgCl_2$, 20mM $NaHCO_3$ in 20mM imidazole buffer, pH 7.5 and that released in a reaction medium containing 4mM $MgCl_2$, 20mM NaCl in 20mM imidazole buffer, pH 7.5.

Fig. 4.10.

HCO_3^--stimulated ATPase activity in the presence of bafilomycin A_1



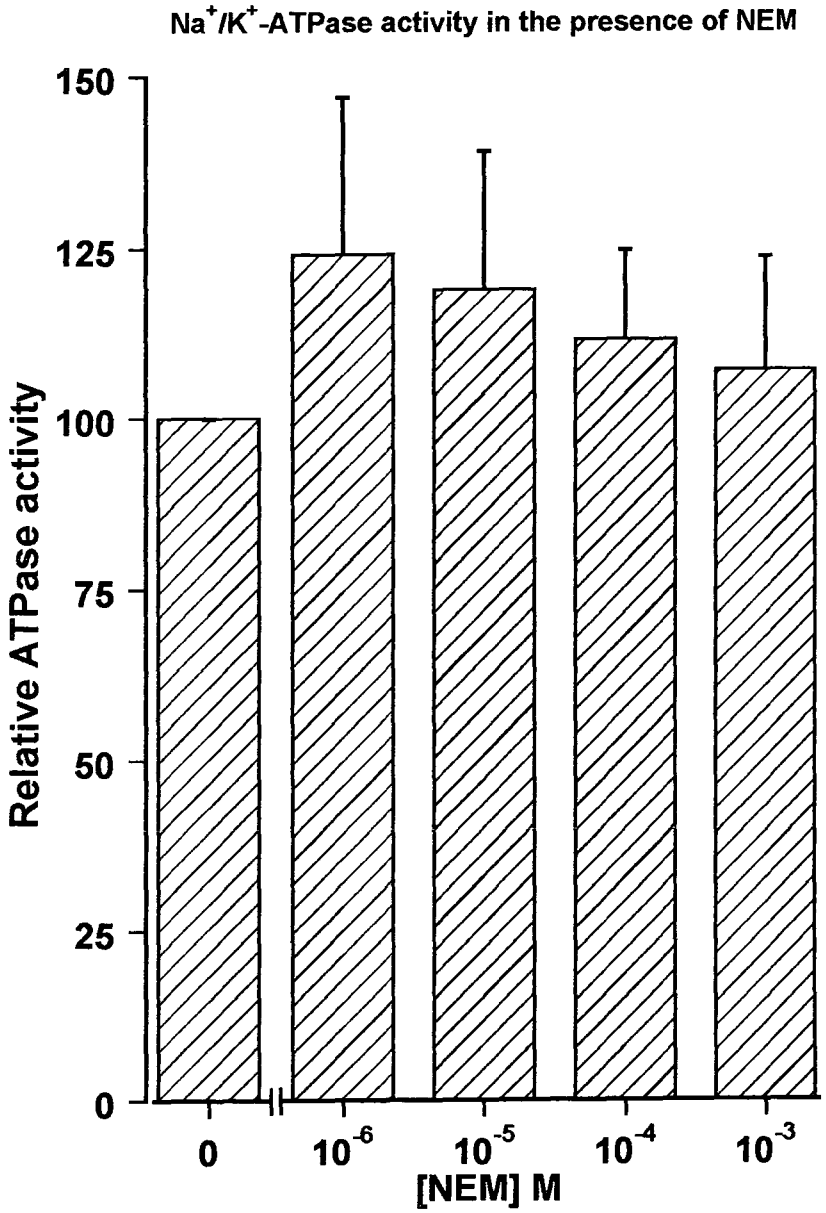
The three bars at each concentration represent the mean ± S.E.M., calculated from three independent preparations

Fig. 4.11. Na^+/K^+-ATPase activity in the presence of NEM.

The Na^+/K^+-ATPase activity associated with the basal membrane-enriched fractions (P_4) of Malpighian tubules from *Locusta* was measured in the presence of different concentrations of NEM. 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.

Na^+/K^+-ATPase activity was determined as the difference in inorganic phosphate liberated in a reaction medium containing 4mM $MgCl_2$, 100mM NaCl, 20mM KCl in 20mM imidazole buffer, pH 7.2. and that released in a reaction medium containing 4mM $MgCl_2$, 100mM NaCl, 20mM KCl in 20mM imidazole buffer, pH 7.2. plus 1mM ouabain.

Fig. 4.11.



Each bar represents the mean ± S.E.M.,
calculated from 3 independent preparations
Specific activity at 0: 194.7±49.1 nmoles P_i/mg prot/min.

Table 4.1. V-type ATPase activities of the apical membrane-enriched fraction in the presence of varying concentrations of K^+.

$[K^+]$ (mM)	Relative specific activity of the apical membrane-enriched pellet (mean ± SEM)
0	100
1	119.9 ± 3.3*
5	146.4 ± 2.0*
10	171.0 ± 4.8*
20	199.2 ± 7.9*
30	226.7 ± 2.8*
40	203.0 ± 5.3*
50	182.2 ± 3.6*

Unstimulated activity ($[K^+]_o$=0) was measured in the assay medium : 1mM $MgCl_2$, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN_3 and 0.1mM Na_3VO_4, pH 7.5. Different concentrations of KCl were included in this medium and stimulation recorded. The stimulation was expressed relative to the unstimulated activity (* significantly different at $P<0.05$).
The specific activity recorded in the presence of 0mM K^+ was 703.0 ± 23.7nmoles P_i/mg prot./min. (n=3)

Influence of salts on azide- and orthovanadate insensitive ATPase activity (V-type ATPase)

The same assay medium as described above was used to investigate the effect of various salts on ATPase activity. K^+ was replaced in the assay medium with Na^+, Rb^+, Li^+, choline or Tris and Cl^- was replaced with various anions, the results of these substitutions are shown in Table 4.2. The effect of NEM and thiocyanate on the ATPase activity was also investigated.

Table 4.2. Influence of salts and inhibitors on V-type ATPase activity

SALT	Relative specific activity of the apical membrane-enriched pellet (mean ± SEM)
0mM salt	100
30mM LiCl	140.3 ± 4.6*
30mM KBr	101.8 ± 3.6
30mM Choline Cl	117.0 ± 1.9*
30mM Tris-HCl	156.2 ± 2.4*
30mM KNO_3	27.0 ± 3.7*
30mM NaCl	133.0 ± 3.5*
30mM $KHC0_3$	190.3 ± 10.1*
30mM KGluconate	155.0 ± 6.0*
30mM RbCl	132.6 ± 4.4*
0mM salt + 10mM SCN^-	24.4 ± 5.7*
0mM salt + 1mM NEM	24.2 ± 1.5*
30mM NaCl + 1mM NEM	23.1 ± 2.2*
30mM KCl + 1mM NEM	27.1 ± 2.4*
30mM $KHC0_3$ + 1mM NEM	43.9 ± 6.3*

The first row (0mM salt) refers to the ATPase activity recorded in assay medium: 1mM $MgCl_2$, 5mM Tris-HCl, 0.1mM EDTA, 0.5mM NaN_3 and 0.1mM Na_3VO_4, pH 7.5. and represents the unstimulated activity The specific activity recorded in this assay medium was 1422.0 ± 46.9nmoles P_i/mg prot./min. (n=8). The effect on this unstimulated activity (0mM salt) produced by various salts and inhibitors was recorded and expressed relative to the activity recorded with 0mM salt (* significantly different at $P<0.05$).

All of the anions stimulated the basic Mg^{2+}-activity, comparing chloride salts Tris was the most potent activator followed by Li^+, Na^+, Rb^+ and choline in descending order. The greatest stimulation produced by an anion when comparing potassium salts was HCO_3^-, followed by gluconate and Br^-. However, nitrate caused an inhibition of

the ATPase activity. Inclusion of 1mM NEM in the assay medium caused a substantial inhibition of the ATPase activity, whether the medium included NaCl, KCl, $KHCO_3$ or only $MgCl_2$. Inclusion of 10mM SCN^- in the assay medium also caused a great reduction in the amount of enzyme activity.

Electron microscopy of membrane pellets prepared from the Malpighian tubules of *Locusta migratoria* by differential centrifugation

P_2, P_3, P_4 and P_5 were prepared for electron microscopy as described previously. Plate 4.1 shows a series of electronmicrographs of these pellets. P_2, which is shown in Plate 4.1A, consisted of a large number of mitochondria (M) and concretions (C) were also observed. P_3 contained a mixture of large and small membrane bound vesicles, this pellet represented a mixture of both basal and apical membranes and is shown in Plate 4.1C. P_4 was also made up of various sized vesicles but no mitochondria were observed (see Plate 4.1B) and P_5, shown in Plate 4.1D, consisted mainly of smaller membrane bound vesicles with no mitochondria present, P_4 and P_5, from biochemical studies, are believed to represent the basal and apical enriched membrane fractions respectively.

Western blots of SDS-PAGE

The different membrane fractions of the Malpighian tubules of *Locusta migratoria* produced by differential centrifugation and a crude homogenate of the midgut of *Manduca sexta* were separated by SDS-PAGE (see Plate 4.2A), transferred to a nitrocellulose membrane and then probed with antiserum, ECL was used for detection.

Antibodies 230-3, 224-3, 90-7 and 221-67 recognized polypeptides 28kDa, 56kDa, 28kDa and 67kDa of *Manduca sexta* midgut respectively (see Plates 4.2B, C, D and E). The Western blot of *Locusta migratoria* enriched membrane fractions probed with antiserum to V-type-ATPase of *Manduca sexta* midgut is shown in Plate 4.2B.

Plates 4.1A-D

Micrographs showing sections through 4 different pellets, representing the end products of different centrifugation steps, obtained during the production of enriched membrane fractions from the Malpighian tubules of *Locusta migratoria*.

Plate 4.1A

This plate shows a section through pellet 2 which was the mitochondria (M)-rich pellet.

Plate 4.1B

This micrograph, showing a section through pellet 4, was believed to represent the basolateral-enriched pellet. Evidence for this has come from biochemical studies.

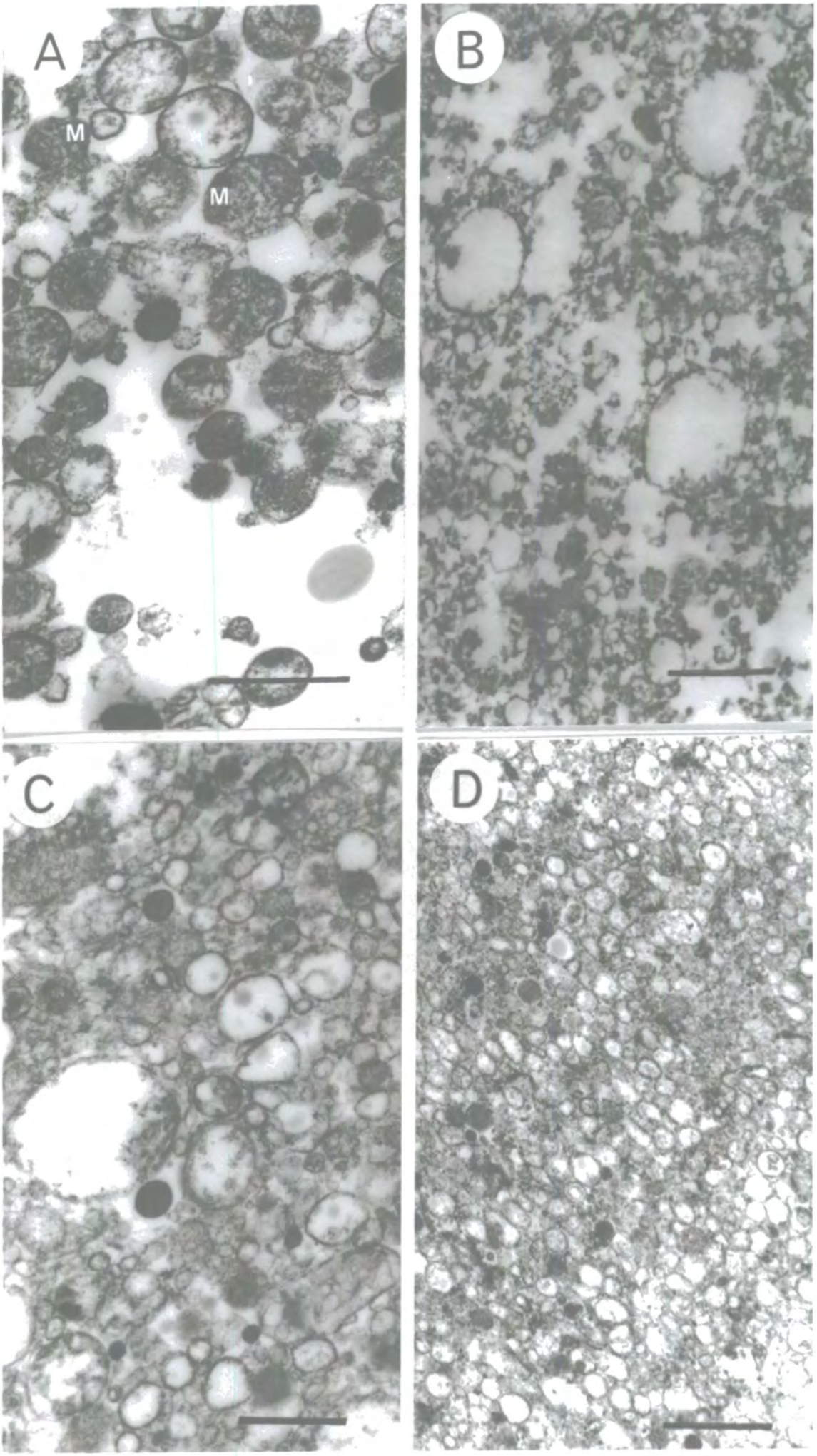
Plate 4.1C

This micrograph shows a section through pellet 3, the pellet which from information derived in biochemical studies was thought to represent both basolateral and apical membranes.

Plate 4.1D

This micrograph shows a section through pellet 5, which from evidence obtained from biochemical studies was believed to represent the apical membrane-rich fraction.

Scale : 1μm


A
M
M
B
C
D

Two gels were prepared in exactly the same way and run simultaneously using the same equipment. One was stained (Plate 4.2A) and one used for Western blotting (Plate 4.2B).

Plate 4.2A

Stained SDS-PAGE gel. Lanes 1-7 represented different protein samples :

1 - Rainbow markers

2 - Crude Malpighian tubule preparation

3 - Enriched apical membrane fraction

4 - Enriched basal membrane fraction

5 - Enriched mitochondrial fraction

6 - Crude homogenate of *Manduca sexta* midgut

7 - Molecular weight markers

Plate 4.2B

Western blot of a gel identical to the one shown above in Plate 4.2A. The blot was probed with a monoclonal antibody 230-3 to the 28kDa subunit of the V-type ATPase present in the midgut 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* (lane 3). The antibody also recognized a protein present in a crude homogenate of midgut from *Manduca sexta* (lane 6).

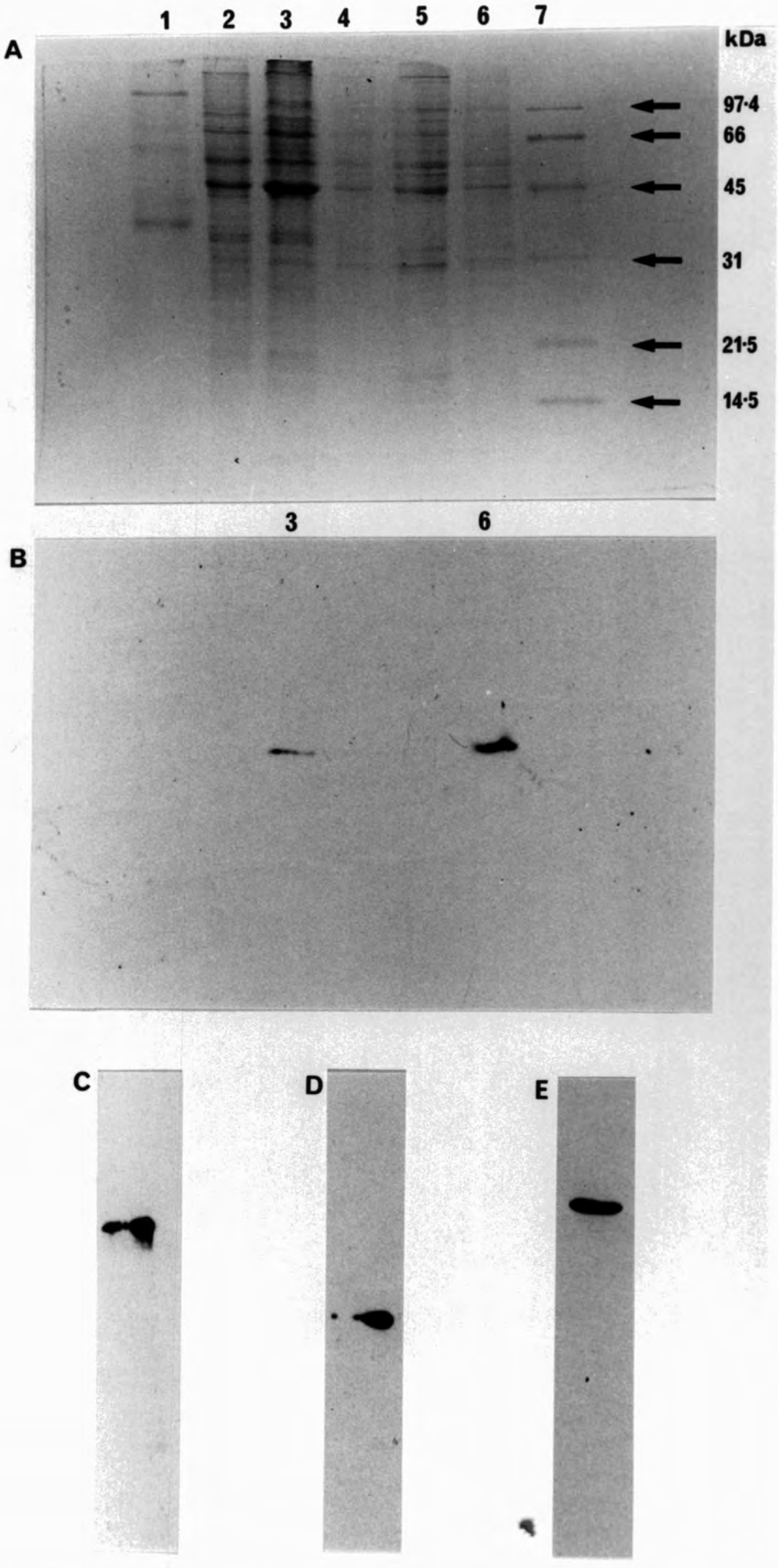
Plate 4 2C-4.2E

Equal protein replicates of crude homogenate of midgut from *Manduca sexta* were run on an SDS-PAGE gel. After Western blotting the nitrocellulose membrane was split into separate lanes to allow probing with different monoclonal antibodies to subunits of the V-type ATPase found in the midgut of *Manduca sexta.*

Plate 4.2C - blot probed with antibody 224-3

Plate 4.2D - blot probed with antibody 90-7

Plate 4.2E - blot probed with antibody clone 221-67


A
1
2
3
4
5
6
7
kDa
97·4
66
45
31
21·5
14·5
B
3
6
C
D
E

Only monoclonal antibody 230-3, which showed immunoreactivity to the 28 kDa subunit of V-type ATPase in *Manduca sexta* midgut recognized a subunit from *Locusta* ATPase, showing similar immunoreactivity to the 28 kDa polypeptide. This was located in lane 3, corresponding to the apical membrane enriched fraction. As expected it also recognized a protein band in the crude midgut preparation of midgut of *Manduca sexta* (shown in lane 6 of Plate 4.2B).

Native micrograident PAGE

Enzyme activities present in the apical-enriched membrane fraction represented by pellet 5 (P_5) produced from the Malpighian tubules of *Locusta migratoria* were separated by native micrograident PAGE. This technique allows enzyme activities to be visualized by incubation in a reaction media containing an appropriate substrate. The reaction product is a white lead salt but addition of ammonium sulphide causes a brown precipitate to form, hence, the bands become visible. When ATP was used as a substrate two enzyme activities were revealed (Plate 4.3A). The use of β-glycerophosphate as a substrate allowed the selective demonstration of alkaline phosphatase activity (Plate 4.3B). This enzyme is an unspecific phosphatase and so will utilize both ATP and β-glycerophosphate as substrates. Therefore, the lower band shown in Plate 4.3A was due to the activity of alkaline phosphatase. Using an identical gel the enzyme activities were transferred to a nitrocellulose membrane and probed with antibody 230-3 (see Plate 4.3C), to a subunit of the midgut V-type ATPase present in *Manduca sexta*, which had previously been shown to cross-react with V-type ATPase present on the apical membrane of the Malpighian tubules of *Locusta migratoria.* The antibody only recognized the upper band shown in Plate 4.3A, indicating the antibody was specific for one apical enzyme activity, the V-type ATPase present in the tubules of *Locusta.*

Plate 4.3A

Native activity microgradient PAGE of the apical-enriched membrane fraction from the tubules of *Locusta*. The gel was stained for ATPase activity by incubation for 1 hour at 35°C in substrate solution containing 5mM ATP, 5mM $MgSO_4$, 100mM Tris-HCl, pH 7.5. At the end of this time the gel was washed in DDW and incubated in a solution containing 2.5mM Pb acetate in 80mM Tris malate, pH 7, for 30 min. To visualize the white lead salt precipitates, gels were rinsed in DDW and incubated with 2% ammonium sulphide which turned precipitates dark brown.

Plate 4.3B

Native activity microgradient PAGE of the apical-enriched membrane fraction from the tubules of *Locusta*. Exactly the same procedure was followed as described above for Plate 4.3A apart from the reaction medium contained 5mM β-glycerophosphate as the substrate.

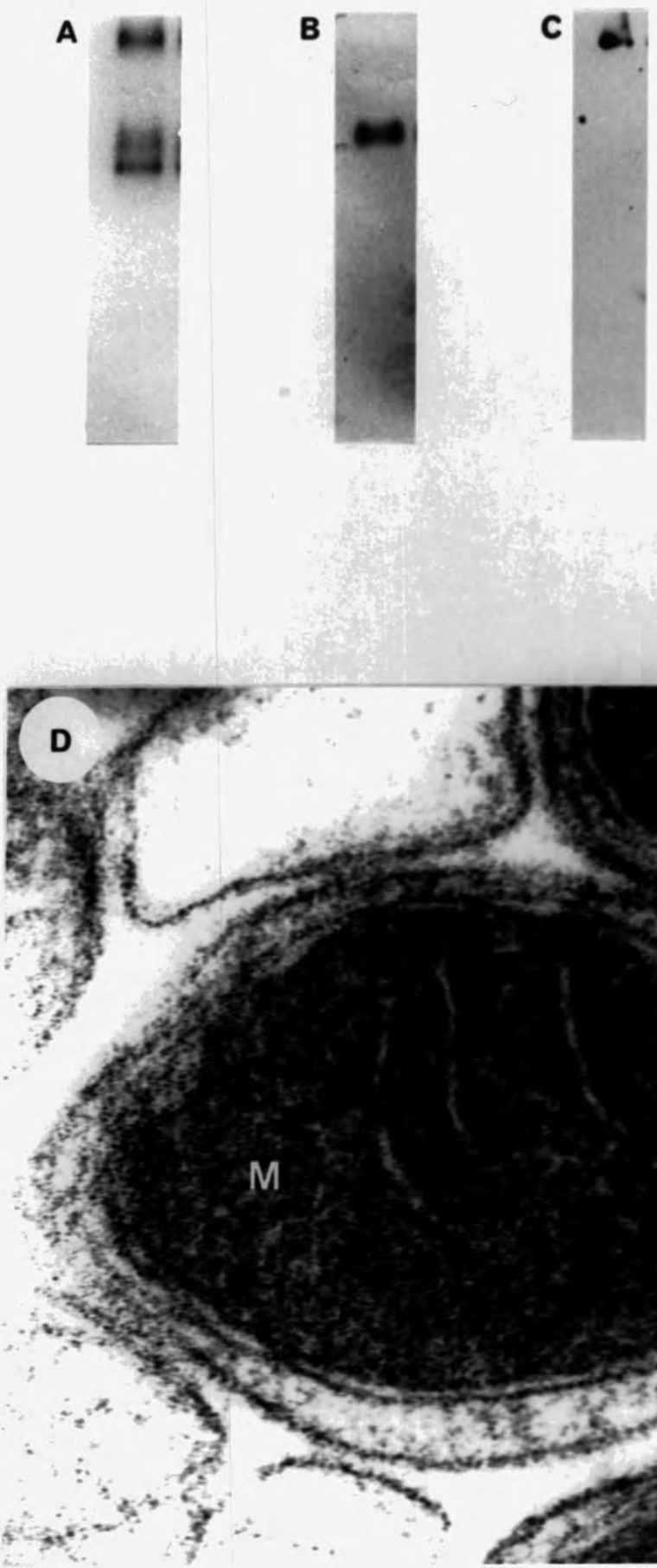
Plate 4.3C

Western blot of a microgradient gel prepared in exactly the same way as those described above. The blot was probed with antibody 230-3, to the 28kDa subunit of the midgut V-type ATPase of *Manduca sexta*. ECL was used for detection.

Plate 4.3D

This micrograph shows a transverse section through a microvillus located on the apical surface of a Malpighian tubule of *Locusta migratoria*. The microvillus contained a mitochondrion (M) and in some regions there appeared to be a "bridge" (arrows) from the outer membrane of the mitochondrion to the plasma membrane. This "bridge" is known as a portasome.

