Studies on ion movements in Malpighian tubules of Locusta migratoria L., with particular reference to their endocrine control

Kay Elizabeth Fogg

STUDIES ON ION MOVEMENTS IN MALPIGHIAN TUBULES OF LOCUSTA MIGRATORIA L., WITH PARTICULAR REFERENCE TO THEIR ENDOCRINE CONTROL.

BY
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University of Durham

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.

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Kay E. Fogg
Durham
September 1990

To my parents

Acknowledgements

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ABSTRACT

Intracellular microelectrodes were used in conjunction with ion substitution, agonists and inhibitors of known transport processes and diuretic hormone (DH), to investigate the mechanisms whereby ions cross the basal and apical cell membranes of the Malpighian tubules of *Locusta migratoria*, and their endocrine control. Values for basal, apical and transepithelial potentials in control saline were -70.4 ± 0.9 mV, -76.1 ± 1.5 mV and +5.5 ± 1.3 mV (lumen positive), respectively (n=73).

Ion substitution experiments, involving K^+, Na^+ and Cl^- in the bathing medium, indicated that the basal membrane is more permeable to K^+ than Na^+ and Cl^-. Ion flux studies suggest that Cl^- entry across the basal membrane is not by direct cotransport with K^+, nor Na^+. Some Cl^- entry also appears to be stimulated under conditions of high $[K^+]_o$. Crude corpus cardiacum (CC) preparations with DH activity effected *ca.* 150% increase in tubular fluid secretion above basal secretion *in vitro*, and *ca.* 106% and 335% increase in net transepithelial Na^+ and Cl^- movement from bathing medium to lumen above basal flux, respectively. Treatment of tubules with CC extract also resulted in a significant increase in levels of the intracellular second messengers, cAMP and the Ca^{2+}-mobilizing Inositol 1,4,5-trisphosphate. Ion substitution experiments using dibutyryl cAMP suggest that cAMP stimulates an apical cation pump, whilst having no effect on Cl^- conductance.

Cytochemical localization and membrane separation techniques used in conjunction with biochemical analyses indicate the presence of $(Na^+ + K^+)$-ATPase on the basolateral membranes, and HCO_3^--stimulated ATPase on both the basolateral and apical membranes.

The results referred to above are discussed, and a hypothetical model is proposed to describe the endocrine control of ion movements across the two cell membranes mediated by changes in intracellular cAMP and Ca^{2+}.

GLOSSARY

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumen (Fraction V, Sigma)
Ca^{2+}-ATPase	magnesium-dependent, calcium-stimulated, adenosine triphosphatase
cAMP	cyclic adenosine 3', 5'-monophosphate
CC extract	corpora cardiaca extract
$(Cl^{-}+HCO_3^{-})$-ATPase	magnesium-dependent, chloride-bicarbonate-stimulated adenosine triphosphatase
E.D.T.A,	ethylene diamine tetra-acetic acid
E.G.T.A.	ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid
HEPES	4-(2-hydroxylethyl)-1-piperazine-ethanesulphonic acid
5-HT	5-hydroxytryptamine
Ins-1,4,5-P_3	D-*myo*-inositol 1,4,5-trisphosphate
K^{+}-ATPase	magnesium-dependent, potassium-stimulated adenosine triphosphatase
$(K^{+}+H^{+})$-ATPase	magnesium-dependent, potassium-hyd*rogen* stimulated adenosine triphosphatase
mM	millimolar
mV	millivolts
$(Na^{+}+K^{+})$-ATPase	magnesium-dependent, sodium-potassium-stimulated adenosine triphosphatase
SEM	standard error of mean
SDH	succinate dehydrogenase
TCP	transcellular potential
TEP	transepithelial potential
Tris	tris (hydroxymethyl) aminoethane
V_A	apical membrane potential
V_B	basal membrane potential

CHAPTER 1

INTRODUCTION

The movement of fluid across a variety of epithelia, whether secretory or absorptive in nature, is a consequence of active ion transport, to which water movement is osmotically linked (Maddrell, 1971, 1977). Most insects regulate the composition of their haemolymph within narrow limits when challenged by extreme environments, tissue water being conserved at the expense of haemolymph volume during periods of severe dehydration (Phillips *et al.*, 1982). Regulation of haemolymph composition and volume (Maddrell, 1980) is largely carried out by the excretory system consisting of the Malpighian tubules, producers of a primary urine, and the hindgut-rectum into which the primary urine is discharged and where selective reabsorption occurs (for review see Phillips *et al.*, 1982). The mechanism of ion and water transport across insect Malpighian tubules was first studied by Ramsay (1953-1958) in the stick insect *Carausius morosus* (then *Dixippus morosus*), leading to subsequent work on tubules from a variety of species, e.g., *Calliphora erythrocephala* (now *Calliphora vicina*) (Berridge, 1968, 1969), *Rhodnius prolixus* (Maddrell, 1969, 1971, 1977), *Locusta migratoria* (Anstee and Bell, 1975, 1978; Morgan and Mordue, 1981, 1983) and *Glossina morsitans* (Gee, 1975, 1976). However, the mechanism of solute-coupled water movement, and its hormonal control, is poorly understood in most insects.

Studies on the Malpighian tubules of insects indicate that whilst some species secrete a slightly hypo-osmotic urine (e.g., *Carausius morosus*, Ramsay, 1954; *Dysdercus fasciatus*, Berridge, 1965), the majority of insects, including *Locusta migratoria*, secrete a fluid which is marginally, but consistently hyperosmotic to the bathing medium over a wide range of osmotic concentrations (Berridge, 1968; Maddrell, 1969, 1977; Phillips, 1964; Anstee *et al.*, 1979) regardless of secretion rate. In most insects, the rates of fluid secretion are inversely proportional to the osmotic concentration of the bathing medium (see Phillips, 1981). These observations are consistent with water movement by some form of "local osmosis" (Maddrell, 1977, 1978, 1980) as proposed by Diamond (1964). This theory proposes that, as a result of solute pumping across the basal and apical cell membranes, the cytoplasm becomes marginally hypertonic to the bathing medium, and the lumen content becomes marginally hypertonic to the cytoplasm. As a result of these small osmotic pressure differences, whose magnitudes are determined by the rates of solute transport and the osmotic permeability of the cell membranes, water moves passively across the epithelial cell.

Several other theories have been proposed to explain how solute transport effects iso- or near iso-osmotic secretion across epithelia. The "standing-gradient" osmotic flow hypothesis for fluid transporting epithelia, originally developed for liver and kidney, was proposed by Diamond and Bossett (1967, 1968). This model is based on the functional geometry of the tissue and depends upon the canals produced between the system of long infoldings of the basal cell membrane and between the elongated microvillar projections of the apical cell membrane being structurally or functionally enclosed at one end. At equilibrium, a standing osmotic gradient is established along the length of each extracellular canal at both the basal and apical surfaces. This osmotic gradient is achieved by the pumping of solute across the region of cell membrane at the closed ends of each canal, such that a local difference in osmotic pressure is developed between the end space and the adjacent cytoplasm, and along the canal itself. As a result, water moves passively along the canals and across the cell membranes so that towards the open end of each canal the fluid produced is iso-osmotic to the cytoplasm. However, it has been argued that the basal cell membrane infoldings and the apical microvilli are too short in insects to permit development of solute gradients along their lengths (Taylor, 1971; Maddrell, 1977). Indeed, the ion gradients predicted by the above model were not observed in the Malpighian tubules of *Rhodnius prolixus* (determinations made by electron-probe X-ray microanalysis, Gupta *et al.*, 1976, 1977). It would also appear that the osmotic permeabilities of cell membranes, required for near iso-osmotic secretion, are impossibly high (Hill, 1975a,b).

Hill (1975b, 1977) suggested that ion movements may entrain water movements in a process of "electro-osmosis". Maddrell (1977), when discussing this theory in relation to insect Malpighian tubules, suggested that apical electrogenic cation pumps produce an electrical potential difference across the membrane. As a result of the electrochemical gradient formed, Cl^- would be drawn out of the cell, and in crossing the apical cell membrane would functionally interact with water molecules, drawing them out of the cell into the lumen. This mechanism relies on the maintenance of a favourable potential gradient across the apical cell membrane.

The simple "local osmosis" theory (Diamond, 1964) was favoured for insect Malpighian tubules by Taylor (1971) and Maddrell (1971). They suggested that the driving force for the movement of water would be the overall osmotic pressure difference between the lumen contents and the bathing medium, which would be effectively increased by the extensive basal infoldings and apical microvilli.

Alternative models to those mentioned previously, propose water movement through paracellular pathways, similar to those seen in gallbladder and proximal tubules of vertebrate kidney (Hill, 1980; Sackin and Boulpaep, 1975). These paracellular pathways

consist mainly of "tight" junctions (for review see Schneeberger and Lynch, 1984) in series with lateral spaces between epithelial cells. The tight junction, or zonula occludens, forms a continuous, gasket-like seal near the apices of adjacent cells in an epithelium at the apical side of the intercellular compartment (Schneeberger and Lynch, 1984). However, exceptions to this include choroid plexus and the pigment epithelium in the retina where tight juntions are near the basal side of the intercellular space (Schneeberger and Lynch, 1984).

It was initially thought that these "tight" junctions would present a barrier to paracellular transport (DiBona, 1972) and also form and maintain structural composition and functional polarity of cells (Schneeberger and Lynch, 1984), but such junctions were subsequently found not to be tight enough to prevent transepithelial transport via this route in some epithelia (DiBona, 1972; Fromter and Diamond, 1972). As a consequence of these findings, "tight" and "leaky" (Fromter and Diamond, 1972) have become common descriptions in epithelial classification (Schneeberger and Lynch, 1984). In "tight" epithelia, the majority of ion and water movements occur transcellularly due to the combined resistances of the apical and basal cell membranes being lower than those of the tight junctions and intercellular spaces. Examples of "tight" epithelia, quantified electrophysiologically, include frog (Schneeberger and Lynch, 1984) and toad (Reuss and Finn, 1974) urinary bladder, rabbit urinary bladder (Lewis *et al.*, 1976) and locust rectum (Hanrahan *et al.*, 1982, 1984). In "leaky" epithelia, paracellular movements of ions and water occur through the leaky "tight" junctions and intercellular spaces (Fromter and Diamond, 1972). Examples of such an epithelium include choroid plexus (Schneeberger and Lynch, 1984), gallbladder (Fromter, 1972) and proximal renal tubule (Guggino *et al.*, 1982) of *Necturus*, and the intestine of freshwater prawns (Ahearn, 1980) and *Aplysia* (Gerencser, 1983).

It is generally thought that iso-osmotic fluid secreting epithelia are "leaky" (Staehelin, 1974; Lord and DiBona, 1976; Lane, 1979), allowing a considerable paracellular movement of ions and water (Sackin and Boulpaep, 1975; Gupta and Hall, 1979). On this basis, one would predict the near iso-osmotic fluid secreting insect Malpighian tubule to be "leaky". However, Maddrell (1980) argued against the paracellular route for water movement across the Malpighian tubules of *Rhodnius prolixus* which, when stimulated, have one of the highest rates of fluid transport yet reported (see Phillips, 1981). Unlike vertebrate gallbladder and proximal renal tubule, intercellular channels in tubules of *Rhodnius prolixus* are relatively rare, comprising only 0.034% of the total surface area (Maddrell, 1980). Furthermore, septate junctions extend along almost the whole length of these intercellular channels (Phillips, 1981). Fluid secretion by a paracellular route should also be indicated by the passive movement of large solutes, e.g., xylose, sucrose and inulin,

which presumably also take this pathway. However, Maddrell (1980) found that the net flux of these sugars remained unchanged when fluid secretion by tubules of *Rhodnius prolixus* was increased approximately 7-fold by the application of the diuretic hormone mimic, 5-hydroxytryptamine (5-HT). Indeed, if inulin could pass through these intercellular junctions, it would be difficult to imagine how these structures could have reflection coefficients for monovalent ions high enough to create the osmotic gradients required for water movement (Maddrell, 1980; Phillips, 1981). Furthermore, if ion movements did take place via a paracellular route, it would be most difficult to explain the difference in composition between haemolymph and luminal fluid. Maddrell (1980) concluded that water movement across the tubules of *Rhodnius prolixus* occurs largely through the cells, and that the static fluid between the apical microvilli need only be 2-3% hyperosmotic to the haemolymph to cause normal rates of fluid secretion by simple osmosis. As a result of these findings, the Malpighian tubules of *Rhodnius prolixus* would be classified as a "tight" epithelium. Similarly, Williams and Beyenbach (1984) have concluded that the Malpighian tubules of *Aedes aegypti* should be classified as a moderately "tight" epithelium, on the basis of measurements of transepithelial concentration gradients and resistance in this epithelium. However, there is good evidence of some paracellular fluid movement in other insect epithelia (O'Donnell and Maddrell, 1983). Paracellular fluid flow has been suggested in the rectal pads of *Periplaneta americana* and *Calliphora erythrocephala* (Wall *et al.*, 1970; Gupta and Hall, 1983) and the salivary glands of *Calliphora erythrocephala* (Gupta *et al.*, 1978).

Whatever the exact mechanism for coupling ion and water movements, the various models agree that fluid secretion by insect Malpighian tubules is a consequence of solute transport. Ion transport across cell membranes requires the presence of various cell membrane proteins (Scoble *et al.*, 1986) such as:

(1) Uniporters, which facilitate the movement of an ion down its concentration gradient.

(2) Symporters, which facilitate the diffusion of an ion down its concentration gradient associated with the movement of a second ion without a concentration gradient, the latter ion undergoing secondary active transport.

(3) Antiporters, which link the diffusion of one ion down its concentration gradient to the movement of a second ion in the opposite direction.

(4) ATPases, which link the movement of ions to ATP hydrolysis.

(5) **Channels, which form pores through the membrane, allowing passive diffusion of ions down their electrochemical gradients.**

Although ion channels selective for Na^+, Cl^- and Ca^{2+} have been characterized in some epithelia (Van Driessche and Zeiske, 1985), many epithelial cells from vertebrate and invertebrate animals possess a large K^+ conductance in their basolateral membrane which enables K^+ entering the cell via the (Na^++K^+)-ATPase (mentioned later) to return to the serosal side (Hanrahan *et al.*, 1986; reviewed by Lewis *et al.*, 1984). Basolateral K^+ conductance is thought to be important in determining flux through basal cell membrane ion channels and electrogenic cotransporters by its influence on the electrical potential across this membrane (Hanrahan *et al.*, 1986). K^+ channels have also been found in many apical membranes of vertebrate epithelia (for review see Van Driessche and Zeiske, 1985).

Demonstration of a specific basolateral Ca^{2+}-activated K^+ channel in a variety of cells (Latorre and Miller, 1983; Van Driessche and Zeiske, 1985) arose from the fact that cellular Ca^{2+} entry leads to an increase in K^+ permeability in red cells (Gárdos, 1958). Ca^{2+}-activated K^+ channels have also been demonstrated in the apical membrane of mammalian cortical collecting duct (Frindt and Palmer, 1987), cultured epithelial cells derived from dog kidney (Bolivar and Cereijido, 1987), and also in cultured human epithelial cells in which they are involved in cell volume regulation (Hazama and Okada, 1988).

The transition between one or more open or closed conformations, known as "gating", is displayed by most ion channels and is triggered by factors such as voltage, channel blockage or opening by interaction with an appropriate substance, e.g., a ligand binding directly to the channel protein, the indirect action of a diffusible second messenger released upon agonist binding to membrane-bound receptors (via G protein activation), through G protein activation which is coupled directly to the ion channel (Barnard *et al.*, 1989), or may simply be spontaneous (Van Driessche and Zeiske, 1985).

Ramsay (1953) was first to recognize the importance of K^+ transport in Malpighian tubule secretion. He deduced that K^+ was transported actively, from haemolymph to lumen, by analysis of ion distributions and electrochemical gradients in tubules of several insects. Subsequent studies confirmed K^+ as the "prime mover" in generating fluid secretion in most insects (Berridge, 1968; Maddrell, 1969; Maddrell and Klunsuwan; 1973), including *Locusta migratoria* which produces a luminal fluid rich in KCl (Anstee *et al.*, 1979). However, the nature of the ion translocation mechanism, and its endocrine control, are still unclear.

It is generally accepted that an electrogenic cation pump is located on the apical cell membrane (Berridge, 1967; Berridge and Oschman, 1969; Gee, 1976; Maddrell, 1971, 1977; O'Donnell and Maddrell, 1984). This pump mainly transports K^+, but appears to be relatively unspecific, readily transporting other cations under the appropriate conditions (Berridge, 1968; Maddrell, 1977). Such unspecificity would suggest that the cation transported will be that which is most abundant in the intracellular compartment, which is in turn determined by the basal membrane permeability. This model was used by Maddrell (1977) and O'Donnell and Maddrell (1984) to explain why the Malpighian tubules of some insects, such as *Glossina, Aedes*, and *Rhodnius*, have Na^+ as the "prime mover", or require Na^+ and K^+ for fluid secretion. For *Rhodnius prolixus*, it was suggested that the apical cation pump has a higher affinity for Na^+ than K^+, maintaining intracellular Na^+ levels lower than those of K^+. The rates at which these two ions are pumped across the epithelium would therefore depend on the affinity of the pump for the two ions and the rates at which they enter the cell. The latter partly depends on the electrochemical gradient across the basal cell membrane and the permeability of this membrane to the two ions. As a result, small changes in relative permeability of the basal cell membrane to Na^+ and K^+ may cause large changes in the ionic composition of the secreted fluid. The secretion of Na^+-rich fluid by the tubules of *Glossina morsitans* (Gee, 1975) could be simply explained by the basal cell membrane having a higher permeability to Na^+ than other tubules. On this basis, Na^+ would enter the cell faster than K^+, and the apical cation pump, having a higher affinity for Na^+ than K^+, would preferentially transport Na^+ across the apical cell membrane. In contrast, the K^+-rich fluid secreted by the majority of insects, including *Locusta migratoria* (Anstee *et al.*, 1979), could be explained on the basis of the basal cell membrane being highly permeable to K^+, but relatively impermeable to Na^+. K^+ would be the major intracellular ion, due to the activity of the $(Na^+ + K^+)$-ATPase, and therefore, would be the main ion transported by the apical cation pump into the lumen. However, it remains to be established to what extent this model applies to other insect Malpighian tubules.

The presence of an apically located K^+ pump in midgut goblet cells of *Manduca sexta* was proposed (Anderson and Harvey, 1966; Harvey *et al.*, 1983) on the basis of net ^{42}K flux measurements in the short-circuited lepidopteran midgut by Harvey and Nedergaard (1964). Evidence also suggests the presence of an apical K^+ pump in dipteran salivary gland (Prince and Berridge, 1972; Berridge *et al.*, 1975a; Gupta *et al.*, 1978), the sensilla of dipteran laballae (Thurm and Küppers, 1980; Wieczorek, 1982) and lepidopteran labial glands (Hakim and Kafatos, 1974). K^+-sensitive ATPase activity has been reported in midgut preparations of *Manduca sexta* (Wolfersberger, 1979; Wolfersberger *et al.*, 1982; Deaton, 1984; Moffett and Koch, 1988a,b) and tentatively suggested in *Hyalophora cecropia* (Zerahn, 1985), the labellum of *Protophormia terraenovae* (Wieczorek, 1982;

Wieczorek and Gnatzy, 1985; Wieczorek *et al.*, 1986), and may be a candidate for the electrogenic K^+ pump. However, no biochemical evidence exists for the presence of a K^+-ATPase associated with the apical membrane of insect Malpighian tubule cells. Wieczorek *et al.* (1986) pointed out that midgut K^+-ATPase activity in *Manduca sexta* is not homologous to (K^++H^+)-ATPase as suggested by Deaton (1984) and English and Cantley (1984). The latter enzyme so far has only been found in the apical regions of vertebrate gastric fundic mucosa cells, possibly providing the driving force for Cl^- transport into the lumen (Rabon *et al.*, 1983), and is not stimulated by HCO_3^-, nor inhibited by ouabain or SCN^- (Schuurmans Stekhoven and Bonting, 1981). Recently, however, Nakagaki and Sasaki (1988) have suggested that a K^+ pump exists on the apical membrane of the posterior silk glands of *Bombyx mori* and that this may be a (K^++H^+)-ATPase.

Berridge (1967) and Berridge and Oschman (1969) suggested that ion movement into the cell across the basal membrane was a result of coupled Na^+/K^+ exchange. In the majority of tissues examined, the enzyme "pump" responsible for such exchange is a Mg^{2+}-dependent (Na^++K^+)-stimulated ATPase, first demonstrated by Skou (1957). This enzyme is ubiquitous in all cells among animal species, but its activity ranges widely (for review see Schuurmans Stekhoven and Bonting, 1981). Indeed, (Na^++K^+)-ATPase activity has been demonstrated in microsomal preparations of Malpighian tubules and hindgut of *Locusta migratoria* (Anstee and Bell, 1975, 1978; Anstee *et al.*, 1979, 1986), tettigoniids *Homorocoryphus nitidulus vicinus* (Peacock *et al.*, 1976) and *Jamaicana flava*, and *Schistocerca gregaria* (Peacock *et al.*, 1972). Other insect tissues exhibiting (Na^++K^+)-ATPase activity include cockroach nerve cord (Grasso, 1967) and honeybee CNS (Cheng and Cutkomp, 1972). Its occurrence is restricted to cell membranes, with hydrolysis of ATP at the inner side of the membrane resulting in the transferral to the extracellular fluid of $3Na^+$/ATP molecule. This occurs in exchange for $2K^+$ in the majority of cases (for review see Schwartz *et al.*, 1975; and Schuurmans Stekhoven and Bonting, 1981; Cantely, 1981). The electrogenic (Na^++K^+)-ATPase enzyme is believed to be responsible for the cytoplasm of most cells having a high (100-160 mM) K^+ concentration, but relatively low (3-30 mM) Na^+ concentration (Bonting, 1970; Schuurmans Stekhoven and Bonting, 1981).

Controversy exists as to the involvement of the (Na^++K^+)-ATPase in relation to fluid secretion by insect Malpighian tubules, due mainly to the failure of some workers to show an effect on tubule function by its specific inhibitor, ouabain (Skou, 1965; Albers *et al.*, 1968; Wallick *et al.*, 1980). Indeed, in Malpighian tubules of *Locusta migratoria*, ouabain (10^{-3}-10^{-6} mM) was found to inhibit fluid secretion (Anstee and Bell, 1975), whilst in *Rhodnius prolixus*, ouabain (>$2x10^{-7}$ mM) stimulated the rate at which these tubules transported Na^+ and fluid into the lumen (Maddrell and Overton, 1988). Some possible explanations for the conflicting reports are given in the review by Anstee and Bowler

(1979). The $(Na^+ + K^+)$-ATPase sensitivity to cardiac glycosides, such as ouabain, also differs markedly between species (Wallick *et al.*, 1980). It has been suggested that such variation in sensitivity results from differences in the rates of dissociation of the glycoside from different source enzymes, but not the rates at which it binds (Wallick *et al.*, 1980; Erdmann and Schoner, 1973). However, some reports suggest that $(Na^+ + K^+)$-ATPase sensitivity cannot be explained solely on the basis of such dissociation rates (Wallick *et al.*, 1980; Pitts *et al.*, 1977).

Maddrell (1971) proposed a model for active ion transport across the Malpighian tubules of *Carausius morosus* and *Calliphora erythrocephala* which excluded the $(Na^+ + K^+)$-ATPase pump in order to overcome the problem of their ouabain-insensitivity. The model suggests that an electrogenic "pump", stimulated by Na^+, actively transports K^+ into the cell across the basal cell membrane. Na^+ and Cl^-, however, enter the cell passively. At the apical surface Cl^- diffuses passively into the lumen, whereas Na^+ and K^+ are transported by electrogenic "pumps".

For *Rhodnius prolixus* (Fig. 1.1), Maddrell (1971, 1980) suggested that Na^+ and K^+ were actively transported (solid lines) across the basal membrane, with Cl^- entry being passive (dashed lines). These ions would then be transported across the apical cell membrane by three electrogenic pumps. The model also indicated that some passive entry of Na^+ and K^+ would occur. However, Maddrell (1971) questioned the possibility of passive K^+ entry on the basis of the intracellular K^+ concentration being higher then that of the extracellular fluid, resulting in conditions being electrochemically unfavourable for such passive entry.

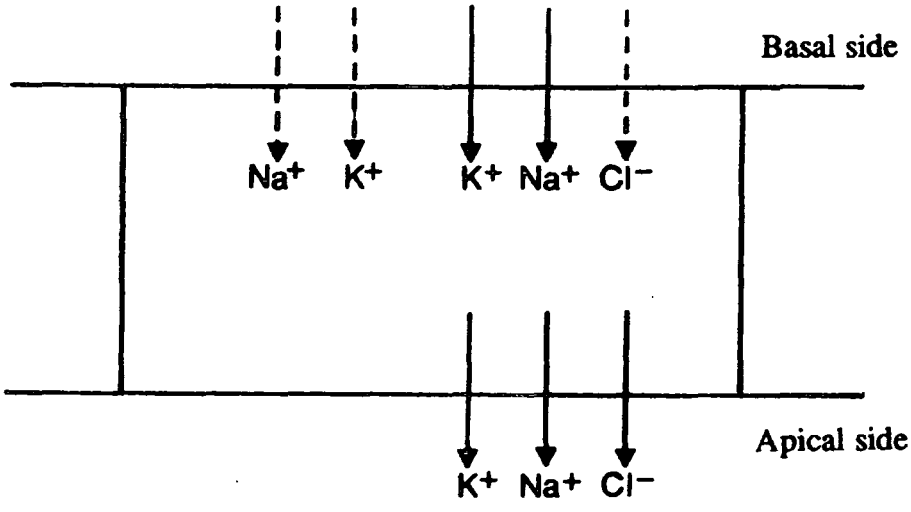


Fig 1.1 (Maddrell, 1971, 1980)

A later model (Fig. 1.2) by Maddrell (1977), mentioned earlier, was proposed to apply to ion transport by any insect Malpighian tubule, regardless of whether K^+ or Na^+ was the "prime mover". This relied on passive ion entry across the basal membrane, with active transport of K^+ and/or Na^+ by an electrogenic cation pump, and passive movement of Cl^-, across the apical membrane . Passive movement of Cl^- across both membranes was also suggested for the Malpighian tubules of *Musca domestica* (Dalton and Windmill, 1980).

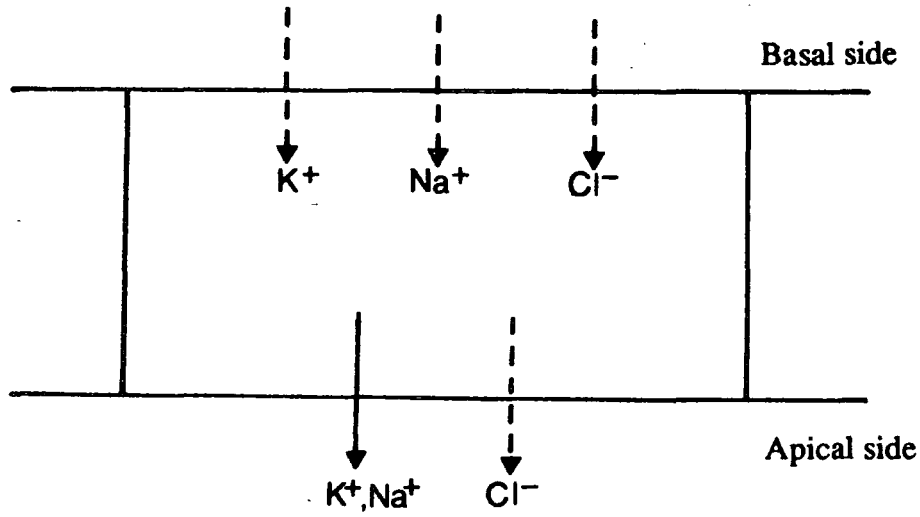


Fig 1.2 (Maddrell, 1977)

The previous model (Maddrell, 1977) was modified by O'Donnell and Maddrell (1984) on finding that the basal cell membrane of Malpighian tubules of *Rhodnius prolixus* was largely impermeable to Na^+ and Cl^-, and that blockers of NaCl entry (Palfrey and Rao, 1983), furosemide and bumetanide, were effective. O'Donnell and Maddrell (1984) (Fig. 1.3) proposed that, during rapid fluid secretion stimulated by 5-HT or the naturally occurring diuretic hormone, a Na^+-K^+-2Cl^- cotransporter, located on the basal membrane, was responsible for the movement of Na^+, K^+ and Cl^- into the cell, thus allowing the movement of K^+ against its concentration gradient. Again, a catholic apical cation pump was proposed for the extrusion of K^+ and/or Na^+ from the cell, with Cl^- exit being passive. Thus, the fluid secreted would contain approximately equal concentrations of K^+ and Na^+ (Maddrell, 1969). However, the fluid secreted by non-stimulated tubules contained only low levels of Na^+ (Maddrell and Overton, 1988). Williams and Beyenbach (1984) also produced evidence of coupled cation-Cl^- movement during stimulated fluid secretion by the Malpighian tubules of *Aedes aegypti*.

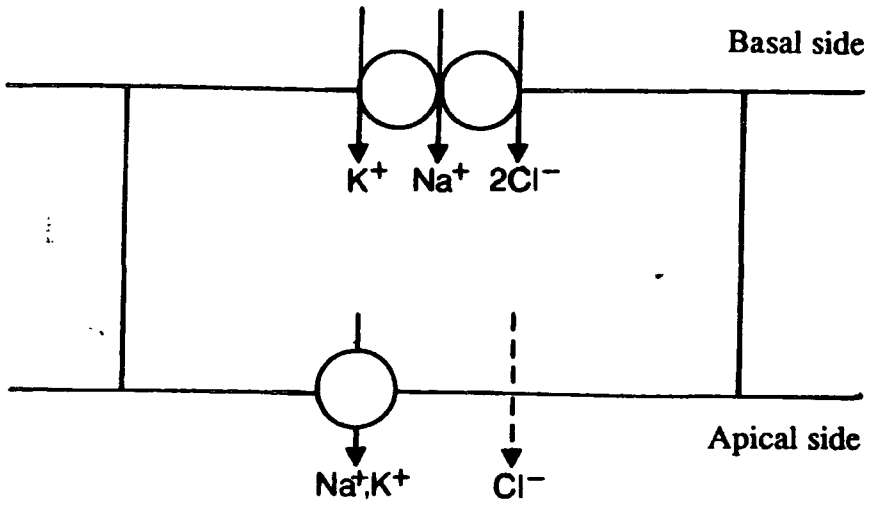


Fig 1.3 (O'Donnell and Maddrell, 1984)

Recently, Maddrell and Overton (1988), from ouabain-binding studies on tubules of *Rhodnius prolixus*, indicated that a ouabain-sensitive (Na^++K^+)-ATPase activity exists on the basal, but not on the apical cell membrane. As mentioned earlier, ouabain was found to stimulate the rate of Na^+ and fluid transport (Maddrell and Overton, 1988). These workers suggested (Fig.1.4) that the Na^+-K^+-$2Cl^-$ cotrantransporter, located on the basal membrane, was responsible for the movement of these ions into the cell, as suggested previously (O'Donnell and Maddrell, 1984), and that under unstimulated conditions the basal (Na^++K^+)-ATPase would serve to regulate intracellular Na^+, keeping levels of this ion lower than those of K^+. Thus, K^+ and Cl^- would be the predominant ions in the cytoplasm, the former being transported by the cation pump, and the latter moving passively, across the apical membrane. Indeed, unstimulated tubules of *Rhodnius prolixus* slowly secrete fluid that is largely a solution of KCl. Gee (1976) has also suggested that a Na^+/K^+ pump, on the basal cell membrane of resting Malpighian tubules of *Glossina morsitans*, may be necessary to maintain intracellular ion concentrations.

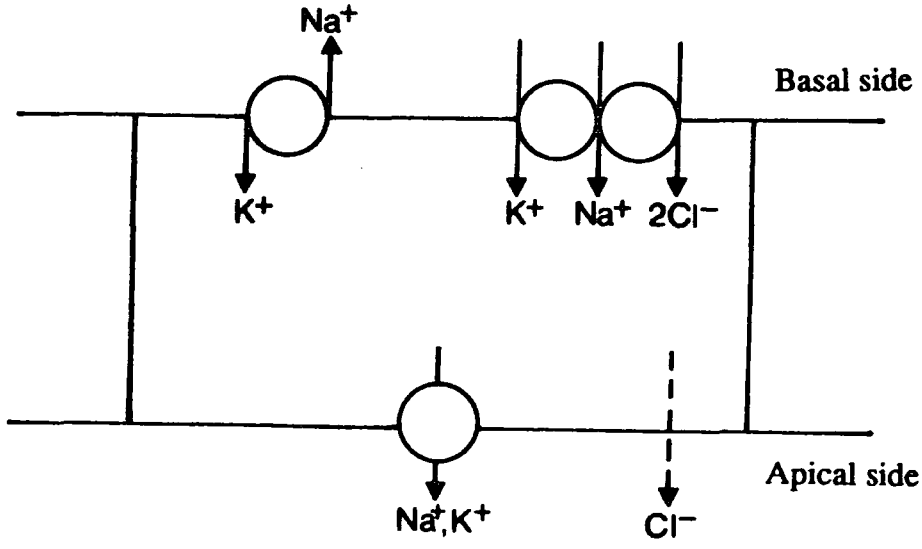


Fig 1.4 (Maddrell and Overton, 1988)

On the basis of the Maddrell and Overton model for *Rhodnius prolixus*, ouabain would inhibit the $(Na^+ + K^+)$-ATPase, but not the cotransporter, resulting in faster ion entry into the main body of the cell, and thus, a faster rate of ion transport into the lumen. As ion movements are followed by water movements, ouabain treatment would be expected to, and indeed does, increase fluid secretion. Inhibition of the $(Na^+ + K^+)$-ATPase would also result in a rise in intracellular Na^+ concentration, and thus, the production of a Na^+-rich urine - as was observed (Maddrell and Overton, 1988). Paracellular diffusion of Na^+ into the lumen was largely discounted on the basis that ouabain made the luminal electrical potential more positive, which would slow down such diffusion (Maddrell and Overton, 1988). During rapid fluid secretion, as occurs on stimulation with the diuretic hormone analogue 5-HT, both the cotransporter and the apical cation pump are believed to operate more than 100 times faster than in the unstimulated tubule (O'Donnell and Maddrell, 1984), with no corresponding increase in Na^+/K^+ exchange (Maddrell and Overton, 1988). As a result, the basal $(Na^+ + K^+)$-ATPase would have no significant effect on the very rapid flow of ions entering the cell, with the result that more Na^+ would be transported across the apical membrane.

Morgan and Mordue (1983) proposed that both Na^+ and K^+ could enter the Malpighian tubule cells of *Locusta migratoria* passively. A K^+-dependent Cl^- pump has been reported in locust rectum (Hanrahan and Phillips, 1984) and was tentatively suggested by Morgan and Mordue (1983) for the active transport of this anion across the basal membrane. An active transport mechanism was proposed to transport Na^+ and K^+ across the apical membrane, followed by passive movement of Cl^- (Fig. 1.5).

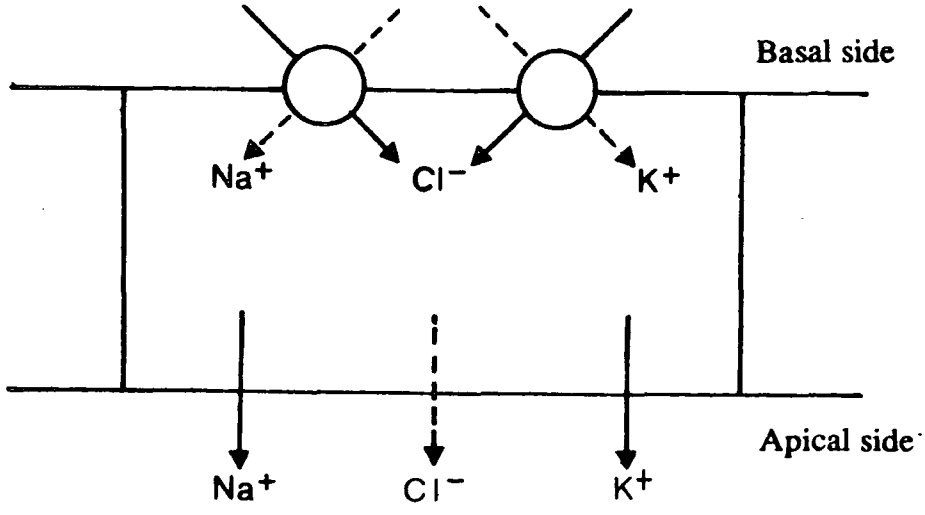


Fig. 1.5 (Morgan and Mordue, 1983)

Baldrick *et al.* (1988), from microelectrode studies on the Malpighian tubules of *Locusta migratoria*, found that the basal cell membrane potential (V_B) was largely accounted for by the selective permeability of this membrane to K^+ and its relative impermeability to Na^+ and Cl^-. These workers suggested (Fig. 1.6) that the considerable concentration and electrical gradients across the basal membrane would provide some Na^+ entry, with Cl^- entry by some electroneutral transport mechanism, possibly stimulated by extracellular K^+. The entry of Cl^- by electroneutral cotransport with K^+ and/or Na^+ seemed feasible on the basis of the effects of bumetanide and furosemide on the electrophysiology of this epithelium, and their inhibition of fluid secretion (Baldrick, 1987). However, the evidence for Na^+-K^+-$2Cl^-$ cotransport in tubules of this insect was less convincing than that established in *Rhodnius prolixus*. Indeed, it was suggested that Cl^- entry was not necessarily dependent on cotransport with Na^+, and the possibility of $2K^+$-$2Cl^-$ cotransport was not ruled out (Baldrick *et al.*, 1988).

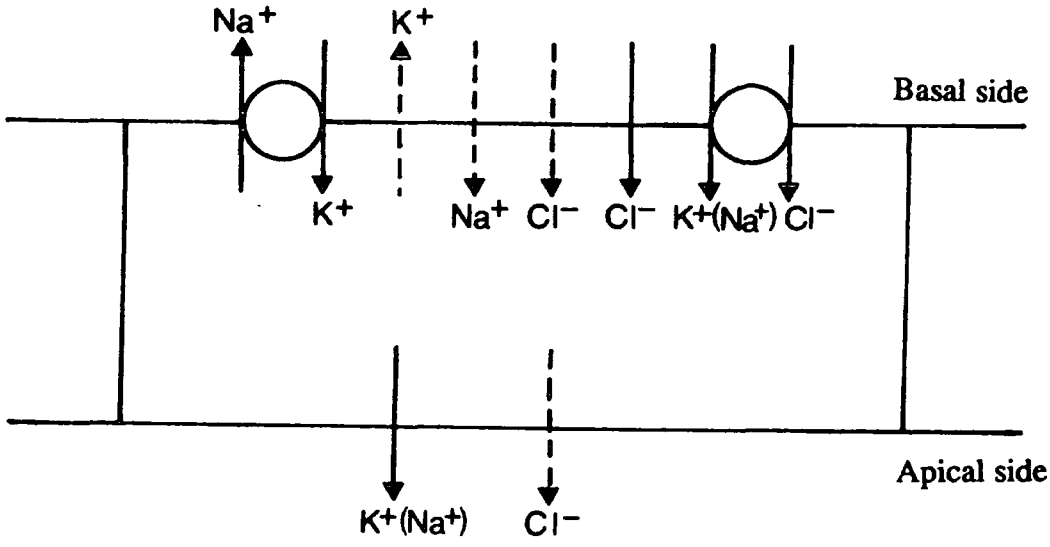


Fig. 1.6 (Baldrick *et al.*, 1988)

It was thought that passive influx of K^+, as suggested by Morgan and Mordue (1983), was unlikely on the grounds that V_B was close, but more positive than the estimated membrane equilibrium potential (E_K). As a result, only small variations in membrane potential would determine whether K^+ would move passively in or out of the cell. Maintenance of the Na^+ gradient across the basal membrane was likely to be achieved by the (Na^++K^+)-ATPase "pump". Baldrick *et al.* (1988) also suggested that K^+, and probably Na^+, would be actively pumped across the apical cell membrane, whilst Cl^- exit to the lumen would occur passively aided by the large favourable electrical gradient. These workers thought that factors which regulate ion movement across the apical membrane could act by stimulation of the cation pump and/or by controlling the supply of Cl^- as counterion.

The passive movement, or active transport of Cl^- across various insect tubules has been mentioned in the previous models. In addition, Cl^- movement across the salivary glands of *Calliphora erythrocephala* (Berridge *et al.*, 1976) is thought to be passive, although the possibility of a Cl^- pump responsible for importing this anion across the basal cell membrane could not be discounted.

Since there seems to be a favourable electrochemical gradient for the movement of Cl^- out of the cell many tissues, Cl^- movement across the exit membrane may be downhill if the conductance is sufficiently high (White, 1986; Frizzell *et al.*, 1979a). Such a passive exit mechanism has been proposed for a wide variety of epithelia including the Malpighian tubules of *Locusta migratoria* (Morgan and Mordue, 1983; Baldrick *et al.*, 1988), shark renal proximal tubules, mammalian nephron, canine tracheal epithelium, frog cornea and rabbit cortical collecting duct (Greger and Schlatter, 1984; Greger, 1985; Sawyer and Beyenbach, 1985b; Welsh *et al.*, 1983; Candia 1980; Sansom *et al.*, 1984).

Various types of Cl^- transport mechanisms have been studied in several vertebrate and invertebrate epithelia. These include electroneutral Na^+-dependent cotransport of Cl^- (or Na^+-K^+-$2Cl^-$ cotransport), $Cl^-/HCO_3^-(OH^-)$ exchange, and electrogenic or electroneutral Cl^- secretion or absorption.

The electroneutral Na^+-K^+-$2Cl^-$ cotransporter is inhibited by sulfamoylbenzoic acid derivatives ("loop diuretics") including furosemide, bumetanide, piretanide and benzmetanide, but unaffected by agents such as amiloride, stilbene disulfonates or thiazide diuretics, thus distinguishing it from Na^+/H^+ or Cl^-/HCO_3^- exchange and thiazide-inhibitable Na^+-Cl^- cotransport found in flounder urinary bladder (for review see O'Grady *et al.*, 1987). Na^+-K^+-$2Cl^-$ cotransport has been identified in epithelia which transport Cl^- against an electrochemical potential difference such as cortical and medullary thick ascending limb of Henle's loop (TALH), shark rectal gland, flounder intestine, MDCK cells, LLC-PK1 cells and T-84 cells, kidney distal tubule of *Amphiuma*, pancreatic acinar cells and parotid acinar cells. It has also been identified in non-epithelial cells; these include avian, human and ferret erythrocytes, Ehrlich ascites tumour cells, human and hamster fibroblasts, vascular smooth muscle and endothelial cells, cultured chick heart cells, BALB/c 3T3 cells and squid axon (for review see O'Grady *et al.*, 1987).