Scale : 100nm


A
B
C
D
M

Ultrastructural observations of the microvilli

Some transverse sections were taken through the microvilli which make up the apical membrane border of the tubule cells (Plate 4.3D). Mitochondria which run along the centre of the microvilli were seen in cross-section. The mitochondria have an inner and outer membrane and in places a narrow "bridge" was observed running from the outer membrane of the mitochondrion to the plasma membrane of the microvillus. These "bridges" have been reported elsewhere in ion transporting epithelia of insects and have been named portasomes (Gupta and Berridge, 1966; Harvey, 1980; Wieczorek, 1982; Bradley *et al.*, 1982; Harvey *et al.*, 1983a; Klein *et al.*, 1991; Just and Walz, 1994). The "bridges" are approximately 15-20nm long and approximately 5-10nm across.

Immunofluorescent localization of V-type ATPase

Tubule structure is shown in Plates 3.1A and 3.1B. The tubules were one cell thick, both surfaces consisted of highly folded cell membranes and the tubule was surrounded by a basement membrane. Antibody 230-3 bound specifically to the apical region of transverse sections of Malpighian tubules (Plates 4.4A and 4.4B) this localization represented the site of the apical infoldings or microvilli, where the portasomes were situated. Labelling of the cells cytoplasm and the basal membrane was negligible (Plates 4.4A, B). Incubations with antibodies 221-67, 224-3 and 90-7 acting as controls also showed negligible labelling (Plate 4.4D, antibody 90-7 shown) and insignificant autofluorescence. However, all of the antibodies clearly labelled the goblet cell cavities of the *Manduca* apical membrane (see Plate 4.4C, antibody 90-7 shown).

Immunofluorescent localization of Na^+/K^+-ATPase

Cryostat sections of Malpighian tubules which have been incubated with monoclonal antibody IgG α 5, specific for the α-subunit of avian sodium pump, are

Plates 4.4A, B

Light micrographs to illustrate the localization of the V-type ATPase in the Malpighian tubules of *Locusta migratoria*. The labelling by antibody 230-3 to the midgut V-type ATPase of *Manduca sexta* was restricted to the apical membrane of *Locusta* Malpighian tubules. The labelling appeared as a bright yellow colour which was not seen in the cytoplasm or on the basal membrane.

Scale : 5μm

Plate 4.4C

Light micrograph of the midgut of *Manduca sexta*. The fluorescent labelling of the goblet cell cavities was provided by antibody 90-7 to the midgut of *Manduca sexta*.

Scale : 5μm

Plate 4.4D

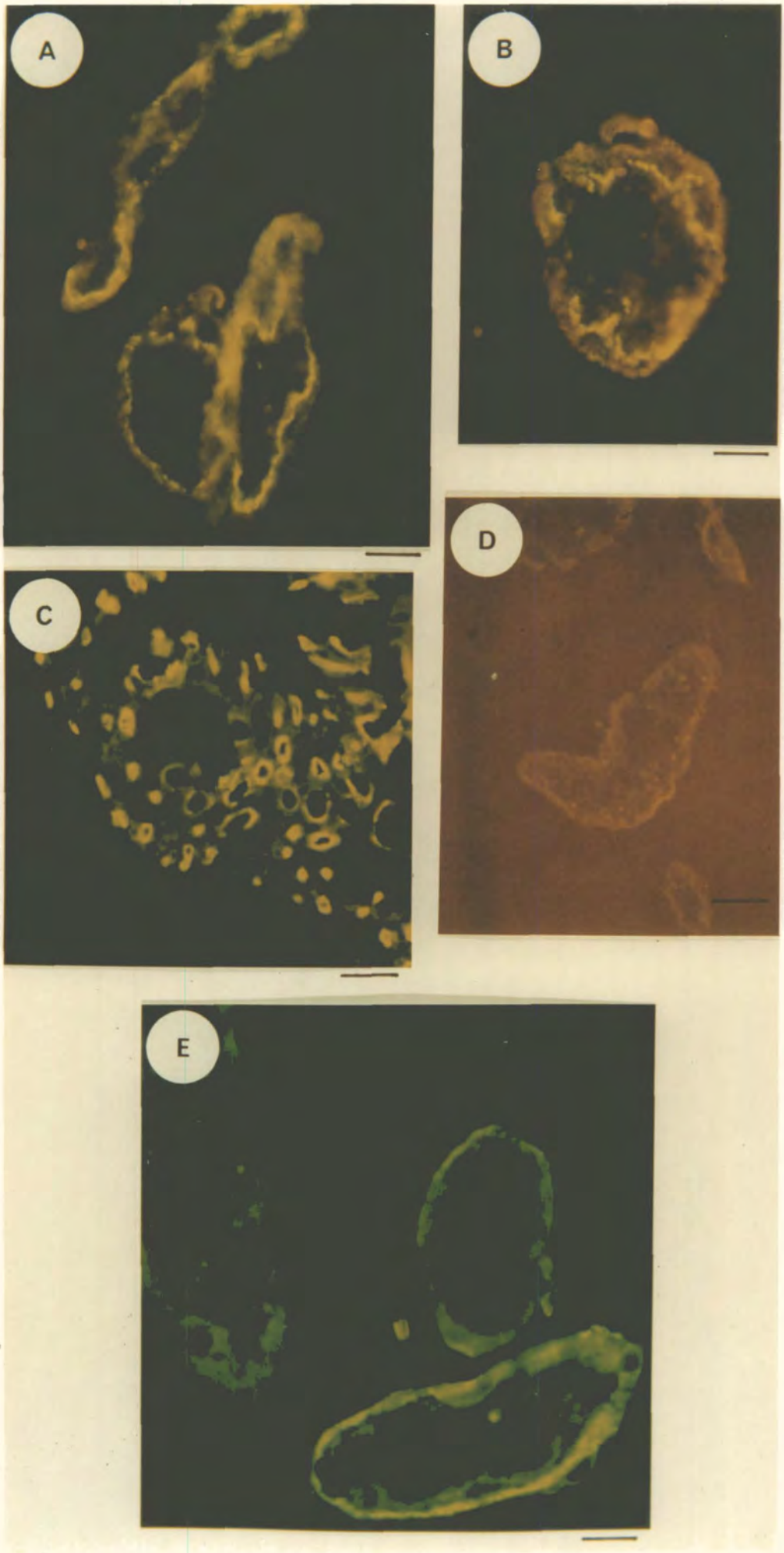
Light micrograph of a Malpighian tubule of *Locusta migratoria* which has been treated with antibody 90-7 to the midgut of *Manduca sexta*. No fluorescent labelling was observed.

Scale : 5μm

Plate 4.4E

Light micrograph of Malpighian tubules of *Locusta migratoria* which have been treated with monoclonal antibody IgG α 5, specific for the α-subunit of avian sodium pump. Fluorescent apple-green labelling can be seen which was localized to the basal surface.

Scale : 5μm


A
B
C
D
E

shown in Plate 4.4E. Cross-sections through the Malpighian tubules show that the fluorescent labelling was confined to the basal region of the tubules, the cytoplasm and apical membrane remaining unlabelled.

DISCUSSION

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 fractions to be assessed. Alkaline phosphatase, succinate dehydrogenase and Na^+/K^+-ATPase were used as markers for apical, mitochondrial and basal membranes respectively.

Assays on pellet 5 demonstrated the greatest mean alkaline phosphatase activity and negligible succinate dehydrogenase and Na^+/K^+-ATPase activities, indicating this represents the apical membrane-rich pellet. In contrast, pellet 4 produced the highest Na^+/K^+-ATPase activity and a low alkaline phosphatase activity indicative of basal membranes.

Mitochondrial contamination has often been a problem when trying to localize enzyme activities. However, the indication from the present study, using marker enzyme assays shows greatest succinate dehydrogenase activity in pellet 2, which corresponds to the mitochondrial pellet in electron microscopic studies (Plate 4.1A) and insignificant succinate dehydrogenase activity in pellet 5 coupled with the apparent absence of mitochondria from pellet 5 as shown by electron microscopy (Plate 4.1D). This suggests that the apical-enriched membrane fraction is relatively free of mitochondrial contamination.

Enzyme assays carried out on the apical membrane-rich pellet 5 revealed an ATPase activity which was insensitive to both azide and orthovanadate. However, this enzyme activity was inhibited by NEM and bafilomycin A_1. These inhibitor characteristics are similar to those reported in other insect secretory tissues (Wieczorek *et al.*, 1989; Bertram *et al.*, 1991; Weltens *et al.*, 1992) where the activity has been attributed to a V-type ATPase. The azide- and orthovanadate-insensitive ATPase activity present in pellet 5 was extremely sensitive to bafilomycin A_1 (~80% inhibition

at 5nM), the activity was also sensitive to NEM (~50% inhibition occurring at 1μM). These values are within the specific concentration range that affects only V-type ATPase activity. Therefore, the azide- and orthovanadate-insensitive activity present in the Malpighian tubules of *Locusta* can be attributed to a V-type ATPase. Bafilomycin A_1 had negligible effect on Na^+/K^+-ATPase activity present in the basolateral membrane-rich fraction (pellet 4); even at 50μM, activity was only inhibited by approximately 20%. The uninhibited ATPase activity could either represent residual Na^+/K^+-ATPase and/or mitochondrial activity or possibly a novel enzyme activity found on the basal membrane which is insensitive to azide, orthovanadate and bafilomycin A_1. The nature of this enzyme activity needs to be investigated further but was beyond the scope of this project. Due to its resistance to inhibition by bafilomycin A_1 it cannot be a V-type ATPase. However, the lack of a V-type ATPase presence on the basal membrane points to a successful separation of the apical and basal cell membranes. V-type ATPase activity was located in the midgut of *Manduca sexta* (Schweikl *et al.*, 1989) by similar procedures. These 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 1μM). 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 100μM NEM.

As outlined in the Introduction, three classes of proton translocating ATPases or ATP-driven cation pumps have been described: P-type, F-type and V-type (reviewed by Al-Awqati, 1986 and Forgac, 1989). P-type ATPases are found in fungal and yeast membranes as well as in gastric microsomal and plasma membranes, they include the ubiquitous Na^+/K^+-ATPase as well as the Ca^{2+}-ATPase of sarcoplasmic reticulum. F-type ATPases are the proton-translocating ATP synthases of mitochondria, chloroplasts and bacterial plasma membranes and V-type ATPases are best known as the acidifiers of vesicles and vacuoles.

The different sensitivities of these ATPases to various inhibitors has been used in the present study to characterize the ATPases present in the Malpighian tubules of *Locusta migratoria.* V-type ATPases are resistant to vanadate unlike the phosphorylated ATPases (Forgac, 1989). Vanadate, a transition metal ion, is able to substitute for phosphate during the phosphorylation cycle forming a stable intermediate preventing dephosphorylation and so inhibiting the phosphorylated ATPase enzyme reaction (Al-Awqati, 1986). Therefore, inclusion of vanadate will only affect P-type ATPases (Pederson and Carafoli, 1987; Macara, 1980). V-type ATPases are also resistant to azide unlike the F-type ATPases which are inhibited (Bowman *et al.*, 1988). Therefore since azide is a specific inhibitor of the mitochondrial ATPases (Bowman *et al.*, 1978) contaminating mitochondrial ATPase activity can be eliminated by the inclusion of azide in the assay medium. Likewise inclusion of vanadate in the assay medium will minimize the contribution P-type ATPases towards the enzyme activity recorded.

The V-type ATPases are, however, extremely sensitive to NEM and bafilomycin A_1. NEM has been shown to inhibit V-type activity at low micromolar amounts, phosphorylated ATPases are inhibited at higher concentrations (100μM-1mM) and F-type ATPases are almost resistant to inhibition by NEM (Forgac, 1989). It 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} < 100μM). 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 Na^+/K^+-ATPase activity. NEM is an alkylating agent and is relatively selective for sulphydryl groups (Forgac, 1989) however it is possible that it could have general toxic effects (Weltens *et al.*, 1992). Bafilomycin 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. Bafilomycin A_1 inhibits V-type ATPases at nanomolar

concentrations and P-type ATPases at micromolar concentrations (Bowman *et al.*, 1988). Mattsson *et al.*, (1991) also reported that bafilomycin A_1 was 10^4 times less potent on P-type than V-type ATPases. Another advantage of using bafilomycin 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.

Strong immunological evidence that the Malpighian tubules of *Locusta migratoria* contain a V-type ATPase has been produced by the present study. V-type ATPases are known to have high homology since polyclonal antibodies against subunits of plant and mammalian V-type ATPases recognise subunits of *Manduca sexta* midgut ATPase (Russell *et al.*, 1992). Furthermore a monoclonal antibody raised against midgut V-type ATPase from *Manduca sexta* also recognizes an apical protein in the Malpighian tubules of the same insect (Klein *et al.*, 1991). 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*. All of these monoclonal antibodies recognized proteins present in the crude homogenate prepared from midgut of *Manduca sexta*. However, using Western blotting, only antibody 230-3 (specific for the 28kDa subunit in *Manduca sexta*) recognized a protein present in the membrane fraction corresponding to the enriched apical membrane pellet prepared from the Malpighian tubules of *Locusta*. The specificity of the antibody to a protein located on the apical membrane of the Malpighian tubules was confirmed by immunocytochemistry of frozen sections. Fluorescent labelling was confined to the apical surface. The specificity of the antibody for V-type ATPase and not other ATPase activities present on the apical membrane was demonstrated by probing a Western blot of apical membrane ATPases with the monoclonal antibody. Two ATPase activities were located when the apical membrane-enriched fraction was subjected to native micrograident PAGE. When the gel was incubated with ATP as a substrate two bands became visible one of which would

represent the V-type ATPase activity. However, when the substrate used was β-glycerophosphate only one band resulted; this band could be attributed to alkaline phosphatase activity. When a Western blot of the gel was probed with monoclonal antibody 230-3 it did not cross-react with the band representing alkaline phosphatase. Therefore it can be concluded that the antibody was specific for the V-type ATPase.

V-type ATPases are multi-subunit proteins composed of at least nine polypeptides of 67, 56, 43, 40, 28, 20, 17, 16 and 14kDa (Wieczorek, 1992; Pannabecker, 1995). 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 (Gräf *et al.*, 1992; Novak *et al.*, 1992; Gräf *et al.*, 1994a). 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 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). The present study 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 (Gräf *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). Gräf *et al.*, (1994a) suggested that this subunit may be involved in membrane targeting. Recent discovery of a novel 14kDa subunit in *Manduca sexta* midgut (Gräf *et al.*, 1994b) lead 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.

The ultrastructure of the apical surface of the Malpighian tubules as revealed by electron microscopy, identified structures resembling portasomes 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 portasomes 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 portasomes 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. Brown *et al.*, (1987) using immunogold labelling were able to localize an H^+-pump to particles studding toad urinary bladder. Just and Walz, (1994) using fluorescence labelling localized an H^+-pump to 10nm-particles on the cytoplasmic surface of microvilli in the salivary glands of the cockroach.

Initially studies on V-type ATPases were limited to the exo- and endocytic pathways of fungi and plants (for reviews see Sze, 1985; Mellman *et al.*, 1986, Forgac, 1989). Nevertheless, V-type ATPases have now been shown to be present in the plasma membrane of vertebrate epithelia. In fact Gluck *et al.*, (1982) stated that CO_2, a stimulator of H^+ secretion, induced fusion of vesicles containing proton pumps with the luminal plasma membrane of turtle urinary bladder. They have also been identified as the ATPase responsible for producing the acid environment by osteoclasts in bone (Blair *et al.*, 1989). More and more they are being identified as components of animal plasma membranes (first confirmed in insects by Schweikl *et al.*, 1989), e.g. midgut of *Manduca sexta* (Wieczorek, 1992); midgut, Malpighian tubules and sensilla of

Manduca sexta (Klein, 1992); sensilla of *Antheraea pernyi* (Klein and Zimmermann, 1991); Malpighian tubules of *Drosophila hydei* (Bertram *et al.*, 1991; Wessing *et al.*, 1993; Bertram and Wessing, 1994); Malpighian tubules of *Formica polyctena* (Weltens *et al.*, 1992; Zhang *et al.*, 1994); insect salivary gland (Just and Walz, 1994) and frog skin (Harvey, 1992). In all of these cases the V-type ATPase was localized to the apical cell membrane.

In plants and fungi the role of the V-type ATPases can be to produce an acidic environment which aids certain processes such as enzymatic hydrolysis in lysosomes (Mellman *et al.*, 1986) or the V-type ATPases can produce a proton gradient which can be used as an energy source to drive secondary active transport (Sze, 1985).

Wieczorek *et al.*, (1989) were the first experimenters to tentatively propose a role for the V-type ATPase as an energizer of secondary active transport which took the form of a H^+/K^+ antiporter in insect epithelia Carrying out studies with a fluorescent indicator on vesicles of goblet cell apical membranes they were able to equate the formation of a pH gradient to ATPase activity recorded in earlier studies (Wieczorek *et al.*, 1986; Schweikl *et al.*, 1989). However, K^+ which was known to stimulate ATPase activity dissipated an established pH gradient or a pH gradient could not be established in the presence of K^+, they explained these findings as due to the presence of an electroneutral antiporter. Further studies supported the existence of an antiporter that represented a different functional entity to the ATPase (Wieczorek *et al.*, 1989). Furthermore these additional studies suggested the antiporter in *Manduca sexta* midgut was electrogenic (Wieczorek *et al.*, 1991; Chao *et al.*, 1991). However, it appears that the nature of the antiporter may vary depending upon which tissue it is found in, as Wessing *et al.*, (1993) working on the Malpighian tubules of *Drosophila hydei* were not able to demonstrate that the antiport was electrogenic and the same conclusion was drawn by Hegarty *et al.*, (1992) studying the tubules of *Aedes aegypti*. Whether H^+ transport and H^+/nK^+ antiport were both carried out by the multipolypeptide V-type ATPase or if antiport was carried out by a separate protein was examined by Lepier *et al.*, (1994). Cholate treatment of vesicles prevented K^+ dissipation of the pH gradient

and SDS-PAGE of cholate treated membranes was consistent with the fact that cholate solubilized the antiporter, since less protein was produced than in control membranes. Further evidence has been provided for the presence of an antiporter in insect secretory epithelia, for example, amiloride, an inhibitor of apical antiporters (Nicolson, 1993) inhibits fluid secretion by Malpighian tubules of *Drosophila hydei*, Bertram (1989); Malpighian tubules of *Glossina morsitans,* Gee, (1976b); Malpighian tubules of *Locusta,* Fathpour, (1980); salivary glands of *Calliphora,* Berridge *et al.*, (1976); Malpighian tubules of *Aedes aegypti,* Hegarty *et al.*, (1992). Furthermore, Maddrell and O'Donnell, (1992) not only showed that amiloride caused a decrease in the rate of fluid secretion but also that it acidified the "urine" produced by *Rhodnius prolixus.* A similar acidification of the "urine" was produced when amiloride was applied to the Malpighian tubules of *Drosophila hydei (*Wessing *et al.*, 1993).

A HCO_3^--stimulated ATPase activity was confirmed in the apical membrane enriched fraction produced from the Malpighian tubules of *Locusta migratoria* as had been previously reported by Fathpour, (1980); Anstee and Fathpour, (1979; 1981) and Fogg *et al.*, (1991). However, a different membrane separation method was used to produce membrane fractions in this study which was believed to result in less cross contamination. When Anstee and Fathpour, (1979) characterized microsomal HCO_3^--stimulated ATPase the degree of mitochondrial contamination was uncertain. The new membrane separation technique described in this study resulted in minimal mitochondrial contamination of the pellet which represented the apical membranes. The apical HCO_3^--stimulated ATPase activity was characterized with respect to pH, $[Mg^{2+}]$ and $[HCO_3^-]$ or $[Cl^-]$. Results were then compared to the original work carried out by Anstee and Fathpour, (1979) and further studies carried out. A HCO_3^--stimulated ATPase activity has also been reported in other insect epithelia, such as, midgut of *Manduca sexta* (Deaton, 1984); rectal epithelium of *Locusta* (Lechleitner and Phillips, 1988); larval midgut of *Hyalophora cecropia* (Turbeck *et al.*, 1968); rectum of dragonfly larvae (Komnick, 1978) and rectum of *Schistocerca gregaria* (Herrera *et al.*,

1978). It has also been recorded in a variety of different animal epithelia (reviewed by Gerencser and Lee, 1983). In the present study the Mg^{2+}-dependent ATPase activity was stimulated maximally by 20mM HCO_3^-, the same value was obtained by Anstee and Fathpour, (1979) for the same tissue. Other studies on different tissues have produced similar findings. In mammalian pancreas 20mM HCO_3^- caused maximal stimulation of the ATPase activity (Simon and Thomas, 1972), 20mM HCO_3^- also produced maximal stimulation of the ATPase activity in the mantle of the clam (Wheeler and Harrison, 1982). Deaton (1984) working on the midgut of *Manduca sexta* found 25mM HCO_3^- caused greatest stimulation of ATPase activity. The same value was obtained by Lechleitner and Phillips, (1988) working on rectal epithelium of *Locusta*, Hegner and Anika, (1975) working on rumen epithelium and Gerencser and Lee, (1985) working on intestine of *Aplysia*. Ivaschchenko *et al.*, (1975) found that maximum stimulation of ATPase activity of a number of different tissues from the rat was produced by a concentration of HCO_3^- between 20 and 30mM. Ho and Chan (1981) obtained a slightly higher value of 30-40mM HCO_3^- for the gill tissue of an eel and Turbeck *et al.*, (1968) discovered the concentration which produced the greatest stimulation of ATPase activity in midgut of *Hyalophora cecropia* was 50mM HCO_3^-. In this study, the inclusion of 20mM HCO_3^- in the reaction medium caused an increase of Mg^{2+}-ATPase activity by approximately 28%. In a previous study by Fathpour, (1980) the increase was larger being approximately 50%. Different degrees of stimulation by HCO_3^- have been recorded in different tissues; Suzuki, (1978) discovered that mouse kidney microsomes were only stimulated by 10-15% when 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 HCO_3^-. A larger 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 HCO_3^-. In red blood cells, Duncan, (1975) recorded a stimulation of 47% when 20mM $NaHCO_3$ was substituted for NaCl.

The optimal pH required for HCO_3^--stimulation seems to vary between organs and organisms. In the present study, the value which caused maximal stimulation was pH 7.5, as was previously determined by Anstee and Fathpour, (1979). In mammalian pancreas, HCO_3^--stimulated activity reached a maximum at pH 7.6 (Simon and Thomas, 1972), in midgut of *Manduca sexta* the maximum was recorded at pH 7.5 (Deaton, 1984), in dog gastric mucosa maximum activity was produced at pH 7.4 and in the intestine of *Aplysia* pH 7.8 was the optimal condition. Ho and Chan, (1981) studying branchial ATPases in the eel *Anguilla japonica* found maximum stimulation of HCO_3^--stimulated ATPase activity was achieved over the pH range 7.5-8.3, and Suzuki, (1978) recorded the optimal pH for HCO_3^--stimulated ATPase from mouse kidney to be pH 9. Finally, Turbeck *et al.*, (1968) recorded HCO_3^--stimulated ATPase maximum activity from the midgut of *Hyalophora cecropia* at pH 8.7.

The optimal [Mg^{2+}] to [ATP] ratio found in the present study was 1:1.5. This was identical to the value obtained by Anstee and Fathpour, (1981) also working on the Malpighian tubules of *Locusta*. It was similar to the ratio used in the study of mouse kidney where optimal conditions were 1.5mM Mg^{2+} and 3mM ATP, a ratio of 1:2 (Suzuki, 1978). In a study by De pont *et al.*, (1972) 2mM ATP and 2mM Mg^{2+} were used to study the ATPase in lizard gastric mucosa. Duncan, (1975) used 2mM ATP and 3mM Mg^{2+} to carry out assays on the ATPase activity of red blood cells and Gerencser and Lee, (1985) working on intestine of Aplysia found maximum activity with 2mM Mg^{2+} (5mM ATP).

However, HCO_3^- was not the only anion which could stimulate the Mg^{2+}-dependent activity, Anstee and Fathpour, (1981) found the ATPase was stimulated by a number of different anions, maximum stimulation was produced by sulphite but selenite and borate also increased activity. Other workers have found similar results: Turbeck *et al.*, (1968); Simon *et al.*, (1972); Blum *et al.*, (1971); Liang and Sacktor, (1976); Ebel and Lardy, (1975) and Gerencser and Lee, (1985). The unspecific nature of the pump lead to it being named an "anion-sensitive" ATPase by Van Amelsvoort *et al.*, (1977).

In the present study, 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 Gerencser and Lee, (1985) working on the intestinal mucosa of the mollusc, *Aplysia*, the membrane pellet which was rich in both alkaline phosphatase and Na^+/K^+-ATPase activity had no detectable cytochrome c oxidase activity and a greatly reduced succinate dehydrogenase activity. Marker enzyme studies also point to a plasma membrane location for HCO_3^--ATPase in the rectum of dragonfly larvae (Komnick *et al.*, 1978) and in rumen epithelium (Hegner and Anika, 1975). For gastric mucosa it was demonstrated that oligomycin, a specific inhibitor of mitochondrial ATPase, inhibited the renal cortex ATPase activity by over 95% whereas the HCO_3^--ATPase from brush border was only inhibited by 36% (Liang and Sacktor, 1976).

In the past, workers were divided into those who believed the HCO_3^--stimulated ATPase activity to be mitochondrial in origin and those who supported the view that it was isolated from the plasma membrane and therefore could be linked to fluid/ion transport. 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 HCO_3^--stimulated activity recorded from the mantle of the clam, *Anodonta cataracta*, 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). Later studies by Izutsu et *al.*, (1977) on red blood cells found a HCO_3^--stimulated activity which had to be non-mitochondrial in origin and so would unquestionably prove the existence of a plasma membrane HCO_3^--ATPase, however, this enzyme activity displayed anomalous properties to the well characterized HCO_3^--ATPase activity studied from a variety of tissues. Ho and Chan (1981) looking at HCO_3^--stimulated activity found it was mainly associated with mitochondria. However, Deaton, (1984) who demonstrated HCO_3^--

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 different distribution of anion-stimulated ATPase and mitochondrial markers in the different cell membrane fractions produced from Locust rectum. Fogg *et al.*, (1991) proposed that since the succinate dehydrogenase and HCO_3^--ATPase showed a different distribution in the different membrane fractions of the Malpighian tubules of *Locusta migratoria,* then the HCO_3^--ATPase activity could not be exclusively mitochondrial in origin.