Various other transport systems for these ions have been postulated which are also inhibitable by these loop diuretics. These include K^+-Cl^-, Na^+-Cl^- and Na^+-K^+ cotransport, the primary active Cl^- pump, and Na^+/Na^+ and K^+/K^+ exchange (for review see Geck and Heinz, 1986). It is believed that these systems are partial aspects of the Na^+-K^+-$2Cl^-$ cotransporter and not distinct systems (Geck and Heinz, 1986). However, there is some

evidence for distinct Na^+-Cl^- and Na^+-K^+-$2Cl^-$ cotransport systems (for review see Eveloff and Warnock, 1987a; Cremaschi *et al.*, 1987a,b; O'Grady *et al.*, 1987). Indeed, as mentioned previously, Na^+-Cl^- cotransport proposed in flounder urinary bladder appears to be pharmacologically distinct from Na^+-K^+-$2Cl^-$ cotransport on the basis of its inhibition by thiazide compounds, but not by furosemide or bumetanide (Stokes, 1984; O'Grady *et al.*, 1987). However, a loop diuretic-sensitive, K^+-independent Na^+-Cl^- cotransport system appears to exist in canine trachea (O'Grady *et al.*, 1987). It has been suggested that the Na^+-K^+-$2Cl^-$ cotransporter may be a multimeric protein complex, dissociating its K^+-dependency and shifting to Na^+-Cl^- cotransport depending on the physiological state of the cell (O'Grady *et al.*, 1987). This might explain situations where Na^+-K^+-$2Cl^-$ and Na^+-Cl^- cotransport have been described in the same preparation, e.g., TALH or Ehrlich ascites cells (for review see O'Grady *et al.*, 1987).

Whether secretory or absorptive, all Na^+-Cl^- cotransport mechanisms postulate active uphill transport of Cl^- into the epithelial cell driven by the inward movement of Na^+ down a favourable electrochemical gradient (Frizzell *et al.*, 1979a; Hanrahan and Phillips, 1983) which is, in turn, maintained by the basal (Na^++K^+)-ATPase pump. Therefore, Cl^- entry is by "secondary active transport" (Frizzell *et al.*, 1979a; Shorofsky *et al.*, 1982). Similarly, the energy required for Cl^- cotransport with Na^+ and K^+ is apparently provided by the cation electrochemical gradient (Hanrahan and Phillips, 1984).

The models for various epithelia differ in the proposed mechanism for Cl^- exit from the cell. K^+- and HCO_3^--coupled steps for Cl^- exit have been suggested where serosal membrane conductance is not sufficient to permit diffusion of Cl^- from the cell (Fig. 1.7, steps ii and iii; for review see Hanrahan and Phillips, 1983). In the presence of electroneutral Na^+-Cl^- coentry, the electrogenicity of transepithelial Cl^- absorption in flounder intestine is explained by some Na^+ leaking back to the mucosal side from the lateral intercellular spaces via tight junctions (Fig. 1.7, step i; Field *et al.*, 1978 ;for review see Hanrahan and Phillips, 1983).

Na^+-coupled electrogenic Cl^- secretion is found in a wide variety of epithelia, e.g., cornea, rat ilium, dogfish rectal gland and killifish operculum (for review see Frizzell *et al.*, 1979a; Hanrahan and Phillips, 1983). In these systems (Fig. 1.8) it is believed that Na^+ is actively recycled at the serosal border while Cl^- moves passively from cell to mucosal side through a cAMP-enhanced Cl^- conductance (see Hanrahan and Phillips, 1983).

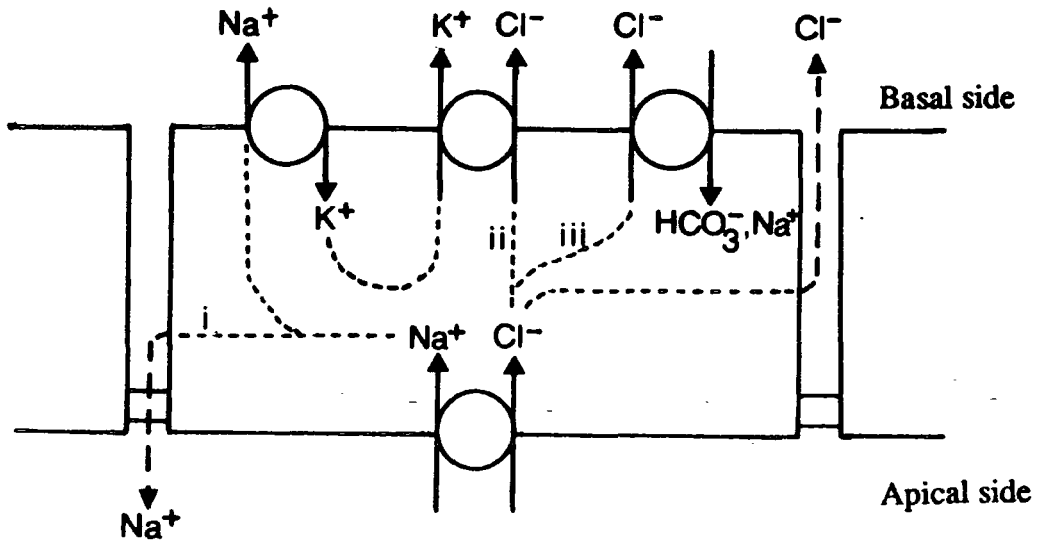


Fig. 1.7 (for review see Hanrahan and Phillips, 1983)

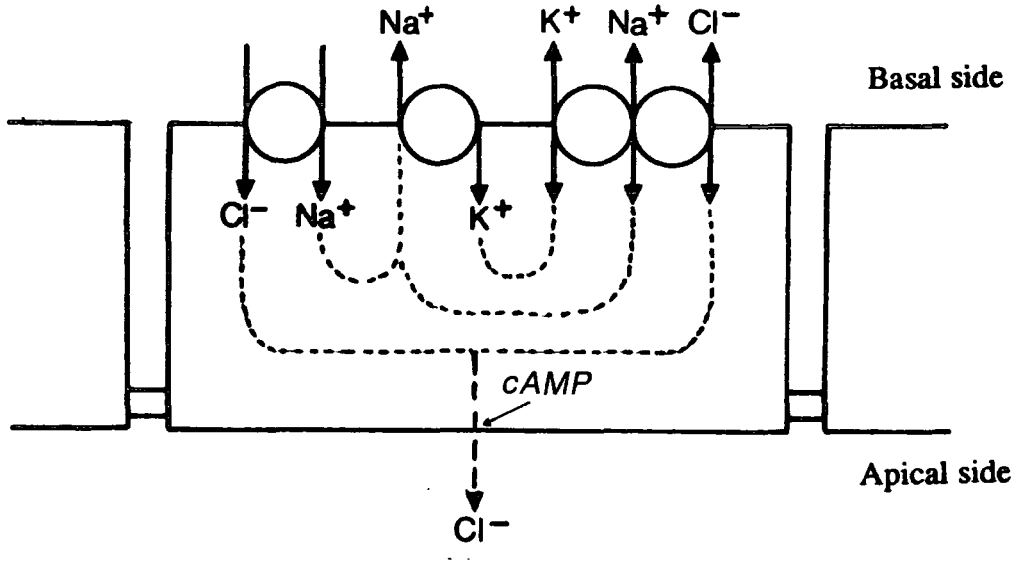


Fig. 1.8 (for review see Hanrahan and Phillips, 1983)

K^+-Cl^- cotransport has been extensively studied in low K^+ sheep red cells and human erythrocyte where it is important in cell volume regulation, being activated by cell swelling or thiol alkylation (for review see O'Grady *et al.*, 1987). However, this cotransport system is relatively insensitive to loop diuretics compared to the Na^+-K^+-$2Cl^-$ cotransport system. The lack in similarity between the electrophysiological response of Malpighian tubules from *Locusta migratoria* (Baldrick *et al.*, 1988) and *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) to furosemide and bumetanide could therefore be explained on the basis of K^+-coupled Cl^- entry in the former insect. K^+-Cl^- cotransport has also been reported in *Necturus* gallbladder, and red blood cells of ducks and dogs (for review see

Eveloff and Warnock, 1987a). The K^+-Cl^- cotransport system in rabbit renal basolateral membrane vesicles is not believed to be a Na^+-K^+-Cl^- cotransport system (Eveloff and Warnock, 1987b). From the various findings outlined it is difficult to establish the relationship between the Na^+-K^+-$2Cl^-$, Na^+-Cl^- and K^+-Cl^- cotransport systems at the present time.

A second widely accepted mechanism of epithelial Cl^- transport involves Cl^-/HCO_3^- exchange at the entry step (Fig. 1.9; see Hanrahan and Phillips, 1983). Cl^-/HCO_3^- exchange has been reported in a number of tissues (Gerencser and Lee, 1983) including frog skin, fish gills, turtle bladder, rabbit colon, anal papillae of *Aedes aegypti* (see Hanrahan and Phillips, 1983), and the rectal salt gland epithelium of *Aedes dorsalis*, where 1:1 exchange of Cl^- for HCO_3^- is thought to occur across the basolateral membrane, with Na^+/H^+ and K^+/H^+ exchange playing no part in transepithelial HCO_3^- transport (Strange and Phillips, 1984). Carbonic anhydrase (c.a.) activity also plays a critical role in salt gland HCO_3^- secretion. Frizzell *et al.* (1979a) suggested that the energy for uphill Cl^- influx may be energized in part by an ATPase or by a passive outflow of HCO_3^- which moves out the cell down its favourable electrochemical gradient. Recently, it was suggested that Cl^- could leave the cells of *Necturus* gallbladder by a diffusive pathway, and a non-diffusive pathway in which Na^+ and HCO_3^- move into the cell in exchange for Cl^- in a process that may or may not be electroneutral (Guggino *et al.*, 1983; see Reuss *et al.*, 1983; Reuss, 1987). This mechanism has also been proposed for intestinal cells of *Amphiuma* (White, 1986).

Early work on both absorptive and secretory epithelia showed a clear link between Na^+ and Cl^- transport which led to the initial hypothesis of Na^+-Cl^- cotransport (see Frizzell *et al.*, 1979a). Such linkage, however, may be direct, with Na^+ and Cl^- transport by the same cotransporter, or indirect via two parallel transporters for Na^+ and Cl^-. The latter scheme constitutes the "parallel exchange hypothesis", in which Na^+/H^+ and Cl^-/HCO_3^- (or Cl^-/OH^-) exchangers located on the same membrane mediate the net flux of Na^+ and Cl^- (Fig. 1.9). Indirect coupling would be provided by cell pH; H^+ exit resulting in the build up of HCO_3^- within the cell, and activation of the Cl^-/HCO_3^- exchanger.

A Na^+/H^+ and Cl^-/HCO_3^- double exchange has been revealed in many epithelia with a coupling between Na^+ and Cl^- due to cellular pH (see Cremaschi *et al.*, 1987a), including rabbit and human intestine (for review see O'Grady *et al.*, 1987), *Necturus* gallbladder, mouse medullary TALH, human lymphocytes, and also in the red blood cells of *Amphiuma* (see Eveloff and Warnock, 1987a), and rabbit caecum (Hatch and Freel, 1988) and gallbladder (Cremaschi *et al.*, 1987a). Indeed, double Na^+/H^+ and Cl^-/HCO_3^- exchange has been described as the predominant or sole mechanism of apical membrane NaCl entry in *Necturus* gallbladder (Reuss, 1984, 1987). There is also evidence of parallel Na^+/H^+ and

$Cl^-/HCO_3^-(OH^-)$ antiporters in rabbit proximal convoluted tubule (Baum, 1987), confirming that the latter exchange mechanism will also except other oxyanions.

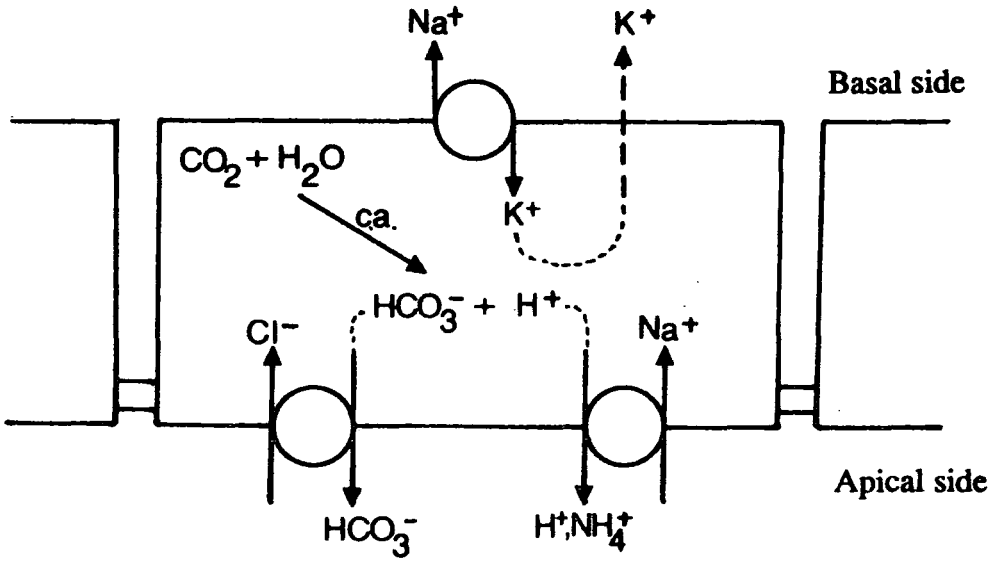


Fig. 1.9 (see Hanrahan and Phillips, 1983)

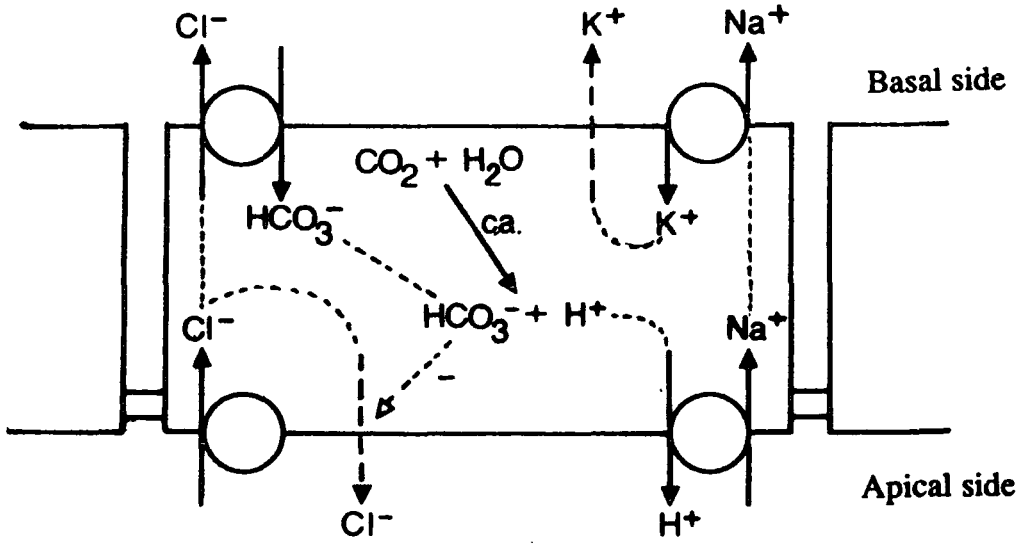


Fig. 1.10 (see Hanrahan and Phillips, 1983)

For electrogenic Cl^- transport at the apical membrane of *Amphiuma* intestine, Na^+/H^+ exchange at the apical membrane, which reduces the backflux of Cl^- from cell to lumen, and Cl^-/HCO_3^- exchange at the basal membrane (Fig. 1.10; see Hanrahan and Phillips, 1983) were proposed by White (1980).

There is evidence that electrogenic Cl^- transport across the apical membrane of locust rectum is not driven by Na^+, K^+, H^+, OH^-, or HCO_3^- gradients (Hanrahan and

Phillips, 1983; reviewed by Phillips *et al.*, 1986; Lechleitner and Phillips, 1988) as proposed by the various cotransport models (Fig, 1.7-1.10). As a result, Hanrahan and Phillips (1983) suggested that active anion transport in locust rectum is a primary transport process probably involving an apical anion-stimulated ATPase. Interestingly, this electrogenic Cl^- transport is activated and stimulated by luminal K^+ (Hanrahan and Phillips, 1983, 1984). Electrogenic Cl^- transport across the integument of *Manduca sexta* is also stimulated by K^+ (Cooper *et al.*, 1980). Indeed, K^+-stimulated Cl^- entry across the basal membrane of the Malpighian tubules of *Locusta migratoria* was indicated, as mentioned previously, by Baldrick *et al.* (1988) and by the present study. Thiocyanate strongly inhibited anion-stimulated ATPase activity in the rectum of *Schistocerca gregaria* (Lechleitner and Phillips, 1988), and Cl^--stimulated ATPase activities and Cl^- transport in *Aplysia* intestine (Gerenscer and Lee, 1985) and larval dragonfly (Komnick *et al.*, 1980).

Since its discovery by Kasbekar *et al.* (1965) anion-sensitive ATPase activity has been demonstrated in microsomal fractions of many tissues (reviewed by Gerenscer and Lee, 1983) including *Necturus* and bullfrog oxyntic cells, dog gastric mucosa and submandibular gland, rat and rabbit salivary gland, mammalian pancreas, rainbow trout gill, midgut of *Hyalophora cecropia*, plasma membrane fractions of rabbit and cat pancreas, cattle rumen and mouse ascites tumour cells, and erythrocyte membranes (for review see Schuurmans Stekhoven and Bonting, 1981). Indeed, such activity has been described in the Malpighian tubules of *Locusta migratoria* (Anstee and Fathpour, 1979, 1981; Fathpour and Anstee, 1981; present study), and recta of dragonfly (*Aeshna* sp., Komnick *et al.*, 1980) and *Schistocerca gregaria* (Lechleitner and Phillips, 1988). It has been proposed that the anion-stimulated ATPase is linked to active transport of anions (Komnick *et al.*, 1980; Bornancin *et al.*, 1980; Gerenscer and Lee, 1983, 1985), especially Cl^- and HCO_3^-, and is referred to as Mg^{2+}-dependent (Cl^-+HCO_3^-)-stimulated ATPase, HCO_3^--stimulated ATPase or Cl^--stimulated ATPase.

The major location of anion-sensitive ATPase activity, however, appears to be on the inner mitochondrial membrane, and it would seem that some early findings of this enzyme activity in plasma membranes was due to mitochondrial membrane contamination (for review see Schuurmans Stekhoven and Bonting, 1981). However, several invertebrate epithelia contain anion-stimulated ATPase in membrane fractions with low mitochondrial contamination (Lechleitner and Phillips, 1988), including microsomal fractions from *Locusta migratoria* (Anstee and Fathpour, 1981). Although Fathpour (1980) hinted at the involvement of HCO_3^--ATPase in fluid secretion by the Malpighian tubules of *Locusta migratoria*, this worker was unable to ascertain whether cellular localization of this enzyme was mitochondrial or non-mitochondrial in origin, or both. Similarly, in rectal tissue *Schistocerca gregaria*, Herrera *et al.* (1978) observed anion-stimulated ATPase activity,

which was stimulated by the addition of Cl^-, sulphate and nitrite, but no attempt to determine the sub-cellular source of this activity was made. Recently, however, Lechleitner and Phillips (1988) provided evidence for an apical plasma membrane anion-stimulated ATPase, which may be responsible for the active transport of Cl^- in locust rectum. An active Cl^- extension process in the basolateral membrane of *Aplysia* intestinal epithelium has also been reported by Gerenscer (1983), and this electrogenic, Na^+-independent mechanism may be a (Cl^-+HCO_3^-)-stimulated ATPase (Gerenscer and Lee, 1985). Whilst anion-sensitive ATPase has been implicated in Cl^-/HCO_3^- exchange in the rectum of *Schistocerca gregaria* (Herrera *et al.*, 1978), H^+/HCO_3^- transport in rat submandibular gland (Simon *et al.*, 1972a) and HCO_3^- transport and Na^+/H^+ exchange in the brush border membrane of the renal proximal tubule (Liang and Sacktor, 1976), its role in ion transport and/or regulation of cellular pH in the Malpighian tubules of *Locusta migratoria* is unclear.

Several theories have been proposed to explain the transport of ions and water through various epithelia, described previously, but definite localization of the cytological structures primarily responsible for active transport, upon which such theories are based, is largely lacking. An attempt was made in the present study to separate and partially purify the basolateral and apical cell membranes of the Malpighian tubules of *Locusta migratoria*, and on the basis of biochemical studies and electron microscopy, evidence was provided for the subcellular localization of the (Na^++K^+)-ATPase and the anion-stimulated ATPase.

Wachstein and Meisel (1957) were first to use a modification of the Gomori Type heavy metal precipitation reaction (Gomori, 1952) for the subcellular localization of Mg^{2+}-activated ATPase (Mg^{2+}-ATPase). This method was subsequently applied to a variety of tissues (Ernst, 1972a). Lead phosphate, the reaction product, was often associated with the extracellular side of the plasma membranes of epithelial cells. On the basis of independent biochemical, physiological and morphological studies, these sites of enzyme activity were thought to function in sodium transport (Wachstein and Meisel, 1957; Ernst and Philpott, 1970; Ernst, 1972a). However, the functional significance of Mg^{2+}-ATPase and its relationship to the transport system remains unclear (Bonting, 1970; Farquhar and Palade, 1966; Tormey, 1966; Ernst, 1972a).

New interest in ATPase cytochemistry was stimulated by the discovery of Mg^{2+}-dependent (Na^++K^+)-stimulated ATPase activity in crab nerves (Skou, 1957). Such activity was abolished by the cardiac glycoside, ouabain (strophanthin-G) (Skou, 1957), an inhibitor of active sodium transport (Schatzmann, 1953). Grasso (1967), as mentioned previously, was first to demonstrate (Na^++K^+)-ATPase activity in insect preparations using nerve microsomal fractions of *Periplaneta americana*. Subsequently, numerous demonstrations of (Na^++K^+)-ATPase activity in insect epithelia have been reported,

including the recta and Malpighian tubules of *Locusta migratoria* (see Anstee and Bowler, 1984).

The involvement of (Na^++K^+)-ATPase in active transport is now well established (Schuurmans Stekhoven and Bonting, 1981), but, with the possible exception of work with red blood cell ghosts (Marchesi and Palade, 1967), attempts to cytochemically localize the enzyme by various modifications of the Wachstein and Meisel (1957) technique have met with little success (Farquhar and Palade, 1966; Tormey, 1966; Ernst and Philpott, 1970; see Ernst, 1972a). As a result the method has been seriously criticized (Moses *et al.*, 1966; Moses and Rosenthal, 1968; see Ernst, 1972a) with much controversy regarding the validity of the technique for demonstration of sites of enzyme activity (see Ernst, 1972a). Several problems were encountered when applying the Wachstein and Meisel (1957) method, summarized by Ernst (1972a,b) as follows:

(1) Lead strongly inhibits Mg^{2+}-ATPase and particularly (Na^++K^+)-ATPase activity.

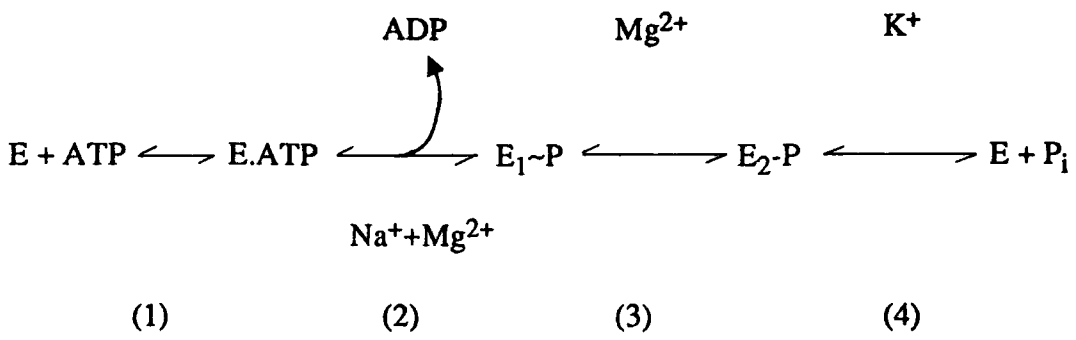
(2) Lead catalyzes a non-enzymatic hydrolysis of ATP which may contribute as a source of inorganic phosphate (P_i) to artificial deposits, but does not in itself account for all of the reaction product localized.

(3) Reaction product contains precipitated nucleotide as well as lead and P_i.

(4) The components of the incubation medium interact such that changes in the lead to ATP ratios in the medium often lead to changes in the pattern of reaction product deposition.

Another problem encountered was insensitivity of reaction product deposition to ouabain (Marchesi and Palade, 1967; Mizuhira *et al.*, 1970). Furthermore, the recommended fixation procedures were also thought to inhibit (Na^++K^+)-ATPase activity (Ernst and Philpott, 1970).

In view of the problems inherent with the Wachstein-Meisel (ATP-Pb) method (1957), an alternative was developed to avoid the use of ATP as the substrate, lead as the capture ion and gluteraldehyde as the fixative (Ernst, 1972a,b). The hydrolysis of ATP by (Na^++K^+)-ATPase is thought to occur in at least four steps (see Schuurmans Stekhoven and Bonting, 1981) as shown overleaf.



(1) The binding of ATP to the enzyme molecule (E).

(2) A Na^+- and Mg^{2+}-dependent phosphorylation of the enzyme, accompanied by breakdown of ATP to ADP.

(3) A conformational change in the enzyme molecule from a state with high Na^+ affinity (E_1) to one with high K^+ affinity (E_2), during which the Na^+ binding sites are turned inside-out.

(4) A K^+-stimulated dephosphorylation, during which the K^+-binding sites turn inward and the enzyme returns to its original conformation.

Thus, (Na^++K^+)-ATPase may be thought of in the dual role of a Na^+-dependent kinase using ATP as substrate, and a K^+-dependent phosphatase (Schuurmans Stekhoven and Bonting, 1981). K^+-dependent phosphatase activity was first described in red cell membranes (Judah *et al.*, 1962), with subsequent studies indicating that such activity was most likely a part (step 4) of the (Na^++K^+)-ATPase transport complex (see Schuurmans Stekhoven and Bonting, 1981). Substrates used to assay K^+-dependent phosphatase activity, e.g., *p*-nitrophenyl phosphate (NPP) and acetylphosphate, lead to the direct formation of E_2-P, which was then dephosphorylated (see Schuurmans Stekhoven and Bonting, 1981).

Ernst (1972a,b), employing the avian salt gland as a model transport tissue, indicated that a cytochemical procedure for K^+-dependent phosphatase activity might provide an alternative method for localizing the (Na^++K^+)-ATPase transport enzyme complex. NPP has provided an excellent substrate for the enzyme since its hydrolysis yields coloured nitrophenol (NP) as well as inorganic phosphate (P_i). The amount of NP produced is determined spectrophotometrically to determine enzyme activity quantitatively under cytochemical conditions, whilst P_i is precipitated with the heavy metal, strontium. Strontium has been found to inhibit the enzyme less than other heavy metals (Ernst, 1972a); attempts to use calcium ions as the capture agent (Padykula and Gauthier, 1963) proved

unsatisfactory as gross diffusion artifacts were produced (Tunik, 1971) unless the calcium concentration was raised to a level at which almost complete enzyme inhibition occurred (Ernst, 1972a). This worker also found that, unlike lead, strontium does not catalyze non-enzymatic hydrolysis in ATP-containing media.

In the Wachstein-Meisel type of incubation medium, the Mg^{2+}-ATPase activity would represent approximately 40% of the total ATPase activity, masking the (Na^++K^+)-ATPase activity, whilst K^+-activated NPPase activity would represent about 80% of the total NPPase activity. However, as much as 87% of the K^+-activated NPPase activity could be inhibited by 20 mM strontium, the concentration used by Ernst (1972a). The pH optimum for the enzyme in the absence of strontium extended over a broad range of pH 8.0-9.0, with maximal activity at pH 8.5 (Ernst, 1972a). Addition of 20 mM strontium to the incubation medium resulted in a shift of the optimum to pH 7.5 (Ernst, 1972a). Therefore, the percentage of inhibition due to strontium could be lowered by altering the pH from alkaline to neutral pH (Ernst, 1972a). However, the alkaline pH, although suboptimal for the enzyme, was prerequisite for the cytochemical localization procedure to ensure adequate trapping of inorganic phosphate by Sr^{2+}. Another problem at the alkaline pH was that alkaline phosphatase activity could also give a positive result. Thus, in rat kidney tubules, the brush border was heavily stained, even in the presence of ouabain (Firth, 1974). That staining was due to alkaline phosphatase activity was confirmed by its abolition by the alkaline phosphatase inhibitor, *L*-tetramisole (Firth, 1974). Staining which occurred on the basal and lateral plasma membranes which was unaffected by *L*-tetramisole, but abolished by ouabain, suggested the presence of ouabain-sensitive ATPase activity in these regions (Firth, 1974).

Fixation of tissues for use in cytochemical studies also caused problems. (Na^++K^+)-ATPase activity was markedly sensitive to aldehyde fixation, although not all aldehydes were equally inhibitory; 0.5% gluteraldehyde completely inhibited (Na^++K^+)-ATPase activity after 40-60 min fixation and reduced the level of Mg^{2+}-ATPase activity by 85%, whilst 2-3% formaldehyde (prepared from paraformaldehyde) fixation for 60-90 min resulted in the loss of only 30% of the (Na^++K^+)-ATPase activity and 65% of the Mg^{2+}-ATPase activity in avian salt gland (Ernst and Philpott, 1970). Similar results were obtained with teleost gill (Ernst and Philpott, 1970). In addition, these workers found that (Na^++K^+)-ATPase activity in formaldehyde-fixed tissue retained an obligatory requirement for Na^+ and K^+, and was fully sensitive to ouabain. However, fixation with formaldehyde alone often resulted in unsatisfactory preservation of tissue fine structure. Ernst (1975), in rat kidney cortex, showed that a combination of fixative consisting of 1% formaldehyde and 0.25% gluteraldehyde would allow acceptable preservation of tissue morphology while preserving adequate enzyme activity for cytochemical localization.

The original technique of Ernst (1972a,b) has been used to localize (Na^++K^+)-ATPase activity in a variety of epithelia (rat cornea, Leuenberger and Novikoff, 1974; alimentary tract of *Locusta migratoria*, Peacock, 1976; lachrymal salt gland of *Malaclemys*, Thompson and Cowan, 1976; rectal gland of the spiny dogfish, Goertmiller and Ellis, 1976; euryhaline teleost gills, Hootman and Philpott, 1979; larval rectum of *Aeshna cyanea*, Komnick and Achenbach, 1979). The modified Ernst (1975) procedure was also successfully utilized in rat liver (Blitzer and Boyer, 1978; Latham and Kashgarian, 1979; Yamamoto *et al.*, 1984) and in human liver (Chamlain *et al.*, 1988). However, insect studies have only met with limited success (Peacock, 1976; Komnick and Achenbach, 1979; Kalule-Sabiti, 1985; present study). The K^+-stimulated NPPase activity was apparent and distinct from alkaline phosphatase activity, but was inconsistently inhibited by ouabain. Similar results were reported for rat kidney cortex (Ernst, 1975). Ernst (1972a) showed that strontium induced a change in K^+-NPPase sensitivity to ouabain. Moreover, the ouabain-sensitivity of the transport ATPase complex was reduced in other tissues when NPP was substituted for ATP (Fujita *et al.*, 1966; Griffith *et al.*, 1968). The combination of these conditions appeared to reduce the ouabain-sensitivity of the rat kidney enzyme to levels which could not be resolved consistently with this cytochemical method. Possible explanations for this are given briefly by Anstee and Bowler (1984).

Guth and Albers (1974) recommended a modification of the Ernst method which included the use of dimethyl sulfoxide (DMSO) in the incubation medium instead of Sr^{2+} (Anstee and Bowler, 1984). This solvent increased the specific activity of K^+-NPPase, shifted the pH optimum for the reaction to pH 9.0 (Albers and Koval, 1972; Firth, 1987) and greatly increased the sensitivity of K^+-NPPase to ouabain (Mayer and Avi-dor, 1970). Mg^{2+} was thought to capture liberated P_i and then be converted to a coloured precipitate by cobalt chloride (Ernst *et al.*, 1980). Such studies by Firth (1987) have shown (Na^++K^+)-ATPase activity to be predominantly in the basal zone of the tubular epithelial cells in medullary ascending thick limbs, distal convoluted tubules, and cortical collecting tubules of mouse and guinea pig kidneys. Although this method has been successfully utilized by Guth and Albers (1974) and Firth (1987) to localize K^+-dependent and ouabain-sensitive NPPase activity, it was only useful in light microscopy. This was due to the localization of K^+-dependent activity being achieved in the absence of Sr^{2+}, and dependent on the observation that, at alkaline pH, P_i was capable of binding to tissue components (Guth, 1973).

The present study used the Ernst (1972b) technique in a further attempt to localize the (Na^++K^+)-ATPase in the basal cell membranes of the Malpighian tubules of *Locusta migratoria* L. In addition, comparative biochemical studies, similar to those carried out by Ernst (1972a), investigated the effect of pH, fixation and strontium on ouabain-sensitive

K^+-NPPase activity in order to access the validity of the technique in locating (Na^++K^+)-ATPase in this epithelium.

Fluid secretion by the Malpighian tubules of many insects has been shown to be under hormonal control (Phillips, 1981, 1982). Diuretic hormone (DH) is a family of neuropeptides which stimulates fluid secretion by isolated Malpighian tubules in at least twenty insect species (reviewed by Gee, 1977; Maddrell, 1981; Phillips, 1982, 1983; Nicolson and Hanrahan, 1986; Wheelock *et al.*, 1988). Since the first demonstration that cAMP acts as an intracellular second messenger (Sutherland and Rall, 1958) mediating the hyperglycaemic action of epinephrine and glucagon in liver cell homogenates, the role of cAMP as such a messenger has become well established in many vertebrate hormone systems (Robinson *et al.*, 1968, 1971) and a similar function has been reported in some insect hormone systems (Prince *et al.*, 1972; Wyatt, 1972; Aston, 1975; Rafaeli *et al.*, 1984; Morgan and Mordue, 1985; Petzel *et al.*, 1987).

The role of cAMP as an intracellular second messenger, mediating the action of DH on insect Malpighian tubules, has been proposed in a number of insects, mainly on the basis that exogenously applied cAMP stimulated an increase in fluid secretion by these tubules *in vitro* (*Rhodnius prolixus* and *Carausius morosus*, Maddrell *et al.*, 1971; *Schistocerca gregaria*, Maddrell and Klunsuwan, 1973; *Glossina morsitans*, Gee, 1976; *Pieris brassicae*, Nicolson, 1976, 1980; *Locusta migratoria*, Anstee *et al.*, 1980; Donkin, 1981; Morgan and Mordue, 1981; Rafaeli and Mordue, 1982; *Papilio demodocus*, Nicolson and Millar, 1983; *Onymacris plana*, Nicolson and Hanrahan, 1986; *Cenocorixa blaisdelli* (Hung.), Cooper *et al.*, 1988; see Phillips, 1982). Furthermore, with the exception of *Musca domestica* (Dalton and Windmill, 1980) and *Onymacris plana* (Nicolson and Hanrahan, 1986), dibutyryl cAMP acts as a secretagogue in all insect tubules studied to date (Maddrell and Phillips, 1978; Szibbo and Scudder, 1979; Donkin, 1981; Rafaeli and Mordue, 1982; Williams and Beyenbach, 1983, 1984). cAMP also stimulates fluid secretion by the salivary glands of *Calliphora erythrocephala* (Berridge, 1980) and fluid absorption in the midgut of *Rhodnius prolixus* (Farmer *et al.*, 1981).

The cellular mode of DH action on Malpighian tubules has been extensively studied in *Rhodnius prolixus* (Maddrell, 1971, 1978; O'Donnell and Maddrell, 1983). in which it has been shown to increase intracellular levels of the cyclic nucleotide, cAMP (Aston, 1975, 1979). Similarly, in the Malpighian tubules of *Locusta migratoria* (Rafaeli *et al.*, 1984; Morgan and Mordue, 1985; present study) and *Aedes aegypti* (Petzel *et al.*, 1987), DH and mosquito natriuretic factor, respectively, have been shown to increase intracellular levels of cAMP.

The biogenic amine, 5-hydroxytryptamine (5-HT or serotonin), has been found to mimic DH action on the Malpighian tubules of some species, but not others (Phillips, 1981). 5-HT stimulation of tubular fluid secretion has been shown in *Rhodnius prolixus*, *Carausius morosus* (Maddrell *et al.*, 1971), *Calliphora vicina* (Schwartz and Reynolds, 1979), *Papilio demodocus* (Nicolson and Millar, 1983), *Locusta migratoria* (Morgan and Mordue, 1984) and *Aedes aegypti* (Veenstra, 1988), and it also stimulates salivary gland activity (Berridge and Patel, 1968; Berridge and Prince, 1972). 5-HT has also been implicated in the activation of cyclic nucleotide generation in many invertebrate tissues (Walker, 1984) and has been shown to increase intracellular levels of cAMP in the salivary glands of *Calliphora erythrocephala* (Berridge and Patel, 1968). However, 5-HT-stimulation of fluid secretion in locust Malpighian tubules has been reported as being independent of cAMP (Morgan and Mordue, 1984). In other studies, fluid secretion by Malpighian tubules was reported to be unaffected by 5-HT (Maddrell and Klunsuwan, 1973; Farquharson, 1974; Dalton and Windmill, 1980). Similarly, Anstee *et al.* (1980) and Baldrick (1987) reported that there was no significant stimulation of fluid secretion in tubules of *Locusta migratoria* by 5-HT, present in control saline, over a concentration range of 10^{-8}-10^{-4} M. Insensitivity of these locust tubules to 5-HT was also reported by Rafaeli and Mordue (1982). These findings were in direct contrast to those of Morgan and Mordue (1984), who found the threshold of 5-HT stimulation of fluid secretion in the tubules of *Locusta migratoria* as lying between 10^{-8}-10^{-7} M, maximum activation occurring at doses greater than 10^{-6} M.

Despite these facts, little is known about the means by which hormonal regulation of ion and fluid secretion by insect Malpighian tubules occurs. However, the receptor mechanisms mediating the action of 5-HT in the salivary glands of *Calliphora erythrocephala* have been examined (Prince and Berridge, 1973; Berridge, 1980; Berridge and Heslop, 1982). In this epithelium, in which it is suggested that 5-HT is the physiological hormone (Trimmer, 1985), it is thought that the information of the 5-HT molecule is transduced into two second messengers, cAMP and Ca^{2+}, through a specific interaction with a cellular receptor (Prince and Berridge, 1973). Thus, a 5-HT_1 receptor activates the opening of channels for Ca^{2+} entry and a 5-HT_2 receptor activates adenylate cyclase to generate cAMP (Berridge and Heslop, 1982). The second messengers cAMP and Ca^{2+} are then responsible for mediating the ability of 5-HT to greatly increase ion and fluid secretion (Berridge, 1980).

Although the Malpighian tubules of *Rhodnius prolixus* and *Carausius morosus* were sensitive to remarkably low concentrations of 5-HT, it is not thought to be the physiological hormone acting on these epithelia as a response to feeding (Maddrell *et al.*, 1971). Therefore it is difficult to apply the model for hormone-stimulated fluid secretion by the salivary glands of *Calliphora erythrocephala* to Malpighian tubules.

Morgan and Mordue (1984) have proposed a hypothetical model for the hormonal control of fluid secretion by the Malpighian tubules of *Locusta migratoria.* This model suggests that two spatially distinct receptors exist on the surface of the tubule cells; one which activates adenylate cyclase activity (R_1) to increase cAMP synthesis and the other to trigger a different secondary cellular event (R_2), possibly increasing intracellular Ca^{2+} concentration. It is suggested that DH is able to stimulate both receptors (R_1 and R_2) simultaneously, thereby activating maximum stimulation. Each receptor can be stimulated independently, with 5-HT stimulating fluid secretion by interaction with R_2 only. Two independent agonists may act at the two receptor sites with maximal activity being achieved by stimulation via each receptor (Morgan and Mordue, 1984). Indeed, it was shown by Morgan and Mordue (1984) that the process of fluid secretion in *Locusta migratoria* was under the control of two distinct diuretic peptides (DP-1 and DP-2), isolated from the corpus cardiacum, which acted at different receptors. DP-1 (*ca.* 6000-7000 daltons) acted at a receptor, resulting in the activation of adenylate cyclase to increase intracellular levels of cAMP, whereas the smaller peptide DP-2 (*ca.* 1000 daltons), acted via an undetermined pathway.

Besides cyclic nucleotides, a new generation of phosphoinositide-derived second messengers have been identified (Berridge, 1984; Berridge and Irvine, 1984; Nishizuka, 1984; Majerus *et al.*, 1986; Michell, 1975; Berridge, 1987). Receptor stimulation triggers the phospholipase C catalyzed hydrolytic cleavage of membrane phosphoinositol 4,5-bisphosphate (PIP_2) to yield two second messenger molecules, inositol 1,4,5-triphosphate (Ins-1,4,5-P_3) and *sn*-1,2-diacylglycerol (DAG), an activator of protein kinase C (Nishizuka, 1984). Once activated by DAG, together with intracellular Ca^{2+}, protein kinase C proceeds to phosphorylate specific proteins that are thought to contribute to the final response (for review see Berridge, 1984, 1986, 1987).

Receptor-mediated hydrolysis of phosphoinositides is a common cellular response to a variety of extracellular stimuli such as hormones, neurotransmitters and certain growth factors (Michell *et al.*, 1981; Berridge, 1984). There is now general agreement that Ins-1,4,5-P_3 formed through the hydrolysis of PIP_2 (Berridge, 1984, 1986, 1987; Berridge and Irvine, 1984) induces the release of Ca^{2+} from the endoplasmic reticulum (Berridge and Irvine, 1984; Berridge, 1986; Williamson, 1986; reviewed by Abdel-Latif, 1986) and thus triggers the biological response to "calcium-mobilizing" hormones and neurotransmitters. Berridge (1986) recently reported that Ins-1,4,5-P_3 was generated by activation of the 5-HT_1 receptor in *Calliphora erythrocephala*, which appeared to be responsible for the mobilization of intracellular Ca^{2+} (Berridge *et al.*, 1984).

The study of the divalent cation Ca^{2+} in transport processes is limited in comparison to studies on monovalent ions, especially in insect epithelia. A central role for Ca^{2+} in secretory processes was proposed by Douglas (1968), and it has since become evident that Ca^{2+} is necessary for secretion in a wide variety of tissues and that the control of intracellular Ca^{2+} is crucial for the regulation of cellular processes (Rasmussen and Goodman, 1977). Ca^{2+} plays an important role as a second messenger conveying signals received at the cell surface to the inside of the cell (Berridge, 1980). It is involved in the regulation of such diverse processes as muscle contraction, secretion of hormones, digestive enzymes and neurotransmitters, and the control of glycogen metabolism in the liver. Indeed, this cation is important in regulating net transport of ions and water in transporting epithelia (Taylor and Windhager, 1979; Windhager and Taylor, 1983).

Intracellular free Ca^{2+} is regulated, in part, by intracellular sequestering organelles, with voltage-dependent and receptor-operated Ca^{2+} channels also being important (Spedding, 1985). The function of Ca^{2+} channels as a major pathway for Ca^{2+} influx is best characterized in excitable tissue, however (Reuter, 1983). The sensitivity of a cell to very small changes in Ca^{2+} concentration reflects the very low intracellular concentration of this cation; the concentration of Ca^{2+} extracellularly being about 104 times greater (Rasmussen, 1989). Maintenance of such a concentration gradient across the plasma membrane depends on its impermeability to Ca^{2+} and the presence of membrane bound transporters which extrude Ca^{2+} from the cell (Rasmussen, 1989). One such transporter is the Ca^{2+}-ATPase discovered by Schatzmann (1966), which has been found in a large number of vertebrate tissues (see Schuurmans Stekhoven and Bonting, 1981; Carafoli and Zurini, 1982; Flik *et al.*, 1985), including the basolateral membrane of kidney proximal tubules (Gmaj *et al.*, 1983; Doucet and Katz, 1982) and of cichlid fish gill (Flik *et al.*, 1985). Interestingly, Ca^{2+} "cycling" across plasma membranes, via Ca^{2+} channels and Ca^{2+} "pumps", has recently been implicated in a complex chain of events by which cells generate sustained responses to stimuli, e.g., the secretion of insulin or the contraction of smooth muscle surrounding blood vessels (Rasmussen, 1989).

Another Ca^{2+} transport system, proposed by Blaustein (1974), present in nerve, muscle, secretory tissues, and intestinal and renal epithelium, consists of a Na^+/Ca^{2+} exchanger which is driven by the Na^+ concentration gradient (Schuurmans Stekhoven and Bonting, 1981). This antiporter operates with a stoichiometry of $3Na^+$ for $1Ca^{2+}$ (Mullins, 1979), and has been documented in basolateral membrane vesicles from a variety of transporting epithelia (Gmaj *et al.*, 1983; Chase and Al-Awqati, 1981; Chase, 1984). It has been suggested that this mechanism is important in the regulation of cytosolic Ca^{2+} in renal epithelia, Ca^{2+} being transported out of the cell across the basal membrane in exchange for Na^+ (Windhager and Taylor, 1983; Scoble *et al.*, 1986). However, the physiological and

functional importance of Na^+/Ca^{2+} exchange in transporting epithelia is still controversial (Snowdowne and Borle, 1985; Mandel and Murphy, 1984).

In insect Malpighian tubules, little information is available regarding Ca^{2+} transport. No evidence of active Ca^{2+} transport was found in the tubules of *Carausius morosus* (Ramsay, 1956) and passive movement of the cation across both basal and apical membranes was proposed for tubules of *Carausius morosus* and *Calliphora erythrocephala* (Maddrell, 1971). However, the involvement of active Ca^{2+} transport in fluid secretion by the Malpighian tubules of *Leucophaea maderae* was inferred by Wright and Cook (1985) on finding large amounts of calmodulin, the Ca^{2+}-binding protein responsible for regulating Ca^{2+}-ATPase activity (Villalobo *et al.*, 1986). In larvae of *Aedes aegypti* a Ca^{2+}-activated ATPase in the gut has been suggested for the transport of Ca^{2+} from the swallowed medium to the haemolymph, and in the rectum for the reabsorption of Ca^{2+} originally secreted by the Malpighian tubules (Barkai and Williams, 1983, 1984). Na^+/Ca^{2+} exchange across the basal membrane has been postulated in cockroach salivary gland for the maintenance of low intracellular Ca^{2+} levels (House and Ginsborg, 1982), with Na^+ linked to feed the $(Na^+ + K^+)$-ATPase on the basal side (Gupta and Hall, 1983). During transcellular Ca^{2+} transport by the midgut of *Calliphora vicini*, Na^+/Ca^{2+} exchange also seems important in Ca^{2+} efflux at the basolateral membrane (Taylor, 1984). However, little is known about the role of Ca^{2+} transport in fluid secretion by the Malpighian tubules of *Locusta migratoria.*

In view of the lack of information concerning the nature and control of the mechanisms of ion and water transport across the Malpighian tubules of insects, the present study was carried out to examine the effect of DH, as a crude corpus cardiacum preparation, on the electrophysiology of, fluid secretion by and the intracellular levels of the known second messengers, cAMP and Ins-1,4,5-P_3, in Malpighian tubules of *Locusta migratoria.* This, together with the information obtained from ion substitution, ion flux and cytochemical localization techniques, was used in the construction of a hypothetical model to describe the ion fluxes which support fluid secretion by the Malpighian tubules of *Locusta migratoria* and their endocrine control.

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. A continuous air exchange was maintained by a fan-driven ventilator (Xpelair) and air circulation was provided by three electric fans. Humidity was controlled by three humidifiers (Lumatic, Humidifier Group, Bromley, Kent, England). Locusts were reared in perspex-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 40 Watt bulb, resulting in cage temperatures varying from 30-40°C depending on the distance from the bulb and the photoperiod. Humidity within each cage also varied with the addition of fresh food and water. Locusts were fed daily on fresh grass, water and bran. Animals were reared at sufficiently high population density to prevent reversion to the solitary phase (Joly and Joly, 1953).

Glassware and Reagents

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

Chemicals

All chemicals and drugs were AnalaR grade or the purest commercially available and were largely supplied by Sigma Co., Poole, Dorset, England. All radiochemicals and PCS scintillation fluid were supplied by Amersham International, England. Sodium acetazolamide (Diamox) was obtained from Lederle (American Cyonamid Company, Pearl River, New York, U.S.A.) and amiloride was a gift from Merck Sharp and Dolme Research Laboratories (Heddesdon, Herefordshire, England). Lubrol was a gift from I.C.I. Dyestuffs Division.

Solutions

The composition of the control insect saline was as follows (mM): NaCl 100, KCl 8.6, $MgCl_2$ 8.5, $CaCl_2$ 2, NaH_2PO_4 4, Glucose 34, HEPES 25, NaOH 11, pH 7.2. In ion substitution experiments alternative salines were used (Table 2.1). All solutions were adjusted to pH 7.2.

In studies involving furosemide it was necessary to dissolve it in spectroscopically pure absolute ethanol (Fison Scientific Apparatus, Loughborough, Leics., England) before adding it to the appropriate saline. In all experiments involving this agent, the same concentration of solvent (<0.1%) was included in the control.

Statistical Analysis

Unless otherwise stated, data are presented as Mean ± SEM, and the significance of difference between means was determined by the application of Student's *t*-test for paired data (Snedecor and Cochran, 1967) with reference to the statistical tables of Fisher and Yates (1963). Values of $P<0.05$ were taken as significant.

Rate of Fluid Secretion

In vitro measurements of the rates of fluid secretion by the Malpighian tubules of *Locusta* were carried out using essentially the same technique as that described by Maddrell and Klunsuwan (1973) (see also Anstee and Bell, 1975; Donkin and Anstee, 1980). Locusts were killed by twisting the head, breaking the neck cuticle from the thorax. The extreme tip of the abdomen was cut off allowing the whole gut, bearing the Malpighian tubules, to be carefully drawn out through the thorax with the head still attached. The gut of the experimental animal was immersed in a small volume of control saline (Table 2.1) in a hollow in a perspex dish. Up to ten tubules were drawn out of the saline bath into the surrounding liquid paraffin and looped around small glass pegs. Each tubule was then partially severed at a convenient point along its length. The rate was determined by measuring the rate of increase in the diameter of the approximately spherical droplet secreted from the cut.

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 30 min to give Rate 1. At the end of this time the saline was replaced by an appropriate fresh solution (either control or experimental, Table 2.1). After a further equilibration period of 20 min, the rate of fluid secretion was redetermined, as above, for a further 30 min, giving Rate 2. The rate of fluid secretion was expressed in nl/min. The effect of treatment was determined

TABLE 2.1 Composition of the Experimental Salines (concentration in mM)

Salt	Control	High $[K^+]$, Na^+-free	Na^+-free	Cl^--free	High $[K^+]$, Na^+- and Cl^--free	Ca^{2+}-free
NaCl	100.0	-	-	-	-	100.0
KCl	8.6	108.6	-	-	-	8.6
$CaCl_2$	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	34.0	34.0
HEPES	25.0	25.0	25.0	25.0	25.0	25.0
KH_2PO_4	-	4.0	4.0	-	4.0	-
$KHCO_3$	-	4.0	4.0	-	4.0	-
KOH	-	11.0	-	-	11.0	-
Na gluconate	-	-	-	100.0	-	-
K gluconate	-	-	-	8.6	8.6	-
Ca gluconate	-	-	-	2.0	2.0	-
Mg gluconate	-	-	-	8.5	8.5	-
Choline chloride	-	-	108.6	-	-	-
Tris base	-	-	11.0	-	-	-
EGTA	-	-	-	-	-	2.0

by comparing Rate 1 to Rate 2; each tubule acting as its own control. This was necessary as secretion rates varied considerably between tubules. The temperature throughout was maintained at 30 ± 0.5°C by placing the perspex dish inside a controlled water-heated temperature chamber.

Corpora Cardiaca Extract Preparation

Extracts were prepared, immediately prior to use, by dissecting corpora cardiaca from the heads of mature male *Locusta migratoria*. The corpora cardiaca were then homogenized in an appropriate ice-cold (0-4°C) buffered saline (Table 2.1) at 1 gland pair per 0.1 ml of saline, unless otherwise stated, (Mordue, 1969; Mordue and Goldsworthy, 1969) and stored on ice until required. Homogenization was carried out in a glass homogenizer with a Teflon pestle (clearance 0.1-0.15 mm) with 20 passes of the plunger at approximately 1000 rev/min. The resulting homogenate was subsequently diluted with the appropriate saline to a final concentration of 1 gland pair per ml. This was acheived by the introduction of the homogenate into the experimental chamber C (Fig. 2.1 and 2.2), described below, via a microsyringe. Control experiments used homogenates of similar amounts of flight muscle.

Electrophysiology

Locusts were killed as described previously. Malpighian tubules were dissected out under control saline (Table 2.1), rinsed and placed in fresh control saline. Tubules were placed under liquid paraffin prior to use to check for leaks (droplets of "urine" emerging from holes in the tubule). Leaking tubules were discarded.

For electrical measurements, tubules were set up in a perspex perfusion chamber (Figs 2.1 and 2.2) based on the "gap" system of Berridge and Prince (1972). The chamber consisted of five connecting baths (A-E), each coated to a depth of approximately 4 mm with the silicon elastomer, Sylgard 184 (Dow-Corning, Seneffe, Belgium). A length of Malpighian tubule (about 2 cm) was drawn through slits connecting baths A,B,C and D, the ends being held under flaps of Sylgard in baths A and D. Baths A and C contained experimental salines, whilst baths B and D were filled with liquid paraffin (LP) to electrically isolate bath A from bath C. Microelectrode recordings took place in the experimental bath C which was perfused via a glass inlet tube F. The tubule passed over a raised Sylgard block in bath C to aid microelectrode penetration. The volume of this bath was 0.5 ml and the perfusion rate was about 4 ml/min. As a result the contents of the bath changed every 7.5 s. Superfusion occurred from bath C to E. Fluid entering the experimental chamber arrived from one of various perfusion bottles. These bottles were

FIGURE 2.1

A schematic diagram of the perspex experimental chamber (PC) used for superfusing and recording membrane and transepithelial potentials of the Malpighian tubules of *Locusta migratoria*. The chamber consists of five connected baths (A-E), a supported glass inlet tube (F) and a supported outlet tube (G). During experimentation, a Malpighian tubule was drawn through slits between baths A to D and supported on a raised Sylgard block (H) in experimental bath C. Microelectrode measurements took place in this bath. Perfusion occurred from bath C to E; this two-chamber arrangement ensured no electrical "pick-up" occurred. Areas I and J represent regions cut away from the perspex into which the 3 M KCl agar bridges were placed (see Fig. 2.2).

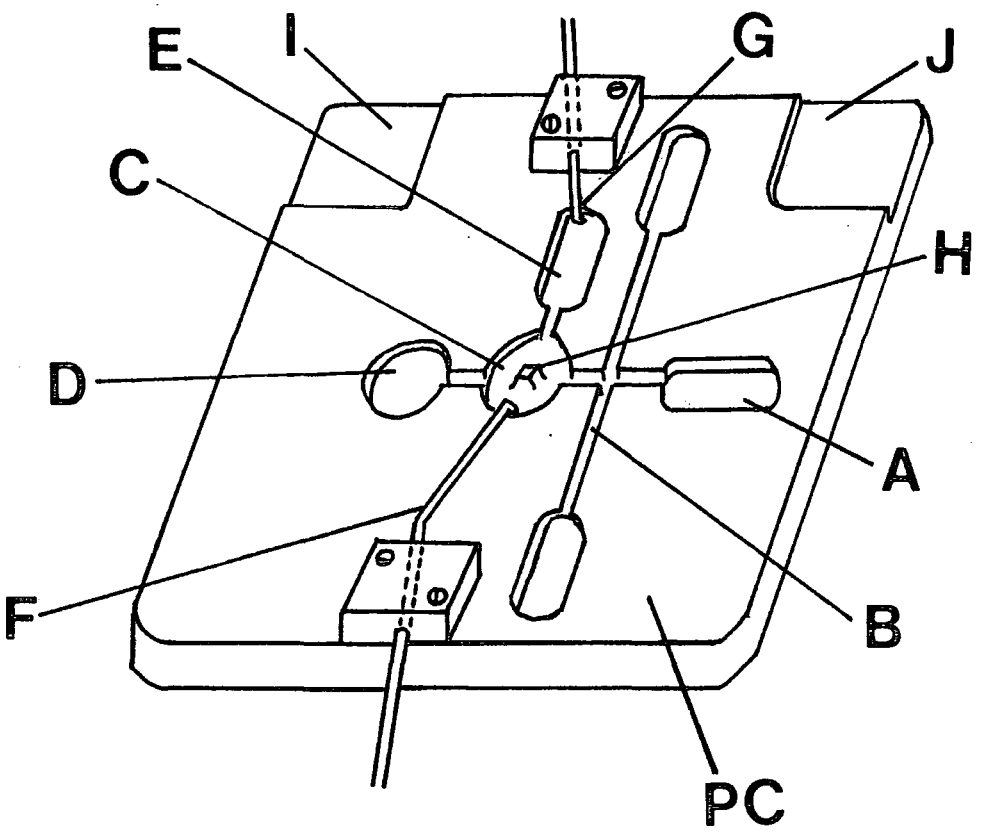

E
I
G
J
C
H
D
A
F
B
PC

FIGURE 2.2

This figure shows the position of the Malpighian tubule in the experimental chamber used for superfusing and the recording of membrane and transepithelial potentials. A tubules of about 2 cm length was drawn taut from baths A to D and fixed under flaps cut into the Sylgard bases of these baths to prevent movement. The tubule rested on a raised block of Sylgard in bath C to aid microelectrode penetration. The portion of tubule in bath A was partially severed using a tungsten needle to ensure that the lumen was continuous with the contents of bath A. Baths A and C contained saline, whilst baths B and D were filled with liquid paraffin (LP) to electrically isolate bath A from bath C. Surface tension prevented the saline and liquid paraffin from mixing.

This figure also shows the circuit diagram of the system used for simultaneous measurement of membrane and transepithelial potentials in the Malpighian tubules of *Locusta migratoria*. The transepithelial potential (TEP) was measured by connecting baths A and C to Ag/AgCl electrodes in 3 M KCl via agar bridges (AB). The Ag/AgCl electrodes were connected to an oscilloscope (CRO) and pen recorder via two components of a high impedance amplifier (A_2 and E_2). TEP was measured by having one AB in bath A and the other in bath C. Membrane potentials were measured by a connecting single-barrelled microelectrode to a second channel of the CRO and pen recorder via two components of an amplifier (A_1 and E_1). The presence of a switch box, and the fact that the two amplifier components A_1 and A_2 were connected, allowed the measurement of the basal cell membrane potential (V_B) and TEP with the perfusate as reference (bath C), and the apical cell membrane potential (V_A) and TEP with the luminal fluid as reference (bath A).

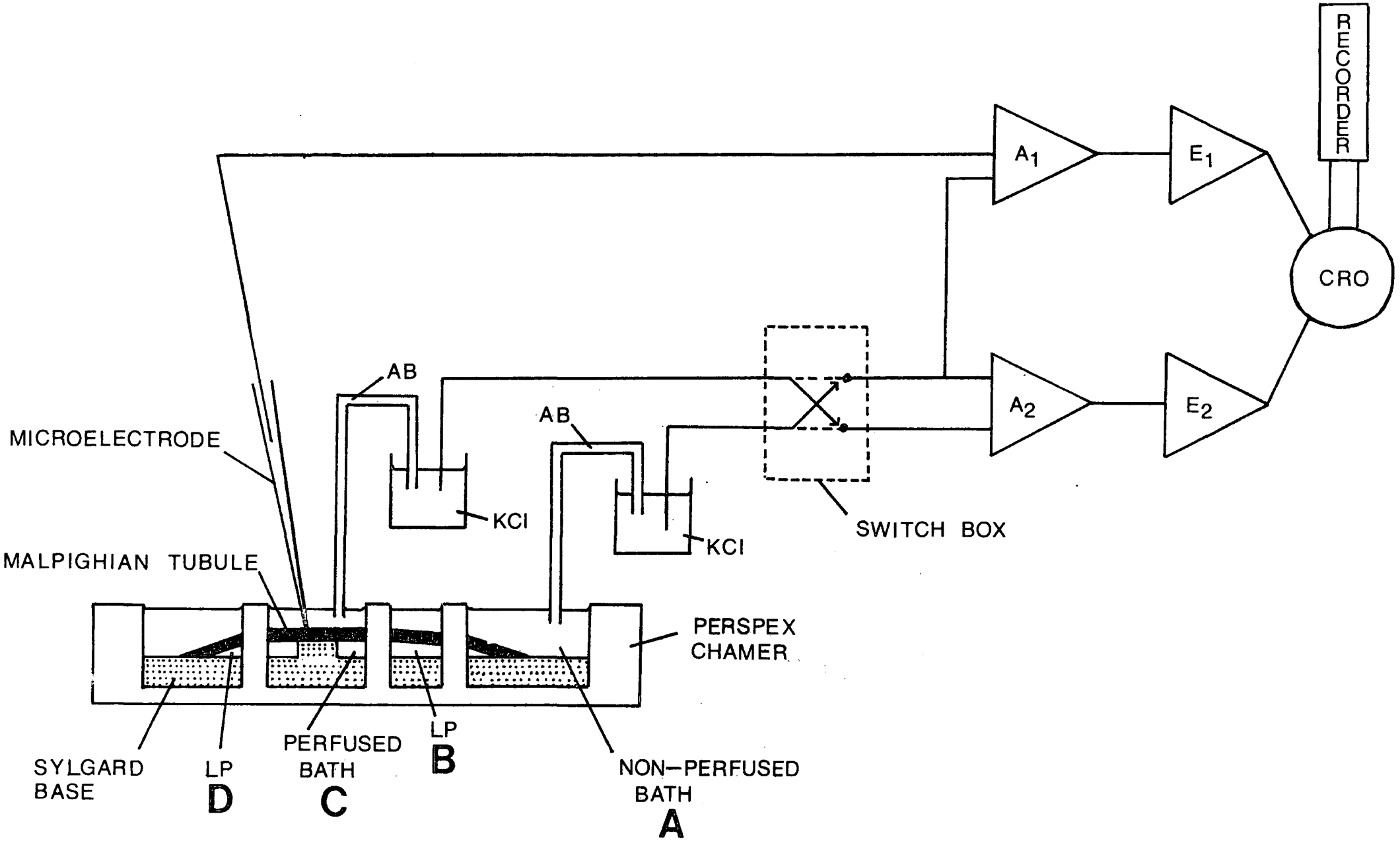
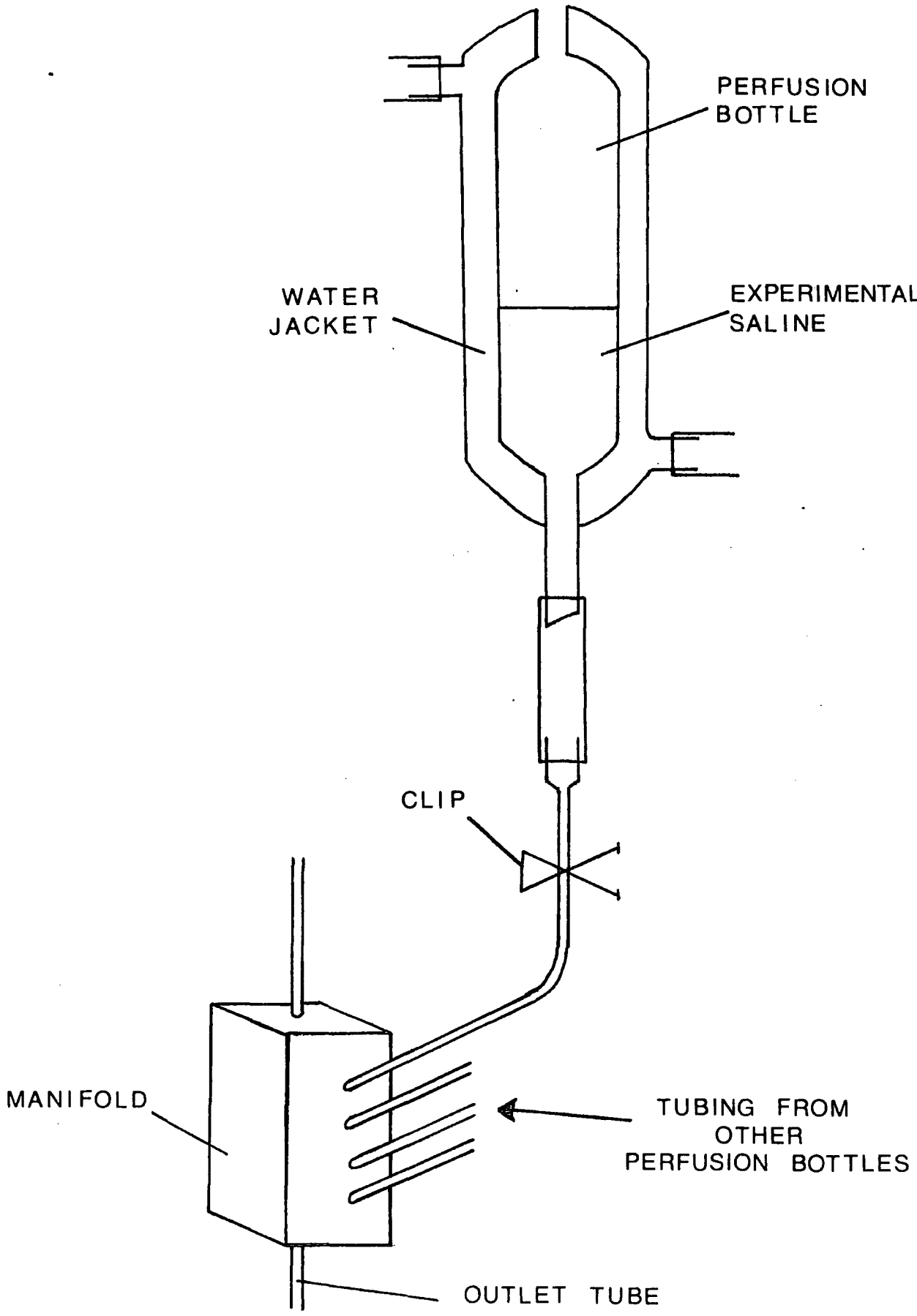

RECORDER
CRO
A1
E1
A2
E2
AB
AB
KCl
KCl
SWITCH BOX
MICROELECTRODE
MALPIGHIAN TUBULE
PERSPEX CHAMER
SYLGARD BASE
LP
D
PERFUSED BATH
C
LP
B
NON–PERFUSED BATH
A

FIGURE 2.3

A schematic diagram of a perfusion bottle and manifold used to superfuse the Malpighian tubules of *Locusta migratoria*. A number of water-jacketed perfusion bottles were connected to a single manifold whose single outlet was connected to the glass inlet tube (F) of the experimental chamber (Fig. 2.1). Perfusing solutions were changed by clipping off the flow from one bottle and opening the clip on the tubing of another bottle.


PERFUSION
BOTTLE
WATER
JACKET
EXPERIMENTAL
SALINE
CLIP
MANIFOLD
TUBING FROM
OTHER
PERFUSION BOTTLES
OUTLET TUBE

jacketed to allow the contained saline to be thermoequilibrated at 30°C and were connected to a perspex manifold (Fig. 2.3). Each bottle could be isolated from the rest of the system by means of a clip at the base. Tests in which amaranth dye was introduced to the system indicated that there was no observable mixing of solutions at manifold junctions. The perfusate was drawn away from the system via a glass outlet tube G, positioned over bath E, by a peristaltic pump (Watson Marlow 502S). Fluid was generally passed to a waste bottle, but recycling during prolonged experiments was possible by removing the pump outlet tube and connecting to the appropriate perfusion bottle.

All tubing used was P.V.C. (Gallenkamp) or silicon (Watson Marlow). The temperature of the experimental bath was kept at 30 ± 0.1°C (with a Gallenkamp Haake DI water bath) by placing the perspex experimental chamber on the surface of a water-heated plate. Thermal contact was maintained between the plate and chamber using liquid paraffin. A fibre optic system (Ealing Beck Ltd., Watford, Herts., England), arranged at one side of the chamber, provided illumination.

Electrical Recording

The arrangement for recording electrical potentials was similar to that of Berridge and Prince (1972). Fig. 2.2 shows that the transepithelial potential (TEP) was measured by connecting baths A and C of the experimental chamber to Ag/AgCl electrodes in 3 M KCl via glass 3 M KCl/4% agar bridges (AB). Each Ag/AgCl electrode was connected to the head-stage (A_2) of a high input impedance field effect amplifier E_2 (design based on that of Colburn and Schwartz, 1972). The amplifier output was displayed on the oscilloscope (CRO) (Telequipment Type D1010) and permanent records were made on a vertical multi-channelled pen recorder (Servogor 460-Metrawatt, Nurnburg, F.R.G.). Initial zeroing of the system was achieved by placing both agar bridges in bath C. Any deviation of the potential from a standardized baseline was due to junction potentials between KCl and Ag/AgCl electrodes or old agar bridges (Barry and Diamond, 1970). This was eliminated by scraping the Ag wires and coating them in AgCl by passing a 25 mA current through a solution of 100 mM HCl for 1 min with the wire to be coated made the anode. Wires were then rinsed and stored until required.

Membrane potentials were measured using single-barrelled microelectrodes fabricated from 1 mm bore diameter thin-walled filamented glass capillary tubing (GC 200F-10, Clarke Electromedical, Reading, England). These were pulled on a horizontal microelectrode puller (Palmer Ltd., London) and back-filled with 3 M potassium acetate (the electrolyte) using a syringe and a 30 swg needle. A thin, chloride-coated silver wire was inserted into the back of the microelectrode and connected to the input stage (A_1)

of the high impedance field-effect amplifier (E_1) whose output was displayed on the second channel of the oscilloscope (CRO) and recorded on the Servogor chart recorder.

As a result of the two amplifiers (E_1 and E_2) being connected at the input stages (A_1 and A_2) simultaneous recording of tubule membrane potential and TEP was possible. The relay switch box connected to a foot pedal (RS 316-939) changed the reference between bath A (luminal fluid) and bath C (the perfusate), enabling alternate measurement of the apical (V_A) and basal (V_B) cell membrane potentials, respectively. TEP was measured throughout.

Microelectrodes were mounted on a micromanipulator (Prior, England) and positioned in the perfusate near the Malpighian tubule. The tip resistances of the microelectrodes inserted in the perfusate were 20-50 MΩ and the tip potentials (potentials between the electrolyte in the microelectrode and the perfusate) were approximately 3 mV. Microelectrode tips were less than 1 μm in diameter; fine tips required for easy cell penetration. A constant current generator system incorporated in the amplifiers (based on the design of Colburn and Schwartz, 1972) was used to measure microelectrode resistance. A 1 nA current passed down a microelectrode resulted in a voltage deflection on the oscilloscope. Using Ohm's Law (V=IR) a value for microelectrode resistance could be calculated.

Before experimentation, the microelectrode was equilibrated in the perfusate and the resistance noted. The microelectrode was then positioned using the fine advance on the micromanipulator, the microelectrode being at an angle of about 60° with respect to the tubule. Gentle tapping of the micromanipulator baseplate caused rapid penetration of a cell, indicated by the rapid registration of a resting potential on both the oscilloscope and the pen recorder. The criteria adopted as indicators of a successful microelectrode penetration were:-

(1) the change in potential from baseline was abrupt,

(2) the intracellular voltage remained constant within 2 mV for 5-15 min after impalement,

(3) the voltage returned to the original baseline, or a value close to it, when the microelectrode was withdrawn from the cell (experiments were not used if voltage "drift" was more than 10 mV over a 1 hr period).

The rationale of these criteria was to exclude leaky impalements from this study.

After a successful penetration, solution changes were carried out as described previously. Experimentation on a single tubule usually did not last longer than 1 hr. After experimentation, the microelectrode was removed from the cell and the microelectrode

resistance measured as a means of checking for microelctrode blocking. Blocking of the tip sometimes caused a slight "drift" of the recorded traces, related to criterion (3), described previously. Changing to a new perfusing solution often led to a liquid junction potential at the agar bridge in experimental bath E. Membrane potentials and TEP measurements were corrected for these junction potentials whose magnitudes (usually only a few mV) were measured by changing perfusion solutions after experimentation, with the micrelectrode withdrawn from the tubule, but remaining in the perfusate.

The perfusion chambers were thoroughly rinsed with deionized water and dried after each experiment. Setting up and penetration of tubules was viewed under a Zeiss microscope. All experiments were carried out in a Faraday cage to omit electrical interference and a vibration-damped bench was used.

This system allowed three voltage measurements to be recorded in the present study, namely basal and apical cell membrane potentials and TEP measurements. In addition, an estimate of transcellular potential (TCP) could also be calculated from TCP = V_B-V_A. For tubules in the various experimental salines (Table 2.1), TEP and TCP were not significantly different, except in high K^+, Na^+-free saline, where there was a small, but significant difference of *ca.* 2.3 mV (see Appendix Table A.1). This difference could be attributed to TCP relating to a single cell, whilst TEP relates to the whole tubule, with variation between cells.

Recently, Aneshansley *et al.* (1988), using the Malpighian tubules of *Aedes aegypti*, suggested that the only reliable method for measuring TEP was by direct penetration of the tubule lumen. This method measured voltages that were exclusively lumen-positive, whereas voltages measured between the secreted droplet and the Ringer bath (method of Ramsay, 1954) were positive and negative. Both methods are supposedly direct indicators of TEP, based on the assumption that the measurements are of open circuit voltages, i.e., there being no closed circuit paths over which the TEP can be divided (Aneshansley *et al.*, 1988). Aneshansley *et al.*(1988) reported that there were such paths in the oil-bathed tubule segment of the Ramsay (1954) method, and that the peritubular water film affected the amplitude of the measured voltage. These workers also reported that the axial lumen resistance and lateral resistance of the epithelial wall affected the amplitude and the polarity of the measured voltages. Such problems would also be expected to affect measurement of V_A, the reference electrode being that in the secreted droplet. Similarly, Isaacson and Nicolson (1989) reported that, in perfused tubules of *Onymacris plana*, the apparent transtubular potential recorded at the collecting end (analogous to the oil gap) was indistinguishable from that previously measured with the oil-gap technique. However, it was invariably lower than that recorded by an intraluminal pipette.

In *Locusta migratoria*, TEP was measured as the potential difference between reference electrodes and compared with that measured by direct microelectrode penetration of the Malpighian tubule lumen. Voltage measurements of TEP by the two methods were not significantly different on the basis of a paired *t*-test (see Appendix Table A.2). In addition, both positive and negative values were obtained using both methods. Measurement of TEP by direct microelectrode penetration of the lumen would require the use of another microelectrode for cell penetration to obtain values of V_B and V_A, which would present considerable difficulty. Due to there being no significant difference between these methods for the measurement of TEP and the difficulty in using a two-microelectrode system, the method of Berridge and Prince (1972) was preferred in the present study.

All potentials were measured in millivolts (mV).

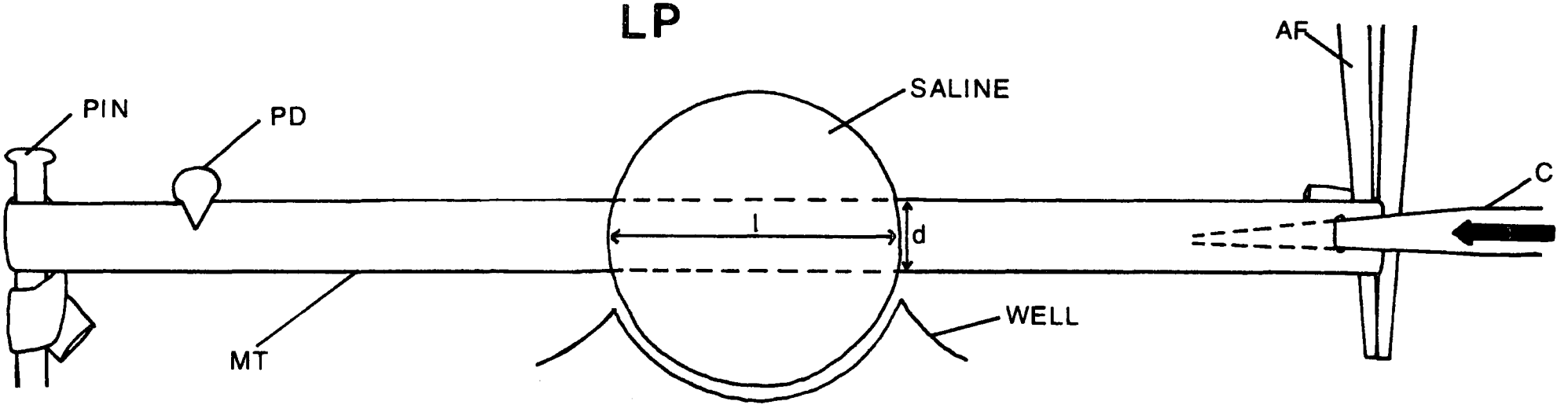
Na^+ and Cl^- Flux Measurements

The method employed was based on that of O'Donnell and Maddrell (1984). Animals were killed as described previously and their tubules suspended in a Sylgard-based liquid paraffin bath (LP), secured at one end by wrapping the tubule around a vertical glass pin, and at the other by a pair of adjustable mounted forceps (AF) (Fig. 2.4). The tubule was positioned such that a known length and diameter (and hence, surface area= Πdl) of its central portion passed through a well containing 100 µl of control buffered saline, pH 7.2. Partial severance of the tubule was made a small distance from the glass pin. A fine cannula (C) (made as described for microelectrodes, using 0.5 mm bore soft capillary tubing) was carefully inserted down the lumen of the tubule close to the forceps. The cannula was filled with perfusion medium (NaCl 9, KCl 99.6, $MgCl_2$ 8.5, $CaCl_2$ 2, NaH_2PO_4 4, $NaHCO_3$ 4, Glucose 40, HEPES 25, NaOH 11 (mM), pH 7.2) and connected to a motor-driven microsyringe. Positive pressure applied to the syringe plunger ejected any lumen contents at the site of partial severance, reducing the possibility of tubule blockage. The tubule was replaced if leakage occurred from any site.

Once a suitable tubule was set up it was perfused at a constant rate of approximately 50 nl/min using the motor-driven microsyringe. Net flux rates of Na^+ and Cl^- from the external well (haemolymph side) across the tubule to the lumen were determined by replacing the saline in the well with control saline containing $^{22}Na^+$ or $^{36}Cl^-$. The perfusion droplet formed at the site of partial severance was collected after 10 min, placed in 10 ml of "Liquiscint" scintillation fluid and the radioactivity was read for 10 min in a Canberra Packard Tri-Carb 300 β-scintillation counter. A 5 µl sample of the radiolabelled saline was counted, as above, to determine the total level of radioactivity in the external well. This level was proportional to the total amount of unlabelled Na^+ or Cl^- in the well. Thus, the level of radioactivity found in the collected perfusate was proportional to the net flux of

FIGURE 2.4

A schematic diagram of the system used to determine the flux rate of Na^+ or Cl^- across the Malpighian tubules of *Locusta migratoria* from bathing medium to lumen. A Malpighian tubule (MT) was suspended in a liquid paraffin (LP) bath, one end secured around a glass pin, the other by adjustable forceps (AF). The central portion of the tubule passes through a well containing 100 µl of an appropriately labelled saline, such that a known surface area (πdl) is exposed to saline. A glass cannula (C) containing a perfusion medium was inserted down the lumen of the tubule close to the forceps. The cannula was connected to a motor-driven syringe which allows the tubule to be perfused in the direction of the arrow. Perfusion leads to the formation of a perfusion droplet (PD) at the site of partial severance of the tubule, which is removed and counted.


LP
PIN
PD
SALINE
AF
C
l
d
MT
WELL

unlabelled Na^+ or Cl^- across the saline-exposed surface area of the tubule. The flux rate was expressed as meq. Na^+ or Cl^- per cm^2 tubule per hr.

All experiments were carried out at 30 ± 0.1°C. The external bathing medium was control saline (Table 2.1) unless otherwise stated. For each tubule, the basal flux was determined prior to treatment. The latter required replacement of the external bathing medium with an appropriate fresh experimental saline (Table 2.1). Controls involved two consecutive basal flux readings, replacing the well contents with the same, but fresh saline between readings.

$^{22}Na^+$ and $^{32}Cl^-$ were both obtained as sodium chloride in aqueous solution from Amersham International plc. "Liquiscint" was supplied by National Diagnostics (196 Rout 206, South Somerville, New Jersey, 05876, U.S.A.).

Protein Determination

Protein determinations were carried out using the Coomassie brilliant blue binding method of Bradford (1976) using BSA Fraction V as standard.

Protein reagent was prepared by dissolving 100mg Coomassie brilliant blue G-250 in 50 ml of 95% ethanol. To this 100 ml of 85% (w/v) orthophosphoric acid was added. The solution was then diluted to 1 litre with deionized water and stored at 4°C until required.

Protein determination were carried out by placing 200 µl test sample in a tube to which is added 20 µl of 1.1 M NaOH and 2 ml protein reagent. Tubes were then left for 10 min at room temperature, after which time the absorbance of each solution was read at 595 nm on a LKB Ultraspec 4050 spectrophotometer.

Protein content was determined with reference to a freshly prepared standard BSA curve with concentration range 0-40 µg BSA/200 µl sample. Standard samples were treated as test samples as described previously. Protein content (µg BSA) was plotted against absorbance to produce a standard calibration curve (Fig. 2.5, typical example).

Extraction of cAMP and Inositol 1,4,5-trisphosphate

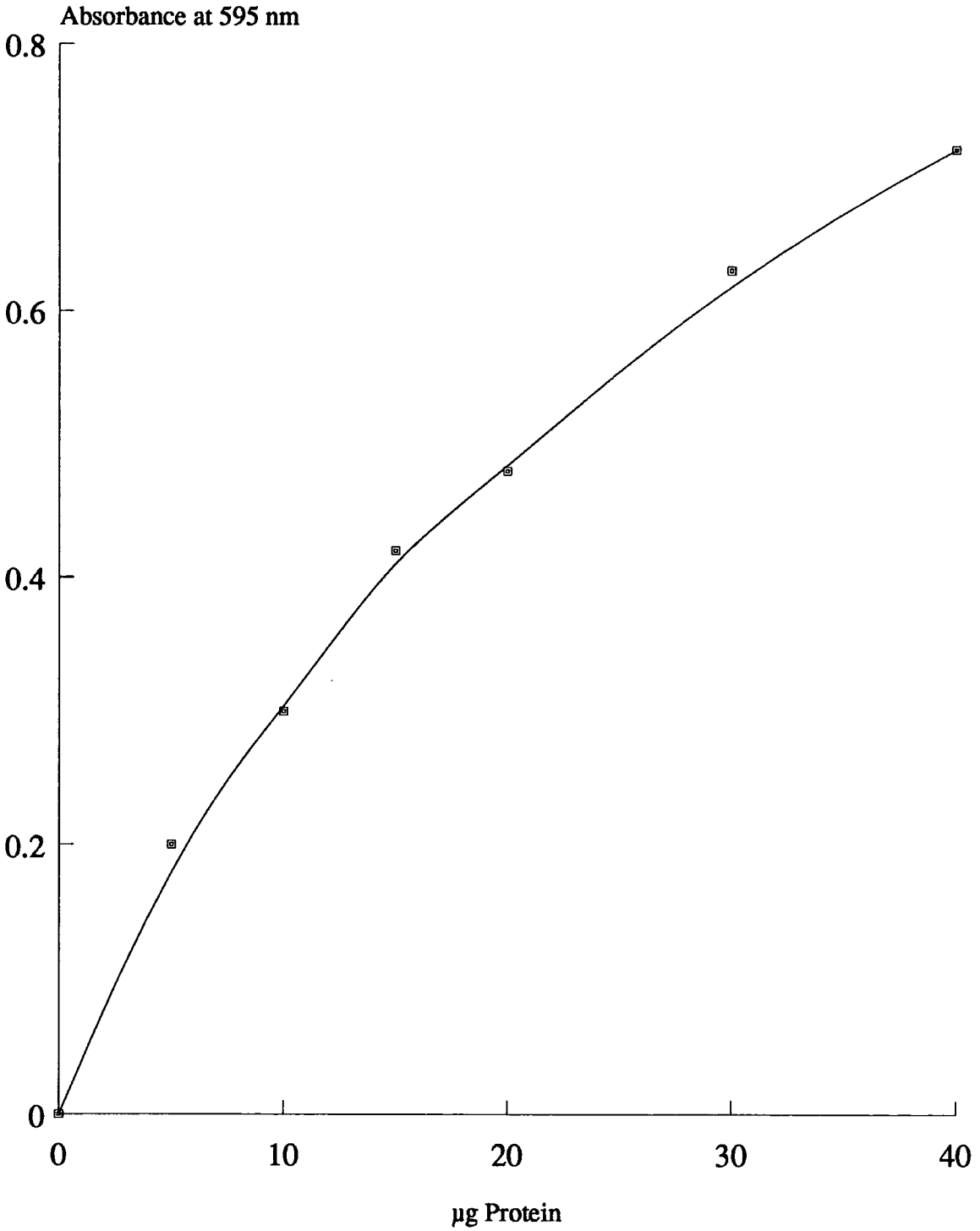
Animals were killed as described previously and the Malpighian tubules kept in ice-cold (0-4°C) control saline (Table 2.1). The method of cAMP and Inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) extraction was essentially that described by Clegg and Mullaney (1985) with some modification (see also, Palmer *et al.*, 1989). Malpighian tubules (40 mg wet weight) taken from a common "pool" were transferred to each of a series of polypropylene tubes containing 400 µl of control saline, pH 7.2 (at room temperature), and a small magnetic stirrer to facilitate mixing. Incubations were carried out at room temperature

FIGURE 2.5

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

Ordinate : Absorbance at 595 nm.