A general feature of the HCO_3^--ATPase is that although it is not inhibited by ouabain it is sensitive to thiocyanate (Turbeck *et al.*, 1968; Van Amelsvoort *et al.*, 1977; Schuurmans Stekhoven and Bonting, 1981) and nitrate (Van Amelsvoort *et al.*, 1977; Anstee and Fathpour, 1981; Ebel and Lardy, 1975; Izutsu and Siegel, 1975; Gerencser and Lee, 1985). 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 these processes (Blum *et al.*, 1971; Simon and Thomas, 1972; Fathpour and Dahlman, 1994). Another possible role for the HCO_3^--stimulated ATPase which has been

suggested is in active Cl^- transport across the apical membrane of locust rectum (Lechleitner and Phillips, 1988). However, since in the Malpighian tubules of *Locusta*, Cl^- transport is most likely to be passive, moving down a favourable electrical gradient, (Baldrick *et al.*, 1988) then an involvement in active Cl^- transport in this tissue seems unlikely.

It is extremely interesting that the V-type ATPase activity located on the apical surface of the Malpighian tubules in the present study displays features characteristic of the anion-stimulated ATPase. It 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).

Nitrate is a well known inhibitor of anion ATPases. Anstee and Fathpour, (1981) working on the Malpighian tubules of *Locusta migratoria*, discovered that 20mM $NaNO_3$ caused the anion-stimulated activity to fall to 71% and 66% of control values in the presence of 20mM NaCl and 20mM $NaHCO_3$ 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 Cl^-. Ebel and Lardy, (1975) reported that 20mM nitrate caused 50% inhibition of ATP hydrolysis in the presence of 20mM 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 carlsbergenesis* 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 inhibition of anion-stimulated ATPase activity by thiocyanate has been referred to previously (see Turbeck *et al.*, 1968; Van Amelsvoort *et al.*, 1977 and Schuurmans, Stekhoven and Bonting, 1981) also Kalule-Sabiti, (1985) working on the Malpighian tubules of *Locusta migratoria* found that thiocyanate inhibited the stimulation due to HCO_3 by 67%. In the present study, inclusion of 10mM thiocyanate in the reaction medium when assaying for V-type activity caused inhibition of the activity. Thiocyanate has previously been reported as an inhibitor of V-type ATPases (Pederson and Carafoli, 1987).

The anion-stimulated ATPase, however, does exhibit some characteristics which are not associated with V-type ATPase activity. Unlike V-type ATPase activity anion-stimulated ATPase activity has been shown to be sensitive to azide. Fathpour, (1980) working on microsomal preparations of tubules from *Locusta* reported that 1mM azide almost completely inhibited the activity of both the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase. Similar results were obtained by Van Amelsvoort *et al.*, (1977) who found that azide produced 90% inhibition of rabbit gastric anion-sensitive Mg^{2+}-ATPase. However azide inhibited erythrocyte Mg^{2+}-ATPase by only 5% (Van Amelsvoort *et al.*, 1978). Furthermore Anand *et al.*, (1977) reported 85% and 8% inhibition by 1mM azide of mitochondrial and microsomal Mg^{2+}-ATPase, respectively, from rat heart. Therefore, perhaps azide is only an inhibitor of mitochondrial Mg^{2+}-ATPase. Another difference emerges when the sensitivity of the anion-stimulated ATPase to bafilomycin A_1 and NEM is compared to that of the V-type ATPase. The V-type ATPase activity is inhibited by nanomolar concentrations of bafilomycin A_1, however, HCO_3^--stimulated ATPase is only substantially inhibited at higher concentrations of 10 and 100μM. 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_3^--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 this difference 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_3^--stimulated ATPase was monitored hence it is possible that there would be a persistent amount of Mg^{2+}-ATPase activity present in the preparation which would not be inhibited by bafilomycin A_1 and NEM.

The present study located a K^+-stimulated activity to the apical membrane of the Malpighian tubules of *Locusta*, maximum stimulation occurring at 40mM KCl. Fogg *et al.*, (1991) had not found an ATPase on the apical membrane of the tubules of *Locusta* that was stimulated by K^+. The finding of Fogg *et al.*, (1991) agreed with that of Keynes, (1973) who could not demonstrate a K^+-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 (Schweikl *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.*, 1983a) which was located in portasomes 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.

CHAPTER 5

Electrophysiological studies on the Malpighian tubules of *Locusta migratoria*

RESULTS

The method used to measure the electrical potential across the basal cell membrane of the Malpighian tubule cells of *Locusta migratoria* was described previously in Chapter 2. The basal membrane potential is expressed in millivolts (mV). Throughout the course of this study V_B was found to be negative with respect to the external reference electrode and was therefore given a negative prefix. The mean value for V_B was -67.28 ± 1.19 mV (n=104). This compares favourably with values reported previously for V_B from Malpighian tubule cells of *Locusta* of -71.6 ± 0.3 mV (Baldrick, 1987; Baldrick *et al.*, 1988) and -70.4 ± 0.9 mV (Fogg, 1990).

Effect of varying $[K^+]_o$ on the basal membrane potential

1) Effect of 128mM $[K^+]$.

Previous studies (Baldrick *et al.*, 1988) established that the basal cell membrane of Malpighian tubule cells of *Locusta* is highly permeable to K^+ compared with Na^+ and Cl^-. Superfusion of the tubules with high $[K^+]$ (128mM), Na^+-free saline (Table 2.1.) resulted in a significant ($P<0.001$) depolarization of V_B by a mean value of 58.50 ± 2.81 mV (n=10) from -68.00 ± 2.67 mV in control saline, to -9.50 ± 1.74 mV.

The results described above, and which are illustrated in Fig. 5.1A., represent the majority of the responses produced to this treatment. Baldrick *et al.*, (1988) named this more common response the "Type A" response and reported it occurring in approximately 80% of cases. As will be explained later this response can be predicted by Nernst. However, in the remaining experiments an unpredictable response was recorded. In these latter experiments the initial depolarization of the basal membrane, produced by

the exposure of the Malpighian tubule cells to high [K^+] saline, was not sustained. An example of this "Type B" response (Baldrick *et al.*, 1988) is shown in Fig. 5.1B. In these cases V_B initially depolarized by a mean value of 35.40 ± 5.31 mV (n=5) to -50.00 ± 3.79 mV from an average resting potential of -85.40 ± 2.09 mV. As shown in Fig. 5.1B the depolarization was followed by a repolarization to a stable value and on the reintroduction of control saline the membrane potential first depolarized before repolarizing to a value not significantly different to the original resting potential. Baldrick, (1987) describes a number of different "Type B" responses.

2) Effect of K^+-free saline on the basal membrane potential.

Fig. 5.2. shows the effect of K^+-free saline (Table 2.1.) on the basal membrane potential. Superfusing the tubules with K^+-free saline produced a significant ($P<0.001$) hyperpolarization of 29.00 ± 3.29mV from a mean resting potential of -58.60 ± 6.66 mV to -87.60 ± 8.80 mV (n=5). Superfusing with control saline produced a repolarization back to a membrane potential not significantly different from the original value. This response of the basal membrane potential to K^+-free saline was similar to that recorded by Baldrick, (1987), for the Malpighian tubules of *Locusta* and by Leyssens *et al.*, (1992) for the Malpighian tubules of *Formica polyctena*.

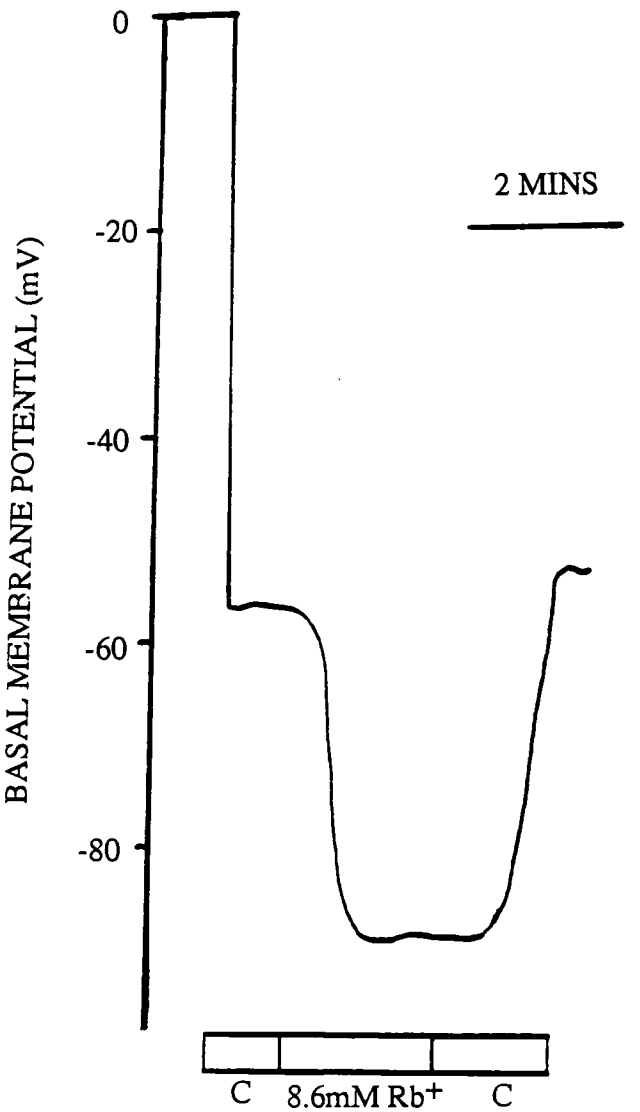


Fig. 5.3.

Typical example of the effect on the basal membrane potential of changing the superfusate from Control saline (8.6mM K^+) to rubidium saline (8.6mM Rb^+, 0mM K^+).

C - Control saline.

Table 5.1. Effect of a variety of Rb^+-containing salines on the basal membrane potential

Basal membrane potential (mV)				
Treatment	Control saline	Rb^+-containing saline	Increase in potential	n
8.6mM Rb^+, 0mM K^+	-68.60 ± 2.08	-91.38 ± 2.09	24.27 ± 2.11	45
100mM Rb^+, 0mM K^+	-55.50 ± 5.32	-91.00 ± 4.71	35.50 ± 2.02	4
2mM Rb^+, 8.6mM K^+	-69.92 ± 1.80	-80.58 ± 1.81	10.67 ± 1.16	24
10mM Rb^+, 8.6mM K^+	-64.81 ± 2.55	-83.13 ± 3.13	18.31 ± 2.62	16

Table 5.1.
Effect on V_B of changing the bathing medium from control saline to a Rb^+-containing saline.
The treatment describes which Rb^+-containing saline was used in a set of experiments.
Values are given as means ± standard error of the mean.
n represents the number of individual experiments, each involving a separate tubule preparation.

Effect of Rb^+ on the basal membrane potential

1) Complete substitution of K^+ with Rb^+.

Substituting the 8.6mM K^+ in control saline with 8.6mM Rb^+ ("Rb^+-saline", Table 2.1.) produced an effect similar to that described for K^+-free saline. The membrane potential hyperpolarized by a mean of 22.78 ± 1.55 mV from a resting potential of -68.60 ± 2.08 mV to a final value of -91.38 ± 2.09 mV (n=45) (see Table 5.1.) which represented a significant increase ($P<0.001$). If following this hyperpolarization the tubules were superfused with control saline there was a depolarization to a value not significantly different to that first recorded in control saline. The initial hyperpolarization was maintained for periods of 1-5 min (Fig. 5.3.) but continued exposure to the Rb^+-saline resulted in a gradual depolarization, after 10 min the potential had depolarized by a mean value of 5.00 ± 1.10 (n=5) (Fig. 5.4.). The initial hyperpolarization was quite variable and ranged from 7 to 47 mV.

2) Effect of exposure of cells to 100mM Rb^+.

Exposing the cells to saline which contained 0mM K^+ but 100mM RbCl (8.6mM NaCl) also produced a hyperpolarization of the basal membrane potential. The potential hyperpolarized from a resting value of -55.50 ± 5.32 to a value of -91.00 ± 4.71mV (n=4) which represented a significant hyperpolarization of 35.50 ± 2.02mV ($P<0.001$). See also Table 5.1. and Fig. 5.5.

3) Effect of inclusion of 2mM or 10mM Rb^+ in control saline.

The unexpected hyperpolarization of the basal cell membrane potential produced in Rb^+-saline was investigated further by studying the response produced in the simultaneous presence of Rb^+ and K^+. As described above complete substitution of K^+ with Rb^+ produced a result similar to that obtained with K^+-free saline. This response would be obtained if Rb^+ was unable to substitute for K^+, investigating the effect on the basal membrane potential in the presence of both K^+ and Rb^+ would reveal if Rb^+ was

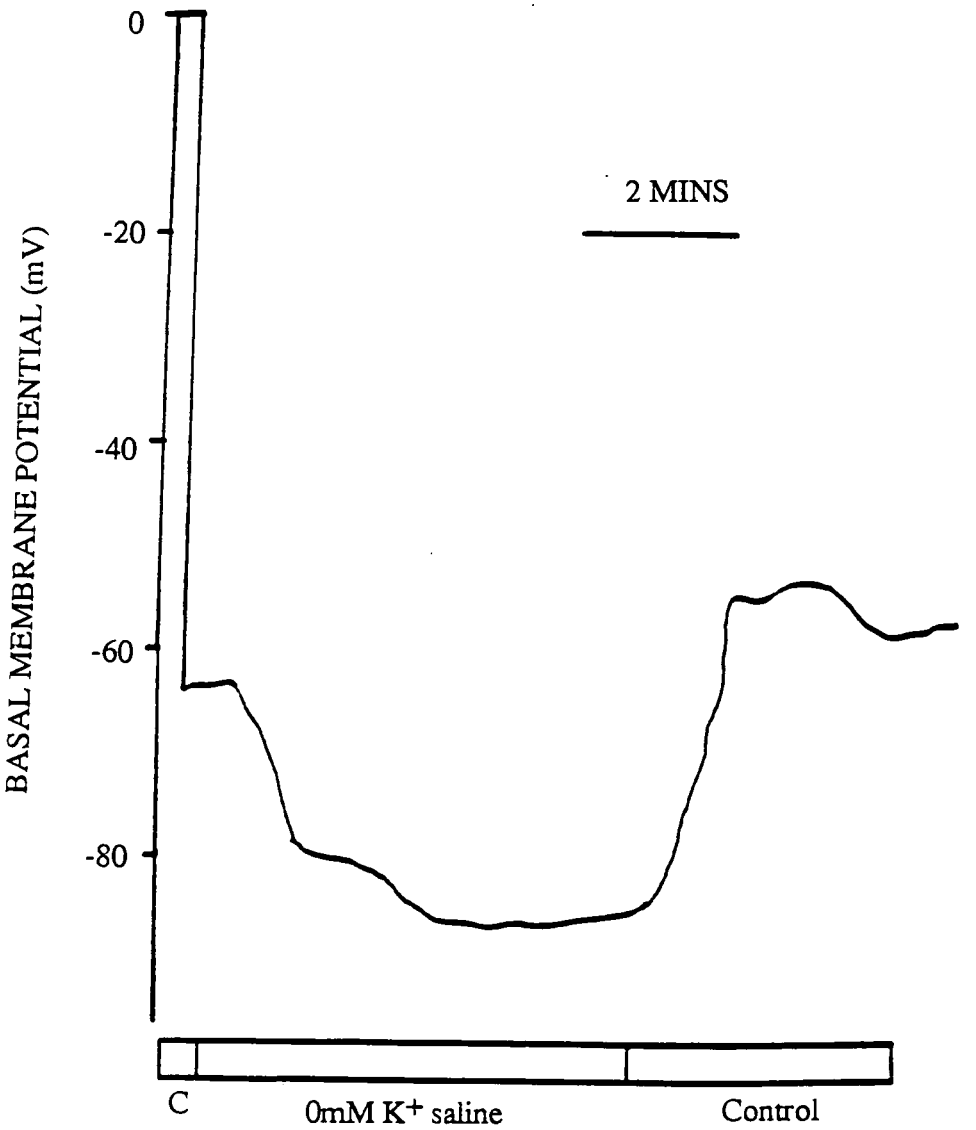
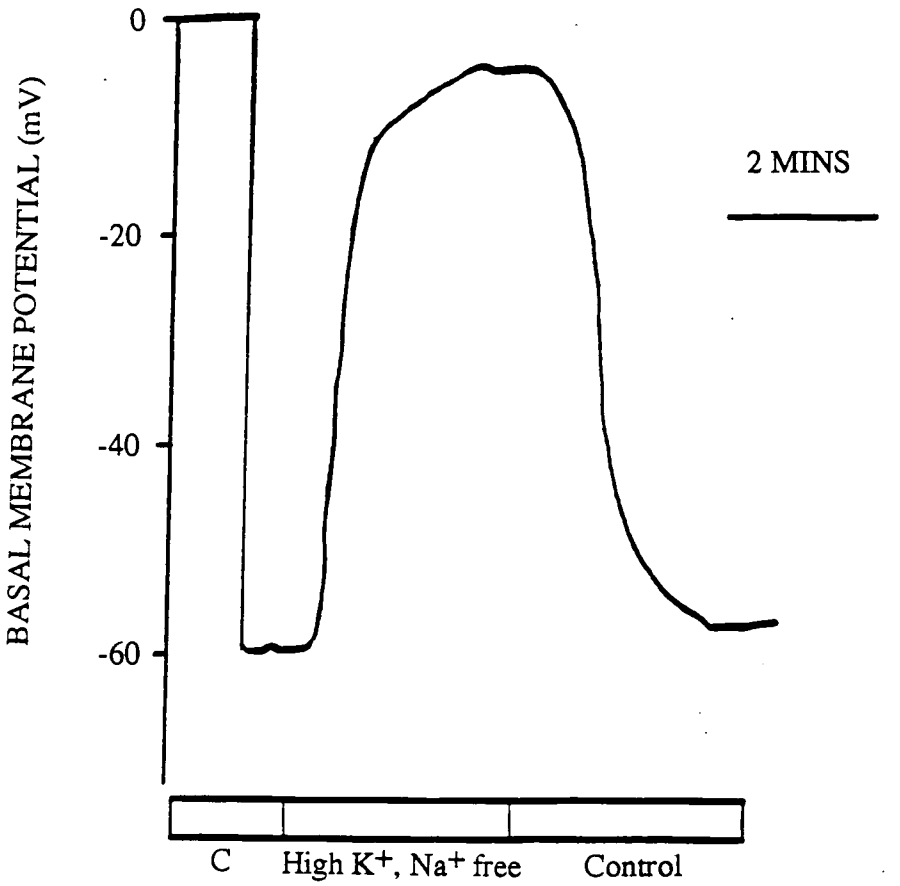


Fig. 5.2.

Typical example of changes in the basal membrane potential when the superfusate is changed from Control (8.6mM K^+) saline to K^+-free (0mM K^+) saline.

C - Control saline.


0
-20
-40
-60
BASAL MEMBRANE POTENTIAL (mV)
2 MINS
C
High K+, Na+ free
Control

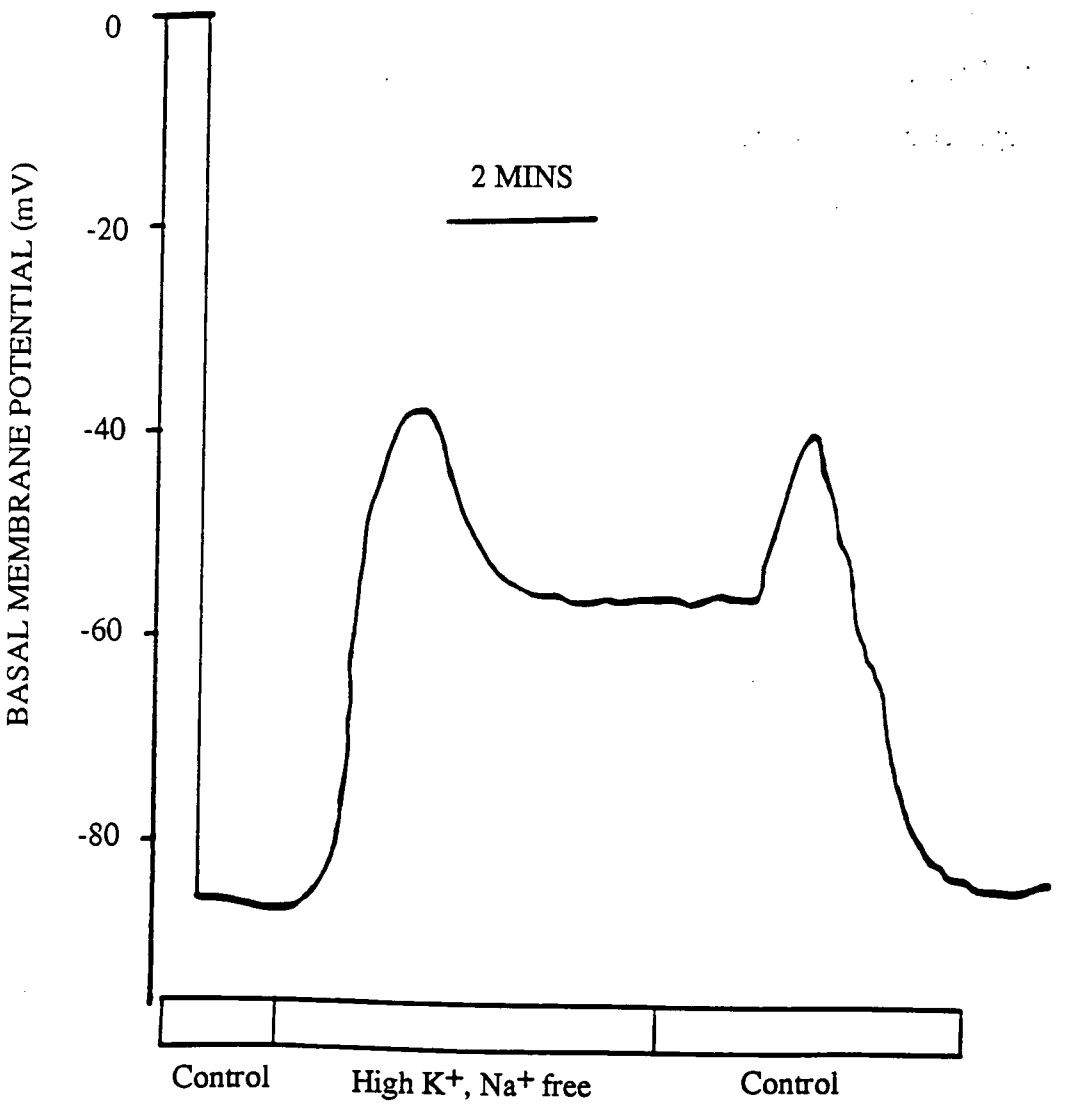

0
-20
-40
-60
-80
BASAL MEMBRANE POTENTIAL (mV)
2 MINS
Control
High K+, Na+ free
Control

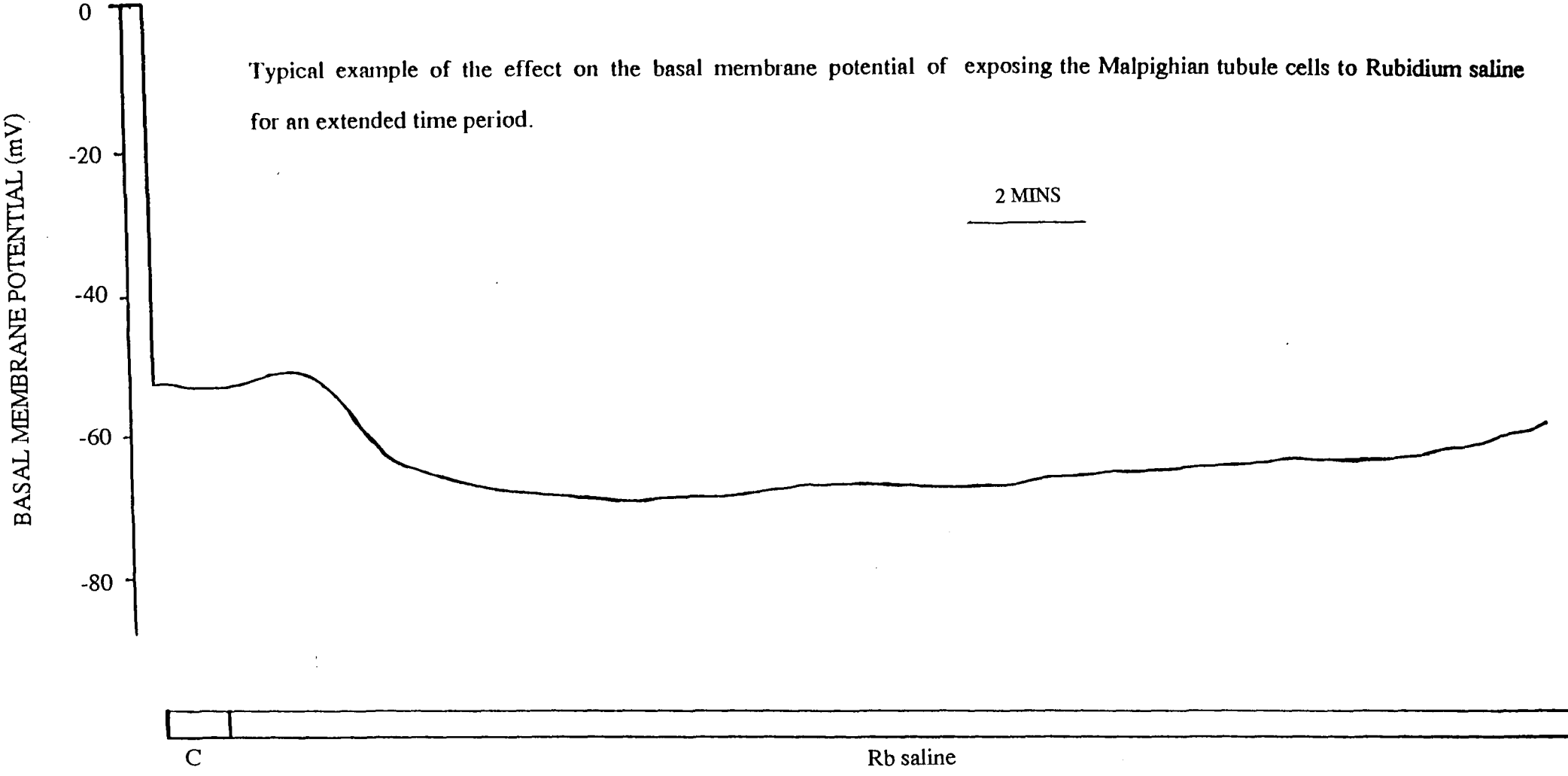
Fig. 5.1 A.