Abscissa : Protein in μg.


Absorbance at 595 nm
0.8
0.6
0.4
0.2
0
0
10
20
30
40
µg Protein

and were started by the addition of 100 µl corpora cardiaca extract (CC extract, preparation described previously) at a concentration of 1 gland pair/0.2 ml saline (final concentration 1 gland pair/ml). The incubations were stopped by the addition of 100 µl ice-cold 20% perchloric acid (PCA) at 2, 4, 6, 8, 10, 15, 30, 60 and 300 second intervals after the addition of the CC extract. Controls were run in which the PCA was added before the CC extract. Samples were then homogenized in a glass homogenizer with a Teflon pestle and kept on ice for 20 min. Precipitated protein was sedimented by centrifugation at 2400 rpm for 15 min at 4°C using an MSE "Coolspin" centrifuge. Supernatants containing soluble cellular components, including cAMP and Ins-1,4,5-P_3, were then transferred to new tubes and titrated to pH 7.5 with 10mM KOH. Precipitated $KClO_4$ was sedimented as above and the supernatants stored frozen until required.

Preparation of cAMP Assay Reagents

The following were allowed to thermoequilibrate to 4°C and were kept on ice until required:-

(1) assay buffer; 50 mM Tris-HCl buffer, pH 7.0, containing 4 mM EDTA,

(2) binding protein; 1 mg protein kinase (3',5'-cylic AMP-dependent binding protein) in 2.5 ml assay buffer containing 12.5 mg bovine serum albumen (BSA) Fraction V,

(3) [^{3}H]cAMP reagent; 0.5 µCi/ml assay buffer,

(4) standard cAMP reagent; 320 pmoles cAMP in 1ml assay buffer, serially diluted with equal volumes of assay buffer to provide additional calibration standards containing 8, 4, 2 and 1 pmoles/50 µl,

(5) activated charcoal absorbent; 520 mg Sigma activated charcoal suspended in 20 ml assay buffer containing 400 mg Fraction V BSA.

Assay of cAMP

The method used was based on that described by Tovey *et al.*(1974) and summarized in Table 2.2. A standard curve (Fig. 2.6) was produced using the following range of cAMP concentrations: 0, 20, 40, 80, 160 and 320 pmol/ml assay buffer. cAMP levels in standards and samples (see extraction) were measured using 50 µl aliquots to which was added 100 µl assay buffer, 40 µl [^{3}H]cAMP reagent and 10 µl binding protein. Following vortex mixing, these solutions were incubated for 3 hr on ice.

At the end of this time, 100 µl activated charcoal absorbent was added to each tube, the latter was vortex mixed for 5 s and then incubated on ice for 1.5 min. A control was run in which 160 µl assay buffer was incubated with 40 µl [^{3}H]cAMP only, to access the absorption of the latter by the charcoal. The tubes were then centrifuged in a cooled Eppendorf centrifuge for 2 min at full speed. It was essential that tubes be spun at least 1 min after the final charcoal addition and within 6 min of the first addition. Hence, assay tubes were dealt with in batches of 10 to facilitate handling.

Following centrifugation, 200 µl of the resulting supernatant was placed in 2 ml PCS liquid scintillant. Radioactivity (dpm) was counted in a Canberra Packard Tri-Carb 300 β-scintillation counter to determine the amount of bound tracer. The value obtained for the control was subtracted from all other data. The zero standard (C_0) dpm and the calibration standards and unknowns (C_x) dpm were used to calculate the percentage tracer bound (C_0/C_x x 100) for each level of cAMP and the unknowns. Results were calculated using a graph (Fig. 2.6) relating the percentage tracer bound for calibration standards to Log_{10}cAMP concentration (pmoles/ml) and expressed as pmoles/mg protein.

Preparation of Ins-1,4,5-trisphosphate Assay Reagents

A D-*myo*-inositol 1,4,5-trisphosphate [^{3}H] assay system (TRK 1000; supplied by Amersham International plc, England) was used. The following were allowed to thermoequilibrate at 4°C and kept on ice until required:-

(1) Ins-1,4,5-P_3-binding protein; not allowed to exceed 4°C during thawing, mixed carefully to avoid frothing,

(2) assay buffer; 0.1 M Tris buffer (pH 9.0) containing 4 mM EDTA and 4 mg BSA/ml,

(3) [^{3}H]Ins-1,4,5-P_3 reagent; 0.75 µCi in 11 ml deionized water (68 µCi/100 ml water),

(4) standard Ins-1,4,5-P_3 reagent; a stock diluted to 250 pmoles/ml deionized water, serially diluted with equal volumes of deionized water to provide additional calibration standards containing 12.5, 6.2, 3.1, 1.5, 0.76, 0.38 and 0.19 pmoles/100 µl.

Assay of Ins-1,4,5-P_3

This is a rapid quantitative binding protein assay for Ins-1,4,5-P_3 and cross-reactivity is reported to be less than 10% for inositol 1,3,4,5-tetrakisphosphate and less than 1% for other inositol phosphates. The method is based on that of Palmer *et al.* (1989), and

TABLE 2.2 Summary of cAMP Assay Protocol (volumes in microlitres)

Reagent	Control	Zero standard	Standards	Samples
Assay buffer	160	150	100	100
Unlabelled cAMP	-	-	50	50
[^{3}H]cAMP	40	40	40	40
Binding protein	-	10	10	10
Vortex mix and incubate for 3 hr on ice				
Activated charcoal	100	100	100	100
Vortex mix, incubate for 1.5 min on ice				
Centrifuge for 2 min (high speed), remove supernatant and count				

TABLE 2.3 Summary of Ins-1,4,5-P_3 Assay Protocol (volumes in microlitres)

Reagent	Total counts (TC)	Non-specific binding (NSB)	Zero Standard (B_o)	Standards	Samples
Assay buffer	100	100	100	100	100
Deionized water	200	-	100	-	-
Unlabelled Ins-1,4,5-P_3	-	100 (stock standard)	-	100	-
[^{3}H]Ins-1,4,5-P_3	100	100	100	100	100
Binding protein	-	100	100	100	100
		Vortex mix and incubate for 15 min on ice			
		Centrifuge at 4^{o}C for 7 min, remove supernatant and count			

FIGURE 2.6

Standard calibration curve for the determination of cAMP.

Ordinate : Percentage tracer bound ($\%C_x/C_o$).

Abscissa : Concentration of cAMP in pmoles/ml

($\log_{10}$ scale).

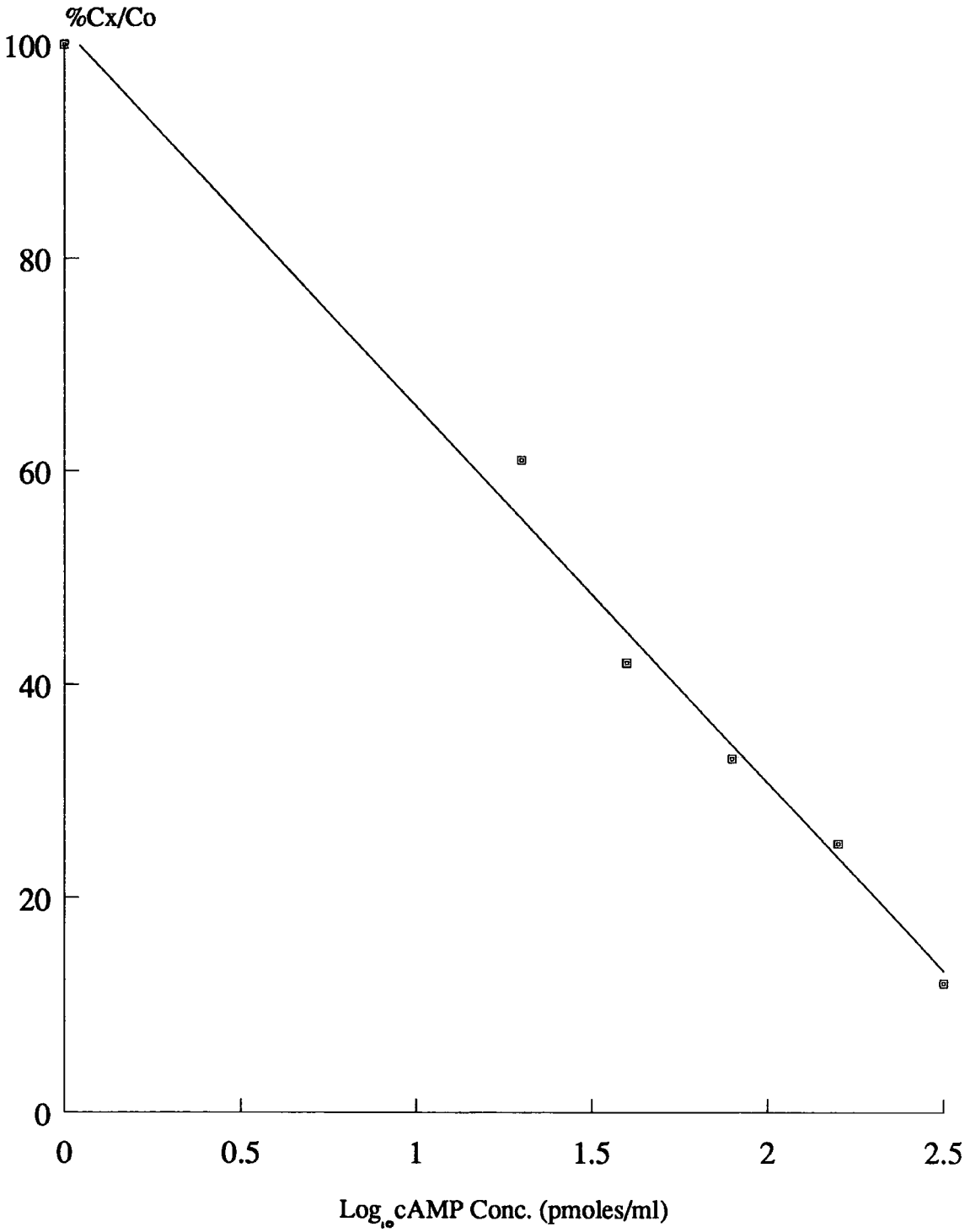

%Cx/Co
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Log10cAMP Conc. (pmoles/ml)

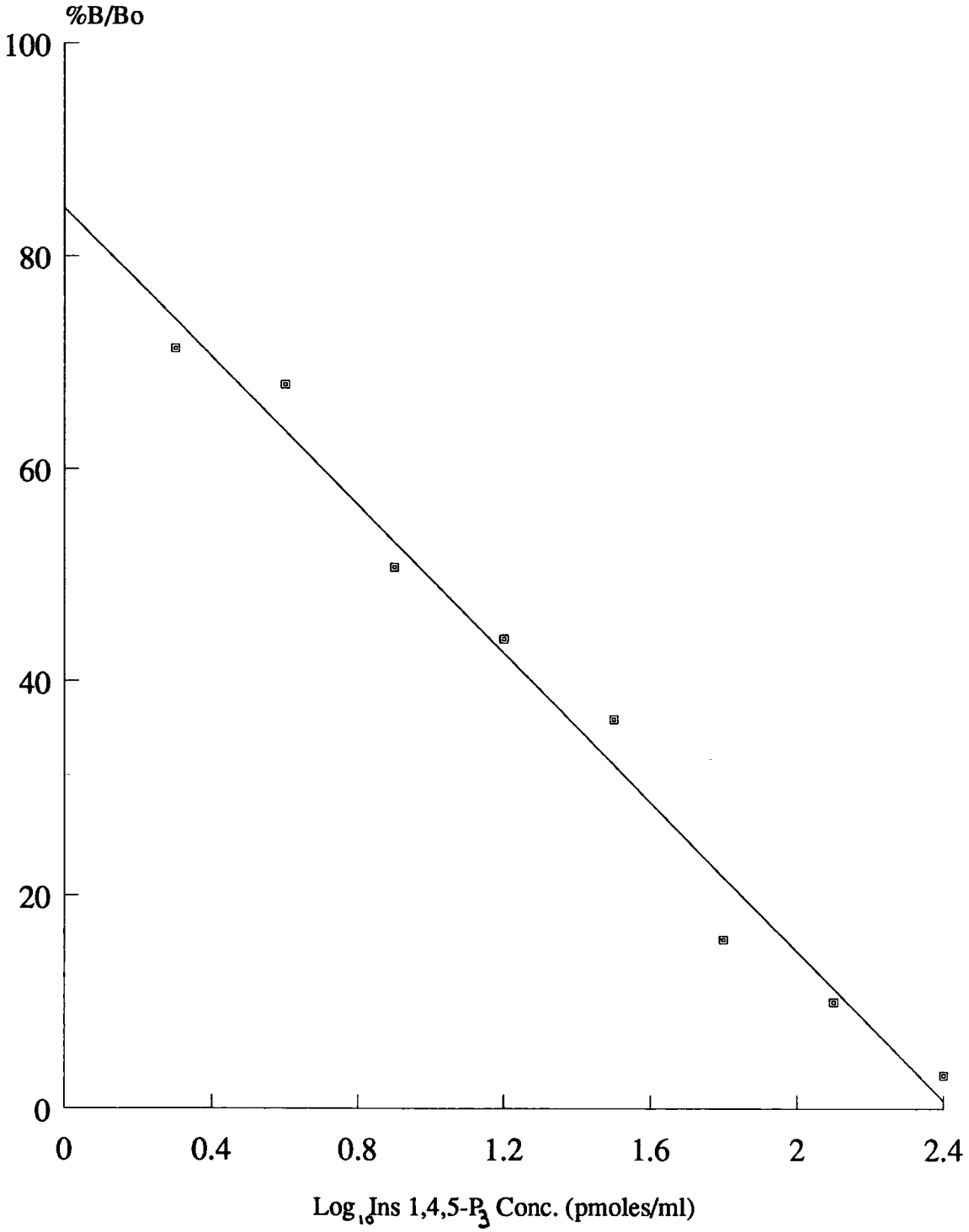
FIGURE 2.7

Standard calibration curve for the determination of Ins-1,4,5-P_3.

Ordinate : Percentage tracer bound (%B.B_o).

Abscissa : Concentration of Ins-1,4,5-P_3 in pmoles/ml

($\log_{10}$ scale).


%B/Bo
100
80
60
40
20
0
0
0.4
0.8
1.2
1.6
2
2.4
Log10Ins 1,4,5-P3 Conc. (pmoles/ml)

summarized in Table 2.3. A standard curve (Fig. 2.7) was produced using the following range of Ins-1,4,5-P_3 concentrations: 0, 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250 pmoles/ml assay buffer. Ins-1,4,5-P_3 levels were measured in standards and samples using 100 µl aliquots to which were added 100 µl of assay buffer, 100 µl [^{3}H]Ins-1,4,5-P_3 reagent and 100 µl binding protein. Following vortex mixing, these tubes were incubated on ice for 15 min. Tubes were then centrifuged at 2400 rpm in an MSE Coolspin centrifuge for 7 min at 4°C. The supernatants were discarded and the pellets resuspended in 200 µl deionized water before being transferred to a liquid scintillation vial containing 2 ml PCS liquid scintillation fluid. Radioactivity (dpm) was counted as described previously to determine the amount of bound tracer.

A total counts (TC) reading was obtained by adding 100 µl assay buffer, 100 µl [^{3}H]Ins-1,4,5-P_3 reagent and 200 µl deionized water directly to the scintillant without centrifugation. A non-specific binding (NSB) and a zero standard (B_0) reading were also obtained (see Table 2.3), and the following calculations made:-

(1) non-specific binding of tracer,

$$\%NSB/TC = 100\ NSB/TC$$

(2) specific binding of tracer,

$$\%B_0/TC = 100\ (B_0 - NSB)/TC$$

(3) normalized percent bound/B_0,

$$\%B/B_0 = 100\ (\text{standard or unknown} - NSB)/(B_0 - NSB)$$

The non-specific binding (1), determined in the presence of excess Ins-1,4,5-P_3, was ca. 7.7%. Specific binding (2) was calculated as 47.4%. Results were calculated using a standard calibration curve (Fig. 2.7) relating the normalized percentage tracer bound (3) to Log_{10}Ins-1,4,5-P_3 concentration (pmoles/ml) and expressed as pmoles/mg protein.

Cytochemical Localization of K^+-dependent, Ouabain-sensitive *p*-nitrophenyl Phosphatase (K^+-NPPase)

The procedure used to localize K^+-NPPase during the present study is essentially the same as that used by Ernst (1972 a,b) in localization of K^+-dependent phosphatase activity in the avian salt gland. Mature adult locusts of both sexes were used throughout these experiments.

Preparation of Tissue for Electron Microscopy

Animals were killed as described earlier. Malpighian tubules were dissected out in ice-cold 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.5, containing 0.25 M sucrose. Tubules were fixed in the same, but fresh solution for 30 min at *ca.* 4°C.

Following paraformaldehyde fixation, tubules were rinsed thoroughly with 0.1 M Tris buffer, pH 7.5, containing 0.25 M sucrose. Cacodylate inhibits ATPase activity and therefore must be washed out completely at this stage. Tubules were then incubated for 60 min at room temperature in the following incubation media (final concentrations):-

(1) standard incubation medium; 100 mM Tris-HCl buffer, pH 9.0, containing 20 mM $SrCl_2$, 10 mM $MgCl_2$, 10 mM KCl and 5 mM NPP (*p*-nitrophenyl phosphate),

(2) control incubation media;

  (a) 10 mM KCl replaced by 10 mM choline chloride,

  (b) 5 mM NPP replaced by 5 mM β-glycerophosphate,

  (c) 10 mM ouabain added to standard incubation medium.

Following incubation in the various media, the tubules were rinsed in three changes of 0.1 M Tris-HCl buffer, pH 9.0, and were treated with two 5 min rinses with 2% $Pb(NO_3)_2$. The latter step is necessary to convert the precipitated strontium phosphate to lead phosphate for visualization in the electron microscope (Ernst, 1972b). Strontium phosphate precipitates are not dense enough for easy viewing in transmission electron microscopy, and are soluble at neutral pH and in osmium solutions even when post-fixation is carried out at alkaline pH.

After rinsing in the lead nitrate solution, the tissue was washed in 0.25 M sucrose to remove any free lead, rinsed in deionized water, dried on filter paper and then post-fixed for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.5.

Following post-fixation, the tubules were rinsed briefly in deionized water and treated according to the following procedure:-

(1) 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,

(2) 20 min in acetone, with 3 changes,

(3) 1-2 hr in acetone mixed with soft "Araldite" (Araldite 10 ml, DDSA 10 ml, dibutyryl phthalate 2ml, D.M.P.30 1 ml) in 1:1 ratio at room temperature,

(4) 30 min in Araldite alone at 45°C,

(5) embedded in fresh Araldite alone for polymerization at 45°C overnight,

(6) preparation moved from 45°C to 60°C for a further 48 hr.

Silver/gold section were cut on a Reichart NK ultratome using glass knives. Sections were expanded with diethyl ether vapour and mounted on uncoated copper grids (3 mm diameter). The sections were then stained in uranyl acetate and lead citrate (Reynolds, 1963) and were examined in a Phillips EM 400T.

Biochemical Assay of K^+-NPPase Activity

Comparative biochemical measurements of *p*-NPPase activity were carried out on both fixed and unfixed material to investigate the effects of fixation and/or incubation in the histochemical reaction medium on enzyme activity in Malpighian tubule preparations of *Locusta migratoria.*

Animals were killed as described previously. Tubules were isolated in ice-cold (0-4°C) 200 mM sucrose isolation medium containing 5 mM Tris-HCl buffer at pH 7.2. Tubules were washed in six changes of the same solution and then blotted on filter paper. The tubules were then divided into approximately equal amounts, one group to be fixed, and the other not. Tissue to be fixed was placed in 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.5, containing 0.25 M sucrose for 30 min, as for the cytochemical procedure. The fixed tissue was subsequently washed on three changes of Tris-HCl buffer, pH 7.5, divided into two sub-groups and homogenized in 10 ml of fresh isolation medium, one at pH 7.2 and the other at pH 9.0. Similarly, unfixed tissue was divided into two and each sub-group transferred to 10 ml of fresh isolation medium, one at pH 7.2 and the other at pH 9.0, and homogenized.

Samples of homogenates were added to the following incubation media (final concentrations) in a 1:1 ratio:-

(1) standard incubation medium; 102.5 mM Tris-HCl buffer, pH 7.2 or 9.0, 100 mM sucrose, 10 mM $MgCl_2$, 10 mM KCl, 5 mM NPP,

(2) control incubation media;

(a) 10 mM KCl replaced by 10 mM choline chloride,

(b) 5 mM ouabain added to (1),

(c) 20 mM $SrCl_2$ added to (1),

(d) 20 mM $SrCl_2$ added to (2a),

(e) 20 mM $SrCl_2$ added to (2b).

The following experimental procedure was used to assess the biochemical activity of K^+-NPPase in the various preparations:-

(1) 1 ml incubation medium + 1 ml homogenate, incubated 30 min at 30°C. A blank was set up for each incubation medium,

(2) reaction stopped by addition of 2 ml ice-cold 20% TCA,

(3) 3 ml of 2 M Tris solution added to each tube,

(4) tubes allowed to stand at room temperature for 10 min to develop the yellow colour, then placed on ice,

(5) tubes centrifuged at 1000 g using an MSE Coolspin centrifuge for 15 min at 4°C,

(6) tubes returned to ice and the absorbance of the supernatant read at 420 nm with the LKB Biochrom Ultraspec 4050.

A standard *p*-nitrophenol calibration curve (Fig. 2.8) was prepared (see below) and the protein concentrations of the various homogenates were determined according to the method of Bradford (1976). The results were calculated and expressed as the amount of *p*-nitrophenol (NP) released in nmoles/mg protein/min.

P-nitrophenol Standard Calibration Curve

Solutions of varying *p*-nitrophenol concentration were prepared by serial dilution of stock 2 M NP to give 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 mM NP. To 2 ml of each solution was added 2 ml of 20% TCA. Then 3 ml of 2 M Tris solution was added to each mixture, tubes allowed to stand for 10 min at room temperature, and read at 420 nm as described previously.

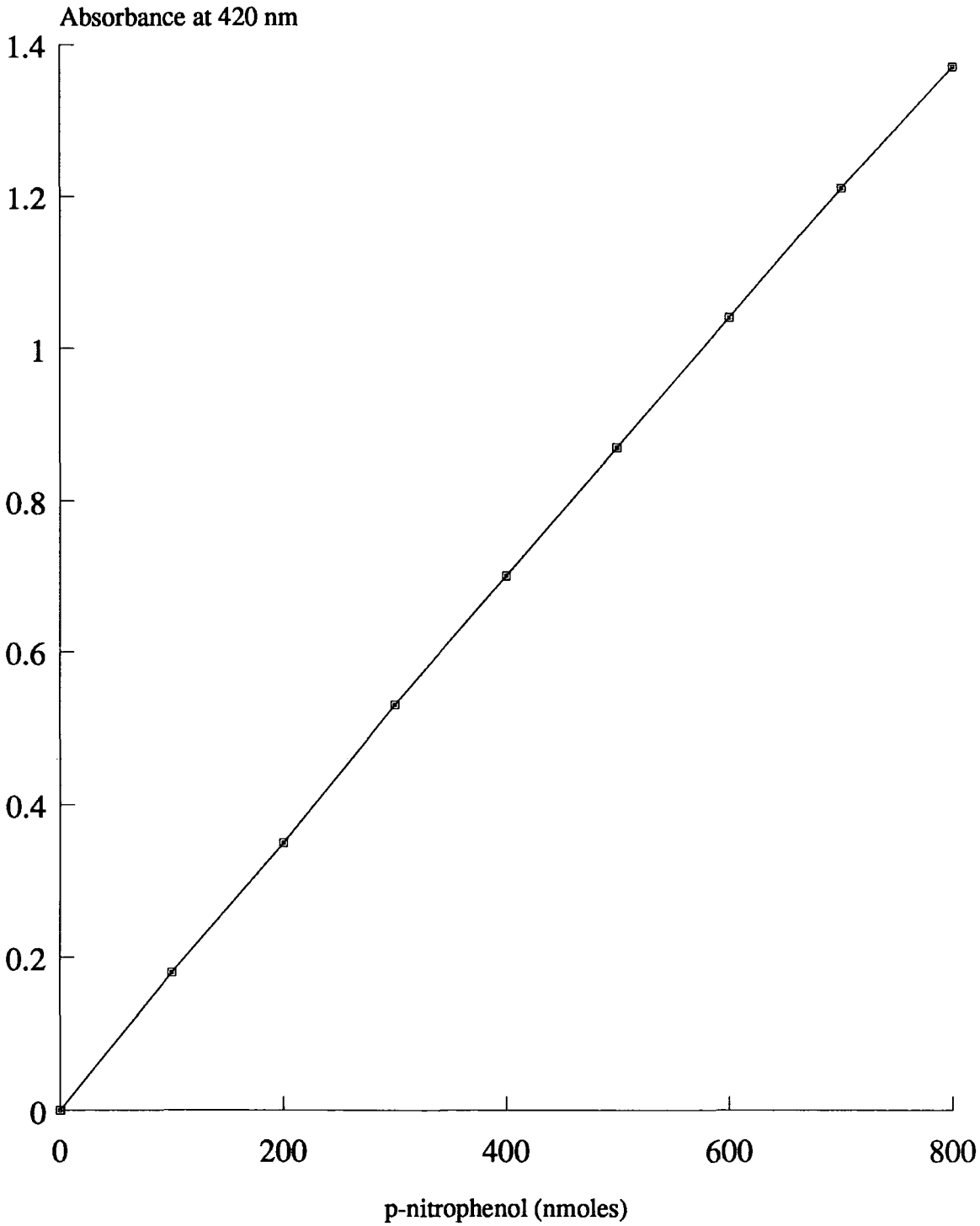
A standard calibration curve was prepared by plotting the amount of NP (moles) against absorbance (Fig. 2.8).

FIGURE 2.8

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

Ordinate : Absorbance at 420 nm.

Abscissa : *p*-nitrophenol in nmoles.


Absorbance at 420 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
p-nitrophenol (nmoles)

Preparation of Non-treated Malpighian Tubules for Electron Microscopy

Locust Malpighian tubules were dissected out as described previously and prepared for electron microscopy by fixation for 1-1.5 hrs in Karnovsky's fixative (Karnovsky, 1965), which comprised of:

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

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

Solutions A and B were mixed in a 1:1 ratio just prior to use. After fixing, material was post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.5) for 1 hr before dehydration through a series of graded ethanols to propylene oxide, prior to embedding in Araldite epoxy resin. Sections were cut and post-stained, as described previously in the histochemical procedure, prior to examination in a Phillips EM 400T.

Membrane Separation for Subsequent ATPase Assays

The method employed was based on that described by Rodriguez and Edelman (1979) with slight modification. During the following procedure a small volume of each fraction was retained on ice for subsequent analysis. Unless otherwise stated, pellets were resuspended and rehomogenized in a known volume of homogenization medium (5 mM imidazole-HCl buffer, pH 7.5, containing 250 mM sucrose). The method is summarized in Fig. 2.9. All steps were carried out at 4°C.

Animals were killed as described previously and their Malpighian tubules dissected out in 10 ml ice-cold homogenization medium. Thorough homogenization was then carried out and the resulting homogenate centrifuged at 600 x g for 10 min using an MSE Coolspin centrifuge. All subsequent centrifugations were carried out using a Fisons MSE "Prepspin 50" Ultracentrifuge, and unless otherwise stated, the 8 x 50 ml aluminium rotor head was used. The pellet (P_1) obtained from the previous centrifugation was retained for assay; the

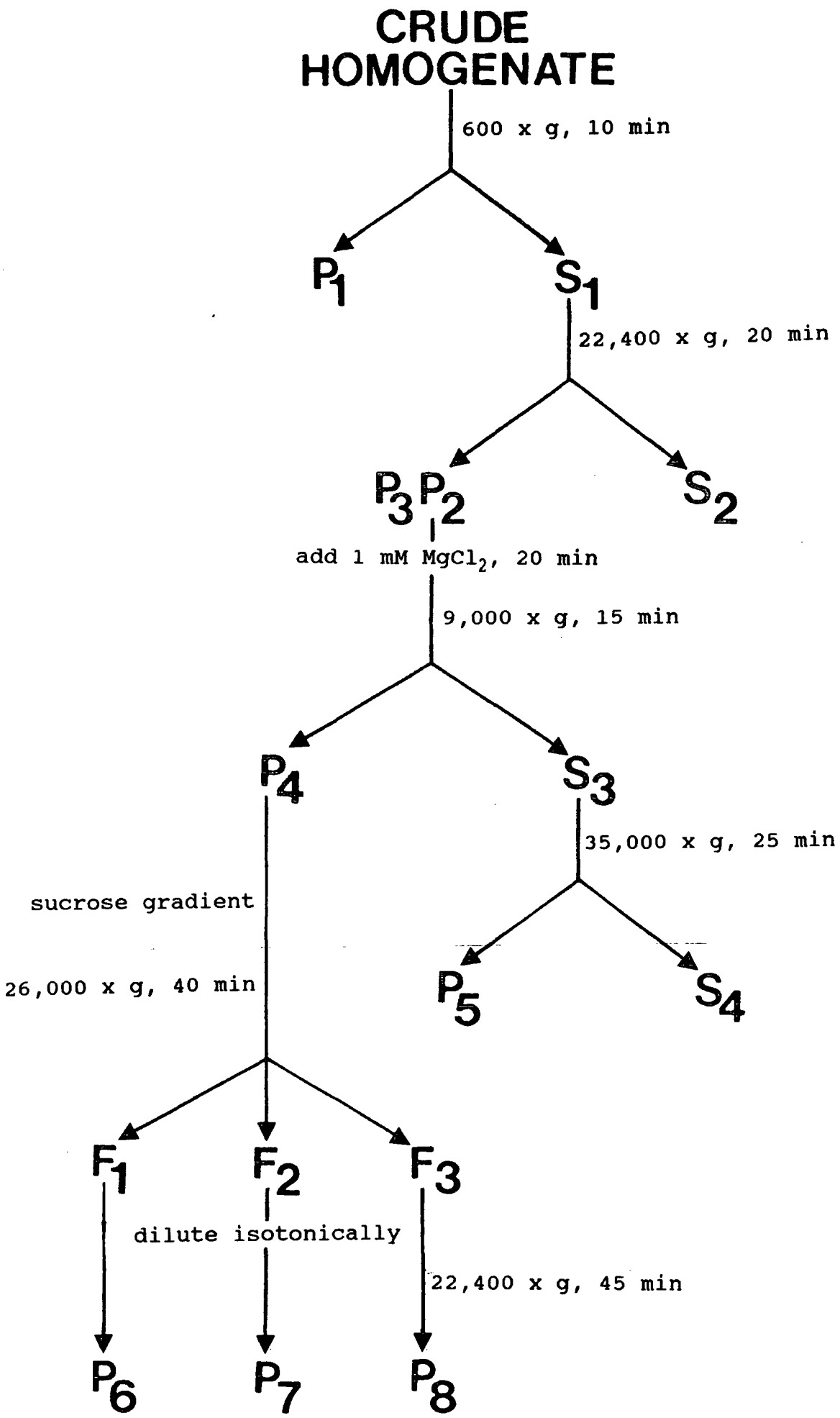
FIGURE 2.9

Flow diagram of the method (Rodreguez and Edelman, 1979) for isolation of the apical and basolateral plasma membranes of the Malpighian tubules of *Locusta migratoria* (see text for details).

Pellets (P)

Supernatants (S)

Intermediate Fraction (F)


CRUDE HOMOGENATE
600 x g, 10 min
P1
S1
22,400 x g, 20 min
P3P2
S2
add 1 mM MgCl2, 20 min
9,000 x g, 15 min
P4
S3
35,000 x g, 25 min
sucrose gradient
26,000 x g, 40 min
P5
S4
F1
F2
F3
dilute isotonically
22,400 x g, 45 min
P6
P7
P8

supernatant (S_1) was subsequently diluted with homogenization medium (if required) and centrifuged at 22400 x g for 20 min. The supernatant (S_2) resulting from this spin was retained for assay. Two pellets result from centrifugation of S_1, the crude plasma membrane pellet (P_2) and the mitochondria-rich pellet (P_3). The "fluffy" P_2 was carefully washed off the more compact P_3 using a minimal volume of homogenization medium, rehomogenized and diluted to a volume of 10 ml with homogenization medium. 1 M $MgCl_2$ was then added to give a final concentration of 10 mM $MgCl_2$ and left to stand on ice for 20 min. P_3 was retained for assay. The resuspended P_2 was subsequently centrifuged at 9000 x g for 15 min. The resulting supernatant (S_3) was then centrifuged at 35000 x g for 25 min. The supernatant (S_4) and the resuspended pellet (P_5) resulting from this spin were retained for assay, P_5 is thought to represent the apical membrane fraction (Rodriguez and Edelman, 1979).

The pellet (P_4) resulting resulting from the centrifugation of resuspended P_2 was resuspended and rehomogenized in a minimal volume of homogenization medium and layered onto a discontinuous sucrose gradient, 7 ml layers of 30, 40, 50 and 60% (w/v) sucrose in 5 mM imidazole-HCl buffer, pH 7.5. The gradient was subsequently centrifuged at 26000 x g for 40 min using a swing-out 6 x 38 ml aluminium rotor head.

Three membrane fractions (F_1, F_2 and F_3) were obtained, suspended at the interface between each sucrose concentration. F_1 is though to represent the basolateral membrane fraction (Rodriguez and Edelman, 1979). Each fraction was carefully removed using a Pasteur pipette, diluted isotonically (i.e., to 250 mM) with 5 mM imidazole-HCl buffer, pH 7.5, then centrifuged at 22400 x g for 45 min to produce the final pellets, P_6, P_7 and P_8 (corresponding to F_1, F_2 and F_3, respectively). Pellets were then resuspended and rehomogenized in a known volume of homogenization medium for assay.

Preparation of Pellets for Electron Microscopy

Pellets obtained from membrane separations were prepared for electron microscopy as described previously for non-treated Malpighian tubules using Karnovsky's fixative (Karnovsky, 1965).

Assay of (Na^++K^+)-ATPase Activity and Analysis of Inorganic Phosphate

Incubations were carried out at 35 ± 0.1°C for a fixed time. Appropriate incubation media were thermoequilibrated for 15 min in disposable polypropylene test tubes. These consisted of 0.5 ml ionic medium and 0.25 ml of 12 mM ATP (Tris salt, see later). Three basis ionic media having the following final concentration of ions were used, unless otherwise stated:-

(1) 4 mM $MgCl_2$ in 20 mM imidazole buffer, pH 7.2,

(2) (1) plus 100 mM NaCl and 20 mM KCl,

(3) (2) plus 1 mM ouabain.

Reactions were started by adding 0.25 ml membrane homogenate (prepared as described previously) and were stopped by the addition of 2 ml of a 1:1 mixture of 1% "Lubrol" and 1% ammonium molybdate in 0.9 M sulphuric acid (Atkinson *et al.*, 1973). Controls were used in each experiment to determine the extent of non-enzymatic hydrolysis of ATP. Tubes were then allowed to stand at room temperature for 10 min to allow the yellow colour to develop.

Following centrifugation at 2000 rpm in an MSE Coolspin centrifuge at 4°C, to spin down any protein that may have precipitated, the supernatants were analysed for inorganic phosphate by reading at 390 nm in a Ultraspec 4050 spectrophotometer. The amount of inorganic phosphate (P_i) released was determined by reference to a standard calibration curve (Fig. 2.10) produced using a stock solution of 0.6 M Na_2HPO_4 which was serially diluted to give a concentration range of 0-0.6 M. To 1 ml of each concentration, 2 ml of the 1:1 Lubrol/acid molybdate (see above) were added, left to stand at room temperature for 10 min and read at 390 nm as above.

Estimation of (Na^++K^+)-ATPase Activity

The enzyme activities of the various membrane fractions prepared previously were determined by measuring the amount of P_i released from ATP hydrolysis (Atkinson *et al.*, 1973). (Na^++K^+)-ATPase activity was determined as the difference in P_i liberated in the reaction medium containing Mg^{2+}, Na^+ and K^+ and that released in the medium containing Mg^{2+}, Na^+, K^+ and ouabain.

Estimation of Mg^{2+}-ATPase Activity

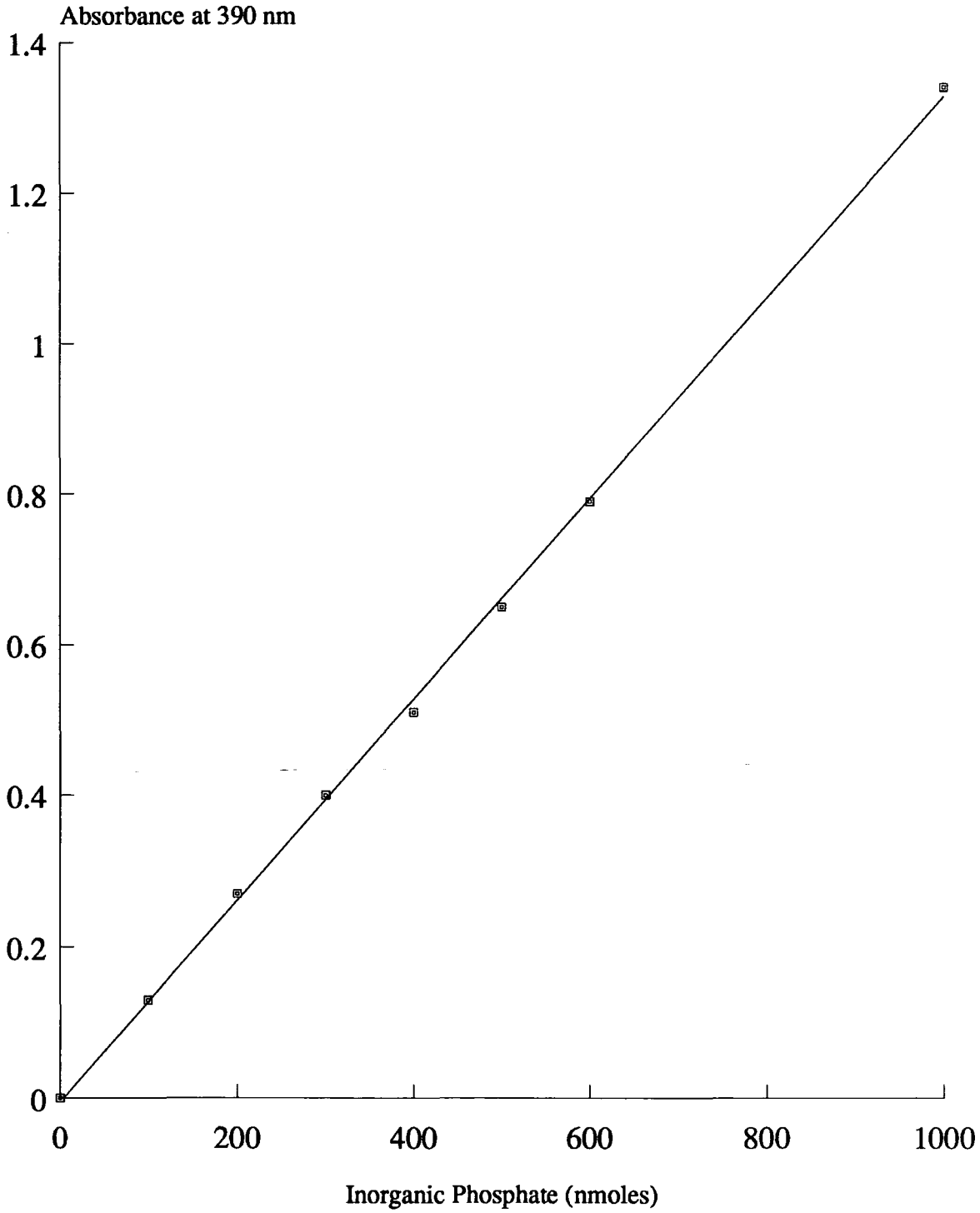
Mg^{2+}-ATPase activity was determined as the difference in P_i liberated in the reaction medium containing Mg^{2+} alone and that released non-enzymatically in the controls.

FIGURE 2.10

Standard calibration curve for the determination of inorganic phosphate.

Ordinate : Absorbance at 390 nm.

Abscissa : Inorganic phosphate (P_i) in nmoles.


Absorbance at 390 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
Inorganic Phosphate (nmoles)

Assay and Estimation of K^+-ATPase Activity

Incubations were carried out as described previously. Two ionic media were used (final concentrations), unless otherwise stated:-

(1) 4 mM $MgCl_2$ and 1 mM ouabain in 1 mM imidazole buffer, pH 7.2,

(2) (1) plus 20 mM KCl.

Liberation of P_i was measured as described previously. K^+-ATPase activity was determined as the difference in P_i liberated in the reaction medium containing Mg^{2+}, K^+ and ouabain and that released in the medium containing Mg^{2+} and ouabain.

Assay and Estimation of Anion-stimulated ATPase Activity

Incubations were carried out as described previously. Three ionic media were used (final concentrations), unless otherwise stated:-

(1) 4 mM $MgCl_2$ in 20 mM imidazole buffer, pH 7.5,

(2) (1) plus 20 mM NaCl,

(3) (1) plus 20 mM $NaHCO_3$.

Liberation of P_i was measured as described previously. Anion-stimulated ATPase activity was determined as the difference in P_i liberated in the reaction medium containing Mg^{2+} and HCO_3^- and that released in the medium containing Mg^{2+} and Na^+.

All ATPase activities were expressed as nmoles P_i liberated/mg protein/min.

Determination of Succinate Dehydrogenase Activity

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

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

$$\text{succinate} + 2\ \text{ferricyanide} \longrightarrow \text{fumarate} + 2\ \text{ferrocyanide} + 2H^+$$

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

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

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 420 nm. The specific activity was expressed as nmoles succinate oxidized/mg protein/min.

Determination of Alkaline Phosphatase Activity

The alkaline phosphatase activities of the various membrane fractions, produced as described previously, were determined using the method described by Bowers and McComb (1966) with slight modification. Reaction media were thermoequilibrated at 35 ± 0.1°C for 15 min. These consisted of 1 ml buffered substrate (1 mM $MgCl_2$ and 25 mM *p*-nitrophenyl phosphate in Sigma 221 buffer, pH 10.3) and 0.5 ml deionized water. The reaction was started by the addition of 0.5 ml membrane homogenate. Changes in absorbance at 420 nm were measured at known intervals over a 20 min period using an Ultraspec 4050 spectrophotometer. Controls were run in parallel in which 0.5 ml deionized water replaced the 0.5 ml homogenate.

The observed changes in absorbency were converted to nmoles *p*-nitrophenol (NP) liberated with reference to a standard *p*-nitrophenol curve (Fig. 2.11) of concentration range 0-800 nmoles NP/ml, prepared by serial dilution from a 2 mM stock solution, plotted against absorbance at 420 nm.

Preparation of 12 mM Tris ATP

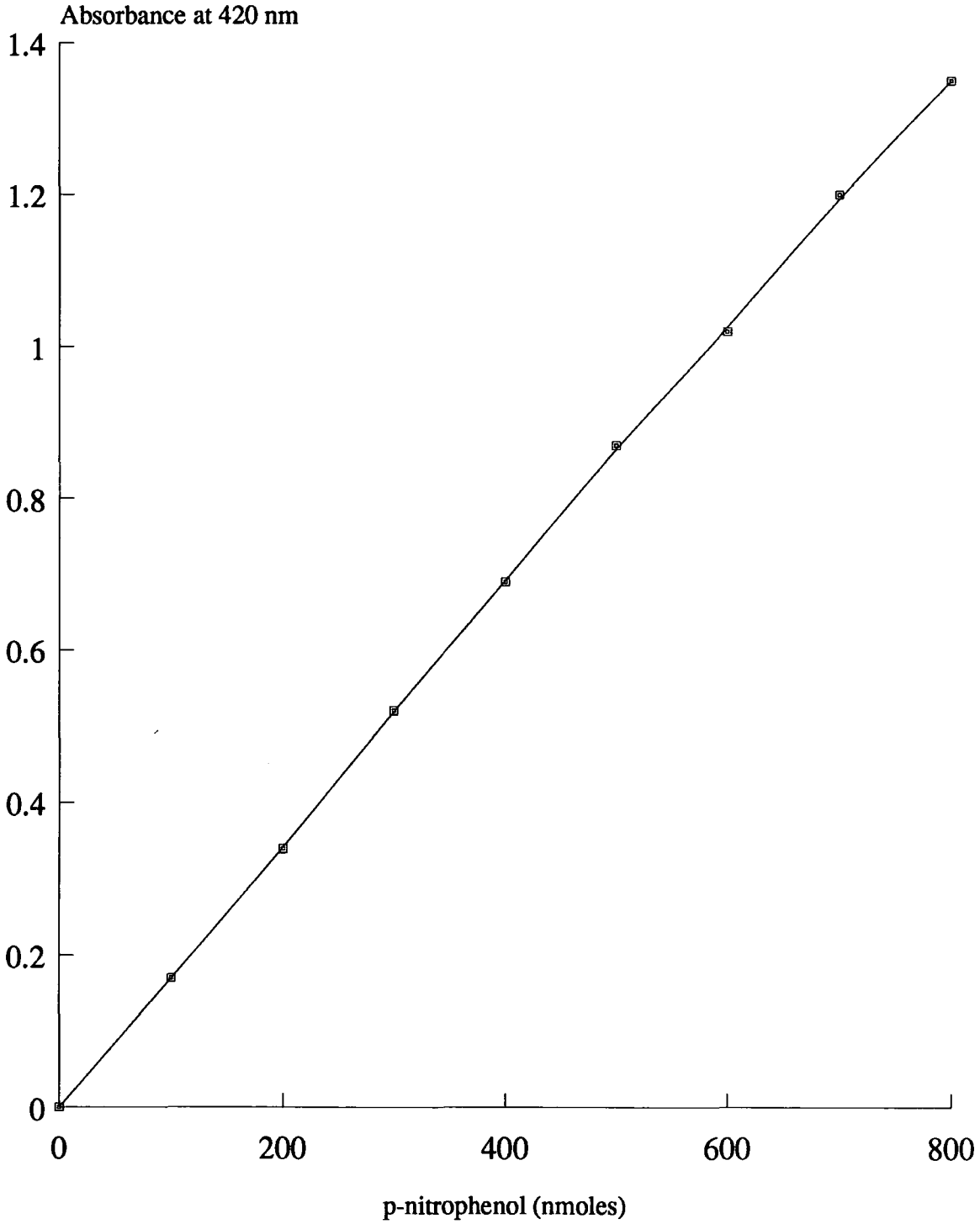
The disodium salt of ATP was converted into Tris ATP by using an ion exchange Dowex 50-X8 resin (H^+ form) according to Schwartz *et al.* (1962). A similar procedure was described by Anstee and Bowler, 1984. 100 mg Dowex resin was first mixed well with 800 ml 5% HCl for 30 min. The resin was then washed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

FIGURE 2.11

Standard calibration curve for the determination of *p*-nitrophenol used in the assay of *p*-NPPase activity.

Ordinate : Absorbance at 420 nm.

Abscissa : *p*-nitrophenol in nmoles.


Absorbance at 420 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
p-nitrophenol (nmoles)

resuspended in its own volume of deionized water and stored at pH 3-4, at room temperature, until required.

When required, a vertically held 5 ml "Volac" glass pipette was packed with resin using a wide mouthed Pasteur pipette. The level of fluid was kept above that of the resin at all times to prevent drying. Air pockets were dislodged with a plunger. Glass wool placed inside the pipette, at its base, prevented the loss of Dowex resin from the column, but allowed the passage of fluids. A silicon tube connected to the base of the column, and a "pinch-clip", enabled the flow of fluid from the column to be controlled.

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

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

CHAPTER 3

RESULTS

All data are expressed as mean values ± SEM. Unless otherwise stated, all statistical comparisons were by application of a paired *t*-test.

Effect of Corpora Cardiaca Extract on Fluid Secretion Rate

The method used to determine the rate of fluid secretion by the Malpighian tubules of *Locusta migratoria, in vitro*, was described previously (Chapter 2).

The mean unstimulated rate of fluid secretion was 1.1 ± 0.2 nl/min (n=24). On addition of corpora cardiaca (CC) extract (final concentration 1 gland pair/ml) to the bathing medium, the fluid secretion rate rose rapidly, within 5 min, to a mean value of 2.5 ± 0.2 nl/min (n=24), representing a significant ($P<0.001$) increase in fluid secretion rate of about 150% above unstimulated. Increased fluid secretion was maintained, in the continued presence of CC extract, for the remaining duration of the experiment (approximately 20 min).

Electrophysiology

The electrophysiological method used to record electrical potentials across the cell membranes of the Malpighian tubules of *Locusta migratoria* was described previously (Chapter 2). The basal (V_B) and apical (V_A) cell membrane potentials and the transepithelial potential (TEP) are expressed in millivolts. Throughout the study, V_B and V_A were found to be negative with respect to the bathing medium and lumen, respectively, and were therefore given a negative prefix. In contrast, TEP, which was measured with respect to the lumen, varied in polarity and was therefore given a positive or negative prefix where necessary.

Membrane Potential Parameters in Control Saline

Mean recorded values for V_B, V_A, TEP and transcellular potential (TCP) are shown in Table 3.1. The mean value for V_B was -70.4 ± 0.9 mV (n=73), cytoplasm negative with respect to the bathing medium, and the mean value for V_A was -76.1 ± 1.5 mV (n=73), cytoplasm negative with respect to the lumen. V_A was significantly different ($P<0.001$)

from V_B. TEP has a mean value of +5.5 ± 1.3 mV (n=73), lumen positive, with a range from -22 to +34 mV. From this range approximately 75% of cells gave a positive TEP value, 23% gave a negative TEP value and only 1% gave a zero TEP value. Thus, in about three quarters of cells examined, V_A was more negative than V_B. There is no significant difference (P>0.1) between TEP and TCP (V_B - V_A), and therefore all subsequent values reported in this study are TEP values.

Cyclic oscillations of V_A, reflected in changes in TEP, occurred in about 50% of cells examined in control saline, as described by Baldrick (1987). In these cases, values for V_A were taken as the mean potential. V_B showed no such oscillations, the potential remaining steady in all cases.

Effects of Corpora Cardiaca Extract in Control Saline

The mean values for potentials measured across the basal (V_B) and apical (V_A) membranes of the tubule cells in control saline were -74.6 ± 2.2 mV (n=13) and -81.0 ± 3.2 mV (n=13) with reference to the bathing medium and the lumen, respectively. The TEP measured was +4.9 ± 2.9 mV (n=13), lumen positive.

Following addition of corpora cardiaca (CC) extract (1 gland pair/ml) to the bathing medium (typical example, Fig. 3.1) there was a slow, but significant (P<0.002) hyperpolarization of V_B by a mean value of 2.3 ± 0.6 mV over a 0.5 min period and by 6.6 ± 1.6 mV (n=13) at 5 min (Table 3.2). In contrast to this slow hyperpolarization of V_B, addition of CC extract resulted in a significant (P<0.01) and rapid depolarization of V_A by a mean value of 9.2 ± 2.3 mV (n=13) over a 0.5 min period. V_A remains relatively stable following this initial rapid depolarization (Table 3.2). Associated with this differential response by V_B and V_A, TEP rapidly changed by a mean value of 9.6 ± 1.9 mV (n=13) from +4.9 ± 2.9 mV, lumen positive, to -4.7 ± 3.2 mV, lumen negative, over a 0.5 min period (Table 3.2). This was followed by a slower change in TEP to a mean value of -7.7 ± 3.2 mV (n=13), lumen negative, at 5 min (Table 3.2). All values for TEP were significantly different to those in control saline alone (P<0.001).

Effects of Corpora Cardiaca Extract in High K^+,Na^+-free Saline

Previous studies (Baldrick *et al.*, 1988) established that the basal cell membrane of Malpighian tubule cells of *Locusta migratoria* is relatively permeable to K^+ compared with Na^+ and Cl^-, which is reflected, in part, in Fig. 3.2a (typical example). Superfusion of tubules with high K^+ (127.6 mM), Na^+-free saline resulted in a significant (P<0.001) near-

Table 3.1 Mean Membrane Potential Parameters in Control Saline

Parameter	Treatment	Mean ± S.E.M. (mV)	Polarity (%) Positive	Zero	Negative	*n*
V_B	Control Saline	-70.4 ± 0.9	-	-	-	73
V_A		-76.1 ± 1.5	-	-	-	73
TCP		+ 5.7 ± 1.4	75	1	23	73
TEP		+ 5.5 ± 1.3	75	1	23	73

V_B and V_A are the basal and apical cell membrane potentials, whilst TCP and TEP are the transcellular (V_B-V_A) and transepithelial potentials, respectively.
n represents the number of independent experiments, each involving separate tubule preparations.

Table 3.2 Effect of Corpora Cardiaca Extract in Various Bathing Salines

Treatment		*n*	V_B (mV)	*P*	V_A (mV)	*P*	TEP (mV)	*P*
a. Control		13	-74.6 ± 2.2	-	-81.0 ± 3.2	-	+4.9 ± 2.9	-
b. Control + Corpora Cardiaca Extract	0.5 min	13	-76.8 ± 2.5	a:b < 0.002	-71.8 ± 2.7	a:b < 0.01	-4.7 ± 3.2	a:b < 0.001
c. " " " "	1.0 min	13	-77.7 ± 2.3	b:c < 0.001	-71.5 ± 2.8	b:c < 0.001	-5.6 ± 3.4	b:c < 0.001
d. " " " "	5.0 min	13	-81.2 ± 2.4	b:d < 0.002	-73.1 ± 3.4	b:d < 0.01	-7.7 ± 3.2	b:d < 0.001
a. Control		4	-70.2 ± 3.5	-	-78.3 ± 5.1	-	+5.9 ± 6.5	-
b. High K^+, Na^+-free		4	-6.9 ± 3.1	a:b < 0.001	-56.0 ± 5.0	a:b < 0.001	+47.1 ± 6.8	a:b < 0.002
c. High K^+, Na^+-free + Corpora Cardiaca Extract	0.5 min	4	-11.4 ± 2.7	b:c < 0.02	-32.3 ± 4.4	b:c < 0.05	+18.4 ± 4.9	b:c < 0.05
d. " " " " "	1.0 min	4	-12.7 ± 3.0	b:d < 0.02	-26.6 ± 5.0	b:d < 0.02	+12.5 ± 3.6	b:d < 0.02
e. " " " " "	5.0 min	4	-15.0 ± 2.6	d:e ns	-20.8 ± 4.7	d:e < 0.001	+5.7 ± 4.3	b:e < 0.002
a. Control		6	-68.6 ± 5.0	-	-76.2 ± 4.6	-	+8.2 ± 4.1	-
b. Cl^--free		6	-65.2 ± 5.4	a:b ns	-85.9 ± 5.0	a:b < 0.01	+21.2 ± 3.3	a:b < 0.001
c. Cl^--free + Corpora Cardiaca Extract	0.5 min	6	-58.6 ± 6.0	b:c < 0.05	-93.5 ± 7.0	b:c ns	+35.7 ± 5.6	b:c < 0.01
d. " " " "	1.0 min	6	-58.1 ± 5.8	b:d < 0.02	-99.8 ± 6.6	b:d < 0.01	+42.2 ± 4.7	b:d < 0.001
e. " " " "	5.0 min	6	-57.5 ± 5.7	b:e < 0.05	-99.1 ± 6.1	b:e < 0.001	+41.7 ± 3.4	b:e < 0.001
a. High K^+		4	-12.6 ± 5.6	-	-59.2 ± 7.2	-	+43.9 ± 6.8	-
b. High K^+, Na^+, Cl^--free		4	-2.5 ± 3.0	a:b < 0.05	-64.5 ± 8.0	a:b ns	+62.2 ± 7.3	a:b < 0.05
c. High K^+, Na^+, Cl^--free + Corpora Cardiaca Extract	0.5 min	4	-3.6 ± 2.9	b:c ns	-68.8 ± 7.9	b:c ns	+65.4 ± 5.9	b:c ns
d. " " " " "	1.0 min	4	-3.7 ± 3.1	b:d ns	-70.3 ± 7.4	b:d ns	+67.5 ± 5.9	b:d ns
e. " " " " "	5.0 min	4	-3.1 ± 2.5	b:e ns	-72.8 ± 6.9	b:e < 0.02	+70.8 ± 5.6	b:e < 0.05
a. Control		7	-70.1 ± 0.9	-	-74.9 ± 6.7	-	+6.2 ± 5.9	-
b. Na^+-free		7	-67.9 ± 1.0	a:b ns	-78.0 ± 6.1	a:b ns	+9.9 ± 6.2	a:b < 0.01
c. Na^+-free + Corpora Cardiaca Extract	0.5 min	7	-69.3 ± 1.4	b:c ns	-72.1 ± 6.1	b:c < 0.05	+3.1 ± 5.8	b:c < 0.05
d. " " " "	1.0 min	7	-69.7 ± 1.6	b:d ns	-72.5 ± 6.4	b:d < 0.05	+2.8 ± 5.7	b:d < 0.05
e. " " " "	5.0 min	7	-71.6 ± 3.3	b:e ns	-71.4 ± 6.0	b:e < 0.05	+0.3 ± 5.2	b:e < 0.02
a. Ca^{2+}-free		7	-58.0 ± 2.5	-	-59.3 ± 3.8	-	+1.6 ± 2.5	-
b. Ca^{2+}-free + Corpora Cardiaca Extract	0.5 min	7	-56.5 ± 2.3	a:b ns	-58.5 ± 4.1	a:b ns	+2.3 ± 2.8	a:b ns
c. " " " "	1.0 min	7	-56.3 ± 2.5	a:c < 0.05	-57.7 ± 3.7	a:c < 0.05	+1.0 ± 2.9	a:c ns
d. " " " "	5.0 min	7	-53.5 ± 3.1	a:d < 0.05	-59.8 ± 5.0	a:d ns	+6.6 ± 3.8	a:d < 0.05

FIGURE 3.1

A typical example of the effects of corpora cardiaca extract (CC extract) on basal (V_B) and apical (V_A) membrane potentials and transepithelial potential (TEP) of a Malpighian tubule cell of *Locusta migratoria*. Resting potentials of V_B and V_A were established in control saline, and CC extract was introduced at the time indicated.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

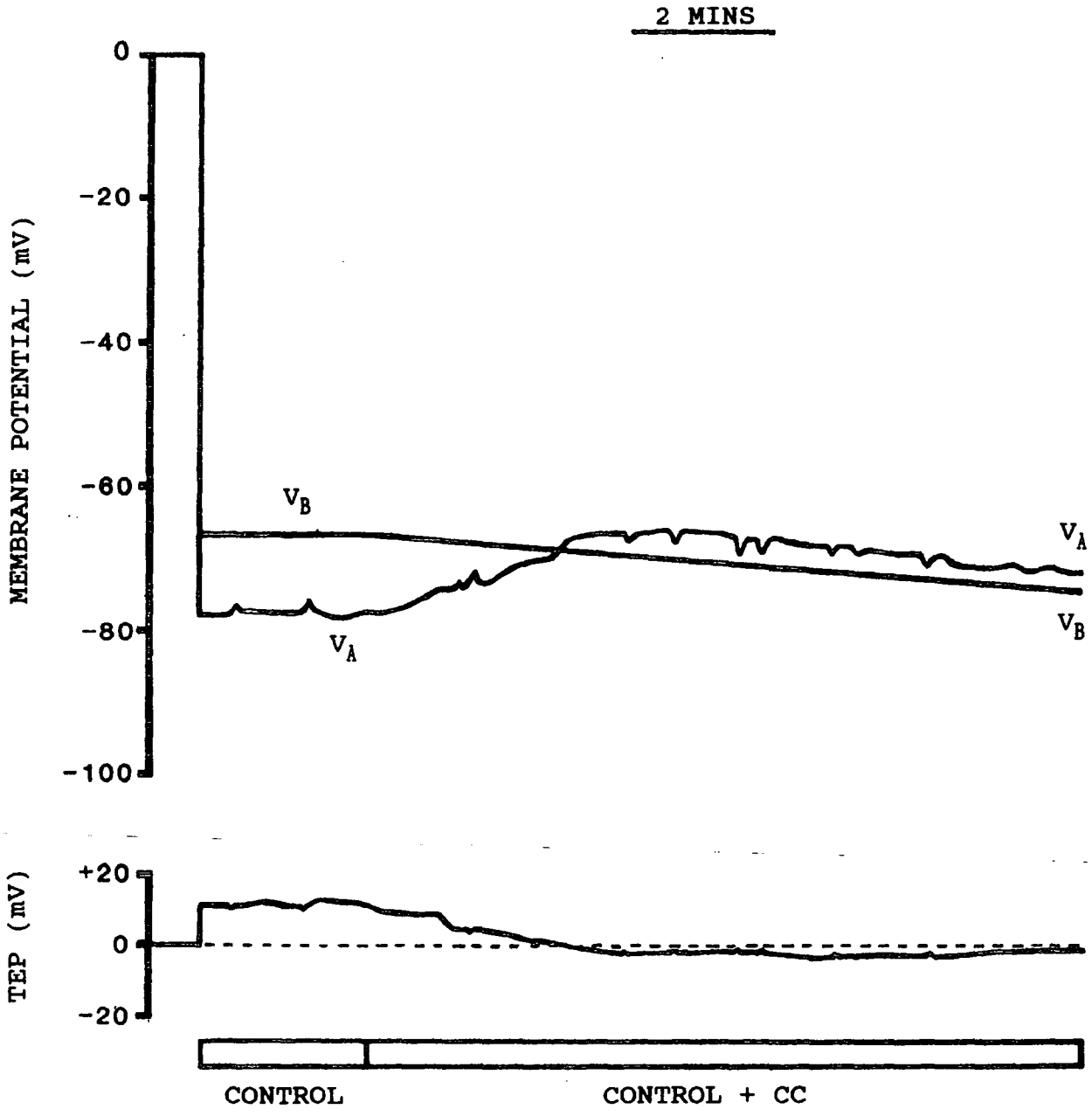

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
V_A
V_B
+20
0
-20
TEP (mV)
CONTROL
CONTROL + CC

FIGURE 3.2

(a) A typical example of the "Type A" response to treatment with 127.6 mM K^+, Na^+-free saline. Resting potentials were established in control saline.

(b) A typical example of the "Type B" response to treatment with 127.6 mM K^+, Na^+-free saline. The experimental protocol was the same as that used to obtain the data in Fig. 3.2a.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

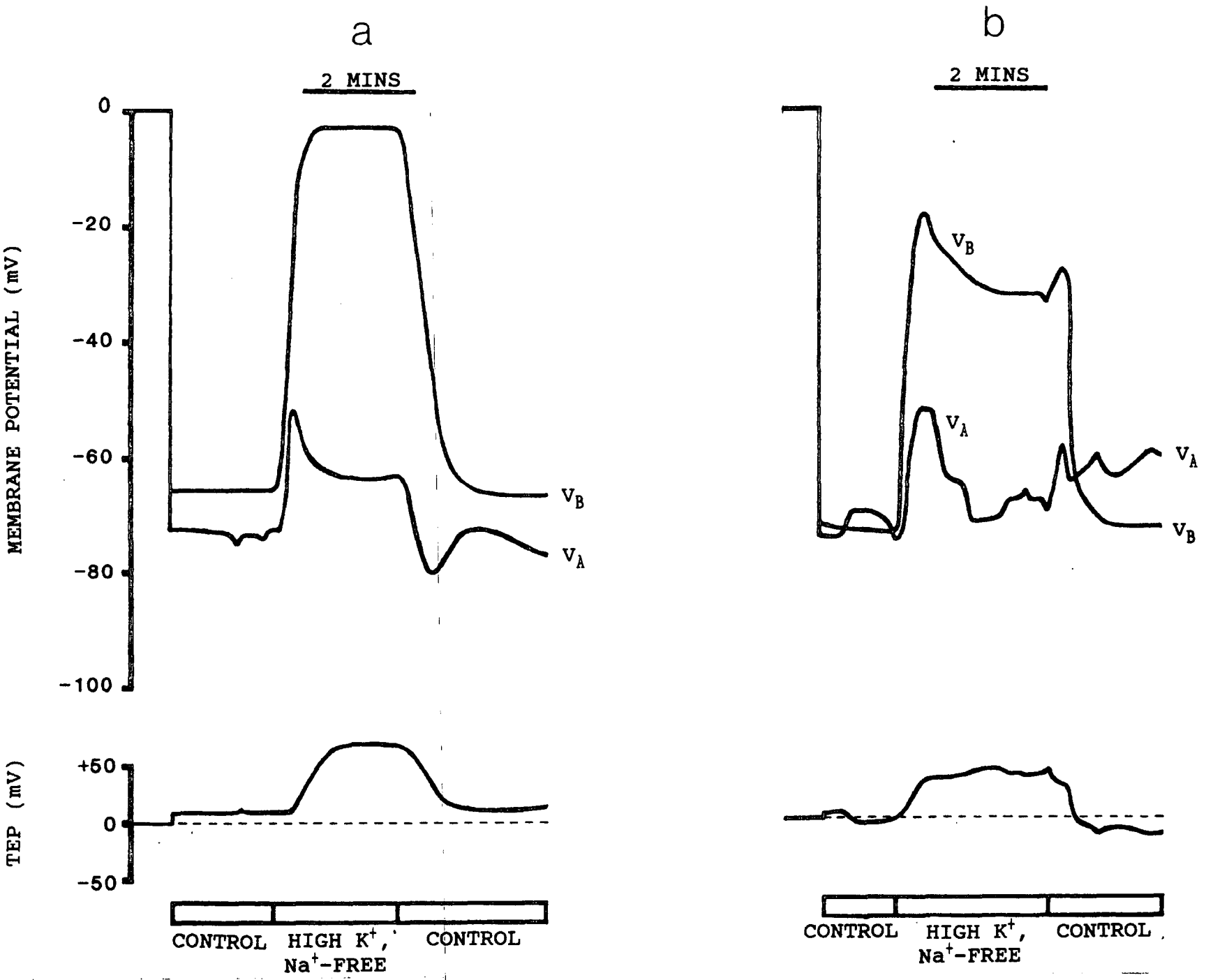

a
b
2 MINS
2 MINS
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
-100
V_B
V_A
V_B
V_A
V_A
V_B
TEP (mV)
+50
0
-50
CONTROL
HIGH K^+, Na^+-FREE
CONTROL
CONTROL
HIGH K^+, Na^+-FREE
CONTROL

FIGURE 3.3

A typical recording of V_B, V_A and TEP following treatment with 127.6 mM K^+, Na^+-free saline and the subsequent addition of CC extract. Resting potentials were initially established in control saline.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

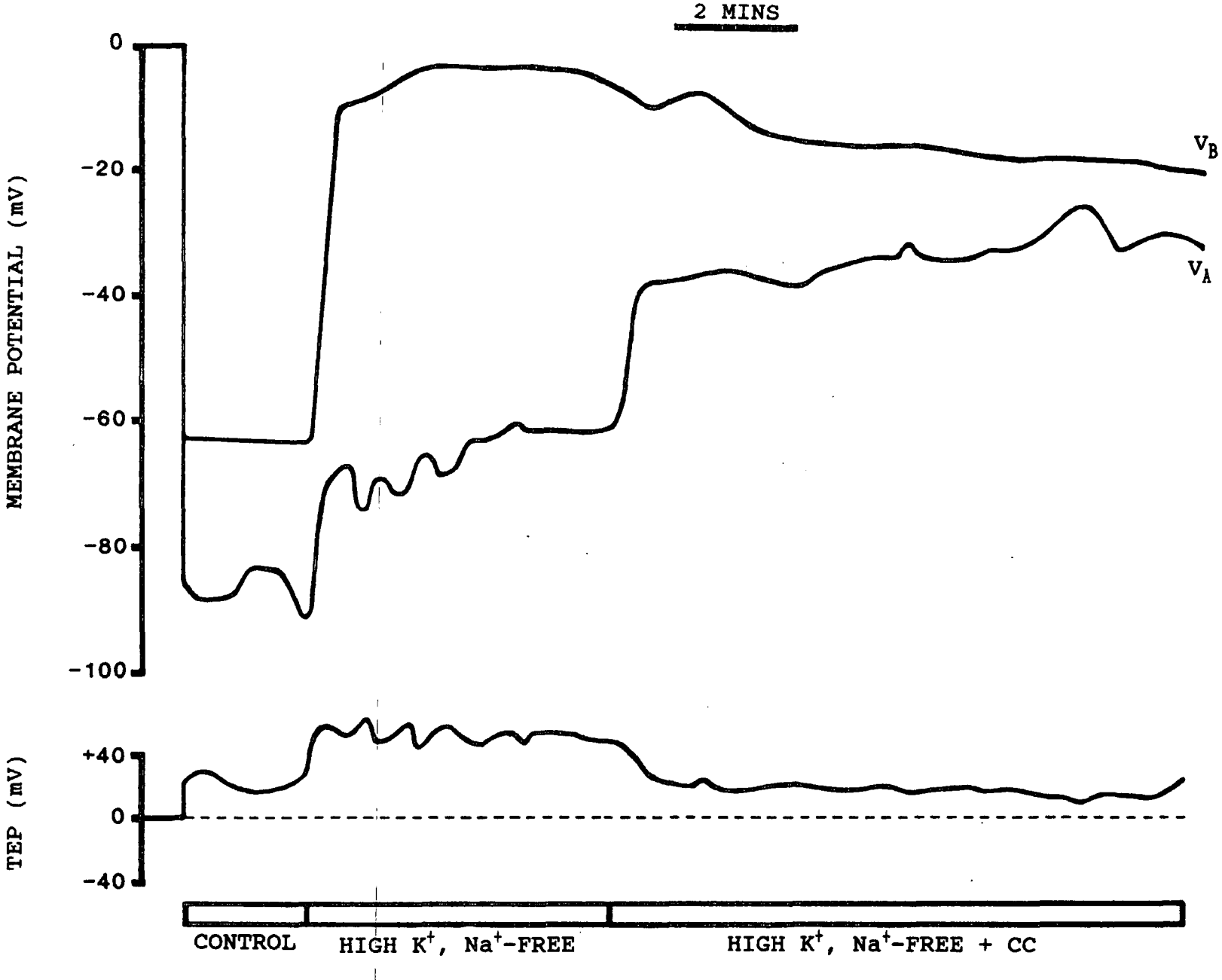

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+40
0
-40
TEP (mV)
CONTROL
HIGH K^+, Na^+-FREE
HIGH K^+, Na^+-FREE + CC

FIGURE 3.4

A typical recording of the response of V_B, V_A and TEP following treatment with Cl^--free saline and the subsequent addition of CC extract. Resting potentials were established in control saline.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

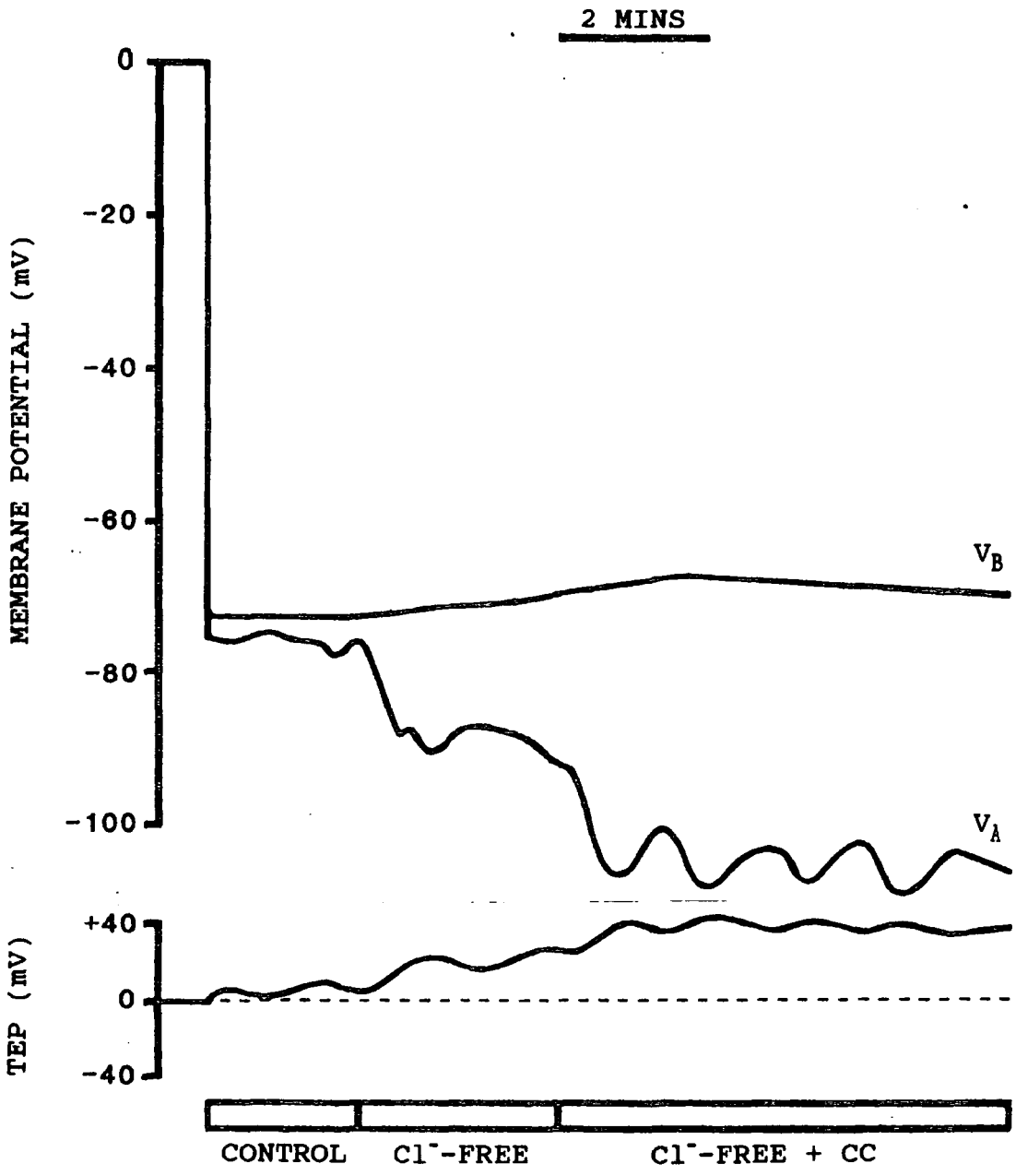

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+40
0
-40
TEP (mV)
CONTROL
Cl^--FREE
Cl^--FREE + CC

FIGURE 3.5

A typical recording of the response of V_B, V_A and TEP following treatment with 127.6 mM K^+, Na^+-free, Cl^--free saline and subsequent addition of CC extract. Resting potentials were established in control saline followed by treatment with 127.6 mM K^+, Na^+-free saline.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

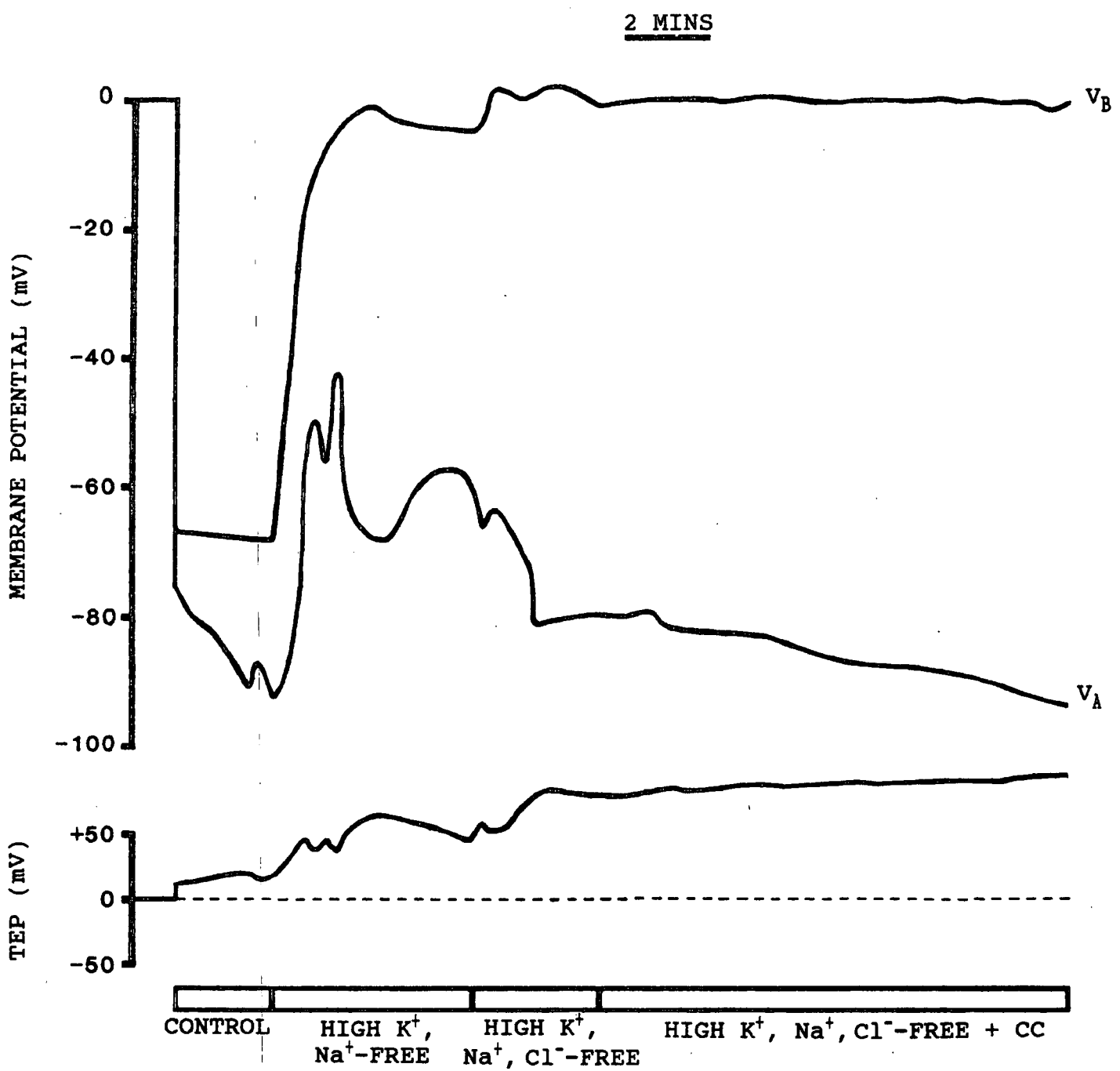
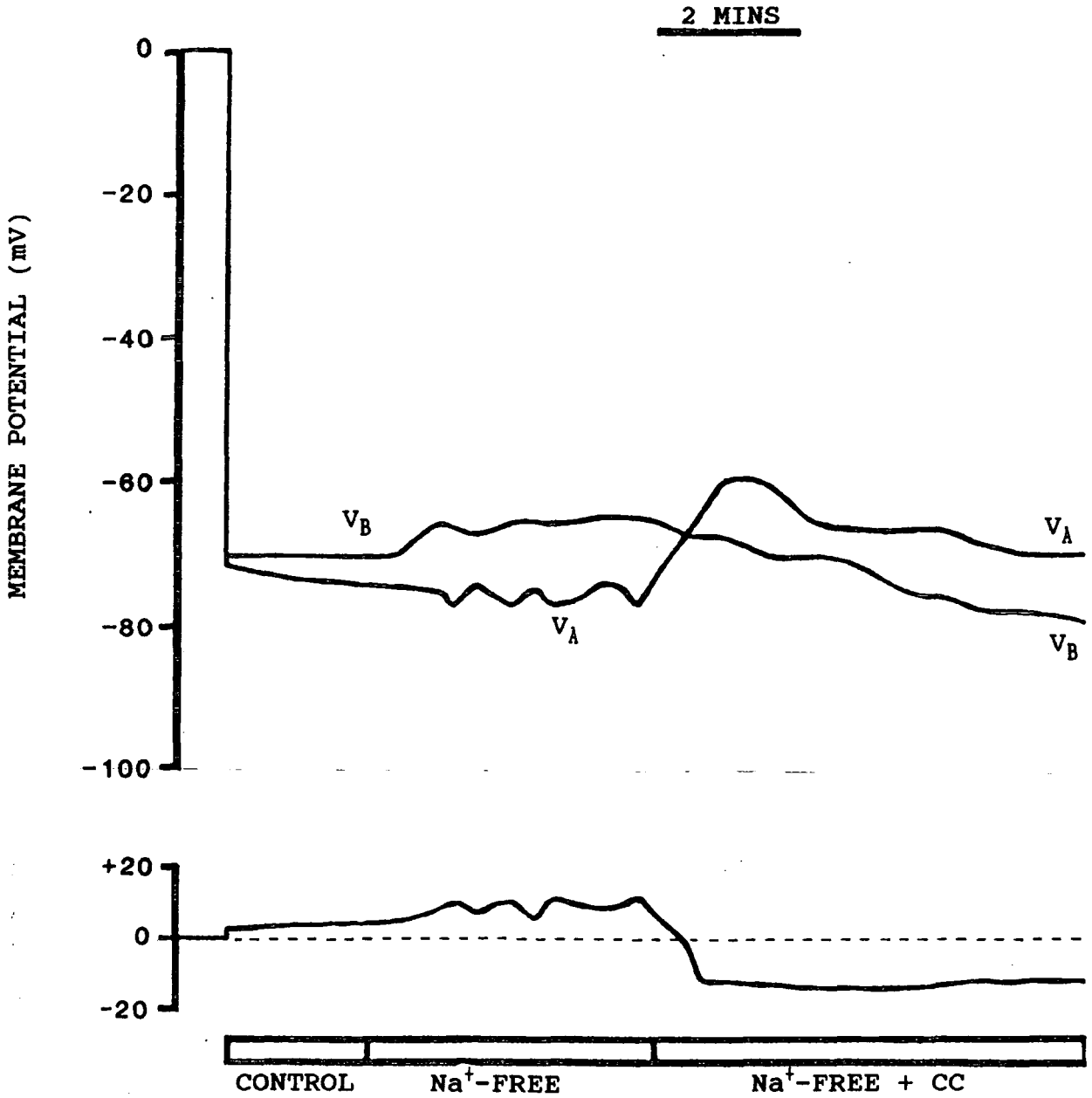

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+50
0
-50
TEP (mV)
CONTROL
HIGH K^+, Na^+-FREE
HIGH K^+, Na^+, Cl^--FREE
HIGH K^+, Na^+, Cl^--FREE + CC

FIGURE 3.6

An example of the response of V_B, V_A and TEP to Na^+-free saline. After establishing resting potentials in control saline, the latter was changed for Na^+-free saline followed by Na^+-free saline containing CC extract.

Ordinate : Potential in mV.

Abscissa : Time in minutes.


2 MINS
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
-100
V_B
V_A
V_A
V_B
+20
0
-20
CONTROL
Na^+-FREE
Na^+-FREE + CC

Nernstian depolarization of V_B by a mean value of 57.8 ± 2.0 mV (n=17) from -67.6 ± 1.9 mV, in control saline, to -9.8 ± 1.8 mV. The depolarization predicted by Nernst is 70.4 mV, based on $[K^+]_i$ remaining constant at 164.5 mM (estimated by Baldrick *et al.*, 1988). There was also a significant ($P<0.001$) change in V_A by a mean value of 22.0 ± 2.5 mV (n=17) from -73.3 ± 2.4 mV, in control saline, to -51.3 ± 3.6 mV.

The results described above, in which tubules are bathed in high K^+, Na^+-free saline (Fig. 3.2a), are representative of approximately 77% of experiments carried out. Similar findings were reported by Baldrick *et al.* (1988), who designated this the "Type A" response. In the remaining experiments a marked variation from this pattern of response was observed (typical example 3.2b), where the initial potential change in V_B on switching to high K^+/Na^+-free saline was not maintained. Baldrick *et al.* (1988) designated this the "Type B" response. Superfusion of these tubules with high K^+, Na^+-free saline resulted in an initial depolarization of V_B by a mean value of 45.8 ± 3.9 mV (n=5) from -74.5 ± 4.2 mV, in control saline, to -31.0 ± 6.8 mV. This was followed by a rapid repolarization to a stable value of -50.4 ± 2.1 mV. Similarly, V_A initially depolarized by 29.0 ± 3.6 mV (n=5) from -76.6 ± 4.9 mV, in control saline, to -51.4 ± 7.6 mV. As with V_B, V_A then repolarized to -76.4 ± 5.1 mV, a value not significantly different to that observed in control saline. Baldrick *et al.* (1988) proposed that the tubules taken from some insects were in such a state that superfusion with high K^+, Na^+-free saline resulted in an overly increased Cl^- entry across the basal membrane effecting the repolarization of V_B and partial short-circuiting of the apical cation pump, with a consequent repolarization of V_A. This study is not concerned with the Type B response. Therefore, all experiments involving the use of high K^+, Na^+-free saline refer to cells exhibiting the Type A response.