A typical example of a "Type A" or Nernstian response of the basal membrane potential on changing the superfusate from Control (8.6mM K^+) saline to high [K^+] (128mM K^+, 0mM Na^+) saline.

Fig. 5.1. B.

An example of a "Type B" response of the basal membrane potential on changing the superfusate from Control (8.6mM K^+) saline to high [K^+] (128mM K^+, 0mM Na^+) saline. The expected depolarization, on exposure of the cells to high [K^+], was followed by a repolarization and on returning to Control saline another depolarization was observed before the potential repolarized back to the resting potential.

Fig. 5.4.



Typical example of the effect on the basal membrane potential of exposing the Malpighian tubule cells to Rubidium saline for an extended time period.

C - Control saline.

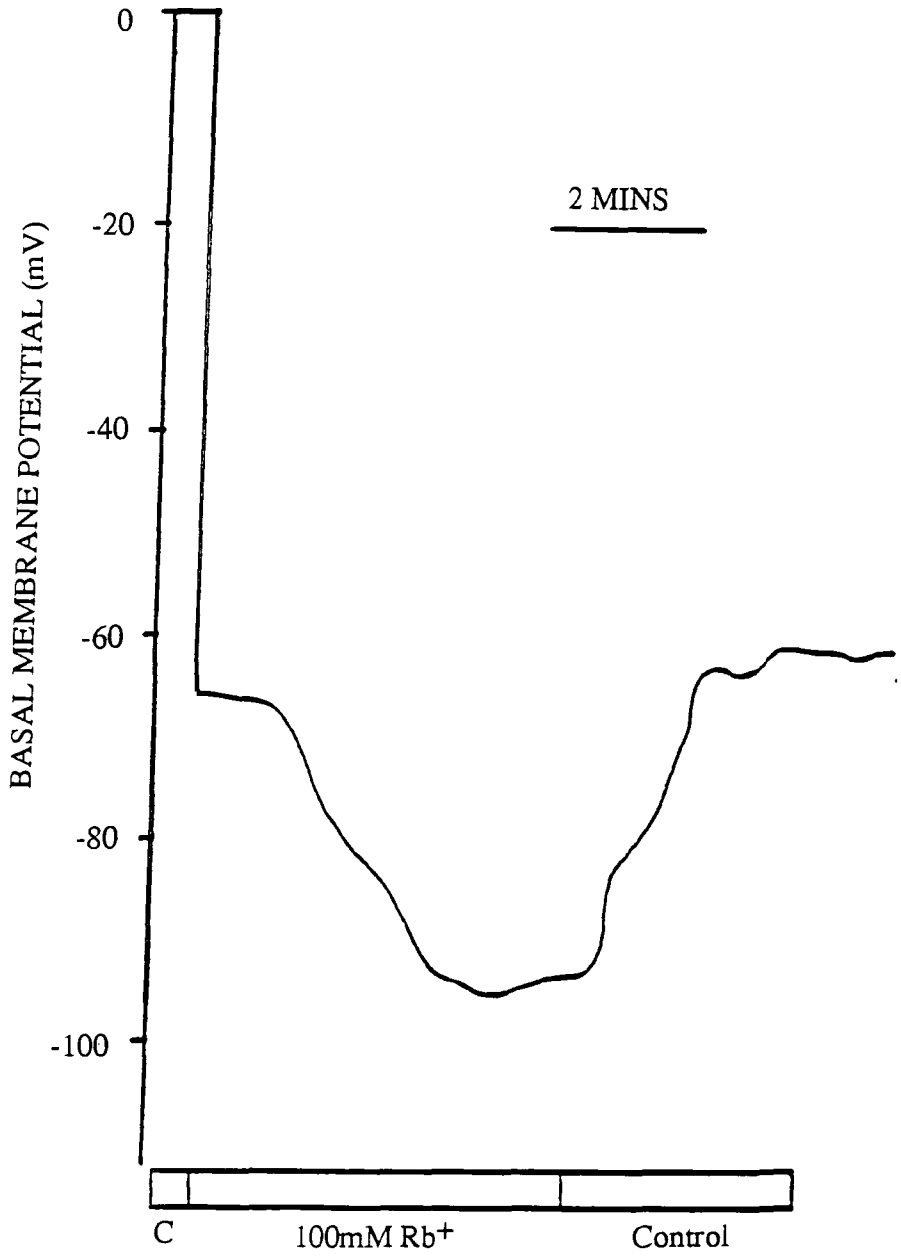


Fig. 5.5.

Typical example of the effect on the basal membrane potential of changing the superfusate from Control saline (8.6mM K^+) to 100mM Rb^+ saline

C - Control saline.

unable to substitute for K^+ or if Rb^+ was acting as a blocking agent. The results are shown in Table 5.1. The addition of 2mM Rb^+ to saline which contained 8.6mM K^+ still resulted in a significant hyperpolarization ($P<0.001$) of the basal membrane potential from -69.92 ± 1.80 to -83.13 ± 3.13mV, an increase of 10.67 ± 1.16mV (see Fig. 5.6.). The inclusion of 10mM Rb^+ in control saline produced a significant hyperpolarization of 18.31 ± 2.62mV from -64.81 ± 2.55 to -83.13 ± 3.13mV ($P<0.001$).

Effect of Rb^+ and high 128mM $[K^+]_0$ on the basal membrane potential

Table 5.2. shows the effect of the introduction of high $[K^+]$ saline containing 2mM Rb^+ on V_B after a period in control saline containing 2mM Rb^+. The results show that the near Nernstian response to high $[K^+]$ under control conditions was reduced when the cell was exposed to 2mM Rb^+previous to and during exposure to high $[K^+]$. As under control conditions, the change in potential from the original resting value, to that produced with high $[K^+]$ only, represents a significantly larger depolarization (Student's *'t'* test, $P<0.05$) to the one produced in the presence of Rb^+. The original resting potentials were not significantly different. If individual cases are studied it can be seen that on some occasions a perfect near Nernstian response was produced (Fig. 5.7.), similar to the majority of Type A responses that were produced under control conditions (see Fig. 5.1A). In some cases transient small depolarizations were measured (Fig. 5.8.) which were more typical of the Type B responses recorded in this study. In the majority of cases the response after exposure to Rb^+ was more similar to the Type B response than the near Nernstian Type A response.

Following on from these studies experiments were carried out where cells were subjected to high $[K^+]$ before being exposed to control saline plus 10mM Rb^+ followed by high $[K^+]$ plus 10mM Rb^+. The results are shown in Table 5.3. When a Type A response resulted from the initial exposure to high $[K^+]$, the result of exposing the cells to control saline plus 10mM Rb^+ followed by high $[K^+]$ plus 10mM Rb^+, produced an initial depolarization, the majority of which resembled Type B responses (Fig. 5.9.),

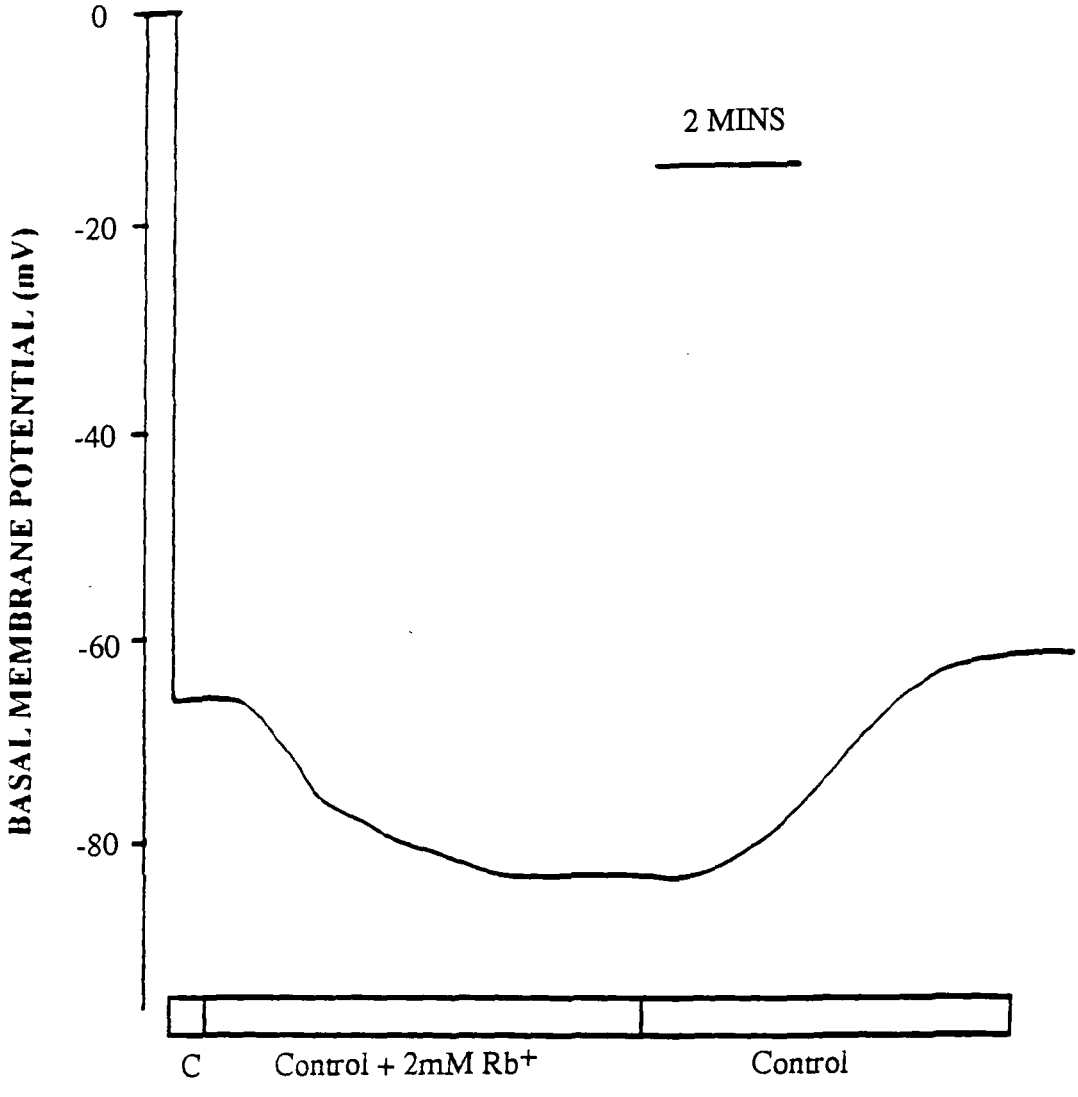


Fig. 5.6.

Typical example of the effect on the basal membrane potential of changing the superfusate from Control saline (8.6mM K^+) to Control plus 2mM Rb^+ saline (i.e., this saline contains both 8.6mM K^+ and 2mM Rb^+).

C - Control saline.

Table 5.2. Effects on V_B of high K^+ or high K^+ plus 2mM Rb^+ saline after pretreatment in control saline or contol plus 2mM Rb^+ respectively

Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Change in potential (mV)	n
A) Control Saline	-73.80 ± 2.88	-	-	High [K^+]	-18.13 ± 3.82	55.67 ± 2.43	15
B) Control Saline	-66.71 ± 3.78	Control Saline plus 2mM Rb^+	-72.88 ± 4.51	High [K^+] plus 2mM Rb^+	-22.13 ± 5.76	44.29 ± 4.20	8

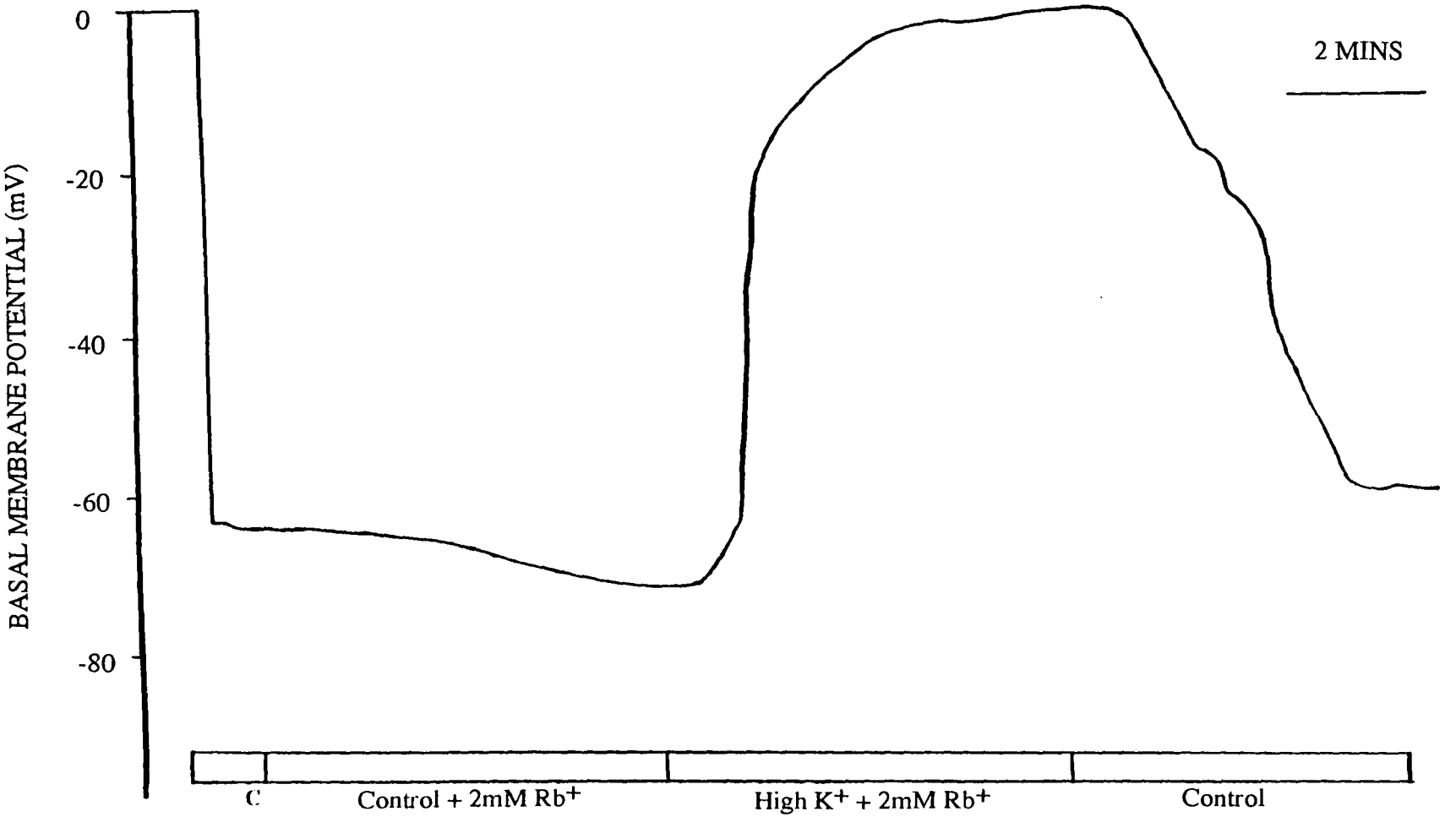
Table 5.2.

A) Effect on V_B of changing the bathing medium from control saline to high [K^+] saline (128mM).

B) Effect on V_B of changing the bathing medium from control saline to control saline plus 2mM Rb^+ to high [K^+] saline plus 2mM Rb^+.

Values are given as means ± standard error of the mean.
n represents the number of individual experiments, each involving a separate tubule preparation.

Fig. 5.7. Example of a typical "Type A" response produced by the basal membrane potential on exposure to high [K^+] (128mM K^+) plus 2mM Rb^+ saline after pretreatment with control plus 2mM Rb^+ saline



C - Control saline.

Fig. 5.8. Example of a typical "Type B" response produced by the basal membrane potential on exposure to High [K^+] (128mM K^+) plus 2mM Rb^+ saline after pretreatment with Control plus 2mM Rb^+ saline.

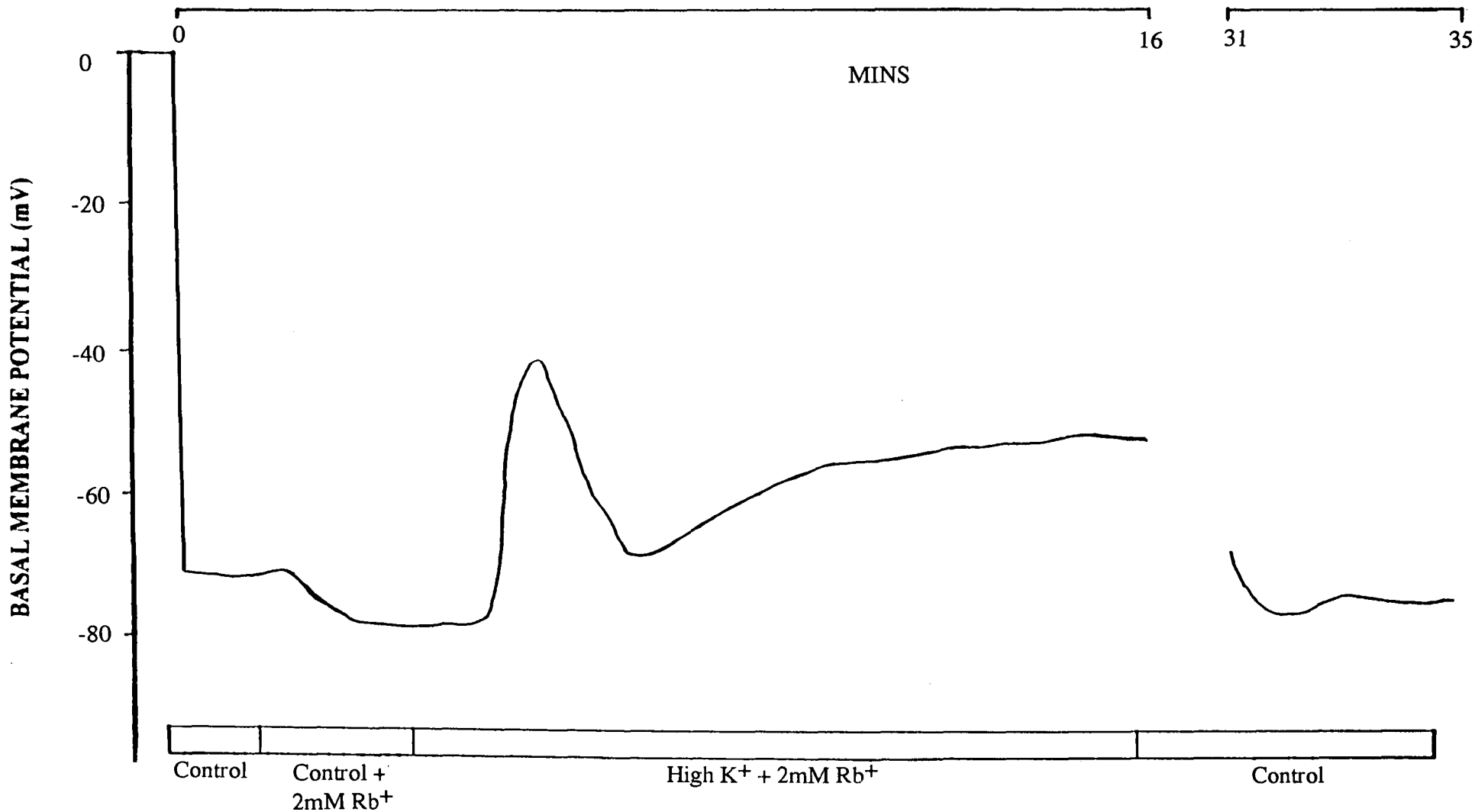


Table 5.3. Effect of changing the superfusate from control saline to control saline plus 10mM Rb^+ followed by high K^+(128mM) plus 10mM Rb^+, after establishing a Type A response by V_B on exposure to high K^+.

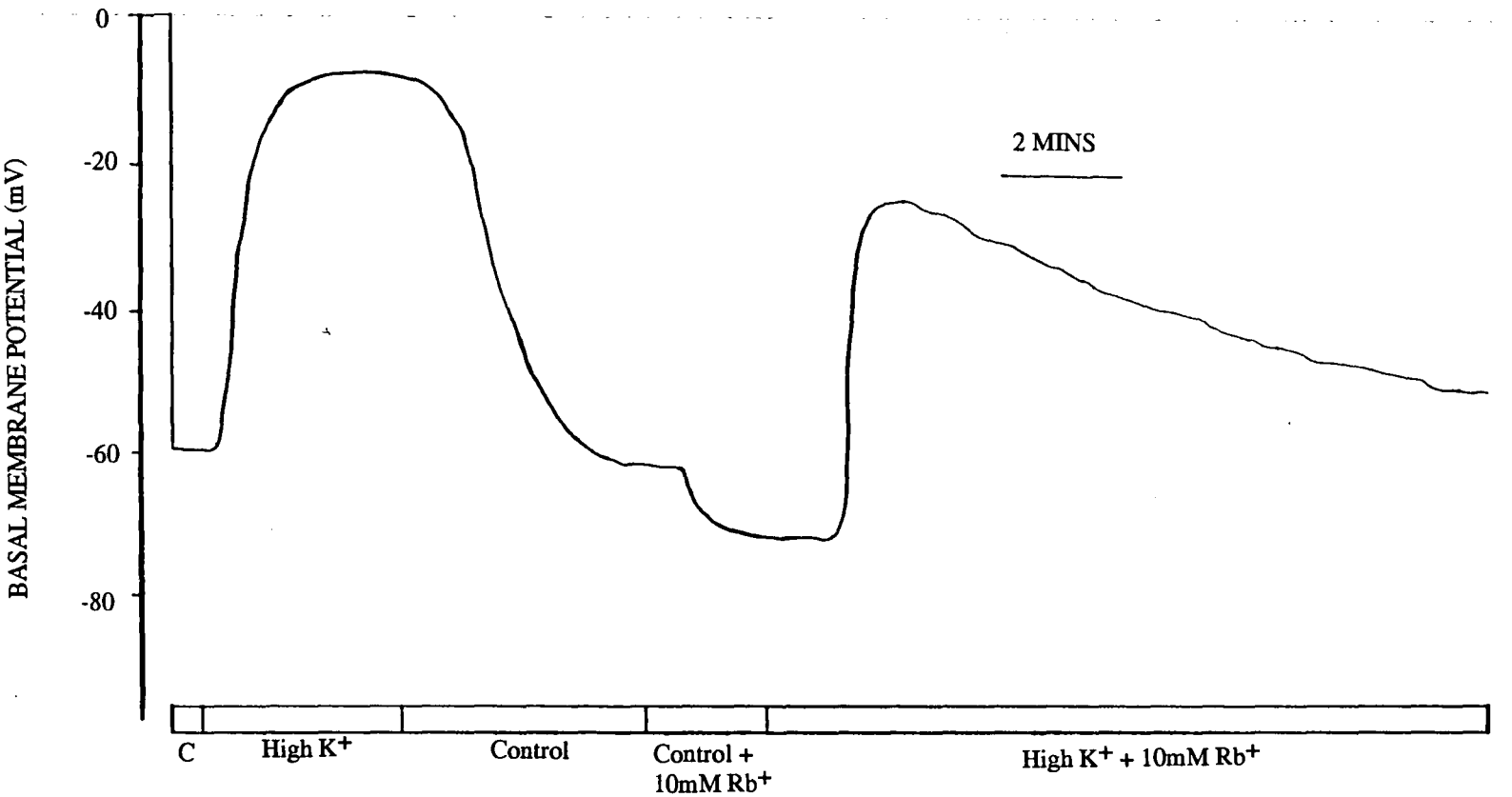
Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	n
Control Saline	-65.25 ± 3.2	High [K^+]	-8.0 ± 2.71	Control Saline	-61.75 ± 3.71	Control saline plus 10mM Rb^+	-88.5 ± 7.68	High [K^+] plus 10mM Rb^+	-18.0 ± 5.48	Control Saline	-80.0 ± 5.34	4

Table 5.3.

A Type A response was established by changing the bathing medium from control saline to high [K^+] saline. The bathing medium was then changed to control saline + 10mM Rb^+ followed by high K^+ plus 10mM Rb^+ to discover if a Type A response still resulted.