Addition of CC extract in the continued presence of high K^+, Na^+-free saline (typical example, Fig. 3.3) resulted in qualitatively similar changes in V_B and V_A as with control saline; a rapid depolarization of V_A and a slow hyperpolarization of V_B, although the latter was not statistically significant ($P>0.05$) at 5 min (Table 3.2). However, the extent of depolarization of V_A, 5 min after the addition of CC extract in the continued presence of high $[K^+]$, Na^+-free saline, was significantly ($P<0.001$) greater (mean depolarization 35.1 ± 2.6 mV, n=4) than that observed in control saline (mean depolarization 7.9 ± 2.4 mV, n=16), on the basis of a Students *t*-test for single data.

Effects of Corpora Cardiaca Extract in Cl^--free Saline

Superfusion of tubules with Cl^--free saline (typical example, Fig. 3.4) resulted in a small, but non-significant depolarization of V_B from a mean value of -68.6 ± 5.0 mV, in control saline, to -65.2 ± 5.4 mV (n=6). In contrast, V_A showed a significant ($P<0.01$)

hyperpolarization by a mean value of 9.6 ± 2.4 mV (n=6) from -76.2 ± 4.6 mV, in control saline, to -85.9 ± 5.0 mV, with a consequent increase in lumen positivity (Table 3.2).

Addition of CC extract in the continued absence of Cl^- (typical example, Fig. 3.4) resulted in a variable initial response by V_B. However, for a total of six experiments there was a significant ($P<0.05$) depolarization of V_B by a mean value of 7.7 ± 2.5 mV during the first 5 min (Table 3.2). In contrast to the depolarization of V_A observed in control saline on addition of CC extract (Fig. 3.1), in Cl^--free saline a significant ($P<0.001$) hyperpolarization of V_A by a mean value of 13.2 ± 1.8 mV (n=6) from -85.9 ± 5.0 mV to -99.1 ± 6.1 mV was observed. The changes in V_B and V_A on addition of CC extract in Cl^--free saline were reflected by TEP, which became more lumen positive by a mean value of 20.6 ± 1.6 mV (n=6).

Effects of Corpora Cardiaca Extract in High K^+, Na^+-free, Cl^--free Saline

Superfusion of tubules with high K^+, Na^+-free, Cl^--free saline (typical example, Fig. 3.5) resulted in a significant ($P<0.05$) depolarization of V_B by a mean value of 10.1 ± 2.6 mV (n=3) from -12.6 ± 5.6 mV, in high K^+, Na^+-free saline, to -2.5 ± 3.0 mV (Table 3.2). There was a small, but non-significant hyperpolarization of V_A. However, in three out of four experiments V_A did hyperpolarize by 6-15mV. The change in V_B was reflected by a significant ($P<0.05$) increase in lumen positivity (Table 3.2).

Addition of CC extract in the continued presence of high K^+, Na^+-free, Cl^--free saline (typical example, Fig. 3.5) had no significant affect on V_B. However, V_A showed a significant hyperpolarization ($P<0.02$) by a mean value of 8.3 ± 1.6 mV (n=4) from -64.5 ± 8.0 mV to -72.8 ± 2.2 mV at 5 min (Table 3.2). As a consequence, there was a significant ($P<0.05$) increase in lumen positivity (Table 3.2) by a mean value of 8.6 ± 2.2 mV (n=4).

Effects of Corpora Cardiaca Extract in Na^+-free Saline

Following superfusion with control saline, Na^+-free saline resulted in a small, but non-significant depolarization of V_B and hyperpolarization of V_A by a mean value of 2.2 ± 1.1 mV and 3.2 ± 1.7 mV (n=7), respectively (typical example, Fig. 3.6). However, the differential responses of V_B and V_A were reflected by a small, but significant ($P<0.01$) increase in lumen positivity (mean increase 3.7 ± 0.7 mV).

Addition of CC extract in the continued absence of Na^+ resulted in qualitatively similar results as in control saline. Thus, there was a rapid depolarization of V_A and a slow hyperpolarization of V_B, although the latter was not statistically significant (Table 3.2).

Effects of Corpora Cardiaca Extract in Ca^{2+}-free Saline

For the purpose of these experiments locust Malpighian tubules were dissected out in Ca^{2+}-free saline to help run down intracellular stores.

The mean value for potentials measured across the basal and apical membranes of tubule cells bathed in Ca^{2+}-free saline were -58.0 $\pm$ 2.5 mV and -59.3 $\pm$ 3.8 mV (n=7) with reference to the bathing medium and lumen, respectively. The mean measured TEP was +1.6 $\pm$ 2.5 mV (n=7), lumen positive.

Following the addition of CC extract, a small, but significant ($P<0.05$) depolarization of V_A was observed during the first minute (mean depolarization 1.6 $\pm$ 0.5 mV, n=7). This was then followed by a slow repolarization of V_A, to a value not significantly different to that in Ca^{2+}-free alone (Table 3.2). Addition of CC extract also effected a slow, but significant ($P<0.05$) depolarization of V_B (Table 3.2) over a 5 min period (mean depolarization 4.5 $\pm$ 1.5 mV, n=7). The change in V_B is reflected in a slow increase in TEP by a mean value of 5.0 $\pm$ 1.9 mV (n=7) over the 5 min period (Table 3.2).

Effect of 10^{-5} M IBMX in Control Saline

Superfusion of tubules with control saline containing the phosphodiesterase inhibitor IBMX at a concentration of 10^{-5} M (typical example, Fig. 3.7) did not significantly affect V_B (Table 3.3). However, such treatment did result in a significant ($P<0.05$) hyperpolarization of V_A by a mean value of 8.8 $\pm$ 2.6 mV (n=4) from -82.2 $\pm$ 2.1 mV, in control saline, to -91.0 $\pm$ 1.3 mV, over a 5 min period (Table 3.3). This change was reflected by a significant ($P<0.02$) increase in lumen positivity (Table 3.3).

Effect of 1 mM Dibutyryl cAMP in Control Saline

Superfusion of tubules with control saline containing 1 mM dibutyryl cAMP (typical example, Fig. 3.8) had no significant effect on V_B (Table 3.3). However, such treatment did result in a significant ($P<0.02$) slow hyperpolarization of V_A by a mean value of 9.4 $\pm$ 2.4 mV (n=6), over a 5 min period, from -84.8 $\pm$ 6.5 mV, in control saline, to -94.2 $\pm$ 4.4 mV. V_A remained relatively stable after this point (Table 3.3). These results are

Table 3.3 Effect of IBMX, cAMP, Thiocyanate, and Acetazolamide in Various Bathing Salines

Treatment		*n*	V_B (mV)	*P*	V_A (mV)	*P*	TEP (mV)	*P*
a. Control		4	-76.8 ± 2.8	-	-82.2 ± 2.1	-	+5.5 ± 1.8	-
b. Control + IBMX (10^{-5} M)	1.0 min	4	-76.7 ± 2.9	a:b ns	-90.9 ± 0.9	a:b < 0.01	+11.3 ± 2.3	a:b < 0.05
c. " "	5.0 min	4	-75.4 ± 2.4	a:c ns	-91.0 ± 1.3	a:c < 0.05	+13.0 ± 2.4	a:c < 0.02
a. Control		6	-72.9 ± 1.6	-	-84.8 ± 6.5	-	+11.4 ± 4.9	-
b. Control + Dibutyryl cAMP (1 mM)	1.0 min	6	-75.6 ± 1.7	a:b ns	-89.0 ± 4.8	a:b ns	+13.0 ± 4.6	a:b ns
c. " " "	5.0 min	6	-75.2 ± 2.0	a:c ns	-94.2 ± 4.4	a:c < 0.02	+18.7 ± 4.5	a:c < 0.02
d. " " "	10.0 min	6	-75.2 ± 2.8	a:d ns	-94.4 ± 6.0	a:d < 0.002	+18.4 ± 5.1	a:d < 0.05
a. Cl^--free		4	-66.9 ± 0.9	-	-82.3 ± 6.5	-	+13.7 ± 7.4	-
b. Cl^--free + Dibutyryl cAMP (1 mM)	1.0 min	4	-65.0 ± 1.1	a:b < 0.02	-87.1 ± 5.8	a:b < 0.05	+21.8 ± 6.8	a:b < 0.05
c. " " "	5.0 min	4	-63.9 ± 1.4	a:c < 0.05	-95.2 ± 6.0	a:c < 0.02	+32.6 ± 6.8	a:c < 0.01
d. " " "	10.0 min	4	-62.3 ± 1.7	a:d < 0.05	-100.0 ± 5.5	a:d < 0.02	+38.4 ± 6.5	a:d < 0.01
a. Ca^{2+}-free		4	-59.8 ± 4.8	-	-62.7 ± 9.9	-	+2.9 ± 7.5	-
b. Ca^{2+}-free + Dibutyryl cAMP (1 mM)	1.0 min	4	-60.1 ± 3.9	a:b ns	-63.3 ± 9.1	a:b ns	+3.3 ± 7.0	a:b ns
c. " " "	5.0 min	4	-60.4 ± 2.8	a:c ns	-68.8 ± 8.7	a:c ns	+9.3 ± 7.3	a:c ns
d. " " "	10.0 min	4	-61.5 ± 1.4	a:d ns	-71.2 ± 8.6	a:d ns	+10.3 ± 7.9	a:d ns

Continued on next page.

Table 3.3 Continued

Treatment		*n*	V_B (mV)	*P*	V_A (mV)	*P*	TEP (mV)	*P*
a. Control		4	-75.3 ± 1.4	-	-90.4 ± 1.7	-	+15.3 ± 2.9	-
b. Control + NaSCN (10 mM)	0.5 min	4	-80.8 ± 1.1	a:b ns	-79.2 ± 1.2	a:b < 0.01	-1.1 ± 1.7	a:b < 0.01
c. " "	1.0 min	4	-80.7 ± 0.9	a:c ns	-84.9 ± 2.0	a:c ns	+2.4 ± 2.6	a:c < 0.05
d. " "	5.0 min	4	-79.0 ± 1.2	a:d < 0.02	-91.3 ± 4.9	a:d ns	+11.9 ± 3.7	a:d ns
a. Cl^--free		3	-70.9 ± 3.6	-	-107.7 ± 3.5	-	+36.3 ± 6.3	-
b. Cl^--free + NaSCN (10 mM)	0.5 min	3	-78.1 ± 5.9	a:b ns	-88.3 ± 2.9	a:b < 0.01	+9.5 ± 8.7	a:b < 0.02
c. " "	1.0 min	3	-78.2 ± 5.9	a:c ns	-90.4 ± 4.1	a:c < 0.05	+11.1 ± 8.4	a:c < 0.05
d. " "	5.0 min	3	-76.3 ± 4.1	a:d < 0.01	-101.6 ± 4.8	a:d ns	+23.7 ± 6.2	a:d < 0.05
a. Control		3	-65.3 ± 1.8	-	-66.5 ± 8.5	-	+1.3 ± 9.8	-
b. " + 1 mM Dibutyryl cAMP + 10 mM NaSCN	0.5 min	3	-71.3 ± 2.0	a:b < 0.01	-53.9 ± 5.8	a:b ns	-18.4 ± 9.4	a:b < 0.01
c. " " " "	1.0 min	3	-72.4 ± 2.7	a:c ns	-57.7 ± 8.2	a:c < 0.05	-14.6 ± 8.7	a:c < 0.01
d. " " " "	5.0 min	3	-72.7 ± 3.0	a:d ns	-77.3 ± 4.9	a:d ns	+4.8 ± 6.4	a:d ns
a. Control		5	-72.2 ± 0.8	-	-71.1 ± 4.8	-	-1.1 ± 4.5	-
b. Control + 1 mM Acetazolamide (Diamox)	1.0 min	5	-75.7 ± 1.5	a:b ns	-72.3 ± 5.3	a:b ns	-3.0 ± 5.1	a:b ns
c. " "	5.0 min	5	-75.7 ± 1.4	a:c ns	-71.8 ± 5.9	a:c ns	-3.9 ± 5.4	a:c ns
d. " "	10.0 min	5	-77.8 ± 1.9	a:d < 0.01	-75.6 ± 6.3	a:d ns	-2.7 ± 5.6	a:d ns

FIGURE 3.7

A typical example of the effects of IBMX (10^{-5} mM) in control saline on V_B, V_A and TEP.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

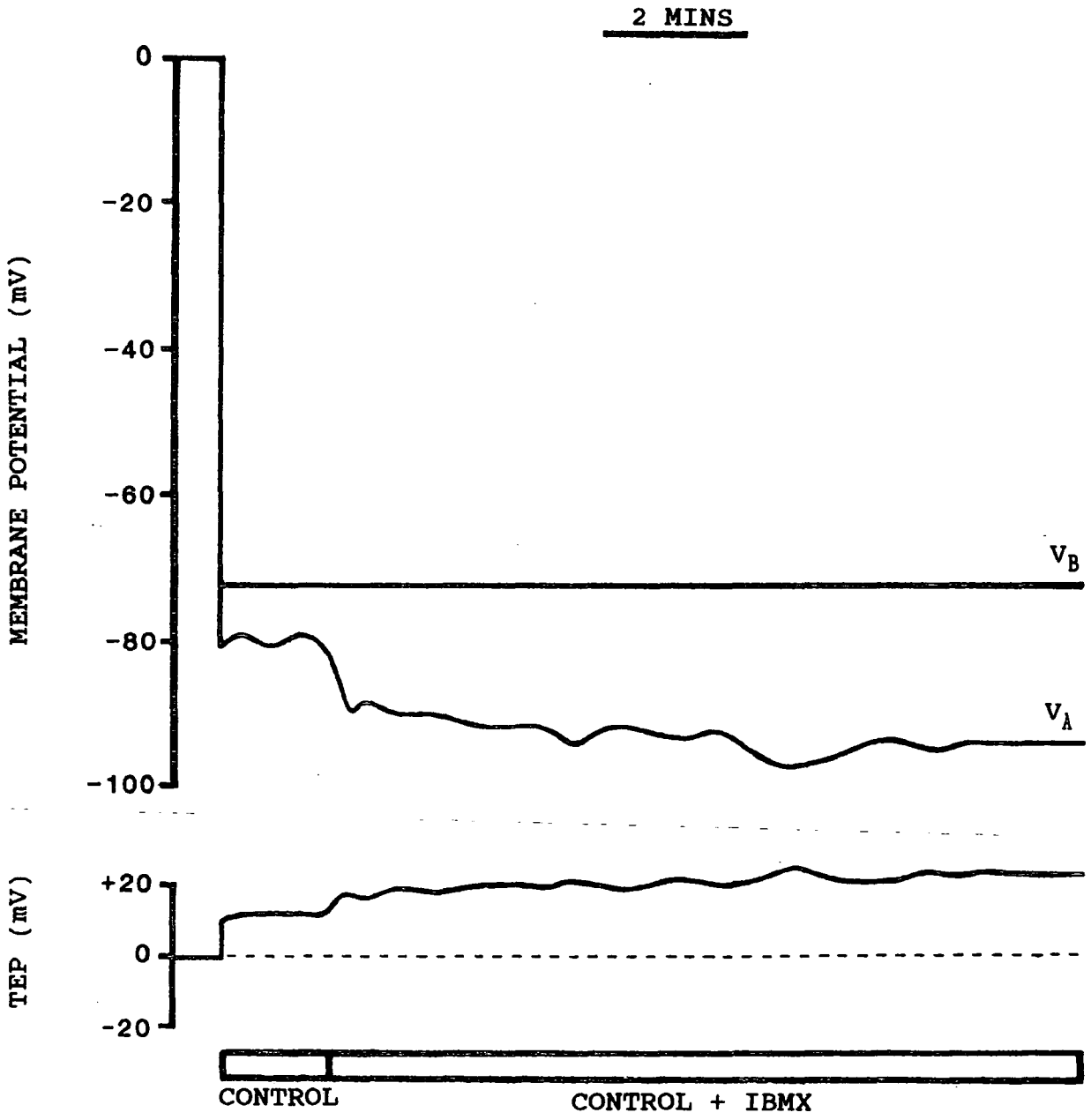

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+20
0
-20
TEP (mV)
CONTROL
CONTROL + IBMX

FIGURE 3.8

A characteristic response of V_B, V_A and TEP to dibutyryl cAMP (1 mM) in control saline.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

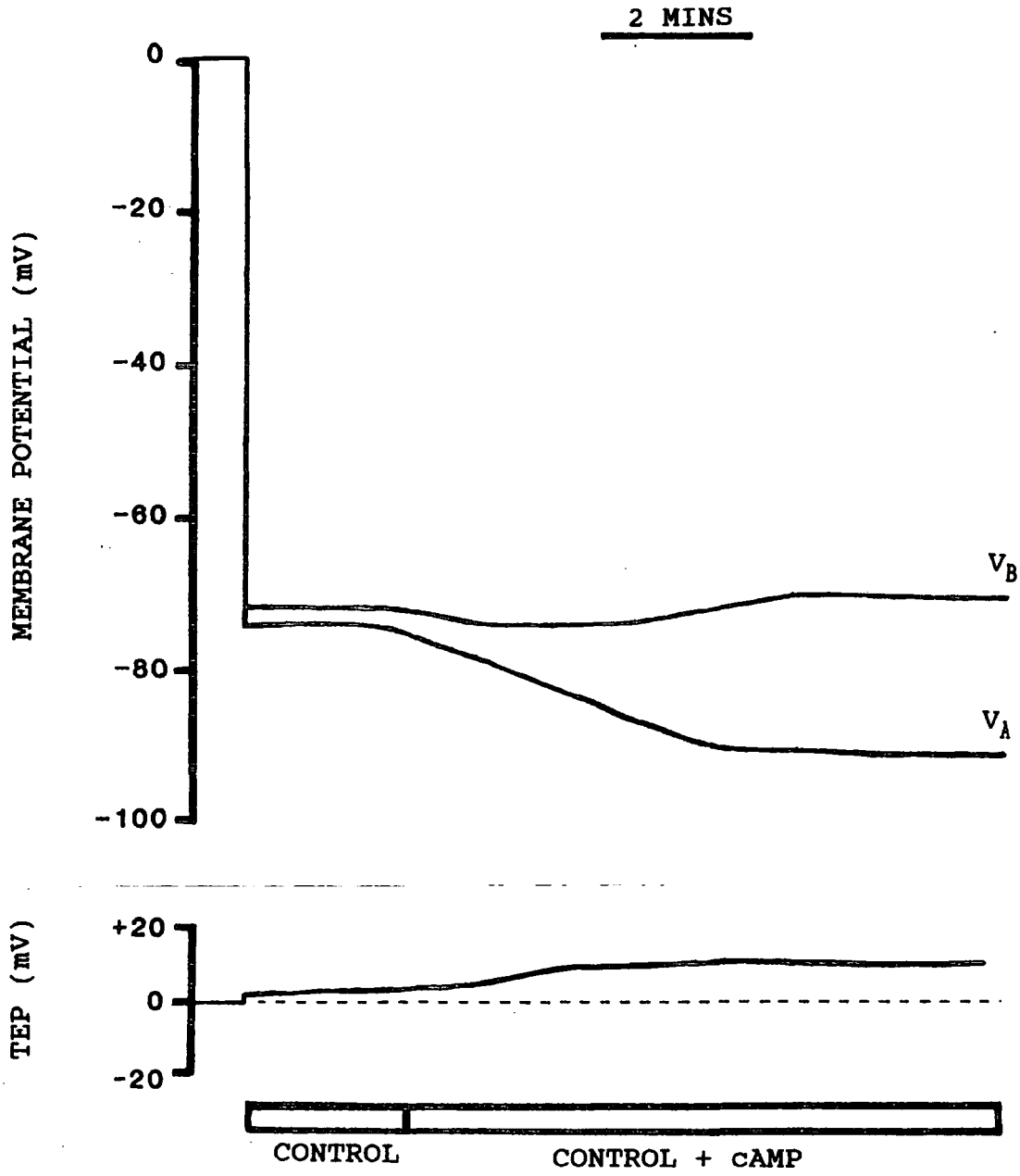

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+20
0
-20
TEP (mV)
CONTROL
CONTROL + cAMP

FIGURE 3.9

An example of the effect of dibutyryl cAMP (1 mM) in Cl^--free saline on V_B, V_A and TEP.

Ordinate : Potential in mV.

Abscissa : Time in minutes.

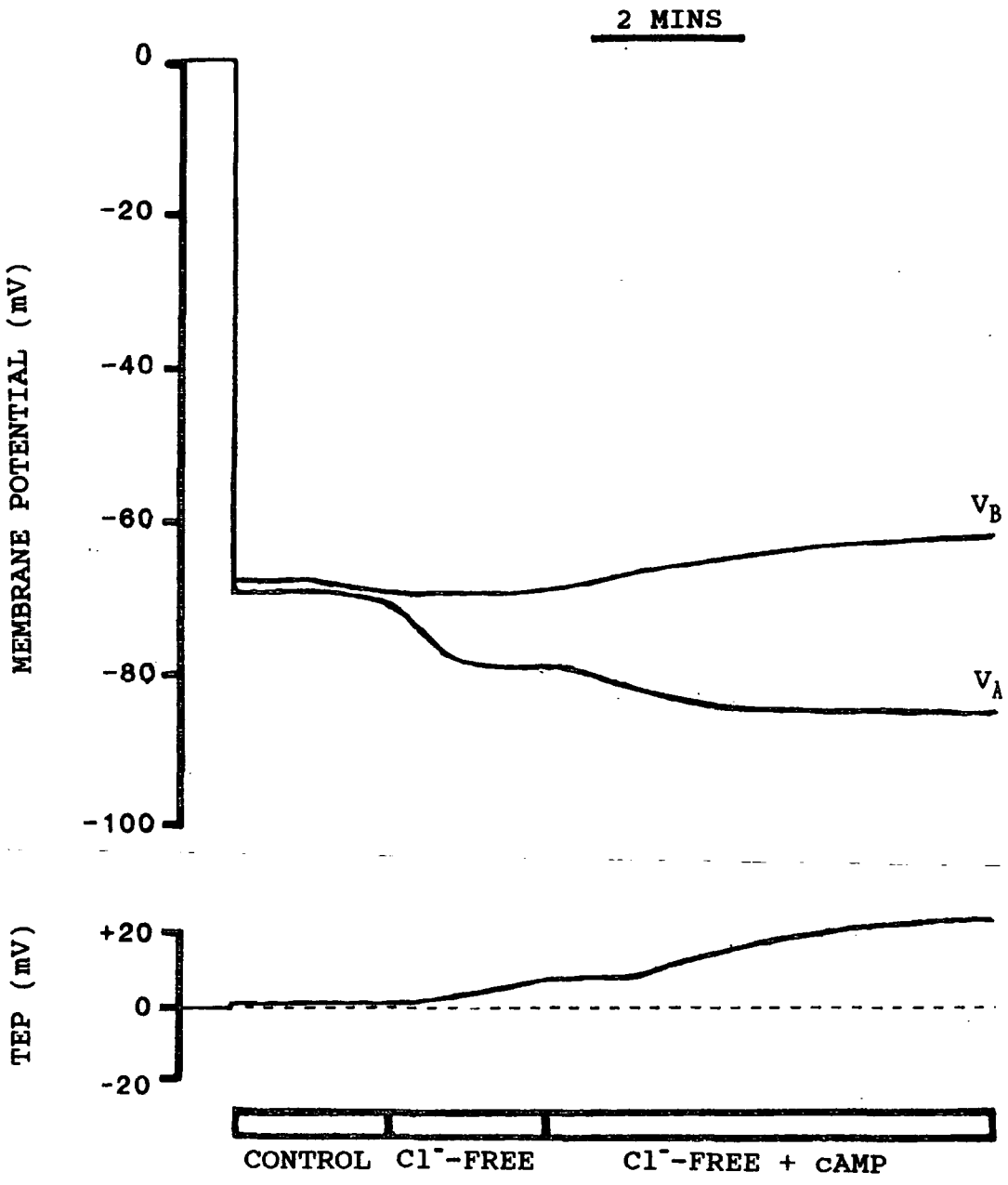
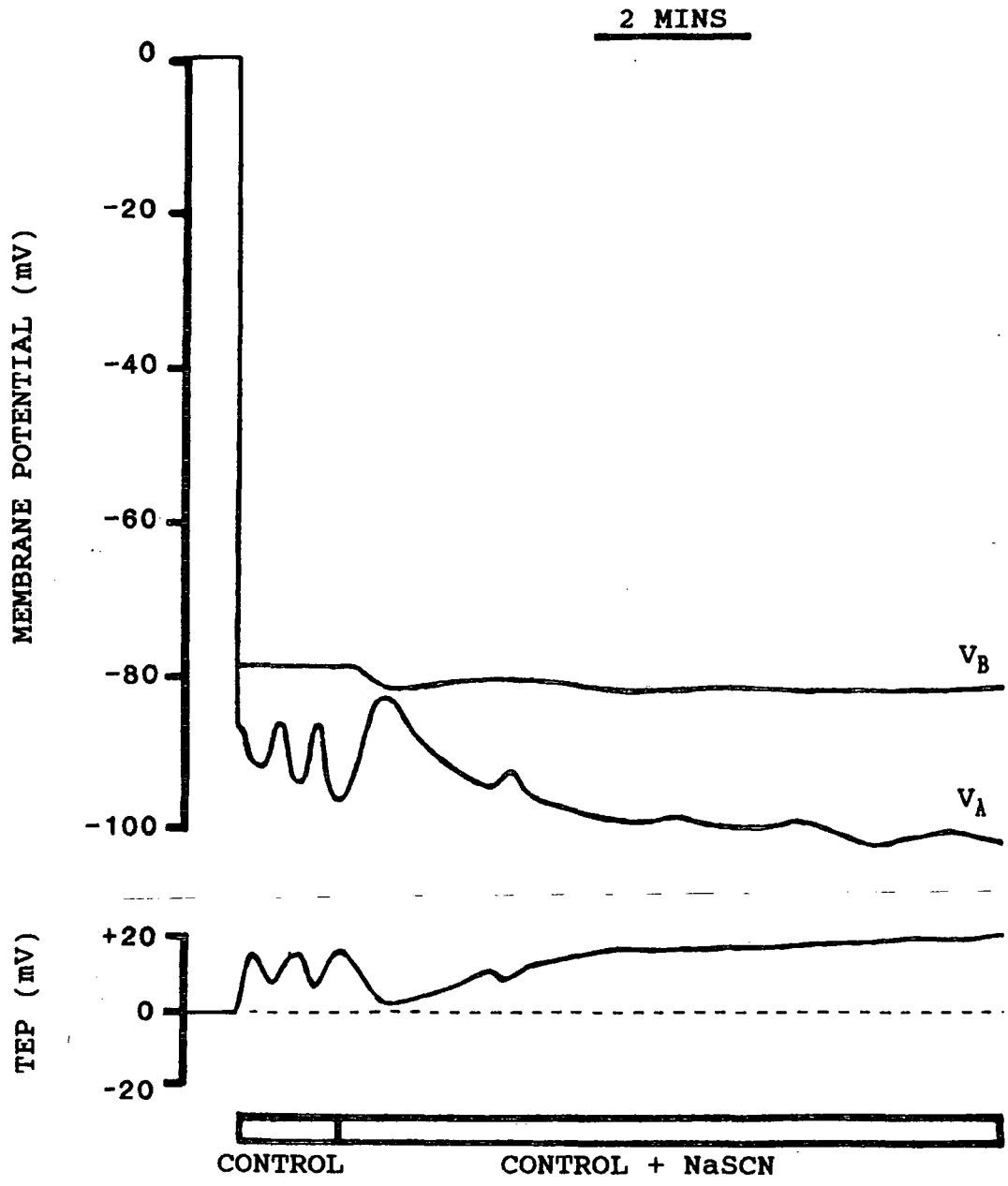

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+20
0
-20
TEP (mV)
CONTROL
Cl⁻-FREE
Cl⁻-FREE + cAMP

FIGURE 3.10

A typical example of the effect of NaSCN (10 mM) in control saline on V_B, V_A and TEP.

Ordinate : Potential in mV.

Abscissa : Time in minutes.


2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
V_B
V_A
+20
0
-20
TEP (mV)
CONTROL
CONTROL + NaSCN

qualitatively similar to those of 10^{-5} M IBMX in control saline (see above). The slower response to cAMP compared with that of IBMX may be due to the lower permeability of the basal membrane to dibutyryl cAMP, with a consequent slower increase in the intracellular messenger level.

Effect of 1 mM Dibutyryl cAMP in Cl^--free Saline

Superfusion of tubules with Cl^--free saline containing 1 mM dibutyryl cAMP (typical example, Fig. 3.9) resulted in a slow and significant ($P<0.02$) hyperpolarization of V_A by a mean value of 12.9 $\pm$ 2.8 mV (n=4) over a 5 min period, similar to the response to this agent in control saline. In contrast, V_B showed a small, but significant ($P<0.05$) depolarization by a mean value of 4.7 $\pm$ 1.3 mV (n=4) from -66.9 $\pm$ 0.9 mV, in Cl^--free saline, to -62.3 $\pm$ 1.7 mV over a ten minute period (Table 3.3).

The affects of dibutyryl cAMP in Cl^--free saline are qualitatively similar to the affects of CC extract in Cl^--free saline.

Effects of 1 mM Dibutyryl cAMP in Ca^{2+}-free Saline

Superfusion of tubules with Ca^{2+}-free saline containing 1 mM dibutyryl cAMP had no significant affect on V_B of V_A over a 10 min period (Table 3.3).

Effects of 10 mM NaSCN in Control Saline

Superfusion of tubules with control saline containing 10 mM NaSCN (typical example, Fig. 3.10) effected a significant ($P<0.01$) depolarization of V_A by a mean value of 11.3 $\pm$ 1.6 mV (n=4) from a mean value of -90.4 $\pm$ 1.7 mV, in control saline, to -79.2 $\pm$ 1.2 mV over a 0.5 min period (Table 3.3). The change in V_A at this time was reflected by a significant ($P<0.01$) decrease in TEP (Fig. 3.10) by a mean value of 16.4 $\pm$ 2.4 mV (n=4) from +15.3 $\pm$ 2.9 mV to -1.1 $\pm$ 1.7 mV, with reference to the bathing medium (Table 3.3). Subsequently, V_A rapidly repolarized, reaching a mean value of -91.3 $\pm$ 4.9 mV (n=4) after 5 min, a value not significantly different to that in control saline alone (Table 3.3).

The inclusion of 10 mM NaSCN in control saline effected a slow, but significant ($P<0.02$) hyperpolarization of V_B by a mean value of 3.7 $\pm$ 0.8 mV (n=4) after 5 min (Table 3.3).

Effects of 10 mM NaSCN in Cl^--free Saline

Superfusion of locust Malpighian tubules with Cl^--free saline containing 10 mM NaSCN had qualitatively similar affects to those seen in control saline containing 10 mM NaSCN; a rapid depolarization of V_A followed by a slower repolarization, and a slow hyperpolarization of V_B (Table 3.3). However, the initial depolarization, within 0.5 min, of V_A (mean depolarization 19.5 $\pm$ 1.3 mV, n=3) was significantly ($P<0.02$) greater than that observed in control saline (mean depolarization 11.3 $\pm$ 1.6 mV, n=4) when compared using a Student's *t*-test for single data.

Effects of 10 mM NaSCN and 1 mM Dibutyryl cAMP in Control Saline

Inclusion of both 10 mM NaSCN and 1 mM dibutyryl cAMP in the control saline bathing the tubule resulted in a significant ($P<0.01$) hyperpolarization of V_B by a mean value of 5.9 $\pm$ 0.5 mV (n=3) during a 0.5 min period. There followed a further small hyperpolarization of V_B, but values were not significantly different to those found on control saline alone (Table 3.3). V_A showed a significant ($P<0.05$) depolarization by a mean value of 8.8 $\pm$ 1.9 mV (n=3), over a 1 min period, from -66.5 $\pm$ 8.5 mV, in control saline alone, to -57.7 $\pm$ 8.2 mV. This was followed by a rapid repolarization leading to a hyperpolarization of V_A by about 10.8 $\pm$ 4.5 mV when compared to the potential in control saline alone, although values were not significantly different to those in control saline alone (Table 3.3).

Effects of 1 mM Acetazolamide (Diamox) in Control Saline

Superfusion of tubules with control saline containing 1 mM acetazolamide resulted in a very slow hyperpolarization of both V_B and V_A, although these data were not significantly different from those in control saline alone, until at 10 min, when V_B showed a significant ($P<0.01$), but small hyperpolarization by a mean value of 5.6 $\pm$ 1.2 mV (n=5) from -72.2 $\pm$ 0.8 mV to -77.8 $\pm$ 1.9 mV (Table 3.3).

Ion Flux Studies

Net Basal Na^+ and Cl^- Flux

Mean values for the net basal flux rates, from bathing medium (haemolymph-side) to lumen (hl), for sodium and chloride ions ($^{hl}Na^+$ and $^{hl}Cl^-$) were 0.64 $\pm$ 0.07 meq./cm^2/h (n=26) and 1.02 $\pm$ 0.19 meq./cm^2/h (n=27), respectively. In both cases, there was no

significant difference between consecutive 10 min flux rate measurements, and so each tubule acts as its own control.

Effect of Corpora Cardiaca Extract on Na^+ and Cl^- Flux in Control Saline

The inclusion of CC extract in the bathing medium (control saline) was found to significantly increase both $^{hl}Na^+$ ($P<0.002$) and $^{hl}Cl^-$ flux ($P<0.05$). $^{hl}Na^+$ flux increased by a mean value of 0.58 $\pm$ 0.20 meq./cm^2/h (n=12) from a basal level of 0.67 $\pm$ 0.11 meq./cm^2/h to 1.25 $\pm$ 0.26 meq./cm^2/h, representing an approximate increase of 106%. $^{hl}Cl^-$ flux increased by 2.30 $\pm$ 0.70 meq./cm^2/h (n=6) from a basal level of 0.79 $\pm$ 0.28 meq./cm^2/h to 3.09 $\pm$ 0.87 meq./cm^2/h, representing an approximate mean increase of 335%.

Na^+ Flux in Cl^- Free Saline

On changing the external bathing medium from control to Cl^--free saline, there was no significant change in $^{hl}Na^+$ flux; 0.73 $\pm$ 0.10 meq./cm^2/h in control saline, and 0.77 $\pm$ 0.11 meq./cm^2/h in Cl^--free saline, suggesting that the movements of these two ions are not linked.

Cl^- Flux in High K^+, Na^+-free Saline

On changing the external bathing medium from control to high K^+, Na^+-free saline, there was a significant ($P<0.01$) increase in $^{hl}Cl^-$ flux by a mean value of 0.45 $\pm$ 0.10 meq./cm^2/h (n=6) from a basal level of 0.50 $\pm$ 0.04 meq./cm^2/h to 0.95 $\pm$ 0.09 meq./cm^2/h, representing an approximate mean increase of 95%.

Effect of 1 mM Furosemide on Cl^- Flux in Control Saline

Inclusion of 1 mM furosemide in the control saline bathing the tubule resulted in a significant ($P<0.02$) decrease in $^{hl}Cl^-$ flux by a mean value of 0.24 $\pm$ 0.25 meq./cm^2/h (n=7) from a basal level of 0.44 $\pm$ 0.09 meq./cm^2/h to 0.20 $\pm$ 0.04 meq./cm^2/h, representing an approximate mean decrease of 48%.

DISCUSSION

The potential profile across the unstimulated Malpighian tubules of *Locusta migratoria* in control saline, in the present study, is compared with that found in various invertebrate and vertebrate tissues in Table 3.4. Whilst the majority of tubules examined exhibited a positive transepithelial potential (TEP) with respect to the lumen, considerable variation in TEP occurred (-22 to +34mV), with about 23% of tubules showing a negative value. Such variation in TEP from tubule to tubule has also been found in *Locusta migratoria* by Ramsay (1953), Morgan and Mordue (1981, 1983) and Baldrick (1987).

Potassium ions must enter the Malpighian tubule cell across the basal cell membrane to provide the basis for the K^+-rich "urine" which is secreted by the Malpighian tubules of many insect species (see Table 3.5), including *Locusta migratoria* (Anstee *et al.*, 1979; Morgan and Mordue, 1983). Notable exceptions are the blood-feeding insects *Rhodnius prolixus* (Maddrell, 1969), *Glossina austeni* (Gee, 1975), *Aedes aegypti* (Beyenbach and Petzel, 1987) and *Libellula quadrimaculata* (Nicholls, 1985), in which a Na^+-rich "urine" is formed (see Table 3.5, reviewed by Maddrell, 1977). For *Locusta migratoria* tubules, Baldrick *et al.* (1988) calculated, on the basis of electrophysiological results, that the intracellular potassium concentration ($[K^+]_i$) should remain relatively constant, at approximately 164.5 mM, for a 1-20 mM range of potassium concentration in the bathing medium ($[K^+]_o$). This estimate agrees well with values reported in various insect tissues (Table 3.6), but is somewhat higher than that of 95 mM reported elsewhere for tubules of *Locusta migratoria* (Morgan and Mordue, 1983). The intracellular sodium and chloride ion concentrations ($[Na^+]_i$ and $[Cl^-]_i$) in various insect tissues are also summarized in Table 3.6. $[Na^+]_i$ has not been measured in tubules of *Locusta migratoria*, but in tubules of *Rhodnius prolixus* such levels were found to be low (13 mM in unstimulated and 42 mM in 5-HT-stimulated tubules) using X-ray probe micro-analysis measuring techniques (Gupta *et al.*, 1976). These findings are in agreement with other transporting epithelia (Table 3.6) which, in general, have low $[Na^+]_i$ values.

The data presented in Tables 3.4, 3.5 and 3.6 give an indication of the ionic and electrical gradients, and of the equilibrium potentials across the basal and apical cell membranes for the various ions in the Malpighian tubules of *Locusta migratoria* (see Table 3.7). For a tubule bathed in control saline (119 mM Na^+, 8.6 mM K^+, 129.6 mM Cl^-), it would appear that K^+ entry across the basal membrane would be against both its concentration and electrical gradient. However, the estimated basal membrane equilibrium potential (E_K) for this ion (Table 3.7), is very similar to the measured basal membrane potential (V_B). This suggests that any slight change in V_B or the K^+ concentration gradient would determine whether K^+ passively enters or exits the cell, if the basal cell membrane

TABLE 3.4 Membrane and Transepithelial Potentials Reported for Various Epithelia from Different Species

Species	Tissue	V_B (mV)	V_A (mV)	TEP (mV)	Reference
Locusta migratoria	Malpighian tubules	-70.4 ± 0.9	-76.1 ± 1.5	+5.5 ± 1.3	Present Study
		-71.6 ± 0.3	-82.7 ± 0.9	+5.7 ± 1.0	Baldrick *et al.*, 1988
		-	-	+10.8 ± 2.1	Anstee *et al.*, 1980
		-	-	+8.7 ± 0.3	Fathpour *et al.*, 1983
		-39.4 ± 0.5	-44.0	+4.4 ± 0.5	Morgan and Mordue, 1983
		-	-	-16	Ramsay,1953
Rhodnius prolixus	Malpighian tubules	-67.0 ± 2.0	-53.0 ± 4.0	-8.0 ± 3.0	O'Donnell and Maddrell, 1984
Aedes aegypti	Malpighian tubules	-65.2 ± 1.5	-118.0	+53.7 ± 5.6	Sawyer and Beyenbach, 1985a
		-	-	+41.1 ± 6.7	Wheelock *et al.*, 1988
Carausius morosus	Malpighian tubules	-	-	+21	Ramsay, 1953
Calpodes ethlius	Malpighian tubules	-	-	+25	Irvine, 1969
Tipula paludosa	Malpighian tubules	-	-	+32	Coast, 1969
Schistocerca gregaria	Malpighian tubules	-	-	+16.6 ± 3.1	Maddrell and Klunsuwan, 1973
	Rectum	-50.7 ± 0.3	-57.8 ± 0.5	+7.2 ± 0.3	Hanrahan and Phillips, 1984
		-50.0 ± 1.3	-61.0 ± 1.6	+10.0 ± 1.1	Black *et al.*, 1987
Calliphora erythrocephala	Salivary glands	-61.1 ± 0.4	-	-	Berridge and Schlue, 1978
Periplaneta americana	Midgut	-	-	-12.0 ± 1.0	O'Riordan, 1969
Squalus acanthias	Proximal tubule	-63.2 ± 3.5	-	+0.4 ± 0.4	Beyenbach and Fromter, 1985
	Rectal gland tubule	-68.0 ± 1.2	-	-12.0 ± 1.1	Greger and Schlatter, 1984
New Zealand White Rabbit	Cortical collecting duct	-69.6 ± 3.0	-68.9 ± 3.1	-0.7 ± 0.3	O'Neil and Sansom, 1984
Swiss mice	Proximal straight tubule	-62.0 ± 1.0	-	-1.7 ± 0.1	Volkl *et al.*, 1986

TABLE 3.5 Ionic Concentration of Secreted Fluid by Various Insect Malpighian Tubules

Species	Treatment	K^+ (mM)	Na^+ (mM)	Cl^- (mM)	Reference
Locusta migratoria	None	140 139-169	35 20-23	- 203	Anstee *et al.*, 1979 Morgan and Mordue, 1983
Schistocerca gregaria	None	135 165	28 47	150 88	Maddrell and Klunsuwan, 1973 Hanrahan and Phillips, 1983
Musca domestica	None	143 ± 4	16 ± 1	95 ± 3	Dalton and Windmill, 1980
Carausius morosus	None	145 154 ± 6	5 5 ± 0	65 -	Ramsay, 1953 Pilcher, 1970
Onymacris plana	None	187 ± 6	22 ± 2	-	Nicolson and Hanrahan, 1986
Conocorixa bifida *Conocorixa blaisdelli*	None	61 75	60 40	88 ± 2 120	Szibbo and Scudder, 1979 Cooper *et al.*, 1988
Rhodnius prolixus	None 5-HT-stimulated	85 146 ± 5 82 ± 24	100 50 ± 1 78 ± 24	180 119 ± 14 181 ± 19	Maddrell, 1969 Gupta *et al.*, 1976 Gupta *et al.*, 1976
Glossina austeni	None	0.5-1.5	160-180	140-160	Gee, 1975
Aedes aegypti	None 5-HT-stimulated cAMP-stimulated	93 ± 5 91 71 ± 5 17	78 ± 24 94 95 ± 4 178	186 ± 3 161 182 ± 3 185	Veenstra, 1988 Beyenbach and Petzel, 1987 Veenstra, 1988 Beyenbach and Petzel, 1987
Libellula quadrimaculata	None	5 ± 2	151 ± 15	127 ± 19	Nicholls, 1985

TABLE 3.6 Intracellular Ion Concentrations from Various Insect Tissues

Species	Tissue	K^+ (mM)	Na^+ (mM)	Cl^- (mM)	Reference
Locusta migratoria	Malpighian tubules	95	-	51	Morgan and Mordue, 1983
		164.5	-	-	Baldrick *et al.*, 1988
	Skeletal muscle	140	-	-	Leech, 1986
Schistocerca gregaria	Malpighian tubules	135	-	-	Maddrell and Klunsuwan, 1973
Rhodnius prolixus	Malpighian tubules (untreated)	103 ± 2	13	31	Gupta *et al.*, 1976
	(5-HT-treated)	102 ± 9	42 ± 2	61 ± 5	Gupta *et al.*, 1976
Calliphora erythrocephala	Salivary glands	143 ± 4	-	39	Gupta *et al.*, 1978
		133 ± 2	-	-	Berridge and Schlue, 1978
		190	-	-	Berridge *et al.*, 1976
Manduca sexta	Midgut	134	-	-	Dow *et al.*, 1984
		140	-	-	Zerahn, 1977
Periplaneta americana	Rectum	93	50	-	Wall, 1977
	Colon	89	48	-	Wall, 1977
Rana esculenta	Proximal tubules	64 ± 2	14 ± 2	14 ± 2	Wang *et al.*, 1984
Wistar Rat	Medullary collecting tubules	98 ± 2	17 ± 1	-	Sudo and Morel, 1984
	Medullary thick ascending tubules	90 ± 2	16 ± 1	-	Sudo and Morel, 1984
Domestic Goose	Salt gland	105 ± 9	62 ± 2	38 ± 5	Peaker, 1971

TABLE 3.7 Estimates of Basal and Apical Membrane Equilibrium Potentials for Various Ions for the Malpighian Tubules of *Locusta migratoria*

Ion	Concentration (mM) in			E_{ion}	
	Bathing medium	Cytoplasm	Lumen	Basal	Apical
K^+	8.6[a]	164.5[b]	140.0[e]	-77.03	-4.21
Na^+	119.0[a]	13.0[c]	35.0[e]	+57.79	+25.85
Cl^-	129.6[a]	51.0[d]	203.0[d]	-24.34	-36.06

Actual measurements of V_B and V_A (present study) are -70.1 $\pm$ 0.9 mV and -76.1 $\pm$ 1.5 mV, respectively.

a. Ionic composition of the bathing saline used in the present study.
b. Estimate of $[K^+]_i$ by Baldrick *et al.*, 1988.
c. Measured by electron-probe X-ray microanalysis by Gupta *et al.*, 1976.
d. Measured using ion selective microelectrodes by Morgan and Mordue, 1983.
e. Measurement by flame photometery by Anstee *et al.*, 1979.

were freely permeable to K^+. Indeed, V_B is largely accounted for by the high permeability of the basal membrane to K^+, and its relative impermeability to Na^+ and Cl^- (Baldrick *et al.*, 1988; present study). If the membrane were permeable, it would appear that Na^+ would enter the cell with both its concentration and electrical gradient, whilst Cl^- entry would be with its concentration gradient, but against its electrical gradient (Table 3.7). Estimated basal membrane equilibrium potentials for these ions (Table 3.7) are in favour of passive Na^+ entry and passive Cl^- exit across this membrane when compared to the measured value of V_B. This indicates that some mechanism must transport Cl^- into the tubule cell across the basal membrane in order to provide a basis for the KCl-rich urine (Anstee *et al.*, 1979).

(Na^++K^+)-ATPase (present in microsomal preparations of *Locusta migratoria*, Anstee and Bell, 1975, 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986; present study) in the basal membrane could be responsible for maintaining high intracellular levels of K^+ and low levels of Na^+ to help maintain these concentration and electrical gradients. The location for (Na^++K^+)-ATPase along the basal membrane in *Locusta migratoria* has been demonstrated in the present study. Indeed, evidence in vertebrates and invertebrates support exclusive basolateral localization of this enzyme in nearly all ion-transporting epithelia (Hootman and Ernst, 1984; Komnick and Achenbach, 1979; Anstee and Bowler, 1984).

As for the basal membrane, comparison of the apical membrane equilibrium potentials for the various ions and the measured potential across the apical membrane (Table 3.7) suggests that passive Cl^- movement into the lumen will be favoured, whilst movements of both K^+ and Na^+ will be against a considerable electrical gradient. This provides indirect evidence for the existence of an apical active transport mechanism for the transport of cations into the lumen. Indeed, an apical cation pump has been proposed in the Malpighian tubules of *Calliphora erythrocephala* (Berridge, 1968), *Carausius morosus* (Maddrell, 1977), *Rhodnius prolixus* (O'Donnell and Maddrell, 1984), *Aedes aegypti* (Beyenbach and Petzel, 1987), *Onymacris plana* (Nicolson and Isaacson, 1987) and *Locusta migratoria* (Baldrick *et al.*, 1988; present study), and in the salivary glands of *Calliphora erythrocephala* (Berridge and Prince, 1972; Berridge *et al.*, 1975a, 1976), the labella of *Protophormia* (Thurm and Kuppers, 1980; Wieczorek, 1982) and the labial glands (Hakim and Kafatos, 1974) and midgut (Harvey *et al.*, 1983; Zeiske *et al.*,1986; Moffet and Koch, 1988a,b) of *Manduca sexta*. Active K^+ secretion by midgut goblet cells of *Manduca sexta* has been convincingly demonstrated by microelectrode studies (Moffet *et al.*, 1982) and X-ray microprobe analysis (Dow *et al.*, 1984).

Recent biochemical studies have revealed the presence of a K^+-stimulated ATPase in the K^+-transporting midgut of *Manduca sexta* (Wolfersberger *et al.*, 1982; Deaton, 1984; Wieczorek *et al.*, 1986) and the labellum of *Protophormia terraenovae* (Wieczorek, 1982;

Wieczorek and Gnatzy, 1985; Wieczorek *et al.*, 1986). It was suggested (Wieczorek and Gnatzy, 1985; Wieczorek *et al.*, 1986) that this may represent the apical electrogenic potassium pump. Previously, no biochemical evidence for K^+-ATPase activity has been demonstrated in insect Malpighian tubules. However, in the present study, some K^+-ATPase activity appears to be associated with the basal, but not the apical cell membrane (see later). Previously, evidence for such a pump was wrongly reported by Harvey *et al.* (1983). These workers incorrectly re-analysed data regarding the report of (Na^++K^+)-ATPase activity in *Locusta migratoria* tubules from the paper by Anstee and Bell (1975), with the resulting conclusion of significant K^+-ATPase activity in this epithelium. In the light of this evidence, the nature of the apical cation pump in insect Malpighian tubules remains unclear.

In insects, information regarding "tight" and "leaky" epithelia is limited. The Malpighian tubules of some species are "tight", water and salt movement being mainly via the transcellular route (*Rhodnius prolixus*, O'Donnell and Maddrell, 1983; O'Donnell *et al*, 1984; *Aedes aegypti*, Williams and Beyenbach, 1984), as it also is in locust rectum (Hanrahan, 1984). In contrast, there is some evidence that solute/solvent coupling occurs paracellularly in other insect epithelia (rectal glands of *Periplaneta americana* and *Calliphora*, Wall *et al.*, 1970; salivary glands of *Periplaneta americana*, Gupta and Hall, 1983, and *Calliphora erythrocephala*, Gupta *et al.*, 1978).

If the Malpighian tubules of *Locusta migratoria* were significantly "leaky", potential changes across both the apical and basal membranes would be coupled due to electrical measurements across the cell being short-circuited by the paracellular pathway. In the present study, tubules bathed in control saline responded to treatment with modified salines, namely high K^+, Na^+-free and high K^+, Na^+- and Cl^--free salines, in a qualitatively similar manner; V_B and V_A depolarizing with both salines. However, in many cases treatment with other modified salines, effected dissimilar changes in V_B and V_A (see Tab. 3.2).

The "leakiness" of an epithelium can explain the similar or dissimilar changes in V_B and V_A. A highly "leaky" epithelium would result in both these potentials changing by similar amounts and in the same direction in, for example, high K^+, Na^+-free saline, whilst in a completely "tight" epithelium V_B would depolarize, but V_A would remain the same. As neither of these extremes occur in tubules of *Locusta migratoria* when the extracellular [K^+] is elevated (present study), it would appear that this epithelium is intermediate in properties through "leaky" tight junctions, as found in many vertebrate systems (Di Bona, 1985). However, other evidence suggests that the Malpighian tubules of *Locusta migratoria* are "tight". In the present study, for example, a large TEP is measured in control, Cl^--free and high K^+ salines in many cases, which is not characteristic of a

"leaky" epithelium (Hanrahan, 1984), and changes in V_B and V_A were different in some modified salines. Thus, in high K^+, Na^+-free saline where changes in V_B and V_A are qualitatively similar, the observed depolarization of V_B may be a Nernstian effect, whilst the depolarization of V_A may be a secondary effect. Indeed, the depolarization of V_A in high K^+, Na^+-free saline was reduced in the absence of Cl^-, which would not be expected if the locust tubule was "leaky", where electrical short-circuiting across the epithelium would take place.

Na^+ Substitution with K^+

In the present study, substitution of Na^+ with K^+ in the bathing medium (127.6 mM K^+, 0 mM Na^+) resulted in a near-Nernstian depolarization of V_B. This was expected on the basis of previous studies (Baldrick, 1987; Baldrick *et al.*, 1988) where it was established that the basal membrane acts essentially as a potassium electrode, being relatively permeable to K^+ compared with Na^+ and Cl^-. In order to establish whether the effects observed with high K^+, Na^+-free saline were a consequence of elevated $[K^+]_0$ rather than Na^+ lack, Baldrick *et al.* (1988) measured V_B and V_A in a high K^+ saline containing 10 mM Na^+. On changing to this solution, V_B and V_A depolarized by about 5 mV and 9 mV less than the corresponding measurements in 128 mM K^+, Na^+-free salines, respectively. Some reduction in the depolarization of V_B was expected on the basis of $[K^+]_0$ being reduced to 118 mM. Apart from this, the inclusion of a little Na^+ in the high K^+ test saline was without any significant effect. Similar selectivity for K^+ occurs in a variety of insect tissues, for example, the salivary glands of *Calliphora erythrocephala* (Prince and Berridge, 1972), the Malpighian tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984), locust oocytes (Wollberg and Cocos, 1981) and locust skeletal muscle (Usherwood, 1978).

Application of the Goldman constant field equation (Goldman, 1943) for a membrane permeable to both K^+ and Na^+, suggested that the basal membrane of *Locusta migratoria* tubules must be 100 times more permeable to K^+ than Na^+ (Baldrick, 1987; Baldrick *et al.*, 1988). This conclusion is consistent with reports for various other epithelia (e.g., *Aplysia* neurones, Sato *et al.*, 1968). In contrast, the basolateral membranes of the Malpighian tubules of *Aedes aegypti* are permeable to both K^+ and Na^+, with membrane conductances for each ion being of similar magnitude under unstimulated conditions (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985a).

In the present study, TEP measurements show a maintained increase in lumen positivity on increasing $[K^+]_0$. Similar findings were reported by Baldrick (1987) and Baldrick *et al.* (1988) for *Locusta migratoria* with TEP values ranging from -43 to +84mV in this saline. Similarly, Fathpour *et al.* (1983) found that increasing $[K^+]_0$ resulted in a

maintained increase in lumen positivity in *Locusta migratoria*, whilst Berridge *et al.* (1975a) reported that in salivary glands of *Calliphora erythrocephala*, treatment with high K^+, low Na^+ saline resulted in a temporary increase in lumen positivity, followed by a gradual shift back towards the resting value (as seen with the "Type B" response in tubules of *Locusta migratoria*, Baldrick *et al.*, 1988). Unstimulated tubules of *Rhodnius prolixus* also responded to high K^+, Na^+-free saline by exhibiting an increase in TEP positivity, although V_A was insensitive to transient changes in $[K^+]_0$ (O'Donnell and Maddrell, 1984). As mentioned previously, the fact that V_B and V_A do not depolarize by exactly the same amount, as indicated by the maintained increase in TEP, is evidence that Malpighian tubules of *Locusta migratoria* are not totally "leaky".

Na^+ Substitution with Choline

In the present study, substitution of Na^+ by choline resulted in a slight, but non-significant depolarization of V_B and hyperpolarization of V_A within the first five minutes. This resulted, however, in a significant, but small increase in lumen positivity. Similar findings were reported by Baldrick (1987), although a gradual depolarization of both membranes began after approximately 10 mins exposure to Na^+-free saline.

The fact that V_B showed a slow, but non-significant depolarization in Na^+-free saline compared to the large and rapid depolarization in high K^+, Na^+-free saline confirms that this membrane is not freely permeable to Na^+, as suggested by Baldrick *et al.* (1988). Furthermore, Morgan and Mordue (1981) found a negligible change in V_B in tubules of *Locusta migratoria* on reduction of $[Na^+]_0$ from 142 to 70 mM. O'Donnell and Maddrell (1984) also reported that the basal membrane of tubules of *Rhodnius prolixus* is permeable to K^+, but not to Na^+ or Cl^-. They proposed that Na^+ and Cl^- movements across this membrane are linked (see later) on the basis of a lumen-positive potential being developed across the apical membrane when either Na^+ or Cl^- is removed from the bathing medium. In contrast, in *Aedes aegypti*, lowering $[Na^+]_0$ resulted in a prompt reduction in TEP positivity (Williams and Beyenbach, 1984), a 5-fold reduction in $[Na^+]_0$ producing a reversible hyperpolarization of V_B (Sawyer and Beyenbach, 1985a). Baldrick (1987) suggested that the initial response to reduced Na^+ in the bathing medium was simply a reflection of a redistribution of ions across the membranes, and their subsequent gradual depolarizations were largely attributed to an inhibition of the $(Na^+ + K^+)$-ATPase, which was consistent with the slow depolarization of V_B observed when tubules of *Locusta migratoria* were treated with the $(Na^+ + K^+)$-ATPase inhibitors, ouabain and orthovanadate (Baldrick *et al.*, 1988). The activity of the Na^+, K^+ pump is dependent on $[Na^+]_i$ in many tissues (Grantham, 1980). Exclusion of Na^+ from the bathing medium may result in a gradual

reduction in $[Na^+]_i$ and a reversal and gradual reduction in the Na^+ gradient across the basal membrane, thus inhibiting the $(Na^+ + K^+)$-ATPase. Indeed, a reduction in $[Na^+]_i$ has been demonstrated in the electric organ of *Electrophorus* when Na^+ was replaced by choline in the ambient medium (Albers *et al.*, 1968). Reduced activity of the $(Na^+ + K^+)$-pump would probably cause a slow reduction in $[K^+]_i$ and the K^+ gradient, resulting in a slow depolarization of V_B, and possibly V_A, due to decreased K^+ transport across the apical membrane.

Cl^- Substitution

In the present study, substitution of Cl^- by gluconate in the bathing medium had no significant affect on V_B, although V_A hyperpolarized rapidly to a new maintained potential, reflected by an increase in lumen positivity, as also shown by Baldrick (1987) and Baldrick *et al.* (1988). Similarly, an increase in the positivity of TEP, in response to reduced $[Cl^-]_o$, was observed in salivary glands of *Calliphora erythrocephala* (Berridge, 1980), in most of the Malpighian tubules of *Aedes aegypti* studied (Williams and Beyenbach, 1984), and also in tubules of *Onymacris plana* on changing from low K^+ (2.5 mM K^+, 149 mM Cl^-) to low K^+, low Cl^- (2.5 mM K^+, 16.5 mM Cl^-) saline, although a considerable depolarization of both V_B and V_A was observed (Nicolson and Isaacson, 1987). In contrast, the TEP of unstimulated tubules of *Rhodnius prolixus* was found to be relatively insensitive to $[Cl^-]_o$ (O'Donnell and Maddrell, 1984).

In *Locusta migratoria*, the apparent insensitivity of V_B to Cl^--free saline suggests that the basal cell membrane is not freely permeable to this anion. Furthermore, any significant passive flux of Cl^- into the cell would require $[Cl^-]_i$ to be less than 9 mM (for V_B = -70.4 mV, $[Cl^-]_o$ = 129.6 mM), which is much lower than the values reported for various insect tissues (Table 3.5). Indeed, in the Malpighian tubules of *Locusta migratoria* (Baldrick, 1987; Baldrick *et al.*, 1988) and 5-HT-stimulated tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984), the basal membrane has been reported as not being freely permeable to Cl^-.

The hyperpolarization of V_A observed on removal of external Cl^- (Baldrick, 1987; Baldrick *et al.*, 1988; present study) can be explained on the basis of continued apical cation extrusion by, for example, the apical K^+ pump in the absence of Cl^- as counter-anion. Such a mechanism has been described for *Locusta migratoria* (Baldrick 1987) and 5-HT-stimulated tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984), and the salivary glands of *Calliphora erythrocephala* (Berridge, 1980), to explain the comparable apical membrane hyperpolarizations observed on removal of Cl^- from the bathing medium. The movement of Cl^- across the apical membrane probably occurs passively, as mentioned

earlier, aided by the large favourable electrical gradient, with only a modest adverse Cl^- concentration gradient (Morgan and Mordue, 1983; Gupta *et al.*, 1976).

In many vertebrate tissues, including the mammalian nephron (Greger and Schlatter, 1983), rabbit cortical collecting duct (Sansom *et al.*, 1984) and human sweat duct (Reddy and Quinton, 1987), reduction in $[Cl^-]_o$ resulted in a depolarization of V_B, consistent with the presence of a Cl^- conductance in the basolateral membrane. In the latter two cases, little change in V_A was observed. However, similar to the present study, reduced basolateral $[Cl^-]$ led to only small changes in V_B in rabbit and *Necturus* proximal tubules (Cardinal *et al.*, 1984; Guggino *et al.*, 1982, 1983).

The insensitivity of V_B to Cl^--free saline in the present study suggests that under normal resting conditions a passive Cl^- conductance does not play an important role in Cl^- entry across the basal membrane in the Malpighian tubules of *Locusta migratoria*. Cl^- transport in the form of a Na^+ (or Na^+-K^+) coupled Cl^- carrier has been postulated in a variety of epithelia (Frizzell *et al.*, 1979a,b; Phillips and Lewis, 1983). Indeed, O'Donnell and Maddrell (1984) have suggested that electroneutral Na^+-K^+-$2Cl^-$ transport occurs across the basal membrane of tubules of *Rhodnius prolixus*. Williams and Beyenbach (1984) have also found evidence for coupled cation-Cl^- movements during stimulated fluid secretion by the tubules of *Aedes aegypti*, whereas Morgan and Mordue (1983) have suggested that active Cl^- entry may be Na^+- and/or K^+-dependent, or that the inward diffusion of Na^+ into the cell may provide the energy for inward movement of Cl^- in tubules of *Locusta migratoria*.

The evidence for Na^+-Cl^- cotransport in *Locusta migratoria* (Baldrick *et al.*, 1988) is less convincing than that established for tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) in which cotransport inhibitors, furosemide and bumetanide, caused a much greater hyperpolarization of V_A. Also, treatment with Na^+-free saline, whether with control or elevated $[K^+]$, produced a dramatic hyperpolarization of V_A in 5-HT-stimulated tubules of *Rhodnius prolixus*, whereas in *Locusta migratoria*, Na^+-free saline caused only a slow depolarization of both V_B and V_A after about 10 mins.

The dissimilarity of effect of Cl^--free and Na^+-free salines on V_A (Baldrick, 1987; Baldrick *et al.*, 1988; present study) suggests that Cl^- entry in *Locusta migratoria* is not necessarily dependent on cotransport with Na^+, as proposed by Baldrick *et al.*(1988). Indeed, net Na^+ flux from bathing medium to lumen, as measured using $^{22}Na^+$ was unaffected by the removal of external Cl^- (present study). Baldrick *et al.* (1988) suggested that some Cl^- entry may be possible through cotransport with K^+ under Na^+-free conditions, as proposed in *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) where, in the absence of either cation, the Na^+-K^+-$2Cl^-$ cotransporter may accept, though less readily, other

stoichiometries such as $2Na^+:2Cl^-$ or $2K^+:2Cl^-$. Indeed, Cl^- flux from bathing medium to lumen, measured using $^{36}Cl^-$, is significantly elevated under high K^+, Na^+-free conditions (present study), which suggests that not only is Cl^- entry independent of Na^+, but that it may also be dependent on $[K^+]_o$. Baldrick (1987) suggested that, for *Locusta migratoria*, some Cl^- entry may be K^+-stimulated, but did not discount the possibility that Cl^- entry may involve other transport mechanisms, such as Cl^--HCO_3^- exchange, or a (Cl^-+HCO_3^-)-stimulated ATPase. Indeed, HCO_3-stimulated ATPase activity associated with the basal cell membrane has been demonstrated in the present study (see later). None of these alternative mechanisms for Cl^- entry can be discounted at the present time.

Na^+ and Cl^- Substitution

In the present study, substitution of Cl^- by gluconate in a high K^+ (127.6mM) bathing medium, producing a high K^+, Na^+-free, Cl^--free saline, resulted in a significant depolarization of V_B, reflected by an increase in TEP positivity, and a small, but non-significant hyperpolarization of V_A. However, in three out of four experiments V_A did hyperpolarize by 6-15mV. Indeed, Baldrick *et al.* (1988) reported that changing from Cl^--free to high K^+, Na^+- and Cl^--free saline resulted in an approximate 20 mV smaller depolarization of V_A compared with that seen in high K^+, Na^+-free saline. As for Cl^--free saline, any hyperpolarization of V_A may be due to continued cation extrusion in the absence of Cl^- as the counter-anion.

As mentioned previously, evidence presented in this and other studies (Baldrick *et al.*, 1988) suggests that a K^+-stimulated Cl^- entry mechanism exists in the Malpighian tubules of *Locusta migratoria*. The depolarization of V_A observed in high K^+, Na^+-free saline can therefore be explained by increased Cl^- availability partially short-circuiting the apical cation pump, as suggested by Baldrick *et al.*(1988). Similarly, the influx of Cl^- in the posterior gills of the Chinese crab is also dependent on the K^+ content of the perfusion saline (Gocha *et al.*, 1987) and transepithelial conductance and unidirectional fluxes of Cl^- were induced by raised serosal $[K^+]$ in canine tracheal epithelium (Stutts *et al.*, 1988).

A K^+-dependent Cl^- pump has been reported in locust rectum (Hanrahan and Phillips, 1984) and tentatively suggested in the Malpighian tubules of *Locusta migratoria* (Morgan and Mordue, 1983). The possibility of Cl^- entry via a K^+-dependent Cl^- pump, cannot be discounted. On elevating $[K^+]_o$, the increased activity of such a pump would increase the transport of Cl^- into the cell which, in turn, would partially short-circuit the depolarizing effect of the inwardly moving K^+. Exclusion of Cl^- from the external medium would therefore remove this short-circuiting effect and lead to a depolarization of V_B, as is observed. Indeed, the mean depolarization of V_B under these circumstances is about

10 mV, which could account for the depolarization of V_B in high K^+, Na^+-free saline only being about 58 mV and not the 70.4 mV expected by application of the Nernst equation. A depolarization of V_B can also be explained on the basis of a K^+-dependent increase in basal Cl^- conductance. The elevated external K^+ would bring V_B below E_{Cl} and so inward passive movement of Cl^- would be favoured. This, in turn, would partially short-circuit the depolarizing effect of the inwardly moving K^+, as previously described for the Cl^- pump. In the absence of external Cl^- this short-circuiting would once again be removed, and Cl^- may, in fact, exit the cell down its concentration gradient resulting in the depolarization of V_B. However, the evidence presented in this study cannot discern between these two mechanisms of Cl^- entry into the Malpighian tubules of *Locusta migratoria*.

In the present study, 1 mM furosemide (the concentration used by Baldrick *et al.*, 1988) was shown to significantly reduce net Cl^- flux from bathing medium to lumen by about 50%. Furosemide is a "high-ceiling" or "loop" diuretic thought to inhibit Na^+-K^+-Cl^- and Na^+-Cl^- cotransport in various epithelia (Frizzell *et al.*, 1979a; Hanrahan and Phillips, 1983, 1984; Palfrey and Rao, 1983; Chipperfield, 1986). Various other transport systems, which are inhibitable by furosemide and other "loop" diuretics, have been postulated for transport of these ions. For example, K^+-Cl^- and Na^+-Cl^- cotransport, the primary active Cl^- pump, Na^+-K^+ cotransport, and Na^+/Na^+ and K^+/K^+ exchange (see review by Geck and Heinz, 1986). A K^+-Cl^- cotransport mechanism has been reported in mammalian erythrocytes (Warnock *et al.*, 1984; O'Grady *et al.*, 1987), *Necturus* gallbladder (Corcia and Armstrong, 1983; Reuss, 1983; Hill and Hill, 1987), proximal tubules (Reuss *et al.*, 1984) and diluting segment of mammalian nephron (Greger, 1985), and rabbit renal cortical basolateral membrane vesicles (Eveloff and Warnock, 1987b), many of which were reported as being furosemide-sensitive. It may be that Cl^- entry into the Malpighian tubule cells of *Locusta migratoria* is by means of such a furosemide-sensitive K^+-Cl^- cotransport. However, the various transport mechanisms mentioned above are believed to be partial aspects of the Na^+-K^+-$2Cl^-$ cotransporter by some workers, not distinct systems (for review see Geck and Heinz, 1986). It may be possible, therefore, that some Cl^- entry into the tubule cells is by a distinct K^+-Cl^- cotransport process or some aspect of the Na^+-K^+-$2Cl^-$ cotransporter. However, in control and Cl^--free salines $[Na^+]$ levels are high (119 mM) compared to $[K^+]$ (8.6 mM), which, according to O'Donnell and Maddrell (1984), would favour Na^+-K^+-$2Cl^-$ or Na^+-Cl^- cotransport. This does not appear to be the case in *Locusta migratoria*. Furthermore, the effect of CC extract on the tubules was to significantly increase both the net $^{hl}Na^+$ and $^{hl}Cl^-$ fluxes by approximately 106% and 335%, respectively. If Na^+ and Cl^- movements were linked, one would expect any stimulation of a Na^+-K^+-$2Cl^-$ cotransporter by CC extract to result in approximately the same percentage increase in net flux for both these ions.

The findings discussed previously appear to provide evidence for the existence of a K^+-Cl^- cotransport mechanism in the basal membrane of the Malpighian tubules of *Locusta migratoria*. However, for tubules bathed in control saline, it is difficult to imagine how such a K^+-Cl^- cotransporter could operate due to the electrochemical gradients for both K^+ and Cl^- favouring their exit from the cell across the basal membrane; i.e., there is no driving force for the transport of Cl^- into the cell by such a mechanism. It may be possible that a K^+-Cl^- cotransporter operates when external [K^+] is elevated. The significant effect of 1 mM furosemide, in control saline, on Cl^- flux across the Malpighian tubules of *Locusta migratoria* must therefore be explained by its inhibition of other Cl^- transporting mechanisms. Indeed, furosemide has been reported as being non-specific in its inhibition of transport processes (Palfrey *et al.*, 1980; for review see Chipperfield, 1986). Cl^-/HCO_3^- exchange is inhibited by furosemide with a $K_{0.5}$ of 2×10^{-4} M in human erythrocytes (Chipperfield, 1986). This inhibition of exchange is not restricted to human erythrocytes (Chipperfield, 1986). Furthermore, at high concentrations (> 1 mM), loop diuretics are non-specific inhibitors of amino acid transport, the Na^+ pump and possibly adenylate cyclase (Chipperfield, 1986). In view of this evidence, it is tempting to suggest that some Cl^- entry across the basal cell membrane of the Malpighian tubules of *Locusta migratoria* is via furosemide-inhibitable Cl^-/HCO_3^- exchange, and possibly by the activity of anion-sensitive ATPase.

Ca^{2+} Substitution

Due to the difficulty of eliminating Ca^{2+} from biological systems, the Malpighian tubules of *Locusta migratoria* used in the present study were dissected out in Ca^{2+}-free saline ($CaCl_2$ being replaced by the Ca^{2+} chelator EGTA) and subsequent microelectrode penetrations were carried out after approximately 30-40 mins in this saline. At this time, measured basal and apical membrane potentials were not significantly different from each other, but both membrane potentials were significantly less than in tubules bathed in control saline, V_B by about 12mV and V_A by about 17mV. It has been suggested that Ca^{2+} enters the Malpighian tubule cells of various insects passively down its electrochemical gradient (Maddrell, 1971). If this is the case in *Locusta migratoria*, removal of Ca^{2+} from the bathing medium would be expected to effect a hyperpolarization of V_B. Indeed, in *Locusta migratoria*, the introduction of Ca^{2+}-free saline in the presence of EGTA effected a hyperpolarization of both V_B and V_A over 1 min, to a maintained value, with no change in TEP, in tubules previously bathed in control saline (Baldrick, 1987), and in 5-HT-stimulated salivary glands of *Calliphora erythrocephala* TEP increased in positivity due to a marked hyperpolarization of V_A (Prince and Berridge, 1973). However, in contrast to the present study, these epithelia spent considerably less time in Ca^{2+}-free media. As a result, the

findings reported here must be treated with caution due to the observation that removal of Ca^{2+} has potentially damaging effects on cellular histology (Donowitz, 1983). Furthermore, intracellular free Ca^{2+} levels are low, 10^{-5} to 10^{-8} M (Schatzmann, 1975), and the absence of Ca^{2+} from the bathing medium may not significantly affect these intracellular levels in the short term.

It is possible that Ca^{2+}-free saline affects the functioning of Ca^{2+}-transporting mechanisms, such as Ca^{2+}-ATPase or a Na^+-Ca^{2+} exchanger. If a (3-5)Na^+-Ca^{2+} exchanger (Mullins, 1979; Chase, 1984), which moved Ca^{2+} out of the cell in exchange for Na^+ movement into the cell (Scoble *et al.*, 1986; Taylor and Windhager, 1979), were to occur in the Malpighian tubules of *Locusta migratoria*, reduction in $[Ca^{2+}]_o$ may lead to a reduction of its activity. Chase (1984) calculated that, in Na^+-transporting epithelia, the electrochemical potential for Na^+ is steep enough to pump Ca^{2+} out of the cell only when $[Na^+]_i$ is < 10mM. When $[Na^+]_i$ is higher, the exchanger reverses and sends Ca^{2+} into the cell. This threshold value is similar to the estimated value of 13mM $[Na^+]_i$ for Malpighian tubules of *Rhodnius prolixus* (Gupta *et al.*, 1976). For a Na^+-Ca^{2+} exchanger that was transporting Ca^{2+} out of the cell, V_B would be expected to hyperpolarize if intracellular free Ca^{2+} levels were to decline in Ca^{2+}-free saline, as Na^+ would no longer be entering the cell. A similar effect would therefore be expected by treatment with Na^+-free saline. The differing effects of Na^+-free (present study) and Ca^{2+}-free salines (Baldrick, 1987; present study) would suggest that this is not the case. In contrast, for the exchanger that was transporting Ca^{2+} into the cell, removal of extracellular Ca^{2+} would be expected to effect a depolarization of V_B as Na^+ would no longer be leaving the cell. This could possibly explain the less negative V_B values in tubules exposed to Ca^{2+}-free saline as opposed to those in control saline. However, the observed less negative V_A values in Ca^{2+}-free, as opposed to those in control saline, are difficult to explain on this basis.

It is also difficult to explain the considerably less negative V_B values obtained in tubules bathed in Ca^{2+}-free saline compared to those in control saline on the basis of a basal Ca^{2+}-ATPase. It is unlikely that this enzyme, which pumps Ca^{2+} out of cells, would contribute significantly to V_B, and therefore the reduction in activity of this pump, even if intracellular free Ca^{2+} levels declined, would most probably have little effect on V_B.

An explanation for the Ca^{2+}-free saline result is further complicated by the fact that Ca^{2+} is involved in the regulation of many cellular processes (Rasmussen and Goodman, 1977).

Chase (1984) suggested that $[Ca^{2+}]_i$ was involved in the regulation of the cell membrane K^+ conductance. The increase in $[Ca^{2+}]_i$ has been shown to hyperpolarize both V_B and V_A, and increase K^+ conductance across both apical and basal membranes in

Necturus gallbladder, suggesting Ca^{2+} directly modulated the K^+ permeability of these plasma membranes (Bello-Reuss *et al.*, 1981). There is also evidence of a similar Ca^{2+}-mediated regulation of K^+ permeability of the apical membrane occurring in cells of the diluting segment and cortical collecting tubule of the kidney (Frindt and Palmer, 1987; Hunter *et al.*, 1988). Most of the Ca^{2+}-activated K^+ channels studied are located in the apical cell membranes of tubule cells and epithelia, whereas K^+ channels in the basolateral membranes of tight epithelia appear to be rather Ca^{2+}-insensitive (Hunter *et al.*, 1988). On this basis, it is unlikely that the absence of external Ca^{2+} would affect V_B in tubules of *Locusta migratoria* (present study).

In conclusion, it is most difficult to interpret the results obtained when Malpighian tubules of *Locusta migratoria* were bathed in Ca^{2+}-free saline in the present study due to the number of different cellular processes in which this cation is involved and the difficulty in eliminating it from biological systems. Further research is necessary to elucidate the role of $[Ca^{2+}]_o$ in this system.

Corpora Cardiaca Extract

In the present study, the introduction of corpora cardiaca (CC) extract into the control bathing medium surrounding the Malpighian tubules of *Locusta migratoria* effected a significant decrease in lumen positivity (see Fogg *et al.*, 1989). Similar results have been recorded for Malpighian tubules of *Onymacris plana* (Nicolson and Isaacson, 1987), whilst Morgan and Mordue (1981) observed that extracts containing diuretic hormone (DH) were inconsistent in their effects on TEP in *Locusta migratoria*. In contrast, Pilcher (1970) showed that lumen positive TEP in tubules of *Carausius morosus* was increased following treatment with diuretic hormone. Similar results to those of Pilcher (1970) were reported in recta of *Romalea microptera* on application of CC extract (Spring, 1986), although potential changes were slow in this tissue compared to those changes observed in Malpighian tubules.

Several substances with diuretic activity, isolated from the heads of various insects, have also been found to affect the TEP of the corresponding Malpighian tubules. Leucokinins isolated from heads of *Leucophaea madera* effected a decrease in tubule lumen positivity (Hayes *et al.*, 1989), as did factors I and II from the heads of *Aedes aegypti* (Beyenbach and Petzel, 1987). However, in contrast to the findings of the latter workers, Veenstra (1988) has shown that partially purified head factors I and II failed to sustain the decrease in lumen positivity, effecting a rapid return to near-prestimulation values. Head factor III, believed to be mosquito natriuretic factor (Beyenbach and Petzel, 1987), resulted in a transient decrease in lumen positivity followed by a rapid and large increase, which was then followed by a steady decline to near-prestimulation levels in tubules of *Aedes aegypti*

(Beyenbach and Petzel, 1987; Veenstra, 1988). Head factor I was not found to stimulate fluid secretion. (Beyenbach and Petzel, 1987).

High potassium salivary gland factor (HPSGF) isolated from the thoracic regions of *Calliphora vicina* (formally *C. erythrocephala*) was also found to decrease lumen positivity in the salivary glands of this insect (Trimmer, 1985), although this factor was shown to be indistinguishable from 5-hydroxytryptamine (5-HT) (Trimmer, 1985), the substance used by several other workers (e.g. Berridge and Prince, 1972; O'Donnell and Maddrell, 1984) as a DH mimic. However, 5-HT is not generally believed to be a physiological hormone in insects. In the salivary glands of *Calliphora erythrocephala* (Berridge and Prince, 1972; Berridge *et al.*, 1975b) and the Malpighian tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984), TEP changed in a negative direction in the presence of 5-HT, comparable to those results with CC extract in the present study.

Morgan and Mordue (1983) and Nicolson and Isaacson (1987) reported that DH had little effect on V_B in tubule cells of *Locusta migratoria* or *Onymacris plana*, respectively. Similarly, no dramatic change in V_B was observed using CC extract in the present study, although a small, but significant hyperpolarization of about 6.6 mV was observed after 5 min exposure. Similarly, Berridge *et al.* (1975, 1976) reported a hyperpolarization of V_B in salivary gland cells of *Calliphora erythrocephala*, whilst O'Donnell and Maddrell (1984) found only small changes in V_B in tubules of *Rhodnius prolixus* following exposure to 5-HT.

In view of the marked stimulation of fluid secretion which treatment with DH promotes, the lack of any large changes in V_B on addition of DH, in this and other studies, is perhaps surprising, particularly when most other transporting epithelial cells show a characteristic potential change in response to endocrine or secretagogue stimulation (Petersen, 1976). Nevertheless, the current observations do not rule out the possibilities of DH-stimulated increases in permeability to K^+ and/or Cl^- or electroneutral cotransport.

The major effect of CC extract on the tubule cells of *Locusta migratoria* is a depolarization of V_A. Similarly, Nicolson and Isaacson (1987) concluded, from studies on V_B and TEP in tubules of *Onymacris plana*, that DH effected changes in TEP as a consequence of changes in V_A, and O'Donnell and Maddrell (1984) showed that, in *Rhodnius prolixus*, the changes in TEP due to the action of 5-HT represented events at the apical membrane. The depolarizing effect of CC extract on V_A in control saline could be accounted for by an increase in apical Cl^- conductance, Cl^- entering the cell across the basal membrane by some electroneutral mechanism, possibly by cotransport with K^+ as discussed previously. Indeed, net Cl^- flux from haemolymph-side to lumen-side (hl) is significantly increased by the action of CC extract.

As mentioned previously, in experiments where control saline was replaced by Cl^--free saline, V_B remained unchanged, whereas V_A hyperpolarized by about 10 mV, as also shown by Baldrick *et al.*(1988). The lack of effect on V_B was taken to indicate low basal membrane permeability to Cl^-, whilst the hyperpolarization of V_A was explained on the basis of continued apical cation extrusion in the absence of a suitable counterion (present study; Baldrick *et al.*, 1988; Fogg *et al.*, 1989). Treatment with CC extract in these Cl^--free conditions resulted in a significant depolarization of V_B and hyperpolarization of V_A, with a corresponding large (about 20 mV) increase in lumen positivity. These results are consistent with CC extract-stimulation of an electrogenic cation pump on the apical cell membrane in the absence of Cl^- as counterion. A similar explanation has been suggested for the effect of 5-HT on V_A in tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). The observed depolarization of V_B in Cl^--free conditions would suggest that CC extract stimulates basal cation entry, again in the absence of Cl^- as counterion. Although K^+ flux was not investigated in the present study, CC extract was found to increase net $^{hl}Na^+$ flux. Such a depolarization of V_B on treatment with CC extract could also be explained on the basis of increased basal permeability to Cl^-, with Cl^- efflux occurring passively in the absence of external Cl^-. As mentioned earlier, passive efflux of Cl^- across the basal cell membrane is favoured even when tubules are bathed in control saline. Therefore, a CC extract-stimulated increase in basal Cl^- permeability under control conditions would also tend to cause a depolarization of this membrane. This is not the case, however. In fact, a small but significant hyperpolarization is observed. Therefore, it would appear that CC extract has no effect on the Cl^- permeability of the basal membrane to Cl^- in tubules of *Locusta migratoria.*

Due to the relative impermeability of the basal membrane to Cl^- in the unstimulated tubule, CC extract must increase Cl^- movement across this membrane for it to act as a counterion. Indeed, net $^{hl}Cl^-$ flux is significantly increased by the action of CC extract. Cl^- entry across the basal membrane, as mentioned earlier, appears not to depend on cotransport with Na^+, and cotransport with K^+ seems possible. In this investigation, both high K^+, Na^+-free saline and CC extract effected a depolarization of V_A. It has previously been proposed that the depolarization of V_A, following superfusion with high K^+, Na^+-free salines, is dependent on K^+-stimulated Cl^- entry across the basal membrane (Baldrick *et al.*, 1988). The current observation that application of CC extract in high K^+, Na^+-free saline effected an enhanced depolarization of V_A, together with its effect in Cl^--free saline, support the hypothesis that some component in the CC extract is modifying Cl^- movement across both the basal and apical membranes. Thus, in control saline, the depolarization of V_A effected by CC extract is explained, in part, on the basis of increased anion movements, with consequent short-circuiting of the apical cation pump.

The depolarization of V_B following the removal of Cl^- from the high K^+, Na^+-free saline, but not the control saline, seems to indicate that Cl^- entry is K^+-stimulated, as proposed by Baldrick *et al.*(1988). Indeed, as mentioned earlier, net $^{hl}Cl^-$ flux is significantly increased on elevation of $[K^+]_o$. Application of CC extract under high $[K^+]$, Na^+-free, Cl^--free conditions has a similar effect to that in Cl^--free saline; little change in V_B, but a significant hyperpolarization of V_A, further supporting the hypothesis that some component of the CC extract results in the stimulation of an apical cation pump in the absence of Cl^- as counterion. The lack of any major response by V_B to CC extract under these conditions further supports the hypothesis that CC extract does not increase basal membrane Cl^- permeability. If this occurred, a hyperpolarization of the basal membrane in the presence of external Cl^- and a depolarization in its absence would be expected.

In experiments where Na^+ was replaced by choline, CC extract effected a quantitatively similar response by V_B and V_A as in control saline, although the small hyperpolarization of V_B was not significant. This would seem to indicate that the movement of Na^+ contributes very little to the basal or apical membrane potentials in CC extract-stimulated tubules, although net $^{hl}Na^+$ flux is increased by such treatment.

In experiments where Ca^{2+} was replaced by the Ca^{2+} chelator EGTA, CC extract resulted in a small, but significant depolarization of V_B, but had no significant effect on V_A. The lack of effect of CC extract on V_A in Ca^{2+}-free saline would seem to indicate that Ca^{2+} is normally involved in the regulation of ion movements across the apical membrane. The small depolarization of V_B, similar to the response in Cl^--free saline, could be taken to be the stimulation of cation movement across the basal membrane without the movement of a suitable counter-anion. An increase of Cl^- movement across the basal membrane, on stimulation of tubule cells with CC extract, may therefore be Ca^{2+}-regulated. However, as mentioned previously, the findings in Ca^{2+}-free saline, reported here, must be treated with caution due to the number of different cellular processes in which this cation is involved, the fact that intracellular Ca^{2+} levels are normally very low and may not be significantly affected in the short term, and also due to the observation that removal of Ca^{2+} has potentially damaging effects on cellular histology (Donowitz, 1983).

cAMP and IBMX

In the present study, treatment of locust Malpighian tubules with dibutyryl cAMP in control saline resulted in a small non-significant hyperpolarization of V_B, and a significant hyperpolarization of V_A (see Fogg *et al.*, 1989). The suggestion that the effect on V_A is a consequence of increased intracellular cAMP is supported by the similar response by V_A observed following treatment with the phosphodiesterase inhibitor IBMX. The slower

response to cAMP compared with that of IBMX may be due to the lower permeability of the basal cell membrane to dibutyryl cAMP, with a consequent slower increase in the intracellular level of this chemical.