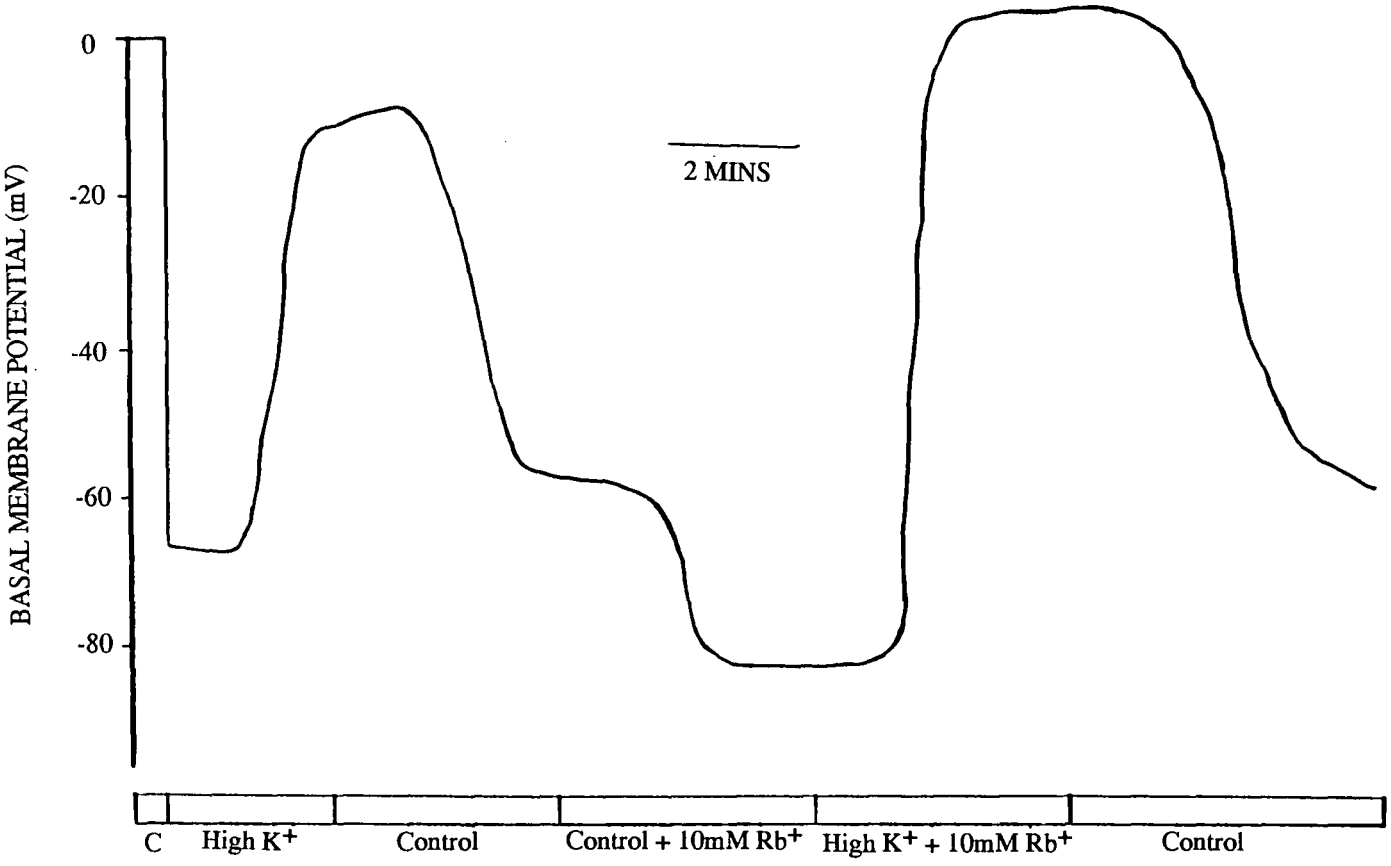
Values are given as means ± standard error of the mean.
n represents the number of individual experiments, each involving a separate tubule preparation.

Fig. 5.9. Example of a typical "Type B" response produced by the basal membrane potential on exposure to high [K^+] (128mM K^+) plus 10mM Rb^+ saline after pretreatment with control plus 10mM Rb^+ saline in cells which had been shown to exhibit the "Type A" response on previous exposure to high [K^+].



C - Control saline.

Fig. 5.10. Example of a typical "Type A" response produced by the basal membrane potential on exposure to high [K^+] (128mM K^+) plus 10mM Rb^+ saline after pretreatment with control plus 10mM Rb^+ saline in cells which had been shown to exhibit the "Type A" response on previous exposure to high [K^+].



C - Control saline.

although a typical Type A response was seen (Fig. 5.10.). As shown in Table 5.3. the initial value recorded in high [K^+] was -8.0 ± 2.71mV compared to the value of -18.0 ± 5.48mV recorded in high [K^+] plus 10mM Rb^+. Although these values are not significantly different it is possible this is due to the small sample size (n=4); the trend does suggest a deviation from Nernst.

Preliminary experiments following the same experimental procedure where a Type B response was produced on initial exposure to high [K^+] also produced a Type B response on subjection to high [K^+] plus 10mM Rb^+.

Effect of bafilomycin A_1 on the basal membrane potential

The effect of 1μM bafilomycin A_1, in control saline, on the basal membrane potential is shown in Table 5.4. and Fig. 5.11. On exposure to 1μM bafilomycin A_1 for 15 minutes the basal membrane potential had depolarized from -62.3 ± 2.7 to -54.0 ± 1.8mV, this represented a significant change of 8.3 ± 1.3mV ($P<0.01$, paired '*t*' test). In a separate set of experiments, shown in Table 5.5., treatment with 2mM Rb^+ caused the basal membrane potential to hyperpolarize to -90.0 ± 5.0 from -76.7 ± 7.7mV. Fifteen minutes after the introduction of 1μM bafilomycin A_1 into this control saline plus 2mM Rb^+ this potential had depolarized to -53.7 ± 4.9mV. This represented a significant change of 36.34 ± 5.24mV ($P<0.05$, paired '*t*' test). An example of this set of experiments is shown in Fig. 5.12. The size of the depolarization caused by 1μM bafilomycin A_1 did depend on the presence of Rb^+, the depolarization in the presence of Rb^+ was significantly ($P<0.05$, Student's '*t*' test) larger.

The effect of NEM on the basal membrane potential

The effect of a variety of different concentrations of NEM was investigated on the basal membrane potential in either control or control + 2mM Rb^+ saline. All the concentrations tested had an effect (1mM, 500μM, 50μM and 15μM), the results of

Fig. 5.11. Typical example of the effect of 1μM bafilomycin A_1 on the basal membrane potential in control saline

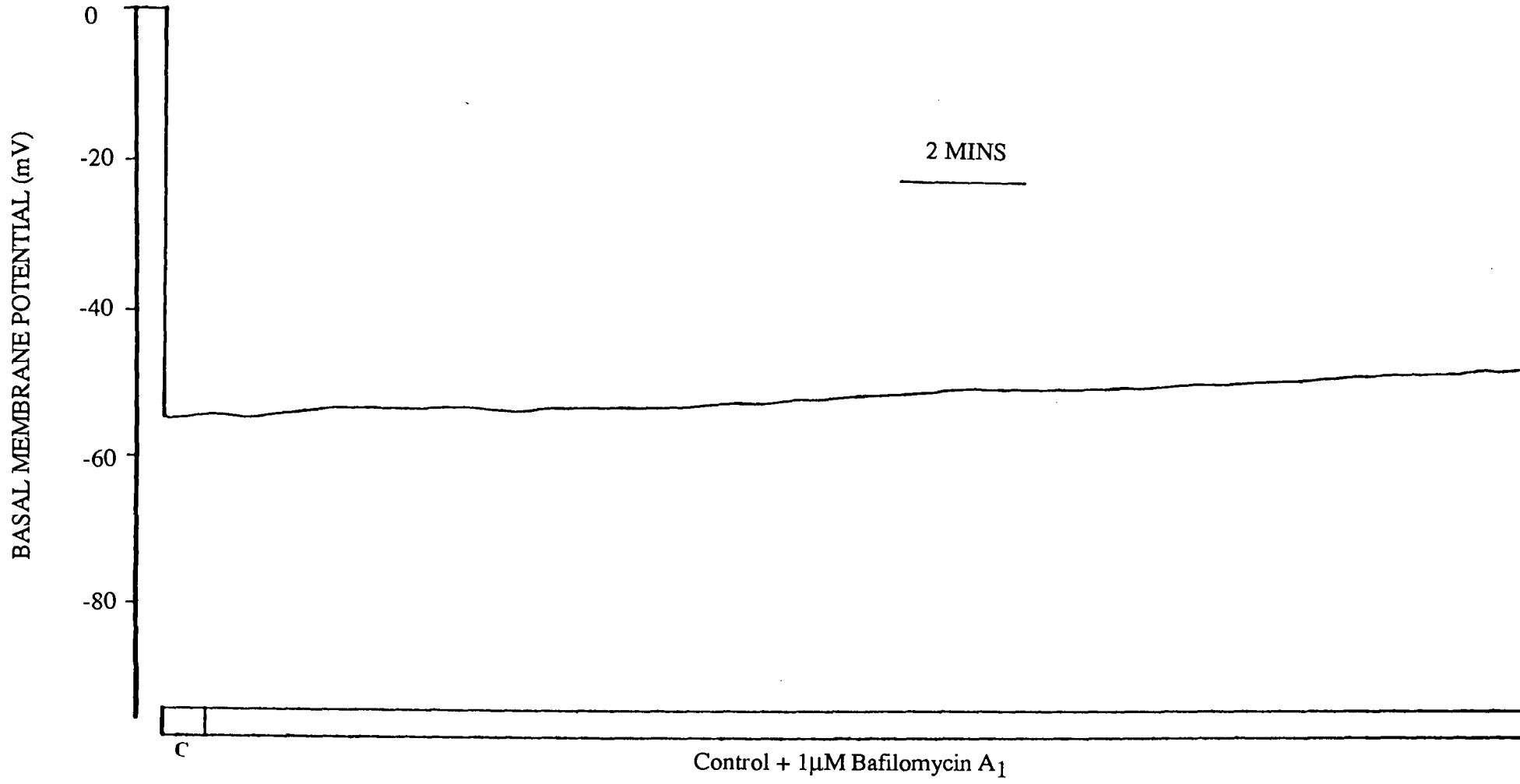


Table 5.4. Effect on the basal membrane potential of changing the superfusate from control saline to control saline containing 1μM bafilomycin A_1

Treatment	P.D. ± S.E.M. (mV)	Time in Control saline and 1μM bafilomycin A_1 in minutes P.D ± S.E.M. (mV)				
		5	10	15	20	25
Control Saline	-62.3 ± 2.7	-60.5 ± 2.5	-56.8 ± 2.2	-54.0 ± 1.8	-51.2 ± 2.7	-44.5 ± 2.5
n	6	6	6	6	5	2

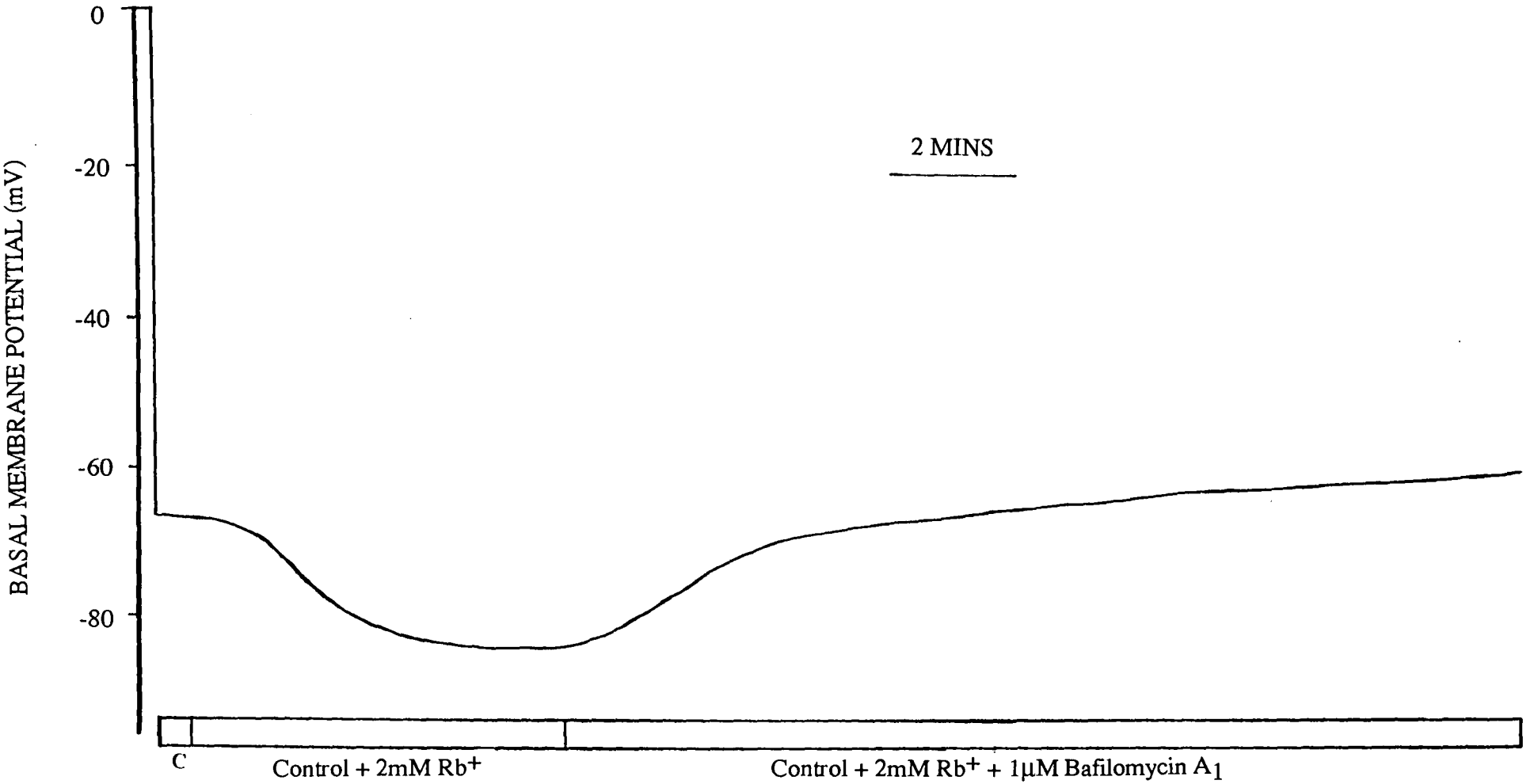
Table 5.4.

Effect on V_B of inclusion of bafilomycin A_1 in control saline, measurements shown were taken at 5 minute intervals.

Values are given as means ± standard error of the mean.

n represents the number of individual experiments, each involving a separate tubule preparation..

Fig. 5.12. Typical example of the effect of 1μM bafilomycin A_1 on the basal membrane potential in control saline plus 2mM Rb^+ saline



C - Control saline.

Table 5.5. Effect on the basal membrane potential of changing the superfusate from control saline plus 2mM Rb^+ to control saline containing 2mM Rb^+ and 1μM bafilomycin A_1

Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Time in Control saline plus 2mM Rb^+ and 1μM bafilomycin A_1 in minutes P.D ± S.E.M. (mV)		
				5	10	15
Control Saline	-76.7 ± 7.7	Control Saline plus 2mM Rb^+	-90.0 ± 5.0	-69.4 ± 8.4	-58.7 ± 7.4	-53.7 ± 4.9
n	3		3	3	3	3

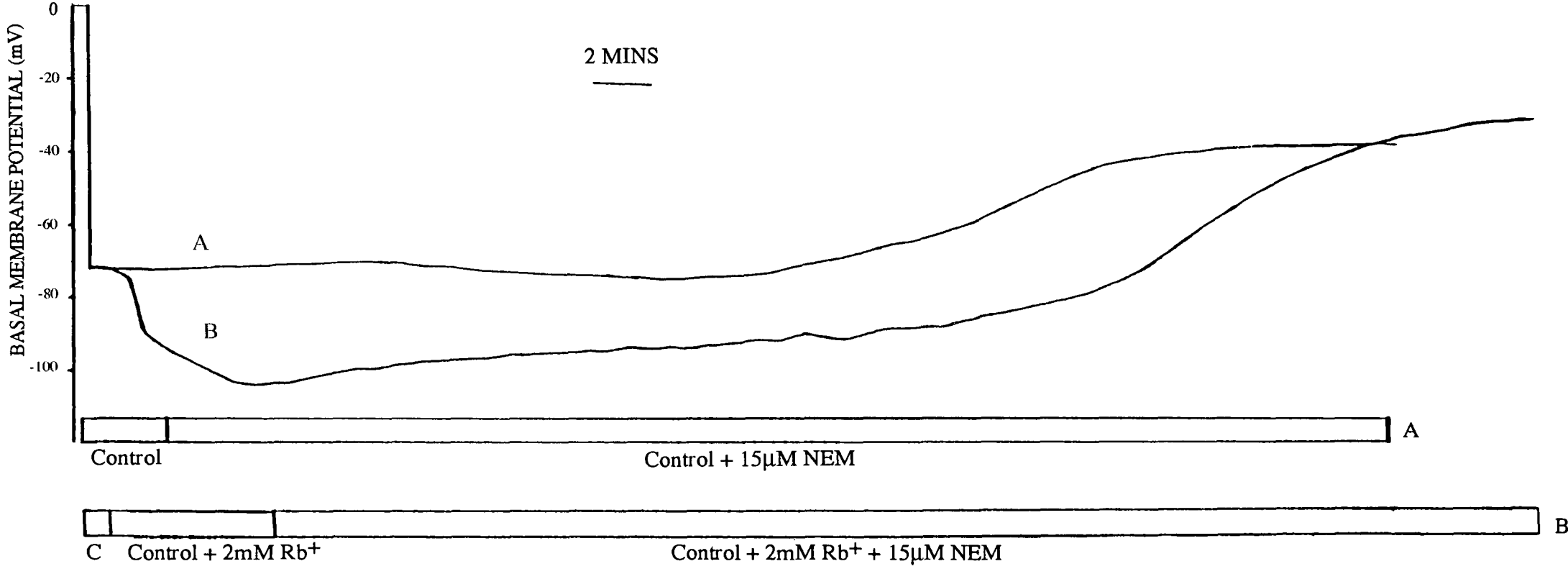
Table 5.5

Effect on V_B of inclusion of bafilomycin A_1 in control saline plus 2mM Rb^+, measurements shown were taken at 5 minute intervals.

Values are given as means ± standard error of the mean.

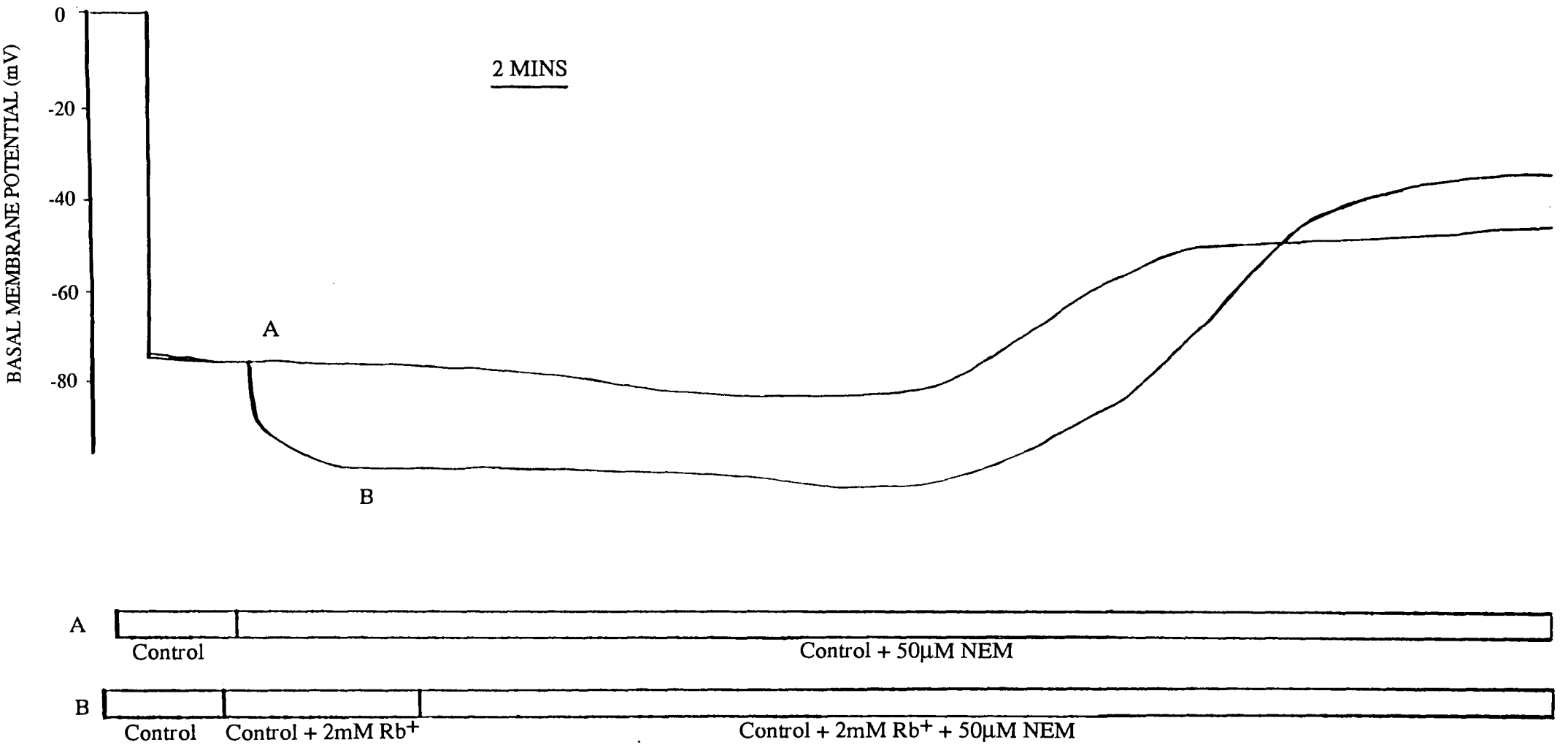
n represents the number of individual experiments, each involving a separate tubule preparation.

Fig. 5.13. Typical example of the effect of 15μM NEM on the basal membrane potential in (A) control saline and (B) rubidium saline



C - Control saline.

Fig. 5.14. Typical example of the effect of 50μM NEM on the basal membrane potential in (A) control saline and (B) rubidium saline



applying 15μM and 50μM NEM in either control saline plus 2mM Rb^+ or control saline alone are shown in Figs 5.13. and 5.14. respectively. As shown in Fig. 5.13., 15μM NEM caused a depolarization of the basal membrane potential in both control and control plus 2mM Rb^+ saline. There was a biphasic response, a gradual depolarization followed by a sharper one, the effect appeared to be slightly quicker in control plus 2mM Rb^+ saline and the depolarization greater. The experiment was repeated using 50μM NEM (Fig. 5.14.). The onset of the rapid depolarization was quicker using this higher concentration of inhibitor, but, again, NEM had a quicker and greater effect in the presence of control plus 2mM Rb^+ saline although the effect in control saline was substantial.

DISCUSSION

Intracellular microelectrode recordings of the basal membrane potential from the Malpighian tubules of *Locusta migratoria* are compared to values of V_B obtained from other invertebrate and vertebrate excretory tissues (see Table 5.6.). V_B is always negative with respect to the bathing medium.

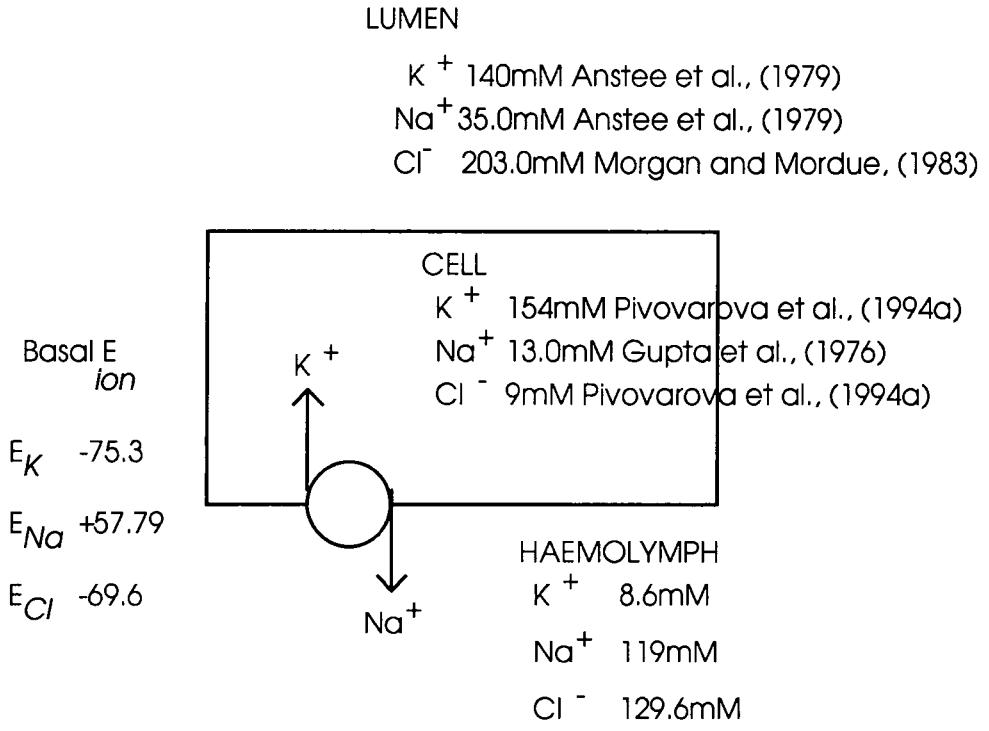
A potassium-rich primary "urine" is produced by many insects (*Locusta migratoria*, Anstee *et al.*, 1979, Morgan and Mordue, 1983; *Schistocerca gregaria*, Maddrell and Klunsuwan, 1973; *Musca domestica*, Dalton and Windmill, 1980; *Carausius morosus*, Pilcher, 1970 and *Onymacris plana*, Nicolson and Hanrahan, 1986). The production of a K^+-rich "urine" requires the passage of K^+ across the basal membrane. Exceptions are the blood-sucking insects which form a Na^+-rich "urine" (*Glossina austeni*, Gee, 1975; *Libellula quadrimaculata*, Nicholls, 1985 and *Aedes aegypti*, Beyenbach and Petzel, 1987).

Fig. 5.15. summarizes the distribution of ions across the Malpighian tubules of *Locusta migratoria*. Na^+/K^+-ATPase which has been demonstrated in this and previous studies (Anstee and Bell, 1975, 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986) has been located to the basal membrane in *Locusta migratoria* Malpighian tubules (present study, Anstee and Bowler, 1984, Fogg, 1990, Fogg *et al.*, 1991) which is in common to findings from other studies on various tissues (Komnick and Achenbach, 1979, Lechleitner and Phillips, 1988, Lebovitz *et al.*, 1989). This location for the Na^+/K^+-ATPase would create concentration and electrical gradients by maintaining high intracellular levels of K^+ and low levels of Na^+. The values reported for the concentrations of K^+ and Na^+ in the "urine" are similar to those obtained by Morgan and Mordue, (1983) also from the Malpighian tubules of *Locusta migratoria* and by Maddrell and Klunsuwan, (1973) for the tubules of *Schistocerca gregaria*.

Table 5.6. Basal membrane potentials recorded from various epithelia of a number of species

Species	Tissue	V_B (mV)	Reference
Locusta migratoria	Malpighian tubules	-67.3 ± 1.2	Present Study
		-70.4 ± 0.9	Fogg (1990)
		-71.6 ± 0.3	Baldrick *et al.*, (1988)
		-39.4 ± 0.5	Morgan and Mordue, (1983)
Aedes aegypti	Malpighian tubules	-62.5 ± 1.5	Sawyer and Beyenbach, (1985)
		-62.9 ± 5.2	Hegarty *et al.*, (1991)
Rhodnius prolixus	Malpighian tubules	-67.0 ± 2.0	O'Donnell and Maddrell, (1984)
Schistocerca gregaria	Malpighian tubules	-50.7 ± 0.3	Hanrahan and Phillips, (1984)
Calliphora erythrocephala	Salivary glands	-61.1 ± 0.4	Berridge and Schlue, (1978)
Formica	Malpighian tubules	-13 ± 2	Dijkstra *et al.*, (1994a)
		-19 ± 1	Zhang *et al.*, (1994)
Drosophila hydei	Malpighian tubules	-58.0	Bertram and Wessing, (1994)
Squalus acanthias (shark)	Proximal tubule	-63.2 ± 3.5	Beyenbach and Fromter, (1985)
	Rectal gland tubule	-68.0 ± 1.2	Greger and Schlatter, (1984)
Swiss mice	Proximal straight tubule	-62.0 ± 1.0	Volkl *et al.*, (1986)
New Zealand white rabbit	Cortical collecting duct	-69.6 ± 3.0	O'Neil and Sansom, (1984)

Fig. 5.15. Distribution of K^+, Na^+ and Cl^- across the Malpighian tubules of *Locusta*.



Using ion selective electrodes, Morgan and Mordue, (1983), recorded a value of 203.0mM for the concentration of Cl^- in the lumen of *Locusta migratoria*, which is slightly higher than the value of 150mM reported for the tubules of *Schistocerca gregaria* (Maddrell and Klunsuwan, 1973). Intracellular values for the levels of Na^+, K^+ and Cl^- in the Malpighian tubule cells of *Locusta migratoria* were calculated by Pivovarova *et al.*, (1994a) from X-ray microanalysis. The value of 154mM reported for K^+ is higher than the concentration recorded using ion selective microelectrodes on this tissue (Morgan and Mordue, 1983) but is similar to the value of 164.5mM calculated from Nernstian plots of microelectrode data attained from the same tissue (Baldrick *et al.*, 1988). It is also similar to values recorded from other insect tissues (140mM, skeletal muscle of *Locusta migratoria*, Leech, 1986; 134mM and 140mM, lepidopteran midgut, Dow *et al.*, 1984, and Zerahn, 1977 respectively; 133mM and 143mM salivary gland of *Calliphora*, Berridge and Schlue, 1978 and Gupta *et al.*, 1978 respectively).

Pivovarova *et al.*, (1994a) were unable to record a value for the intracellular concentration of Na^+ as it was present at levels below the detection limit of the measuring system (therefore present at levels less than 17mM). Intracellular Na^+ has been found at low levels in most insect tissues (13mM in the Malpighian tubules of *Rhodnius prolixus*, Gupta *et al.*, 1976; 16mM in the midgut caeca of *Schistocerca gregaria*, Dow *et al.*, 1981 and 11mM in the midgut of *Periplaneta americana*, O'Riordan, 1969). In line with other transporting epithelia (Diez de los Rios *et al.*, 1981 and Wang *et al.*, 1984).

Intracellular Cl^- levels were also low, the concentration recorded by Pivovarova *et al.*, (1994a) in the cytoplasm being approximately 9mM. This can be compared to values of 31mM in the Malpighian tubule cells of *Rhodnius* (Gupta *et al.*, 1976) and 33mM recorded from the midgut caecal cells of *Schistocerca gregaria* (Dow *et al.*, 1981).

The data presented in Fig. 5.15. can be used to estimate the equilibrium potentials for the ions across the basal and apical membranes of *Locusta migratoria*. Concentrating on K^+ entry, the estimated basal membrane equilibrium potential (E_K) for this ion is more negative than V_B and so both concentration and electrical gradients are not favourable for K^+ entry. But, as E_K is close to the measured V_B, if the basal membrane is permeable to K^+ a small change in V_B or the K^+ concentration gradient would determine if K^+ passively enters or exits the cell. It has already been shown that V_B is mainly due to the high permeability of the basal membrane to K^+ and relative impermeability to Na^+ and Cl^- (Baldrick, 1987; Baldrick *et al.*, 1988 and Fogg, 1990).

If the membrane were permeable Na^+ entry would take place moving down both an electrical and concentration gradient whilst Cl^- entry would be against an electrical gradient but with a concentration gradient. The equilibrium potentials for these ions are in favour of passive Na^+ entry and passive Cl^- exit, leading to the conclusion that some mechanism is needed for Cl^- entry (Anstee *et al.*, 1979).

The effect of high $[K^+]_o$ on the basal membrane potential

The present study found that substitution of Na^+ with K^+ resulted in a depolarization of the basal membrane potential. A previous study by Baldrick, (1988) confirmed that this depolarization was near-Nernstian as the relationship between $[K^+]_o$ and V_B could be quite accurately predicted using the Nernst equation for values of K^+ greater than 8.6mM. At high $[K^+]_o$ the basal membrane is acting as a K^+ electrode (For a perfect K^+ electrode if V_B equalled zero then $[K^+]_o = [K^+]_i$). This behaviour is found in other tissues, e.g. salivary gland of *Calliphora* (Prince and Berridge, 1972; Berridge *et al.*, 1976), Malpighian tubules of *Rhodnius* (O'Donnell and Maddrell, 1984), oocytes of *Locusta migratoria* (Wollberg and Cocos, 1981), muscle fibres of locust and cockroach (Usherwood, 1978), the Malpighian tubules of *Onymacris plana* (Nicolson and Isaacson, 1987), the Malpighian tubules of *Carausius morosus* (Pilcher, 1970) and the Malpighian tubules of *Formica polyctena* (Leyssens *et al.*, 1992). At lower values of $[K^+]_o$, however, V_B began to deviate from the Nernstian prediction, Baldrick, (1987), concluded that this was due to other ions having an influence on V_B. As V_B deviated from Nernst in a positive direction Baldrick, (1987) concluded that Na^+ was having an influence on V_B. Applying the Goldman constant field equation (Goldman, 1943) Baldrick, (1987), calculated V_B taking into account its dependence on ionic concentration gradients and selective permeabilities to Na^+ and K^+, and found that the membrane was approximately 100 times more permeable to K^+ than Na^+. This preferential permeability to K^+ over Na^+ is found for neurones in *Aplysia* (Sato *et al.*, 1968) and in squid axon (Hodgkin and Katz, 1949). However, in the blood-sucking insect *Aedes aegypti* the basal membrane is permeable to Na^+ and K^+ to the same degree (Sawyer and Beyenbach, 1985).

Effect of K^+-free saline on the basal membrane potential

Superfusing the tubules with a K^+-free solution caused the basal membrane potential to hyperpolarize as is predicted by Nernst. Hyperpolarization of the basal membrane potential in response to a K^+-free bathing solution has also been reported by Baldrick, (1987) and Fathpour *et al.*, (1983) in the Malpighian tubules of *Locusta migratoria*, by Leyssens *et al.*, (1992) for the Malpighian tubules of *Formica polyctena* and by Berridge *et al.*, (1976) and Berridge and Schlue, (1978) in the salivary glands of *Calliphora*.

Effect of Rb^+ on the basal membrane potential

Rb^+ has been used as a marker for K^+ in various studies as it often substitutes for K^+ on transport pathways (Warden *et al.*, 1989), (see also Schafer and Troutman, 1986; Beck *et al.*, 1988 and Harvey *et al.*, 1968). During the course of this study Rb^+ had been used as a K^+ substitute in both fluid secretion and biochemical studies, it had also been used as a marker for K^+ in an X-ray microanalysis investigation of the same tissue (Pivovarova *et al.*, 1994a), and so it was thought appropriate to investigate the effect Rb^+ replacement of K^+ had on the basal membrane potential.

If Rb^+ did act as a K^+ mimic then on changing the superfusate to Rb^+-saline no change in the basal membrane potential would be expected. However, it was discovered that superfusion with Rb^+-saline caused the basal membrane potential to hyperpolarize. Exposure to high $[Rb^+]_o$ (100mM) also caused a hyperpolarization of V_B even though the concentration gradient for Rb^+ entry was extremely favourable; this suggested Rb^+ was either unable to substitute for K^+ or it was blocking basal K^+ channels. The next step in this study was to investigate the effect on the basal membrane potential when it was superfused with a solution containing both K^+ and Rb^+. Both the superfusate containing 2mM Rb^+ in control saline and 10mM Rb^+ in control saline caused the basal membrane potential to hyperpolarize. V_B responded in a similar manner to when it was

exposed to K^+-free saline, suggesting that rubidium was blocking/preventing K^+ entry into the cell. The value of V_B produced on exposure to Rb^+ was more negative than E_K, therefore this potential cannot be attributed to an equilibrium potential.

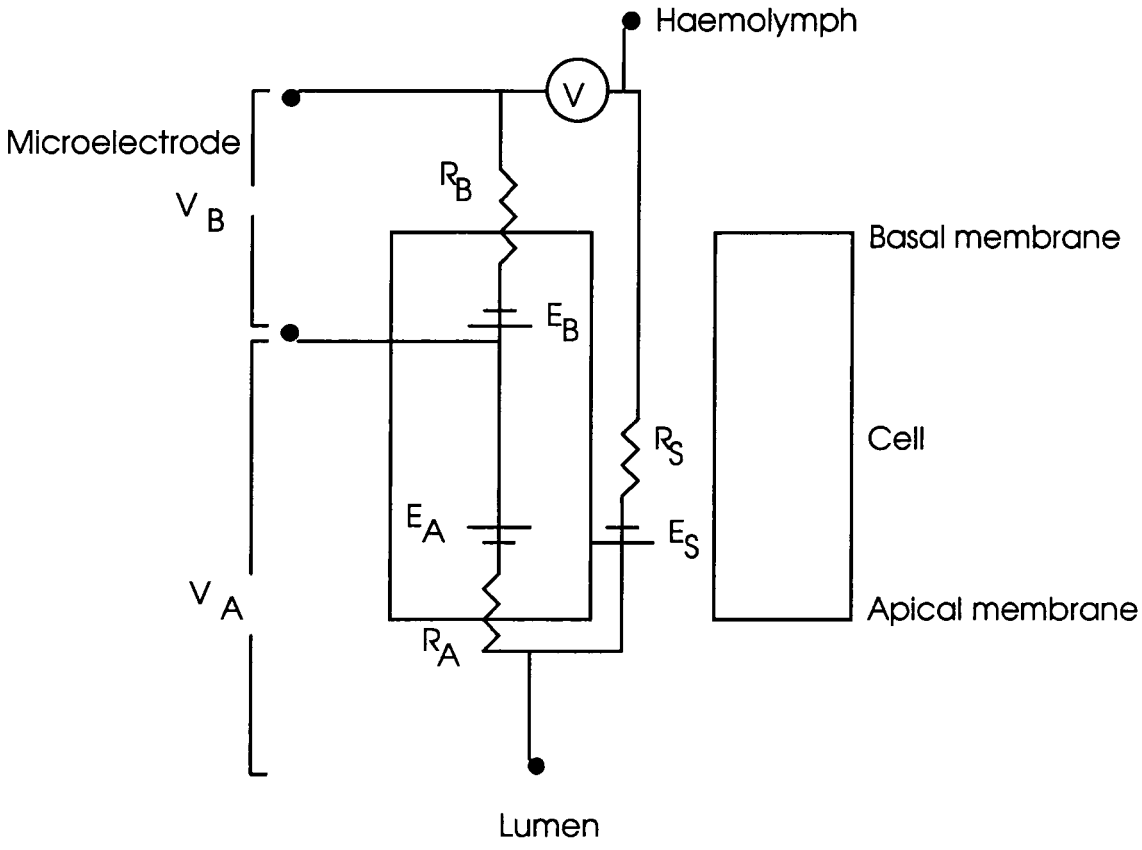
The effect that Rb^+ had on V_B is similar to that produced by barium. Ba^{2+} has been used as a K^+ channel blocker, inhibiting K^+ conductance in many studies on different tissues, e.g. Locust rectum (Hanrahan *et al.*, 1986), cortical collecting tubule (Hunter *et al.*, 1984), frog skin (Nagel, 1979) and squid axon (Armstrong *et al.*, 1982). It has also been found to cause hyperpolarizations of V_B in insect Malpighian tubules; in *Locusta migratoria* inclusion of 1mM BaCl in control saline caused a hyperpolarization of V_B of between 3 and 34mV (Baldrick, 1987). Weltens *et al.*, (1992), recorded a strong hyperpolarization of V_B from -16 ± 1mV to -84 ± 4mV caused by Ba^{2+} in the Malpighian tubules of *Formica polyctena.* Leyssens *et al.*, (1993b) have also recorded a Ba^{2+}-induced hyperpolarization of V_B in the tubules of *Formica.* The midgut of *Manduca sexta* also hyperpolarizes on exposure to Ba^{2+} (Moffett and Koch, 1988a; Chao *et al.*, 1990). Weltens *et al.*, (1992) suggested that Ba^{2+} treatment revealed the apical membrane potential.

Epithelial tissues can be modelled using electrical equivalent circuits (Boulpaep and Sackin, 1979). The circuit has three barriers, the apical and basal membranes and the paracellular pathway. The transcellular pathway (incorporating electromotive forces across the apical and basal membranes) and shunt pathway are electrically coupled; via contact with the electrolyte solutions of the haemolymph and luminal fluid (Beyenbach, 1995).

In Fig. 5.16. R_A, R_B and R_S represent resistances created by the apical and basal membranes and shunt pathway respectively. E_A, E_B and E_S represent the electromotive forces (emf) at the apical and basal membranes and shunt pathway respectively. If the emf of the basal membrane were solely due to events at this surface, then as the basal membrane is highly permeable to K^+ but relatively impermeable to other ions, the emf at this membrane would be equal to E_K. Similarly, at the apical surface, E_A would mainly be due to the electrogenic pump. However, the voltage (V_B) which is actually recorded

by the microelectrode will be influenced by E_A and E_S. Therefore, $V_B = E_B \pm \Delta E_A+E_S$.

Fig. 5.16. Circuit diagram of a Malpighian tubule, modified from Leyssens *et al.*, (1992).



Evidence from studies on *Locusta* do point to the tubules being a moderately tight epithelium. If the epithelium were leaky then electrical measurements across the cell would be short circuited by the paracellular pathway, and similar potential changes would occur across both membranes. Baldrick, (1987), found large differences in the responses of V_A and V_B in some cases, but, also some "intermediate" responses, i.e. V_A does also depolarize in high $[K^+]$ but not by the exact amount that V_B does. It is possible, therefore, that the tissue exhibits "leaky" tight junctions (Baldrick, 1987).

As described above the voltage which is recorded will have contributions from all barriers/resistance pathways. The principal contribution will come from the barrier with the lowest resistance as current preferentially flows across the lowest resistance pathway. The voltage divider ratio (VDR) is a measurement of the apical over basal membrane resistance, it has been calculated for the Malpighian tubules of *Formica polyctena* (Weltens *et al.*, 1992 and Leyssens *et al.*, 1992) and recorded as 47 ± 9 (n=6) and 44 ± 8 (n=6) respectively. In *Locusta*, V_B essentially responded to changes in $[K^+]_o$, however, factors which influenced V_A had negligible affect on V_B (Baldrick *et al.*, 1988; Fogg *et al.*, 1989) these findings led Leyssens *et al.*, (1992) to conclude that the voltage divider ratio is of a similar order of magnitude in *Locusta*. The voltage divider ratio has also been measured in *Aedes aegypti* (Aneshansley *et al.*, 1988) and was found to be low, only 0.6. It is possible that as a blood feeder Na^+ is probably the prime mover in fluid secretion and so the basal conductance might be lower than in *Formica polyctena*. Because the basal membrane potential of both *Formica* and *Locusta* is close to E_K (Leyssens *et al.*, 1992; Baldrick, 1987) the basal membrane potential therefore reflects the relatively high permeability of the membrane to K^+ and relatively low to permeability other ions. The apical membrane possibly contains an electrogenic cation pump with little ionic conductance. This combination produces the high voltage divider ratio. A consequence of a high voltage ratio is that the electromotive force of the apical membrane, as seen in measurements of V_B, is masked. Lowering the VDR by increasing the resistance of the basal membrane would unmask effects occurring at the apical membrane. Weltens *et al.*, (1992) found that Ba^{2+} caused a large increase in the resistance of V_B, producing a drop in the VDR from 47 to 1.4 and so they were able to visualize across the basal membrane electrical events occurring at the apical membrane. Dijkstra *et al.*, (1994a) also working on the Malpighian tubules of *Formica* reported a decrease in the VDR caused by Ba^{2+} increasing the resistance of V_B. Likewise Moffett and Koch, (1988a) working on the midgut of *Manduca sexta* recorded a decrease in the conductance of the basal membrane after the application of Ba^{2+}. They state that the effect of reducing the conductance of the basal membrane by blocking the K^+ channels

with Ba^{2+} drives the potential of the membrane towards the electromotive force of V_A, as a result of the change in the VDR.

Considering this information it seems possible that Rb^+ is also acting as a K^+ channel blocker which results in a fraction of V_A to be measured across the basal membrane. Rb^+, unlike Ba^{2+}, which blocks a number of different types of K^+ channels (Rudy, 1988; Castle *et al.*, 1989; Hille, 1992), is known to block a certain type of K^+ channel known as the anomalous or inward rectifier, more will be said about this later. Previous results obtained by this study have produced evidence for the presence of a V-type ATPase on the apical membrane, therefore, the effect of two V-type ATPase blocking agents on the potential recorded across the basal membrane in the presence and absence of Rb^+ were investigated. When applying bafilomycin A_1 (the most specific inhibitor of V-type ATPases known at present) in control saline the potential recorded depolarized by 8.3 ± 1.3mV. In the presence of Rb^+ the depolarization is significantly larger (36.3 ± 5.2mV, $P<0.05$). Therefore, it would seem that Rb^+ does reveal a component of V_B which is made up by E_A, and so the inhibitory affect of bafilomycin A_1 on the V-type ATPase is exposed. The effect of NEM on the membrane potential recorded in the presence and absence of Rb^+ was less conclusive although the onset of the depolarization in the presence of Rb^+ was quicker and stronger. In conclusion, the hyperpolarization of V_B which is produced on the introduction of Rb^+ to the superfusing saline is due to a contribution from the apical membrane potential. Inclusion of V-type ATPase inhibitors suggest that an electrogenic proton pump is present on this apical surface. That the hyperpolarization was due to an active process was also revealed by Weltens *et al.*, (1992) who demonstrated its decline in the presence of metabolic inhibitors and Moffett and Koch, (1988a) who observed that hypoxia reduced the hyperpolarization in the midgut of *Manduca sexta.*

The Type A and Type B response

In the presence of high [K^+] (128mM) saline the basal membrane potential depolarized to a maintained potential which was similar to that predicted by Nernst. This was named the Type A response (Baldrick, 1987) and was found to occur in the majority of experiments (approximately 80%). However, a Type B response was also recorded, where the depolarization produced was non-Nernstian. Baldrick, (1987) suggested that superfusing the tubules with high [K^+] resulted in V_B beginning to depolarize in a Nernstian fashion but as the membrane depolarized a hyperpolarized component "switched on" driving V_B away from the value expected from a Nernstian depolarization. Pretreatment with K^+-free saline induced the Type B response. Baldrick, (1987) concluded that the Type B response may be related to changes in $[Na^+]_i$ as treatment with K^+-free saline probably leads to an increase in $[Na^+]_i$.

Effect of Rb^+ on the Type A and Type B response

Superfusion of tubules with high [K^+] plus 2mM Rb^+-saline after pretreatment with control + 2mM Rb^+ induced the Type B response for V_B. A similar result was obtained by Baldrick, (1987) when the tubules were exposed to 1mM Ba^{2+} and high [K^+] after treatment with control saline plus 1mM Ba^{2+}. However, the responses of V_B on exposure to 2mM Rb^+ or 1mM Ba^{2+}, in the presence of high [K^+] were different in the degree to which the Type B response was induced. In the presence of 2mM Rb^+ and high [K^+] a typical Nernstian Type A response was sometimes produced although the majority of responses represented the Type B response. However, in the presence of 1mM Ba^{2+} and high [K^+] an "enhanced" Type B response was produced; the initial depolarized potential was more negative that the equivalent value found in the naturally occurring Type B response (Baldrick, 1987). Because Rb^+ is a relatively weak K^+ channel blocker (Silver *et al.*, 1994) another set of experiments were carried out to investigate if the Type B response could be induced in the presence of high [K^+] and

10mM Rb^+. It was found that although the response to high $[K^+]$ did show a deviation from Nernst that Type A responses could still be produced. The different results obtained using Rb^+ and Ba^{2+} can be explained as Rb^+ is known to be only a weak channel blocker (Silver *et al.*, 1994) and also does not block all types of K^+ channels unlike Ba^{2+} (Hille, 1992).

The inward rectifier

That Rb^+ interferes with the movement of K^+ has been established for a number of years (Bolingbroke *et al.*, 1961; Solomon, 1952; Sjodin, 1959). Adrian, (1964) concluded that Rb^+ interfered with the inward movement of K^+, by reducing the membranes permeability to K^+ and that Rb^+ did not pass through the channel for anomalous rectification. Rb^+ is now considered a blocker of anomalous or inward rectifiers (Hille, 1992; Benson and Levitan, 1983), although it does not abolish inward rectification completely as it can be reduced further with Ba^{2+} (Silver *et al.*, 1994). Examples of tissues which exhibit inward rectifiers are the egg cell membrane of a starfish (Hagiwara *et al.*, 1976), frog skin epithelium (Urbach *et al.*, 1994), *Necturus* proximal tubule (Kawahara *et al.*, 1987) and rabbit proximal tubule (Parent *et al.*, 1988).

K^+ channels appear to be present in every eukaryotic cell with more than one type existing (Rudy, 1988). The main characteristic of the inward rectifier is that conductance increases with hyperpolarization allowing K^+ entry and on depolarization there is little K^+ exit (Rudy, 1988). There is evidence that the rectification of the channel is a result of intracellular Mg^{2+} blocking the channel (Vandenburg, 1987; Urbach *et al.*, 1994). Inward rectifiers are an important target of modulation by second messengers (Rudy, 1988; Benson and Levitan, 1983).

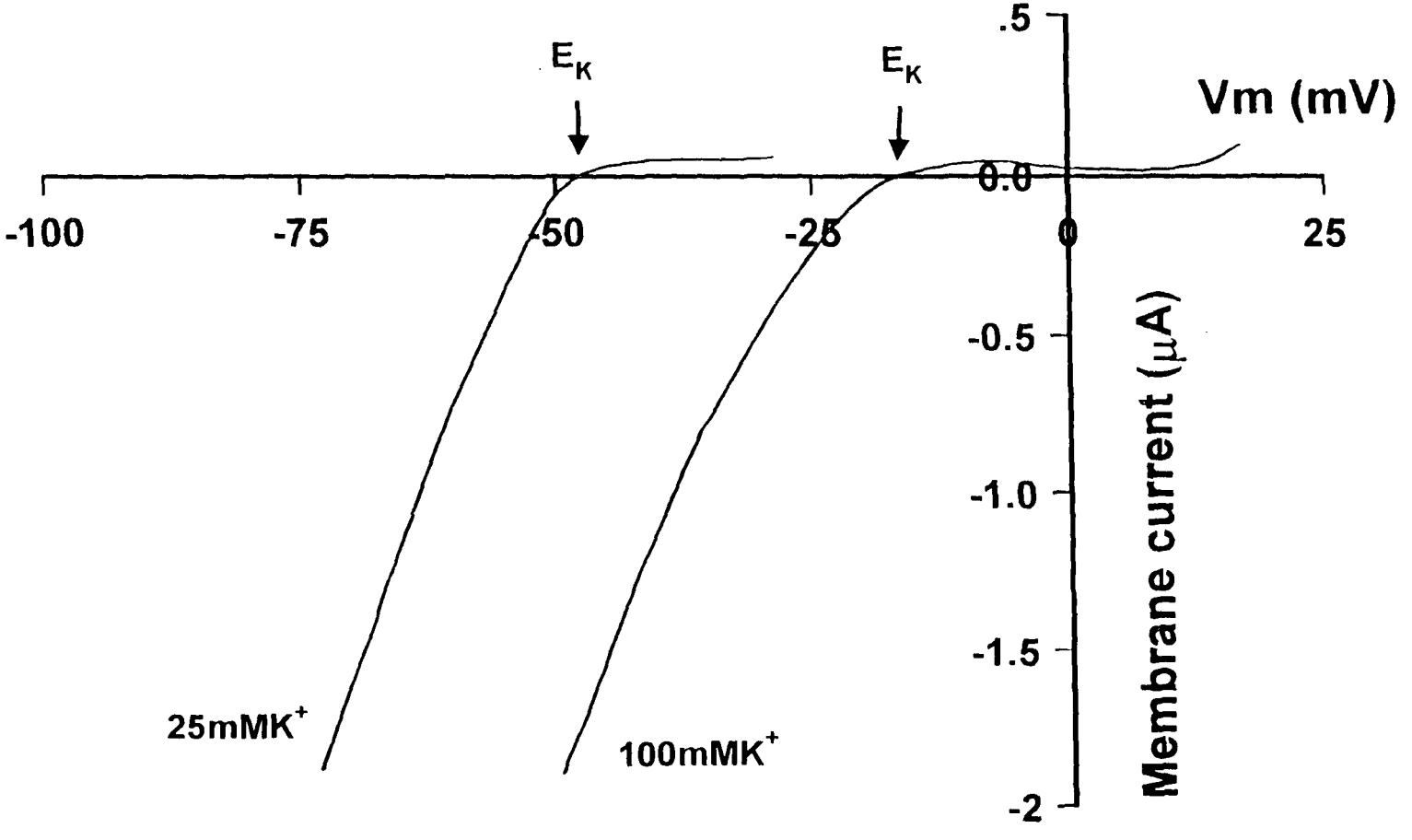
Fig. 5.17. shows hypothetical current-voltage relations displayed by an inward rectifier bathed in different K^+ concentrations. This graph demonstrates an important property of the inward rectifier, that is, the voltage around which the current rectifies depends on $[K^+]_o$, closely following E_K (Vandenburg, 1987: Hille, 1992). A

Fig. 5.17.

Schematic diagram of the current-voltage relations of the egg cell membrane of a starfish at two different K^+ concentrations (25 and 100mM). Modified from Hagiwara *et al.*, (1976). The characteristic features of the inward rectifier are illustrated in this figure. They open on hyperpolarization and large inward currents are produced, by contrast, voltage-clamp depolarizations produce only small outward currents. Also the voltage dependence of their gating depends on $[K^+]_o$, moving along the voltage axis to rectify around the new E_K (Hille, 1992).

E_K - K^+ equilibrium potential (mV).

Vm - Membrane potential (mV).


E_K
E_K
Vm (mV)
.5
0.0
-100
-75
-50
-25
0
25
-0.5
-1.0
-1.5
-2
Membrane current (μA)
25mMK^+
100mMK^+

consequence of this is the K^+ permeability remains relatively constant independent of $[K^+]_o$. The question arises as to why this would be important in Malpighian tubules. K^+ is the prime mover in fluid secretion, actively being extruded across the apical membrane. This K^+ extrusion must be balanced by an intake of K^+ across the basal membrane. The level of K^+ in the haemolymph has been shown to vary with feeding (Bernays and Chapman, 1974) and also the action of the apical pump will lower $[K^+]_i$, both of which will affect E_K. However, if the permeability of the basal membrane to K^+ tracked E_K then fluctuations in $[K^+]_o$ or $[K^+]_i$ would not diminish the K^+ supply to the apical membrane and secretion could proceed.

An inward rectifier could also prevent electrogenic pump currents from greatly changing the membrane potential. If the Na^+/K^+-ATPase is electrogenic, pumping $3Na^+$ out of the cell in exchange for $2K^+$, then this can be thought of as a current flowing out of the cell, some of this current will flow back across the membrane and so will change the voltage across the membrane. However, the hyperpolarization of the membrane potential will have opened the inward rectifiers, the resistance of the membrane will have decreased so the change in the voltage will be minimized. The inward rectifier short circuits the current to prevent a large hyperpolarization. Also a hyperpolarization of the membrane increases the permeability of the membrane to K^+ and hence clamps the membrane potential at E_K even more effectively.

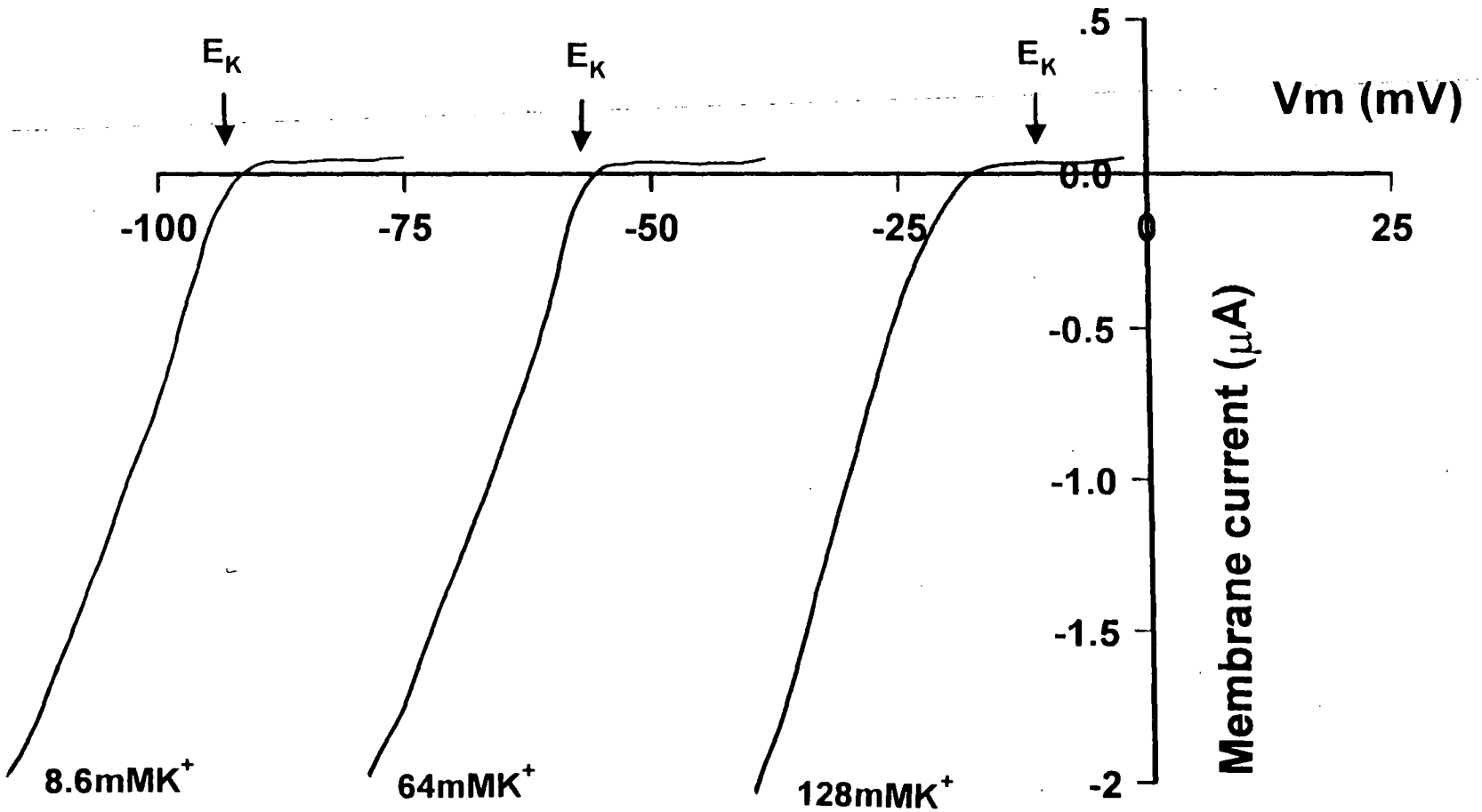
Although the reasons for the existence of an inward rectifier on the basal membrane of Malpighian tubules are not clear it provides a possible explanation for the Type B response which is sometimes recorded when the cells are exposed to high $[K^+]$ (see Fig. 5.18). As shown in Fig. 5.10. at high concentrations of K^+, Rb^+ can no longer block K^+ channels effectively as a depolarization is produced on exposure to high $[K^+]$. This is probably due to a combination of Rb^+ being a relatively weak K^+ channel blocker (Silver *et al.*, 1994), and the much higher concentration of K^+ "out-competing" the Rb^+, it is also possible other K^+ channels exist in the membrane which Rb^+ does not block (unlike Ba^{2+} which blocks a variety of K^+ channels).

Fig. 5.18.

Hypothetical diagram of current-voltage relations at three different K^+ concentrations (8.6, 64 and 128mM). The current-voltage curves produced in 8.6 and 64mM K^+ represent the "normal situation" where E_K is slightly to the left of the point where the current rectifies, meaning that at E_K the membrane is highly permeable to K^+. In some cases it is postulated that if the cell is exposed to high $[K^+]_o$ that the curve does not match the movement of E_K, in these situations the membrane would be impermeable to K^+ at E_K (as shown by the curve at 128mM K^+).

E_K - K^+ equilibrium potential (mV).

Vm - Membrane potential (mV).


E_K
E_K
E_K
Vm (mV)
.5
0.0
-0.5
-1.0
-1.5
-2
-100
-75
-50
-25
0
25
Membrane current (μA)
8.6mMK+
64mMK+
128mMK+

If the superfusate is changed from 8.6mM K^+ to 128mMK^+ the membrane potential responds to the increase in K^+ and the current-voltage curve tracks towards the new E_K and the membrane potential begins to depolarize. If the current-voltage curve is unable to reach the new E_K, then at this new membrane potential, the membrane will be relatively impermeable to K^+, in other words the basal membrane resistance will be high. This change in VDR will cause a greater and greater proportion of V_A to be measured across the basal membrane and so the trace produced will deviate from the typical Type A response (see Fig. 5.1B.) producing a "hyperpolarization". On changing the superfusate back to control saline as the curve moves back along the X-axis it assumes the normal characteristics, that is, the membrane becomes permeable to K^+ again and as $[K^+]_o$ is still relatively high the membrane will depolarize again (see Fig. 5.1B.) before hyperpolarizing as the concentration of K^+ in the superfusate falls gradually back to 8.6mM. It seems as though exposure of the cells to Rb^+ (or Ba^{2+}) increases the probability of this anomalous Type B response being produced possibly by interfering with the inward rectifier and so increasing the probability that the voltage-current curve does not match the movement of E_K.

CHAPTER 6

Preliminary studies on the cell culture of Malpighian tubules of *Locusta migratoria*

RESULTS

Single cell structure

Single cells were produced from Malpighian tubules following incubation with dispase as described in the Methods section. The single cell in Plate 6.1A was produced from the Malpighian tubules of a 4th instar locust. Two large nuclei were seen and the majority of the cells produced were binucleate. The cells were mainly oval-shaped and approximately 40μm across, no other structural details were observed but the cells did appear to have a 'granular' appearance possibly due to microvilli projecting from the surface. Projections and/or invaginations could clearly be seen when cells were observed with the SEM (Plate 6.1B) it is possible that these represented either microvilli or basal infoldings. This cell was produced from the Malpighian tubules of a 1st instar locust and was approximately 20μm across.

Cell culture of Malpighian tubule cells

Malpighian tubule cells were kept in culture as described in the Methods section. Plate 6.2A shows cells which had been in culture for 6 days. The cells were long and flat and most of them were binucleate. Plate 6.2B shows cells which had been kept in culture for 6 days also but in this dish spherical, membrane bound bodies were also visible. To confirm that the cells in culture were living a culture dish containing cells which had been kept in culture for 21 days was selected and trypan blue vital dye was added (as described previously). The cells did not appear to take up the dye, as shown in Plate 6.2C, the nuclei and cytoplasm remained the same colour, indicating that the cells were

Plate 6.1A

A high power light micrograph of a single Malpighian tubule cell prepared from Malpighian tubules of a 4^{th} instar locust. The cell was binucleate.

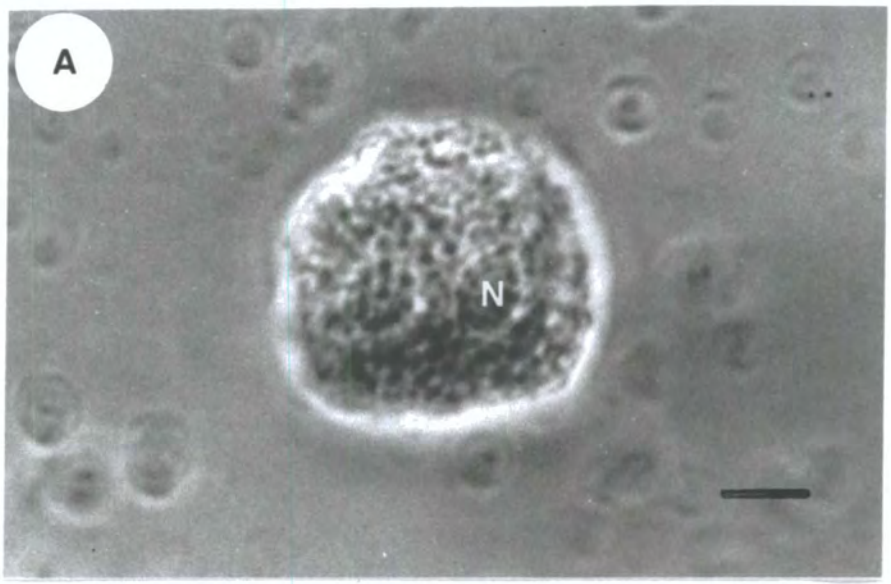
N = nucleus

Scale : 10μm

Plate 6.1B

A scanning electron micrograph of a single Malpighian tubule cell from a 1st instar locust. It is possible that the projections and ridges were due to microvilli and basal infoldings.

Scale : 10μm


A
N

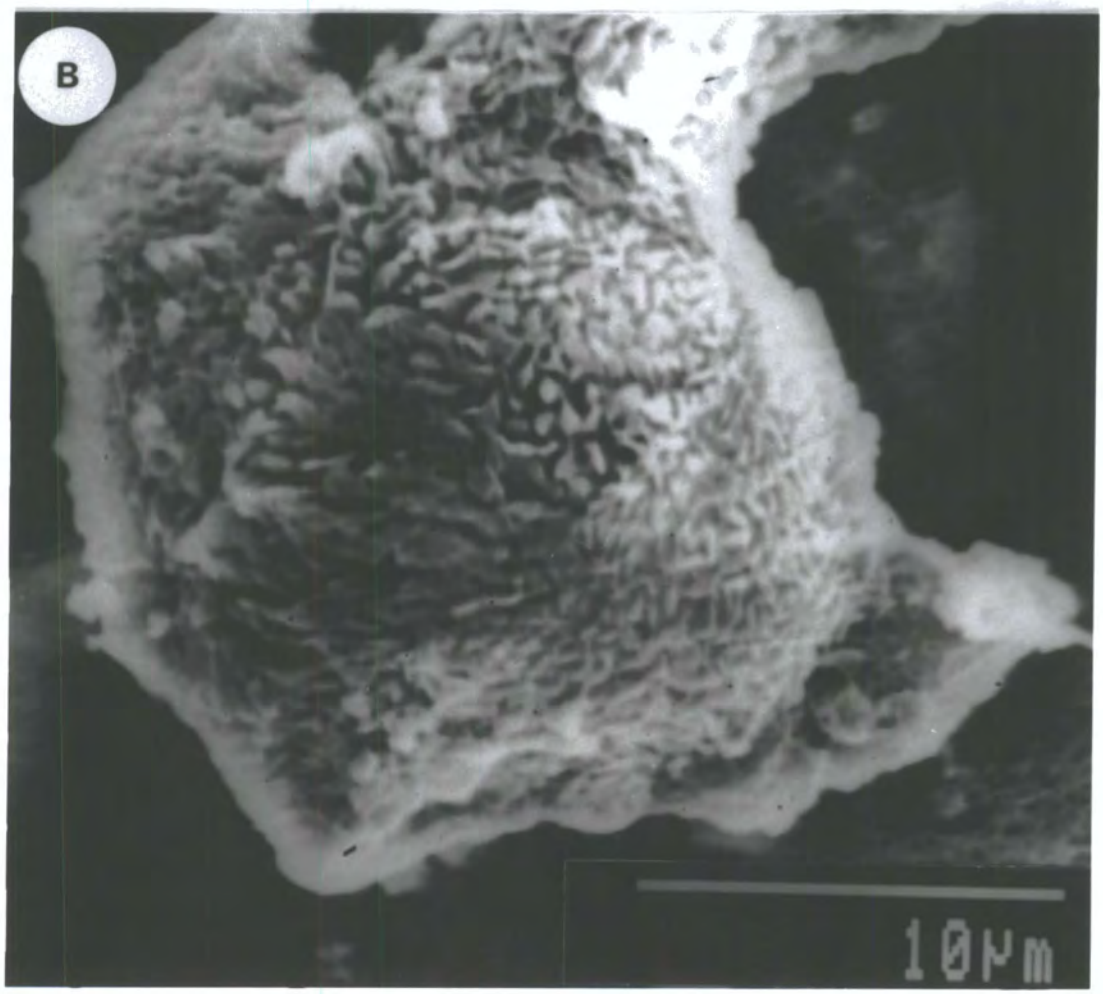

B
10µm

Plate 6.2A

Phase-contrast light micrograph of Malpighian tubule cells (C) which had been kept in culture 6 days. The cells were no longer held in a tubule-like structure and were well spread out on the bottom of the culture dish. The majority of the cells were binucleate.

Scale : 6.25μm

Plate 6.2B

Phase-contrast light micrograph of Malpighian tubule cells which had been kept in culture 6 days. In this culture dish long, flat Malpighian tubule cells (C) and spherical membrane bound bodies (S) were observed

Scale : 3.1μm

Plate 6.2C

Light micrograph of cells which had been in culture for 21 days and were exposed to trypan blue to determine their viability. The nuclei of the cells were still clearly seen and the cytoplasm did not appear to take up any dye. (A piece of stained debris (D) was included in the photograph for comparison). Microvilli (MV) appeared to be visible on the border of one cell.

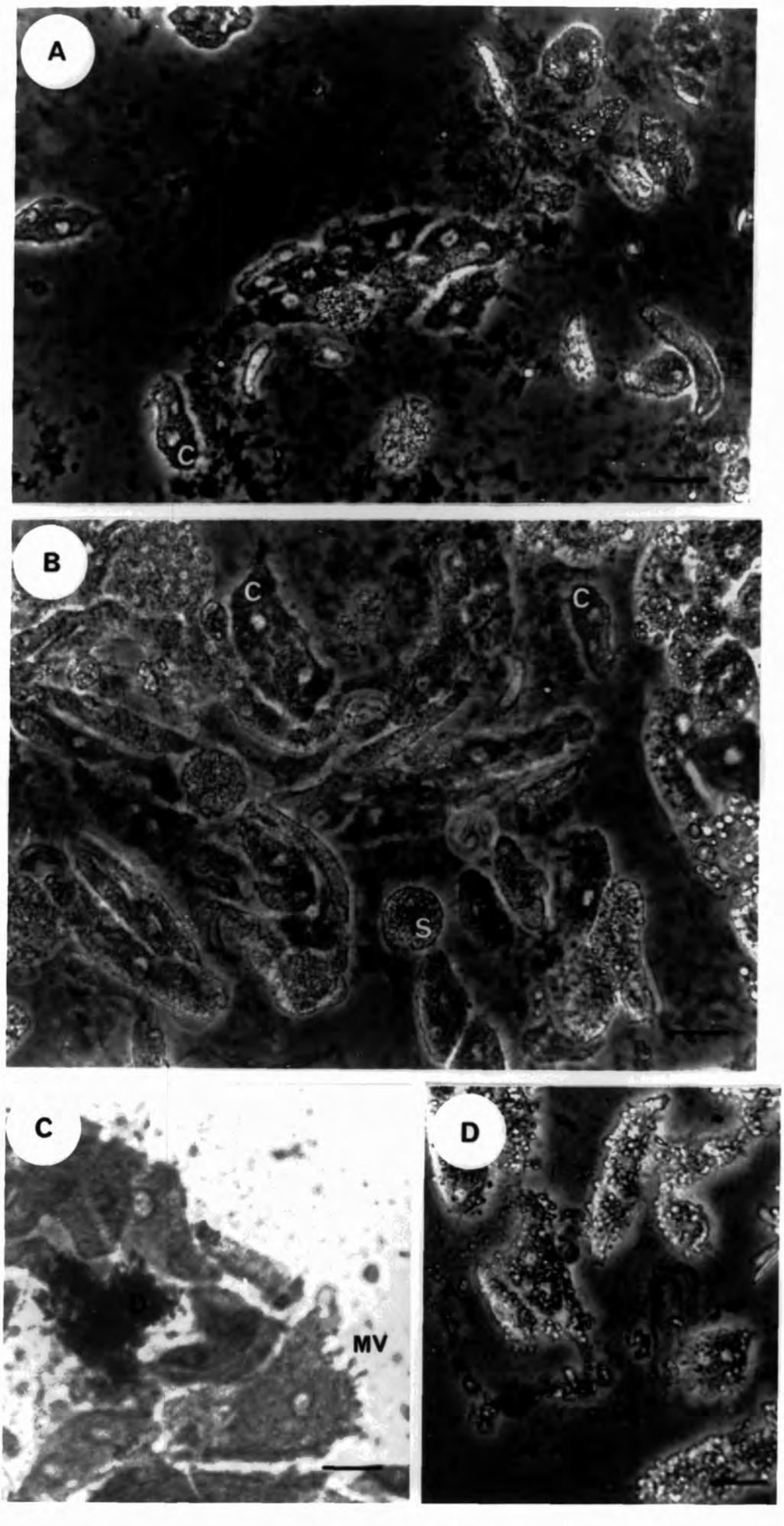
MV = microvilli

Scale : 3.1μm

Plate 6.2D

Phase-contrast light micrograph of cells which had been kept in culture for 11 days. The surface of the cells appeared "granular", possibly due to the presence of microvilli.

Scale : 3.1μm


A
C
B
C
C
S
C
MV
D

viable. One of the cells appeared to have projections from one surface, these could possibly be microvilli.

The cells did not divide and they did not form a monolayer. However, disruption and migration of cells did occur. Partially digested tubules had been placed onto the Matrigel and a coverslip had been placed on top of them. In time single cells did emerge from the tubule mass and some had migrated from underneath the coverslip. Cells which had been in culture for 11 days are shown in Plate 6.2D. The cells had a very 'granular' appearance which could be due to microvilli.

DISCUSSION

In the past attempts have been made to produce cultures of Malpighian tubule cells. Hirumi and Maramorosch (1964) (cited by Aizawa, 1971) endeavoured to produce cultures from the tubules of the monarch butterfly. Large epithelial cells did appear from tubule fragments by the fifth day, attached to the glass surface and produced cell sheets. Over the next 10 days they grew actively but after 15 days began to degenerate. Larsen (1967) attempted to produce cultures from a number of insect tissues, including Malpighian tubules, but found growth was slow, however, inclusion of prothoracic glands in the culture medium did seem to increase the rate of growth. Ting and Brooks (1965) (cited by Quoit, 1971) tried to establish cultures from Malpighian tubules. They found that the tubules continued to contract *in vitro* but showed no cell migration. Quoit, (1971) reported that most attempts at Orthopteran cell culture did not produce true cell cultures with mitotic activity. A more recent attempt to produce a culture of Malpighian tubule cells was carried out by Satmary and Bradley, (1984). They managed to produce single cells which adhered weakly to glass or plastic and which survived for 7 days in culture. However, the cells did not divide.

In the present study a viable culture of Malpighian tubule cells was established for up to at least 21 days. During this time the cells became dissociated from partially digested tubules and appeared to migrate outwards occupying a larger area of the culture dishes. However a cell sheet or monolayer was not established. This result was similar to that observed by former investigators (Satmary and Bradley, 1984; Larsen, 1967) who report success at maintaining viable cultures but no evidence of cell division. Perhaps failure to obtain successful division was due to a deficiency in a vital growth/development substance, if so, inclusion of this component in the culture medium may produce cell division. Support for this argument comes from the work carried out by Larsen, (1967) who found that inclusion of the prothoracic glands in the culture medium increased the growth of the cell cultures. Also Stengl and Hildebrand, (1990) discovered that 20-hydroxyecdysone prolonged survival and stimulated formation of

processes from some cells of cultured antennal flagella of *Manduca sexta*. However, Grace, (1962) found that incorporation of insect hormones in cultures did not stimulate proliferation. Jones, (1962) proposed that capacity to grow is present in the cell itself but this is only realized if the culture medium contains all of the components necessary for nuclear synthesis. Another possible explanation for the absence of any cell division comes from a common property of Malpighian tubule cells, that is, that the cells reach a certain stage and then cease to divide, nuclear replication takes place without cell division (Sehnal, 1985). This is the case in the Hemipteran bug *Rhodnius prolixus*, throughout the life cycle the number of cells in the Malpighian tubules remains constant, cell division does not take place but at each larval moult the DNA content of the cell doubles (Maddrell *et al.*, 1985). This condition was reported for two other Hemipteran bugs *Dysdercus fasciatus* and *Oncopeltus fasciatus*. It is possible that the same condition exists in the Malpighian tubules of *Locusta migratoria* and perhaps this would explain why cell division was not observed in cultures of tubule cells, if the cells do not undergo mitosis *in vivo* perhaps they would be unable to undergo mitosis *in vitro*. It seems a feasible explanation that the reason for this condition is that excretion can carry on undisturbed. Also cell growth without mitosis is advantageous for rapidly developing organisms as both time and energy are conserved. The larger cells that result possibly maintain internal homeostasis at a lower energetic cost and are more effective at excretion (Sehnal, 1985).

In order for the cells to form a monolayer they need to attach to a substrate. In the present study adhesion to glass or plastic was minimal and so a solubilized basement membrane preparation was used to coat the plastic culture dishes, furthermore, the cells were encouraged to attach by placing a coverslip on top of them and exerting a little pressure. By using Matrigel the cells were presented with an *in vitro* attachment matrix which provided a better *in vivo* substitute than glass or plastic. As previously mentioned the cells did not form a monolayer, Maddrell and Overton, (1985), found that isolated cells of *Rhodnius prolixus* were able to function independently of contact with other cells, even though in Malpighian tubules as in other transporting epithelia the cells

normally form a sheet. From this observation it can be concluded that physical contact with other cells is not crucial for the cells to excrete so maybe the isolated culture cells could be functional. Also Matrigel is believed to promote cells' polarization into apical and basolateral regions. The present study did observe a cell with apparent microvilli (Plate 6.2C).

The majority of the cells maintained in culture were long and flattened, however, some spherical membrane bound structures were also observed (Plate 6.2B). It is possible that these are artefacts. Satmary and Bradley, (1984) also reported the presence of "blebs" on the surface of cultured Malpighian tubule cells. They interpreted this structure as basal membrane in origin which had become "un-infolded" and so had expanded into a bleb-like structure, it is possible that the same thing has occurred in this study. Another possibility is that the spherical structures, which are in the minority, represent the type II cell population but this is highly speculative.

An interesting feature of the single cells that were produced was that the majority of them were binucleate, this is also the case for the Malpighian tubules of the Hemipteran bugs *Rhodnius prolixus*, *Oncopeltus fasciatus* and *Dysdercus fasciatus* (Maddrell *et al.*, 1985). In fact up to now these were the only species believed to possess Malpighian tubules with two nuclei, although the mature follicle cells of a cockroach and some Hemipterans are also binucleate (Bonhag, 1955; Nath *et al.*, 1959). Also recently "granular" cells have been reported in the initial segment of the Malpighian tubules of *Locusta migratoria* and *Schistocerca gregaria* that were sometimes binucleate (Prado *et al.*, 1992). Because not all of the cells of the Malpighian tubules of *Locusta* are binucleate or indeed any of the cells of other species means it cannot be vital for their function. Having two spherical smaller nuclei rather than one large one would increase the surface area of the nucleus in contact with the cytoplasm.

CHAPTER 7

GENERAL DISCUSSION

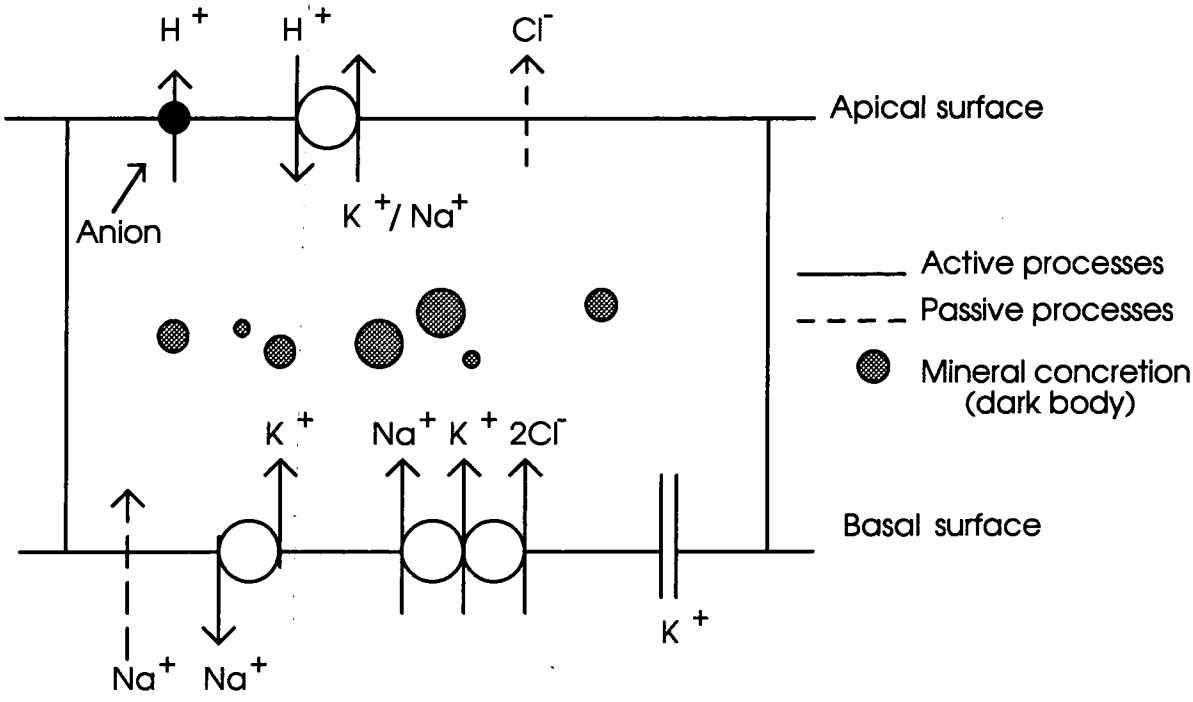
In the present study, clear biochemical evidence has been obtained which demonstrates the presence of V-type ATPase activity in the Malpighian tubules of *Locusta migratoria*. This activity is associated with the fraction containing mainly apical plasma membranes. The ATPase activity was found to be stimulated by K^+ and to exhibit similar salt selectivities to an ATPase enzyme located in another insect secretory tissue, the goblet cell apical membrane of the midgut of *Manduca sexta* (Schweikl *et al.*, 1989). Furthermore, 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 immunofluorescent microscopy. Additionally, electrophysiological studies revealed an activity on the apical membrane which is inhibited by specific V-type ATPase inhibitors. In a further set of experiments these inhibitors were shown to inhibit fluid secretion. Finally, an ultrastructural investigation of the microvillar border demonstrated the presence of portasomes which are believed to represent the site of the V-type ATPase (Klein *et al.*, 1991).

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); *Formica polyctena* (Weltens *et al.*, 1992; Dijkstra *et al.*, 1994b) and *Manduca sexta* (Klein *et al.*, 1991; Klein, 1992; Zeiske, 1992). However, I believe that this is the first study to demonstrate biochemically a V-type ATPase 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. Fig. 7.1. represents a hypothetical model which seeks to explain the mechanisms of ion and fluid movements across the Malpighian tubules of *Locusta migratoria*. It is based upon information generated in the present study and in previous studies carried out in this laboratory and elsewhere. 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). The demonstration that amiloride, an inhibitor of antiporters, reduces fluid secretion by the Malpighian tubules of *Locusta migratoria* (Fathpour, 1980; Fathpour and Dahlman, 1994) provides evidence that an antiporter is involved in ion and fluid secretion in this tissue. Experiments investigating the effect of substances on the ionic composition of the "urine" suggested that if Na^+ and K^+ used a common apical exit mechanism that it had a much higher affinity for K^+. However, separate exit mechanisms for $Na^+$and K^+ cannot be discounted.

The proposal of a V-type ATPase and antiporter in the Malpighian tubules of *Locusta* does not drastically alter previous models proposed to explain fluid secretion in this tissue. The two components simply replace the common cation pump which had been postulated to exist on the apical membrane. It can, however, explain some results which could not have been accounted for by the common cation pump. Maddrell and O'Donnell, (1992) working on *Rhodnius prolixus* revealed that whilst furosemide caused the fluid secreted to become more alkaline, amiloride caused it to become more acidic. Slowing cation transport by inhibiting the antiporter with amiloride would mean that the

Fig 7.1. Schematic diagram of the model proposed to explain the mechanisms of ion and fluid transport across the Malpighian tubule cells of *Locusta migratoria*.



It is proposed that a basal Na^+/K^+-ATPase is responsible for K^+ entry into the tubule and for maintaining low intracellular Na^+. K^+ channels in the basal membrane allow passive movement of K^+, under control conditions these channels will 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. Some passive entry of Na^+ is proposed to occur at the basal surface down concentration and electrical gradients. The action of a co-transporter at the basal surface allows linked Na^+, K^+, and Cl^- entry, although it is possible that under Na^+-free conditions the co-transporter operates as a KCl transporter.

At the apical surface K^+ exit is postulated to occur via an antiporter which is energized by a V-type ATPase. 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.

Under conditions of K^+ deprivation, mineral concretions which may act as intracellular K^+ stores, release K^+ to allow fluid secretion to proceed.

lumen would become more negative due to the relatively fast movement of Cl^- into the lumen, this would reduce the potential gradient for driving the antiporter, H^+ and Cl^- would accumulate in the lumen and so the fluid would be more acidic. Slowing Cl^- movement into the cell by using the co-transporter inhibitor, furosemide will cause the lumen to become more and more positive; this will either drive H^+ out of the lumen into the cell or drive OH^- in the other direction from the cell into the lumen leading to a more alkaline secretion (Maddrell and O'Donnell, 1992).

The present study has produced evidence (Chapter 4) for the existence of both an HCO_3^--stimulated ATPase and V-type ATPase on the apical membrane. The V-type ATPase activity displayed some characteristic features of the 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 HCO_3^--stimulated ATPase was not sensitive to NEM and bafilomycin A_1 at levels that V-type ATPase activity was affected. Nevertheless, it is possible that a component of the HCO_3^--stimulated ATPase preparation is bafilomycin A_1-sensitive at concentrations specific for V-type ATPases and therefore can be ascribed to V-type ATPase activity. This is an area which requires further investigation. Fogg *et al.*, (1991) proposed that the active extrusion of 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, their proposal was speculative.

Why would H^+-ATPases, typical constituents of intracellular membranes, be found in insect transporting plasma membranes? Especially when H^+-ATPases in other animal tissues are closely associated with the endomembrane system (Gluck *et al.*, 1982; Brown *et al.*, 1987 and Swallow *et al.*, 1990). In the Malpighian tubules of *Locusta* a number of different vesicles were observed (Chapter 3). It could be possible that these too contain V-type ATPases in their membranes. However there is no evidence that these vesicles fuse with the plasma membrane and so insert H^+-translocating ATPases. Bradley and Satir, (1981) did suggest that the apical plasma membrane of the Malpighian tubules of *Rhodnius prolixus* could increase its

dimensions by incorporating intracellular vesicles. They have evidence that an increase in the rate of fluid secretion causes an increase in the volume and surface area of the microvilli. Later Bradley, (1989) using freeze fracture studies was able to reveal the presence of "studs" that cover the surface of the microvilli, the density of these "studs" increased when the tubules were exposed to 5-HT. He proposed that the studs might be the site of ion translocating complexes. In *Manduca sexta* an association between the plasma membrane and intracellular vesicles has been found. During embryogenesis of the goblet cell in the midgut the goblet cavity is produced by the incorporation of an intracellular vesicle (Hakim *et al.*, 1988).

Basal Na^+/K^+-ATPase activity was demonstrated using immunocytochemical techniques and membrane separation methods in conjunction with biochemical studies. Ouabain inhibition of fluid secretion, accompanied by increased and decreased intracellular levels of Na^+ and K^+ respectively (Pivovarova *et al.*, 1994b) and increased levels of Na^+ in the "urine" (present study) indicated a role in for this enzyme in fluid secretion. Other related studies support the existence of a Na^+/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 K^+ transport could be carried out by the Na^+/K^+-ATPase (Anstee *et al.*, 1986). Hence it is proposed that the basal Na^+/K^+-ATPase is responsible for K^+ entry into the cell, in exchange for Na^+, thus, maintaining the Na^+ gradient. This function for the Na^+/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 Na^+ gradient established by the Na^+/K^+-ATPase can be used to drive the basal co-transporter.

Evidence from microelectrode studies suggests that passive entry of K^+ into the Malpighian tubules cannot be discounted. Under "normal" conditions because E_K is

more negative than V_B any passive K^+ movement will be out of the cell. However the present study has produced some evidence for the existence of an anomalous rectifier on the basal surface of the Malpighian tubules of *Locusta migratoria*. Such a K^+ channel would open when the membrane is hyperpolarized and would result in passive K^+ entry. The membrane potential would then move back in the direction of E_K and the anomalous rectifier would close. Because the voltage dependence of this channel's gating depends on extracellular K^+, the hyperpolarization would not have to be caused by a change in extracellular K^+. This would only result in the current-voltage curve rectifying around the new E_K and so potassium permeability remaining the same. However an electrogenic pump current as produced by the Na^+/K^+-ATPase could successfully hyperpolarize the basal membrane and so open anomalous rectifiers. A rectifier offers a possible mechanism to control basal K^+ permeability. In secretory epithelia control of transport across the basal membrane is often the mechanism for controlling transport across the whole epithelium (Beyenbach, 1995). Setting the membrane potential at a point on the current-voltage curve close to the transition point between permeable and impermeable would mean that a change in the membrane potential of only a few millivolts could cause a large change in the permeability of the basal membrane. This offers a possible mechanism by which to control fluid secretion. Indeed application of corpora cardiaca to the tubules of *Locusta* led to a small but significant hyperpolarization of V_B (Fogg *et al.*, 1989). cAMP was also found to cause a small hyperpolarization but this was not statistically significant (Fogg *et al.*, 1989). Morgan and Mordue, (1983), working on the tubules of *Locusta*, reported that cAMP and diuretic hormone had no effect on V_B. However in one case cAMP was found to cause a small hyperpolarization (n=10). A hyperpolarization of V_B in the salivary glands of *Calliphora* in response to 5-HT and cAMP was reported by Berridge *et al.*, (1976) and Berridge and Prince, (1972b) respectively. O'Donnell and Maddrell, (1984) found exposure of the Malpighian tubules of *Rhodnius* to 5-HT usually resulted in a small depolarization of V_B although hyperpolarizations were occasionally produced.

Microelectrode studies indicated that the basal membrane of *Locusta migratoria* was permeable to K^+, but relatively impermeable to Na^+ and Cl^- (Baldrick *et al.*, 1988; Fogg, 1990). O'Donnell and Maddrell, (1984) finding that the basal cell membrane of *Rhodnius prolixus* was impermeable to Na^+ and Cl^- proposed that these ions entered cells via a Na^+-K^+-$2Cl^-$ co-transporter, although in the absence of either cation other stoichiometries could be accepted. Baldrick *et al.*, (1988) suggested that such a co-transporter may exist in the Malpighian tubules of *Locusta migratoria* providing a mechanism for Cl^- entry. However, it was also suggested that under Na^+-free conditions Cl^- could be transported with K^+ only. Effects of co-transporter inhibitors on the tubules of *Locusta migratoria* were weak (Baldrick *et al.*, 1988) when compared to effects seen on the tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). Fogg, (1990) discovered that Na^+ transport was unaffected by the absence of Cl^- from the bathing medium but did find that under high $[K^+]_o$ conditions that Cl^- entry could be explained by KCl co-transport. Under control conditions Fogg, (1990) suggested Cl^- entry might occur in exchange for HCO_3^- or by the activity of a (Cl^- + HCO_3^-)-ATPase. The present study demonstrated a HCO_3^--stimulated activity associated with the apical membrane this activity is also known to be associated with the basal membrane (Fogg, 1990) and has previously been implicated in various transport processes including Cl^-/HCO_3^- exchange (Herrera *et al.*, 1978). However, most evidence from a variety of insect species does point to the existence of a co-transporter in Malpighian tubules (O'Donnell and Maddrell, 1984; Maddrell and Overton, 1988; Hegarty *et al.*, 1991; Leyssens *et al.*, 1994). As furosemide does inhibit fluid secretion in *Locusta migratoria* (Kalule-Sabiti, 1985), and the inclusion of cAMP, a stimulator of co-transporters (Audsley *et al.*, 1993; Dijkstra *et al.*, 1995), in the bathing medium does increase the amount of intracellular Na^+ and Cl^-, but not K^+ (possibly as cAMP also stimulates the exit mechanism for K^+) in the Malpighian tubules of *Locusta migratoria* (Pivovarova *et al.*, 1994b). It is proposed that a co-transporter does exist on the basal surface of the Malpighian tubules of *Locusta migratoria.*

Locusta migratoria secretes a KCl-rich "urine". In a previous study a large negative apical membrane potential was recorded (Fogg, 1990), hence, it is proposed that Cl^- exits the cell passively down a favourable electrical gradient. This has previously been proposed in *Locusta migratoria* (Fogg, 1990; Morgan and Mordue, 1983; Baldrick *et al.*, 1988) and *Rhodnius prolixus* (O'Donnell and Maddrell, 1984).

An interesting finding of the present study was the possible involvement in fluid secretion of the mineral concretions distributed throughout the cytoplasm of the Malpighian tubule cells. It would appear that they act as intracellular K^+ stores as if the tubules are subjected to K^+-free or K^+-poor conditions K^+ still appears at substantial concentrations in the "urine". This suggests that in *Locusta migratoria* fluid secretion is strongly dependent on K^+ secretion.

In conclusion, the results obtained from the present study and described above can be used to construct a hypothetical model (Fig. 7.1.) to explain the mechanisms involved in ion and fluid transport across the Malpighian tubules of *Locusta migratoria.*

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APPENDIX

Table A.1. Effect of [Cl^-] or [HCO_3^-] on ATPase activity

[Cl^-] or [HCO_3^-] (mM)	Mg^{2+}-dependent ATPase activity	
	+NaCl	+$NaHCO_3$
2.5	243.6 ± 25.2	271.6 ± 30.5
5	253.7 ± 18.1	282.1 ± 29.9
10	259.1 ± 24.0	290.9 ± 23.1
15	249.9 ± 24.9	288.9 ± 26.0
20	236.7 ± 25.4	295.0 ± 32.7
25	236.7 ± 20.2	261.1 ± 21.9
30	243.8 ± 25.9	258.7 ± 17.6

Activity was measured in nmoles P_i liberated/mg. protein/min.

Mg^{2+}-dependent ATPase activity was 231.4 ± 25.2 nmoles P_i liberated/mg prot./min.

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

Table A.2. Effect of pH on ATPase activity

pH	Mg^{2+}-dependent ATPase activity (nmoles P_i liberated/mg. protein/min.)		
	Mg^{2+}	Mg^{2+} +NaCl	Mg^{2+} +$NaHCO_3$
7.0	235.4 ± 26.8	282.2 ± 32.1	245.5 ± 20.8
7.5	243.6 ± 27.3	291.4 ± 29.5	348.3 ± 32.2
8.0	196.7 ± 28.3	238.0 ± 17.5	305.6 ± 22.8
8.5	184.8 ± 33.8	175.0 ± 16.1	234.1 ± 3.9
9.0	146.5 ± 24.8	107.7 ± 38.5	191.5 ± 16.5

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

Table A.3. Effect of $[Mg^{2+}]$ on ATPase activity

$[Mg^{2+}]$ (mM)	Mg^{2+}-dependent ATPase activity (nmoles P_i liberated/mg. protein/min.)	
	+20mM NaCl	+20mM $NaHCO_3$
0.5	149.0 ± 7.6	189.5 ± 19.9
1.5	229.7 ± 9.0	272.9 ± 23.6
2	229.2 ± 9.7	306.9 ± 22.0
2.5	226.6 ± 6.9	266.1 ± 24.1
5	223.6 ± 5.5	244.0 ± 26.9
7.5	216.6 ± 10.1	237.0 ± 25.7
10	186.4 ± 20.4	210.8 ± 18.4

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

Fig. A.1.

Calibration curve for NaOH concentration (mM) against % emission read at 595nm.

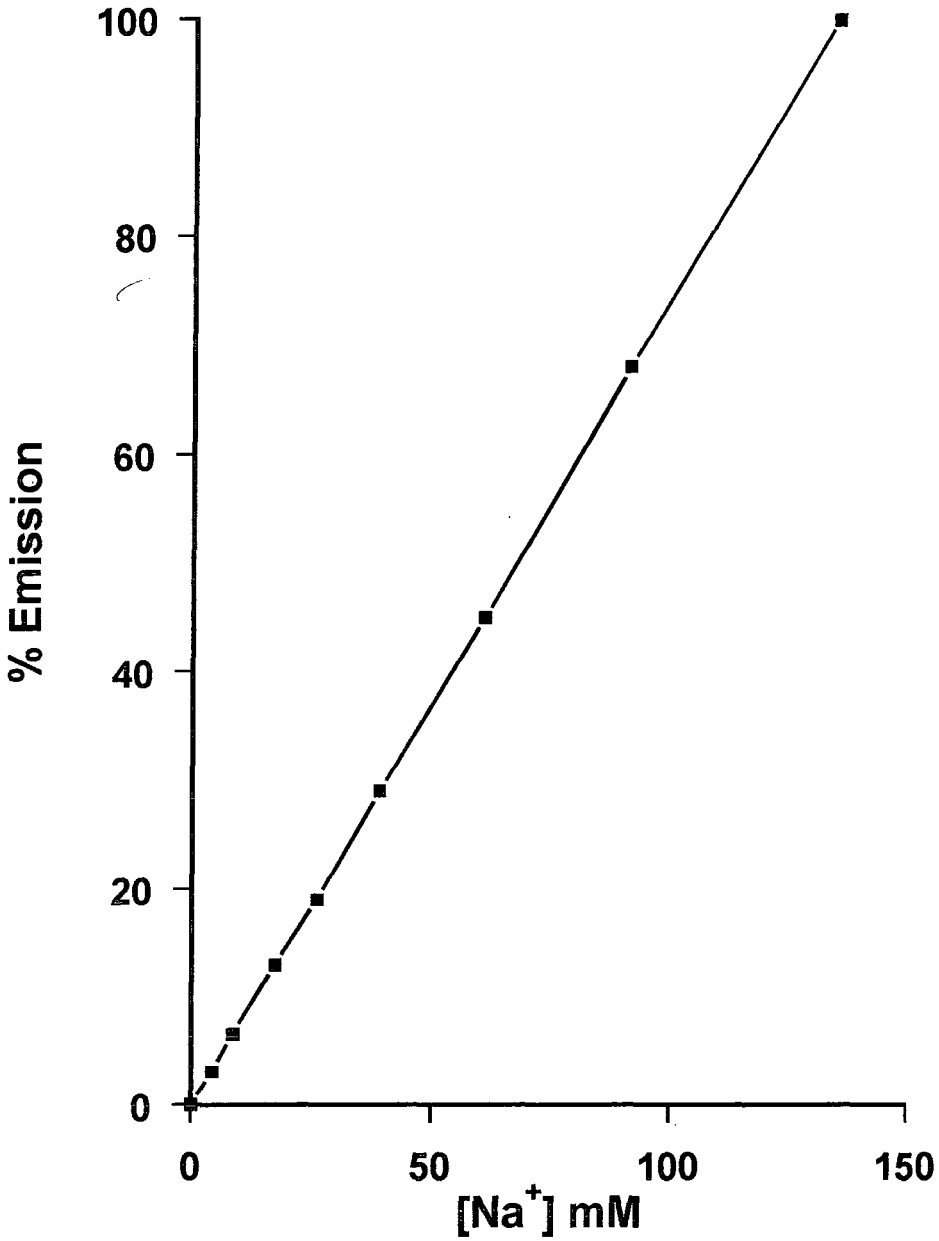

Fig. A.2.

Calibration curve for KOH concentration (mM) against % emission read at 766.5nm.

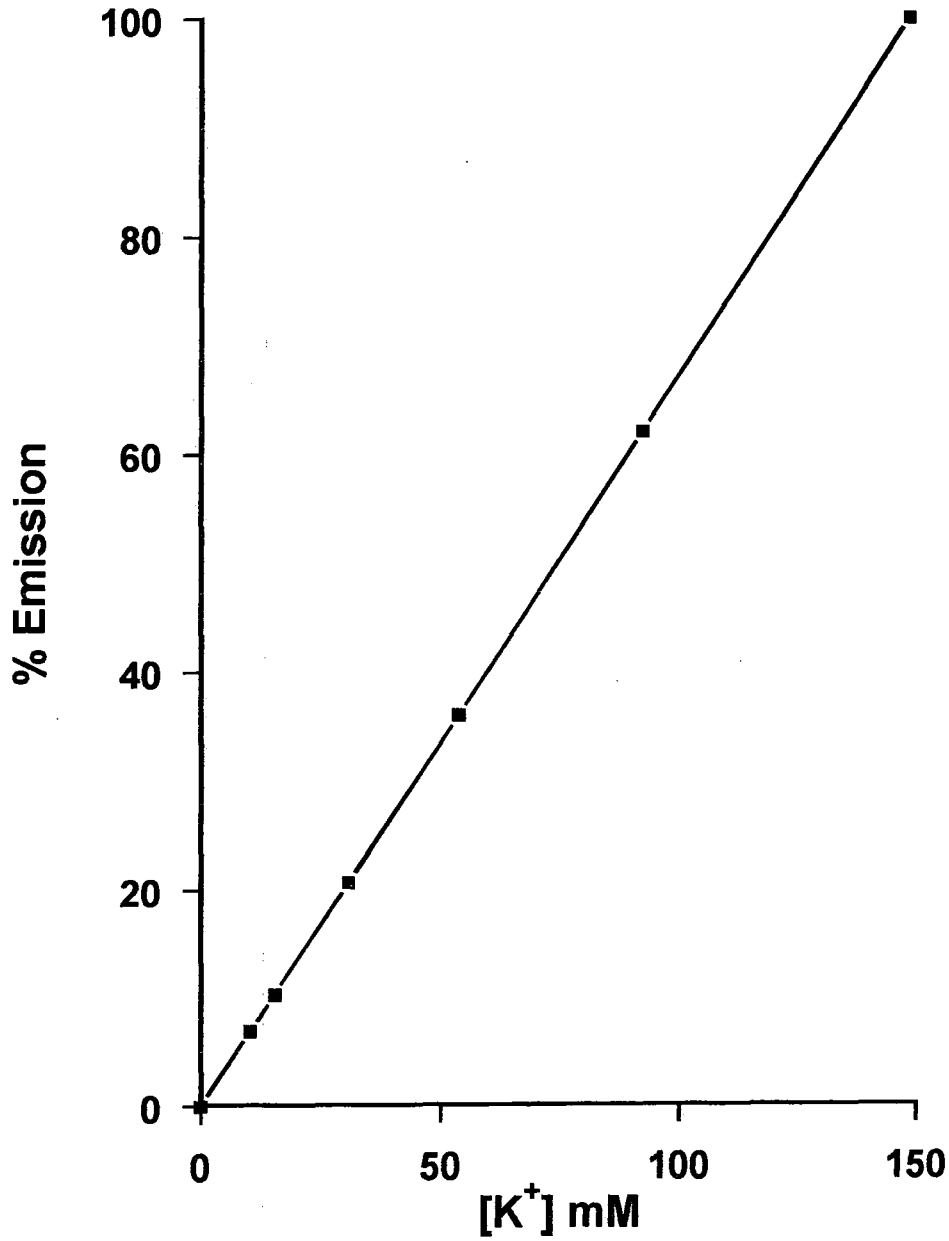

Fig. A.3.

Calibration curve for RbCl concentration (mM)
against % emission read at 780nm.