A number of workers (Prince and Berridge, 1972; Berridge and Prince, 1972; Berridge *et al.*, 1975b; Maddrell, 1980) have proposed models for the endocrine control of ion translocation by epithelial cells in insects in which cAMP acts as intracellular second messenger. Indeed, in tubules of *Locusta migratoria* an AVP-like insect diuretic hormone, extracted from suboesophageal and thoracic ganglia, has been shown to increase intracellular levels of cAMP (Proux and Herault, 1988). A similar response is observed in tubules of *Rhodnius prolixus* following treatment with that insect's DH (Aston, 1975). Similarly, in tubules of *Aedes aegypti*, mosquito natriuretic factors have been shown to increase intracellular levels of cAMP *in vitro* and *in vivo* (Petzel *et al.*, 1987). Indeed, in the present study, CC extracts with diuretic activity have been shown to increase the levels of this second messenger (see later) in the Malpighian tubule cells of *Locusta migratoria, in vitro* (Fogg *et al.*, accepted for publication, 1990). However, the difference in response by V_A to CC extract and cAMP, observed in the present study, implies that cAMP cannot mediate the full effects of DH in Malpighian tubules of *Locusta migratoria.*

The biogenic amine, 5-HT, which mimics the effects of DH on Malpighian tubules of some insect species, has also been shown to increase intracellular levels of cAMP in salivary glands of *Calliphora erythrocephala* (Berridge and Patel, 1968), in which cAMP is thought to stimulate an apical cation pump (Berridge and Prince, 1972; Berridge *et al.*, 1975). The stimulation of such a pump by increased intracellular levels of cAMP in the Malpighian tubules of *Locusta migratoria* would be consistent with the observed hyperpolarization of V_A in both control and Cl^-free saline containing dibutyryl cAMP, and in control saline containing IBMX. A quantitatively similar effect of cAMP on V_A to that in control and Cl^--free saline was observed in Ca^{2+}-free saline, which suggests that Ca^{2+} is not involved in the cAMP-mediated cellular response. The observation of essentially the same effects of cAMP and CC extract in Cl^--free media, viz. hyperpolarization of V_A, is consistent with cAMP- and CC extract-stimulation of an electrogenic cation pump on the apical cell membrane in the absence of Cl^-. A similar explanation has been suggested for the effect of 5-HT on V_A in tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984).

As mentioned previously, the depolarization of V_A in control saline effected by CC extract, but not cAMP, is explained, in part, on the basis of increased anion movements with consequent short-circuiting of the apical cation pump. If this is so, then it implies that such increased anion conductance must be independent of cAMP. In salivary glands of *Calliphora erythrocephala*, cAMP is thought to stimulate an apical cation pump whereas

Ca^{2+} increases anion conductance across both the basal and apical cell membranes (Berridge and Prince, 1972; Berridge *et al.*, 1975b). The results presented above are consistent with the suggestion that such a mechanism of control may also operate in Malpighian tubules of *Locusta migratoria* in response to some factor(s) present in the crude CC extracts. Indeed, in the present study, CC extract has been shown to increase the intracellular levels of the calcium-mobilizing second messenger D-*myo*-Inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) in the tubule cells of *Locusta migratoria, in vitro* (see later).

Acetazolamide

In the present study, treatment of locust tubules bathed in control saline with acetazolamide (Diamox), at a concentration of 1 mM, had no significant effect on either V_B or V_A. Acetazolamide acts by inhibiting carbonic anhydrase (Rector *et al.*, 1965), the enzyme which catalyses the reversible dehydration of carbonic acid (Gay, 1982). Carbonic acid dissociates into H^+ and HCO_3^- in solution. Acetazolamide will therefore prevent or reduce the production of intracellular HCO_3^-. Electroneutral Cl^--HCO_3^- exchange has been shown in a variety of epithelia (Gerencser and Lee, 1983). It is possible that Cl^- transport by the Malpighian tubules of *Locusta migratoria* is through such an exchange, as suggested by Baldrick (1987). If this agent were inhibiting Cl^--HCO_3^- exchange across the basal membrane by reducing intracellular HCO_3^- in tubule cells of *Locusta migratoria*, a hyperpolarization of V_A might be expected on the basis of continued cation extrusion with the reduced availability or absence of Cl^- as counter-anion, similar to the effect observed in Cl^--free saline. No change in V_B would be expected due to the electroneutral nature of this exchange mechanism. Indeed, removal of basolateral CO_2 and HCO_3^- or addition of acetazolamide hyperpolarized V_A, but did not change V_B, in the rectal glands of *Aedes dorsalis* (Strange and Phillips, 1984).

In the present study, the lack of effect of acetazolamide in control saline on either V_B or V_A would seem to indicate that Cl^- entry into the Malpighian tubule cells is not via such a Cl^- entry mechanism, although it is possible that some other anion may substitute for Cl^-, or that HCO_3^- has access into the cell from the bathing medium. Indeed, work by Baldrick (1987), in which acetazolamide was applied in HCO_3^--free saline to the tubules of *Locusta migratoria*, resulted in a hyperpolarization of V_A by about 3 mV after 15 min exposure. Such a hyperpolarization could be taken to be the result of inhibition of Cl^--HCO_3^- exchange, although the extent of this hyperpolariztion and the lack of similarity between this and the effect of Cl^--free saline would suggest that any Cl^--HCO_3^- exchange that may occur does not contribute greatly to the movement of Cl^- across this epithelium.

Thiocyanate

Sodium thiocyanate (10 mM) has been shown to inhibit fluid secretion by the Malpighian tubules of *Locusta migratoria* (Fathpour, 1980; Kalule-Sabiti, 1985; Baldrick, 1987), whilst in *Musca domestica* fluid secretion was stimulated (Dalton and Windmill, 1980). This compound has been shown to inhibit anion transport; Na^+-dependent active Cl^- transport (Epstein *et al.*, 1973; Zadunaisky *et al.*, 1971) and anion-stimulated ATPase activity across a variety of tissues (Gerenscer and Lee, 1983; see later). Anion-stimulated ATPase activity, associated with both basal and apical membrane fractions, was demonstrated in the present study (see later). Also in this study, SCN^- (10 mM) in control saline effected a rapid (within 0.5 min) and significant depolarization of V_A by about 11.3 mV followed by an equally rapid repolarization to pre-treatment values. In contrast V_B slowly hyperpolarized, but this 3-5 mV hyperpolarization was only significant 5 min after treatment, and is difficult to explain on the basis of reduced anion entry. Qualitatively similar responses by V_B and V_A to those in control saline were observed in Cl^--free saline on addition of SCN^-, although this agent effected a greater initial depolarization of V_A than in control saline. In the absence of Cl^- it may be expected that SCN^- would have little effect on either V_B or V_A. On this basis it is difficult to explain the similar effects of SCN^- on V_A in Cl^--free and control saline. Inhibition of Cl^- entry across the basal membrane by SCN^- in control saline would be expected to lead to the hyperpolarization of V_A due to the reduced availability of Cl^- to follow the active extrusion of cations across the apical membrane. The initial depolarization of V_A in all the salines tested is especially difficult to explain on these grounds.

When SCN^- (10 mM) was applied together with dibutyryl cAMP (1 mM) in control saline, there was no significant change in V_B. V_A showed a similar initial response, over the first minute, to that of SCN^- in the absence of dibutyryl cAMP. However, after 5 min, V_A had hyperpolarized, although not significantly, to values approximately 10.8 mV more negative that those in control saline alone. This would be expected if cAMP were stimulating an apical cation pump for which there was reduced availability of a suitable counter-anion. The mean hyperpolarization of V_A after 5 min is similar to the approximate 9.4 mV observed in control saline in the presence of dibutyryl cAMP alone. Therefore, it is difficult to explain the role of anion-stimulated ATPase in fluid secretion by the Malpighian tubules of *Locusta migratoria* at this time. Future experiments, where SCN^- is applied to tubules that are CC extract-stimulated, may shed further light on anion transport across the basal cell membrane.

RESULTS

Effect of Corpora Cardiaca Extract on Intracellular cAMP

Figure 3.11 is an example of a set of experimental data showing the effect of CC extract on the levels of cAMP in cells of Malpighian tubules of *Locusta migratoria* at various times after treatment with the agonist. Following application of CC extract, there was a rapid increase in the amount of intracellular cAMP. Thus cAMP increased from an unstimulated control level of 28.2 ± 6.7 pmoles/mg protein (n=3) to yield a transitory peak of 312.1 pmoles/mg protein at 8 sec (Fig. 3.11). Thereafter, there was a rapid decline to 26.1 pmoles/mg protein within the next 2 sec. In the continued presence of CC extract, there followed a more gradual increase in intracellular cAMP to 209.1 pmoles/mg protein at 1 min and to 407.5 pmoles/mg protein at 5 min. A similar pattern of response to CC extract was observed in all experiments. However, examination of the mean data in Table 3.8 suggests considerable variation in the levels of cAMP over the first 2-8 sec. This is to a large extent a consequence of the differences in the times of appearance of the initial transitory peaks (mean time after CC extract application was 6.5 ± 1.0 sec, n=4) and reflects, in part, difficulties encountered in rapidly terminating the short-time incubations. This variation is shown in the data presented in Table 3.8. If one ignores the time differences, the mean value for the initial peak was 472.0 ± 66.0 pmoles/mg protein (n=4) and this was attained within 4-8 sec of treatment with CC extract; a significant increase above the basal intracellular levels of cAMP ($P<0.01$; based on a Student's *t*-test for single data). Despite individual variation, the levels of intracellular cAMP measured at 1 min and 5 min, following application of CC extract, were significantly above ($P<0.05$; based on a Student's *t*-test for single data) those of the unstimulated controls.

Effect of Corpora Cardiaca Extract on Intracellular Ins-1,4,5-P_3

Figure 3.12 shows a set of data representing the effect of CC extract on Ins-1,4,5-P_3 levels in cells of Malpighian tubules of *Locusta migratoria*. The measurements were made on the same tissue extracts as shown for cAMP in Fig. 3.11 to permit comparison between the responses of the two second messengers. It can be seen that treatment with CC extract (Fig. 3.12) effected a rapid rise in Ins-1,4,5-P_3 within 10 sec, from a non-stimulated level of 37.9 ± 4.4 pmoles/mg protein (n=23) to 172.4 pmoles/mg protein at 8 sec. This initial response, resembling that observed for cAMP, was followed by a rapid decline to 34.2 pmoles/mg protein at 10 sec. Thereafter, there was a progressive rise in Ins-1,4,5-P_3 over the next 20 sec to 101.5 pmoles/mg protein, at 30 sec post-treatment, followed by a

decline to 27.6 pmoles/mg protein at 1 min. Four minutes later, intracellular Ins-1,4,5-P_3 had risen again to 76.1 pmoles/mg protein. As with cAMP measurements, all independent determinations showed a similar pattern of response to CC extract over the first 10 sec. There was again, however, some variation in the timing of the initial peaks for the reasons mentioned previously for cAMP; mean peak time being 5.2 $\pm$ 1.0 sec, n=5. Ignoring these time differences, the mean initial peak was 170.0 $\pm$ 26.7 pmoles/mg protein (n=5), attained 2-8 sec after CC extract application; a significant increase above the non-stimulated control level ($P<0.01$; based on a Student's *t*-test for single data). Table 3.9 shows that Ins-1,4,5-P_3 levels were significantly increased by treatment with CC extract and that following the initial peak the second messenger level fell rapidly to near non-stimulated control values at 10 sec. Some variation was noted in the subsequent pattern of change in Ins-1,4,5-P_3 content as reflected by the SEM values of the data in Table 3.9. Nevertheless, the mean levels of Ins-1,4,5-P_3 were significantly higher in treated than in the non-stimulated controls from 15 sec post-treatment ($P<0.01$; based on a Student's *t*-test for single data).

FIGURE 3.11

An example of the effect of CC extract on intracellular levels of cAMP.

Ordinate : cAMP in pmoles/mg protein.

Abscissa :Time in seconds.

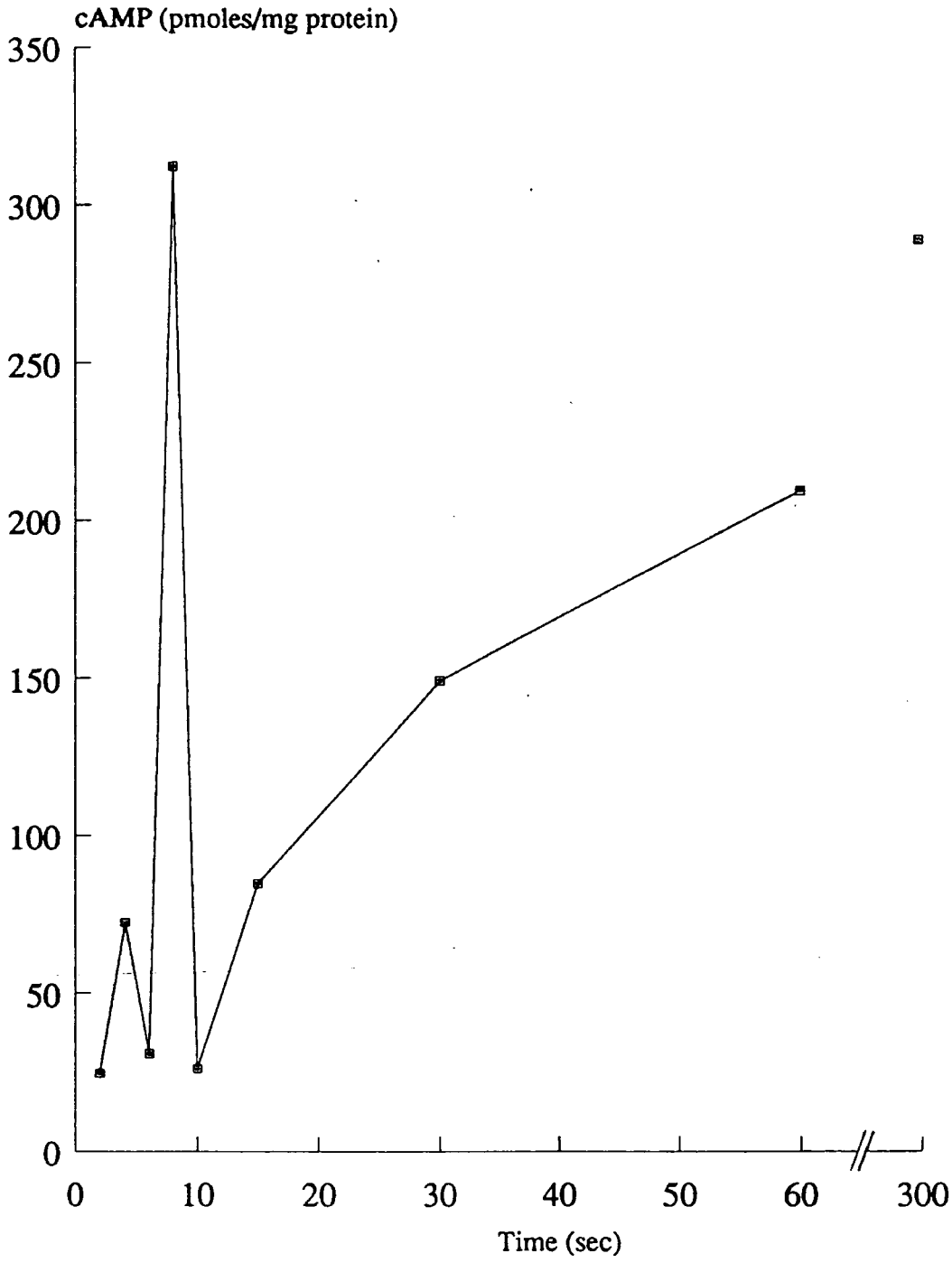

cAMP (pmoles/mg protein)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
300
Time (sec)

FIGURE 3.12

An example of the effect of CC extract on intracellular levels of Ins-1,4,5-P_3.

Ordinate : Ins-1,4,5-P_3 in pmoles/mg protein.

Abscissa : Time in seconds.

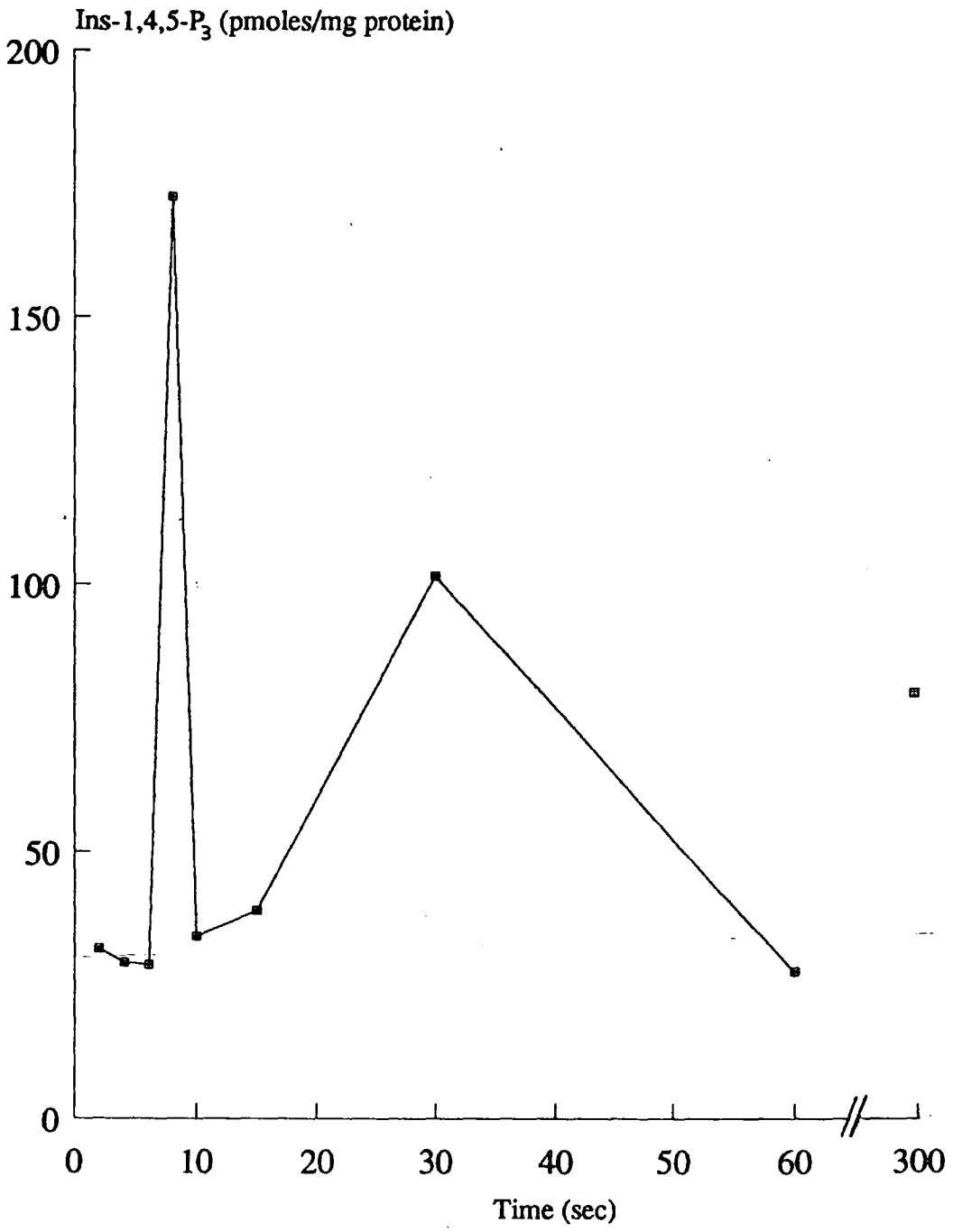

Ins-1,4,5-P3 (pmoles/mg protein)
200
150
100
50
0
0
10
20
30
40
50
60
300
Time (sec)

Table 3.8 Effect of Corpora Cardiaca Extract on Intracellular cAMP Levels

Time after treatment (sec)	Mean level of intracellular cAMP (pmoles/ mg protein)	*n*	*P*
2	39.6 ± 10.3	4	ns
4	192.6 ± 136.5	4	ns
6	143.8 ± 92.3	4	ns
8	309.1 ± 143.8	3	ns
10	38.1 ± 11.8	4	ns
15	136.5 ± 45.7	4	ns
30	179.9 ± 105.9	3	ns
60	141.1 ± 34.7	4	< 0.05
300	386.7 ± 104.8	4	< 0.05

Mean values are quoted ± SEM.
The mean level of cAMP in non-stimulated control tubules was 28.2 ± 6.7 pmoles/mg protein (n=3).
Values of *P* were obtained by application of a Student's *t*-test for single data between control and experimental data.
n represents the number of independent determinations.
The relatively large SEM values reflect, in part, changes in second messenger levels occurring at different times in individual experiments.

Table 3.9 Effect of Corpora Cardiaca Extract on Intracellular Inositol 1,4,5-trisphosphate Levels

Time after treatment (sec)	Mean level of intracellular Ins-1,4,5-P_3 (pmoles/mg protein)	*n*	*P*
2	58.1 ± 22.7	5	ns
4	79.9 ± 33.7	5	< 0.05
6	103.0 ± 37.0	5	< 0.01
8	131.1 ± 27.3	5	< 0.01
10	42.0 ± 17.9	5	ns
15	118.2 ± 52.1	5	< 0.01
30	94.4 ± 19.8	5	< 0.01
60	73.5 ± 13.9	5	< 0.01
300	95.2 ± 30.5	5	< 0.01

Mean values are quoted ± SEM.
Non-stimulated controls were monitored at various times throughout the experimental period; the mean overall level of Ins-1,4,5-P_3 was 37.9 ± 4.4 pmoles/mg protein (n=23).
Values of *P* were obtained by application of a Student's *t*-test for single data between control and experimental data.
n represents the number of independent determinations.
The relatively large SEM values reflect, in part, changes in second messenger levels occurring at different times in individual experiments.

DISCUSSION

The application of CC extract resulted in marked increases in intracellular levels of both cAMP and Ins-1,4,5-P_3 in Malpighian tubules of *Locusta migratoria*. At 1 min and 5 min after CC extract was applied, the mean levels of cAMP had increased approximately 5-fold and 14-fold, respectively, compared with the controls (Table 3.8). Correspondingly, levels of Ins-1,4,5-P_3 had increased approximately 2-fold after 1 min and 2.5-fold after 5 min post-treatment (Table 3.9); no such changes were observed in non-stimulated controls run over the experimental period. This, together with the near-coincidence of the timing of the initial peaks for both second messengers in the independent experiments, indicates that the changes are corpora cardiaca-stimulated. Thus, it is clear that a factor or factors present in crude homogenates of corpora cardiaca can induce changes in the activities of adenylate cyclase and phospholipase C in Malpighian tubules of *Locusta migratoria*.

The present study appears to be the first to demonstrate the increase in Ins-1,4,5-P_3 levels in Malpighian tubule cells of *Locusta migratoria* exposed to CC extract with diuretic activity, although a number of workers have reported increased levels of cAMP. For example, Aston (1975) reported that, in *Rhodnius prolixus*, cAMP was increased nearly 4-fold, 3 min after stimulation with DH extract. Morgan and Mordue (1985) reported DH-stimulation of intracellular levels of cAMP by 7 to 8-fold in Malpighian tubules of *Locusta migratoria*, after 3 min at 30°C. It should be noted that the non-stimulated control levels of cAMP found in the present study were considerably greater (approximately 10-fold) than those reported in the same insect by Morgan and Mordue (1985). In contrast, the basal levels of intracellular cAMP found in the Malpighian tubules of non-fed female *Aedes aegypti* (Petzel *et al.*, 1987) were considerably greater (approximately 12-fold) than those reported here for *Locusta migratoria*. However, 5 min after the commencement of post-blood meal diuresis in the female mosquito, intracellular cAMP levels were only about 1.4 times (Petzel *et al.*, 1987) those found here for CC extract-stimulated tubules of *Locusta migratoria* at 5 min. In *Aedes aegypti*, tubule fluid secretion has been correlated with an increase in intracellular cAMP over a range of about 600 pmoles/mg protein; fluid secretion rates saturating at about 2nl/min at 900 pmoles cAMP/mg protein (Petzel *et al.*, 1987).

In the present study, it was shown that the generation of increased levels of the two second messenger molecules by CC extract was extremely rapid; an initial peak being produced at 6.5 $\pm$ 1.0 sec for cAMP and 5.2 $\pm$ 1.0 sec for Ins-1,4,5-P_3. These peaks represented an approximate 17-fold and 4.5-fold increase in cAMP and Ins-1,4,5-P_3 levels, respectively, compared to controls. Following a fall to near non-stimulated levels, at approximately 10 sec post-treatment, there was a slower increase in second messenger levels.

To date, few studies on early second messenger response to agonists have been reported for insect tissue, a notable exception being the study on salivary glands of *Calliphora erythrocephala* by Berridge and Heslop (1982) in which stimulation with 5-HT resulted in a rapid increase in intracellular cAMP (measurements being taken every 10 sec) reaching a peak at 30-40 sec. After this initial peak, the levels fell to near basal values by 130 sec and the began to rise again. Berridge and Heslop (1982) proposed, from work using the phosphodiesterase inhibitor theophylline, that the intracellular level of cAMP may be determined by changes in the activity of both adenylate cyclase and phosphodiesterase.

Similar results to those reported in the present study have been described for vertebrate tissues. For example, Flitney and Singh (1980) reported that exogenous application of ATP to frog heart induced a 4.5-fold increase in intracellular cAMP within 5-8 sec and that this was followed by an abrupt fall to a level below that of the control. The levels of cAMP then rose again, but more slowly, to reach a secondary maximum of 3.5-fold control values after 140-160 sec. The sudden fall in the level of cAMP after the initial peak is probably due to activation of phosphodiesterase (Berridge and Heslop, 1982). However, the mechanism controlling the latter enzyme is uncertain. Clayberger *et al.* (1981), suggested that membrane-bound phosphodiesterase in rat erythrocytes is activated through a Ca^{2+}-calmodulin system, whereas in the frog heart cGMP has been implicated (Flitney and Singh, 1980).

In the present study, the observed rapid fall in intracellular cAMP levels in CC extract-stimulated tubules of *Locusta migratoria* following the initial peak could be due to rapid activation of phosphodiesterase, possibly through Ca^{2+}, with subsequent levels being regulated, at concentrations sufficient to maintain fluid secretion, by adenylate cyclase and phosphodiesterase activities. On this basis, the initial peak of intracellular cAMP occurring 4-8 sec after CC extract addition could simply be an overshoot, rapidly brought under control by the activation of phosphodiesterase.

The role of cAMP as an intracellular second messenger, mediating the action of DH on insect Malpighian tubules, has been speculated upon mainly on the basis that exogenously applied cAMP stimulated an increase of fluid secretion, *in vitro*, in a number of different species, including *Locusta migratoria* (Anstee *et al.*, 1980; Donkin, 1981; Morgan and Mordue, 1981; Rafaeli and Mordue, 1982). Furthermore, it is generally recognised that the regulation of intracellular cAMP depends upon the balance between the rates of production by adenylate cyclase and degradation by phosphodiesterase. On this basis, inhibition of phosphodiesterase should elevate intracellular cAMP levels and thereby result in increased fluid secretion in hormonally unstimulated cells. Indeed, theophylline not only resulted in increased fluid secretion by the salivary glands of *Calliphora erythrocephala* (Berridge,

1970), but also in a corresponding increase in intracelluair levels of cAMP (Prince *et al.*, 1972). Similar results were found using both theophylline and forskolin (a stimulator of the adenylate cyclase system) on Malpighian tubules of *Aedes aegypti* (Petzel *et al.*, 1987), and with IBMX on tubules of *Locusta migratoria* (Morgan and Mordue, 1985). Maddrell *et al.* (1971), reported that aminophylline (theophylline ethylene diamine) stimulated fluid secretion by isolated Malpighian tubules of *Carausius morosus*, but neither this nor theophylline nor caffeine would stimulate tubules of *Rhodnius prolixus*. Similarly, theophylline had no affect on tubular fluid secretion by *Locusta migratoria* (Rafaeli and Mordue, 1982), whilst Morgan and Mordue (1985) reported a slight stimulatory effect, but no corresponding increase in intracellular cAMP levels in tubules of this insect.

In *Rhodnius prolixus*, the lack of stimulation by the various methyl xanthines was attributed to the very slow rate of fluid secretion by unstimulated tubules, reflecting a low adenylate cyclase activity (Maddrell *et al.*, 1971). A similar explanation was given by Morgan and Mordue (1985) to account for the variation in intracellular levels of cAMP found in tubules of *Locusta migratoria* in response to IBMX treatment. They suggested that unstimulated cells have a low adenylate cyclase activity, and hence, a low rate of cAMP production, whilst tubules partially stimulated on removal from the insect would have a higher rate of cAMP production. Rafaeli and Mordue (1982) suggested that in *Locusta migratoria* the lack of effect of theophylline was due to its being treated as a toxin and excreted rapidly.

Direct evidence of the involvement of intracellular cAMP in the action of DH only came when Aston (1975) demonstrated that DH resulted in the elevation of this second messenger in the Malpighian tubule cells of *Rhodnius prolixus*. Similar findings in *Aedes aegypti* (Petzel *et al.*, 1987) and *Locusta migratoria* (Rafaeli *et al.*, 1984; Morgan and Mordue, 1985; present study), together with the effects of the phosphodiesterase inhibitors (Maddrell *et al.*, 1971; Morgan and Mordue, 1985; Petzel *et al.*, 1987) and forskolin (Petzel *et al.*, 1987), seem to provide sufficient evidence for the involvement of cAMP in the coupling of hormonal stimulus to fluid secretion by these Malpighian tubules (Rafaeli *et al.*, 1984). The presence of a cAMP-dependent kinase in tubules of *Locusta migratoria* further substantiates such coupling (see Rafaeli *et al.*, 1984).

Rafaeli *et al.* (1984), reported that in tubules of *Locusta migratoria* cAMP was generated rapidly in response to DH stimulation, reaching a peak after 20 min, followed by a rapid decline. The peak level represented an approximate 13-fold increase in cAMP compared with basal levels. It also appeared that after only 5 min the intracellular cAMP levels reached were sufficient for maximal stimulation of fluid secretion. Any further increases in cAMP were not reflected by a corresponding fluid secretory response to DH,

and were regarded as an overshoot effect (Rafaeli *et al.*, 1984). Such an excess of cAMP was excreted by the tubules, a mechanism not unlike the parathyroid hormone-stimulated urinary excretion of cAMP found in vertebrates (human, Alston *et al.*, 1980; rat, Carnes *et al.*, 1980; chick, Pines *et al.*, 1983). It was also noted that the basal levels of both fluid secretion and intracellular cAMP concentrations (Rafaeli and Applebaum, 1980) govern the extent of stimulation obtained in the presence of DH; the lower the basal level, the higher the level of stimulation obtained (Rafaeli *et al.*, 1984). In contrast, Morgan and Mordue (1985) found a substantial increase in intracellular cAMP in response to DH in *Locusta migratoria*, but higher levels of cAMP were observed in tubules stimulated for only 3 min, when compared with those at 10 min, although these levels were only significantly different at the P=0.2 level of significance. However, these observations were consistent with those made in stimulated Malpighian tubules of *Rhodnius prolixus* (Aston, 1975) and the salivary glands of *Calliphora erythrocephala* (Prince *et al.*, 1972); maximal levels of intracellular cAMP being observed after 2-3 min, followed by a decline to a slightly lower equilibrium level.

It may be possible that intracellular levels of cAMP are regulated not only by the activities of adenylate cyclase and phosphodiesterase, but also by the excretory process itself when produced in excess (Rafaeli *et al.*, 1984). Following the initial 4-8 sec peak after CC extract addition, the rapidity (within 2 sec) of intracellular cAMP decrease would seem to indicate phosphodiesterase activation, not excretion by the tubule. However, this does not rule out the possibility of some cAMP-excretory mechanism being involved in intracellular cAMP regulation in the continued presence of the agonist.

It has been shown that fluid secretion by locust Malpighian tubules is under the control of two distinct diuretic peptides (DP-1 and DP-2), extracted from the corpus cardiacum, which act at different receptors (Morgan and Mordue, 1984; Morgan *et al.*, 1987). DP-1 (*ca.* 6000-7000 daltons) acts at one receptor resulting in the activation of adenylate cyclase and the production of cAMP, whilst DP-2 (*ca.* 1000 daltons) acts via an undetermined transduction pathway (Morgan and Mordue, 1984). In blowfly salivary glands, two distinct types of 5-HT receptor have been demonstrated, one activating adenylate cyclase, and the other stimulating the Ca^{2+}-phosphoinositol cascade (Berridge, 1981; Berridge and Heslop, 1981,1982; Berridge *et al.*, 1984).

In the present study, intracellular levels of inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) rose rapidly, with no apparent time lag, reaching a peak 5.2 $\pm$ 1.0 sec after the addition of CC extract, representing an approximate increase of 4.5-fold non-stimulated control levels. Following a fall to near non-stimulated levels, at about 10 sec post-treatment, there was a slower increase in Ins-1,4,5-P_3 level. The almost immediate change in Ins-1,4,5-P_3 levels in

response to agonist appears to be a common feature of this type of second messenger system, with an initial peak being attained within 2-15 sec in a variety of tissues from different species (e.g. salivary glands of *Calliphora erythrocephala*, Berridge *et al.*, 1984; rat cerebral cortical slices, Batty *et al.*, 1985; rat parotid glands, Irvine *et al.*, 1985; rabbit peritoneal neutrophils, Bradford and Rubin, 1986; rat pancreatic islets, Biden *et al.*, 1987; A431 human epidermoid carcinoma cells, Hepler *et al.*, 1987; Tilly *et al.*, 1987; giunea-pig hepatocytes, Tennes *et al.*, 1987). Berridge *et al.* (1984) reported that on addition of 5-HT to salivary glands of *Calliphora erythrocephala*, intracellular Ins-1,4,5-P_3 levels rose rapidly, with no apparent time lag, reaching a peak at approximately 4 sec, followed by a gradual decline to basal levels over 1 min and then by a gradual increase over the next 5 min. This pattern of response is similar to that recorded in the present study in the Malpighian tubules of *Locusta migratoria*.

The role of Ins-1,4,5-P_3 as a Ca^{2+}-mobilizing intracellular messenger is well established in a number of cells and tissues (Berridge and Irvine, 1984; Abdel-Latif, 1986; Berridge, 1987). Bradford and Rubin, (1986) reported that the peak level of intracellular Ins-1,4,5-P_3 occurring 5-10 sec after the addition of *N*-formyl-methionyl-leucyl-phenylalamine to rabbit peritoneal neutrophils, was in excess of that needed to release Ca^{2+} from intracellular stores. On this basis, as suggested for cAMP, the rapidly formed peak of Ins-1,4,5-P_3 observed in the present study could simply be an overshoot that is quickly brought under control by feedback mechanisms, which then regulate Ins-1,4,5-P_3 at levels sufficient to maintain fluid secretion.

Rossier *et al.* (1987), have suggested that Ins-1,4,5-P_3 is metabolized via two distinct pathways in rat aortic smooth muscle cells: (1) a dephosphorylation pathway, leading to formation of inositol bis- and mono-phosphate, and (2) a Ca^{2+}-sensitive phosphorylation/dephosphorylation pathway, involving the formation of inositol 1,3,4,5-tetrakisphosphate (Ins P_4) and leading to the formation of Ins-1,3,4-P_3. A Ca^{2+}-sensitive Ins-1,4,5-P_3-kinase activity has been demonstrated in intact HL-60 cells (Lew *et al.*, 1986), permeabilized adrenal glomerulosa cells (Rossier *et al.*, 1986), and subcellular fractions from insulin-secreting RINm5F cells (Biden and Wollheim, 1986). Thus, it would seem that Ins-1,4,5-P_3 may control its own metabolism through Ca^{2+} by modulating its own degradation.

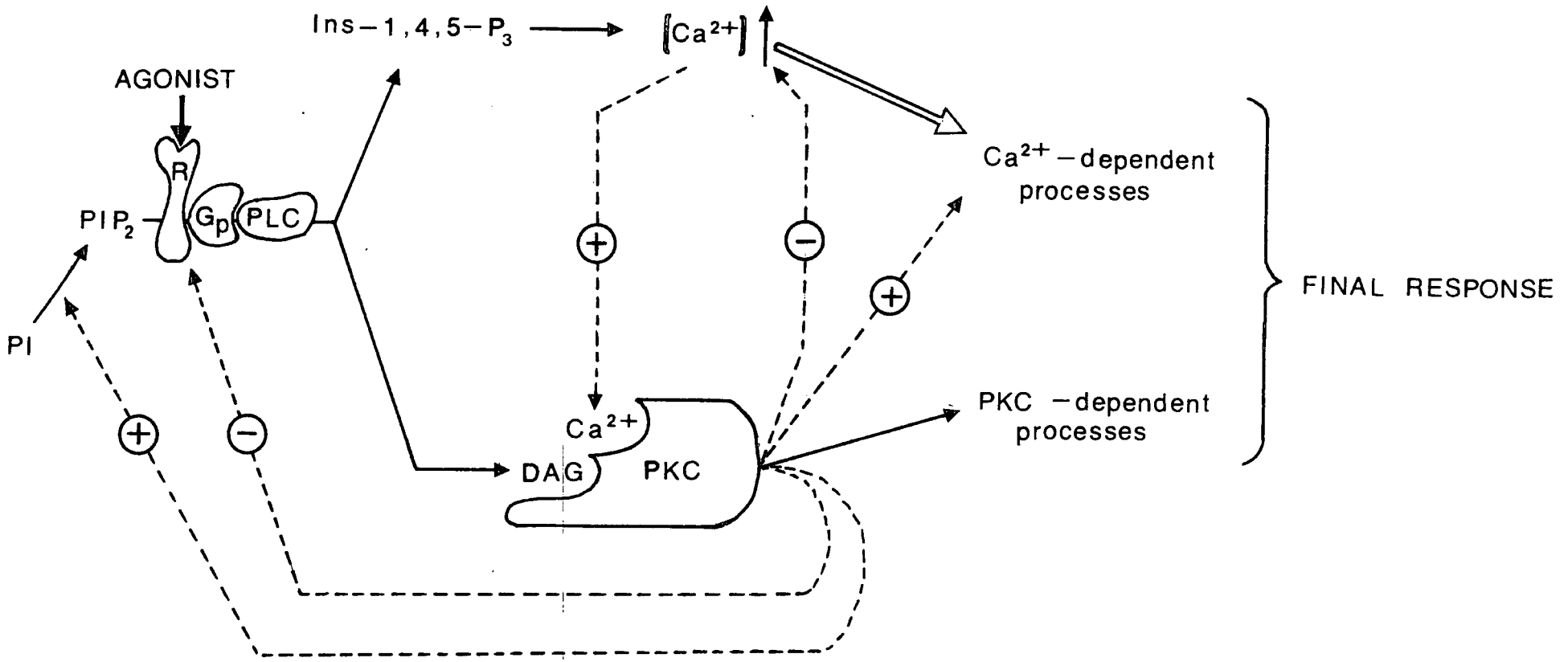
Figure 3.13, shows the "dual signal hypothesis" (Berridge, 1987), which describes how the Ins-1,4,5-P_3/Ca^{2+} and diacylglycerol (DAG)/protein kinase C (PKC) signal pathways, of the inositol signalling system, interact to control a large range of cellular processes. The two pathways appear to act synergistically in many cells (Nishizuka, 1984). It is proposed that the Ins-1,4,5-P_3/Ca^{2+} pathway plays a major and direct role in initiating

FIGURE 3.13

The "dual signal hypothesis" (Berridge, 1987). The dashed lines represent the homologous interactions operating between the Ins-1,4,5-P_3/Ca^{2+} and diacylglycerol/protein kinase C (DAG/PKC) pathways. A key feature of the inositol lipid signalling system is that both products of phosphatydyl inositol-4,5-P_2 (PIP_2) hydrolysis by phospholipase C (PLC) function as second messengers, thus forming a bifurcating pathway for transferring information into the cell.

When an agonist occupies its receptor (R), it activates a G-protein (G_p), which then binds GTP leading to the stimulation PLC. This enzyme is responsible for the hydrolysis of PIP_2, leading to the formation of Ins-1,4,5-P_3 and DAG. Ins-1,4,5-P_3 effects the release of Ca^{2+} from intracellular stores, whilst DAG and Ca^{2+} act by stimulating PKC. Both the increase in intracellular free Ca^{2+} and the activation of PKC contribute to the final response.

PI represents phosphatydyl inositol.


Ins−1,4,5−P3
[Ca2+]
AGONIST
R
PIP2
Gp
PLC
PI
Ca2+
DAG
PKC
Ca2+ −dependent processes
PKC −dependent processes
FINAL RESPONSE

cellular responses. This is based mainly on its control of the level of intracellular Ca^{2+}, which is recognized as a major second messenger for stimulating a broad spectrum of cellular responses. Indeed, Ins-1,4,5-P_3 added to permeabilized cells or injected into intact cells can result in a number of complex physiological responses (Berridge, 1987). The DAG/PKC pathway may also contribute directly to the final response, but appears mainly to modulate either the Ca^{2+}-signalling pathway or other signal pathways (Berridge, 1987).

The mechanism of cellular control is further complicated by the feedback relationship between intracellular Ca^{2+} and cAMP. The first indication of such an interaction came when Ca^{2+} was found to inhibit adenylate cyclase in broken cell preparations (e.g. kidney, Streeto, 1969; Taunton *et al.*, 1969; foetal rat calvaria, Chase *et al.*, 1969; heart, Drummond and Duncan, 1970). Ca^{2+} was further implicated as a modulator of cAMP levels when, in the rat parotid gland, the normal increase in cAMP levels induced by α-adrenergic agents was severely reduced in glands simultaneously treated with a β-adrenergic agent (Butcher, 1975), which was thought to act by increasing Ca^{2+} influx (Selinger *et al.*, 1974). Similarly, a depression of cAMP levels was observed with the use of the Ca^{2+} ionophore A23187 (Butcher, 1975).

The actions of both 5-HT and cAMP on insect salivary glands are dependent on Ca^{2+} (Berridge and Prince, 1972). The onset of the secretory response of these glands to 5-HT, and particularly to cAMP, is slower than normal in the absence of Ca^{2+} from the bathing medium. In both cases, the brief stimulation was followed by a decline to very low rates of secretion; a dramatic recovery in secretion rate being observed on returning Ca^{2+} to the bathing medium.

Irvine and Houslay (1988) reported that the desensitization of adenylate cyclase following glucagon-stimulation in intact rat hepatocytes was a cAMP-independent process which appeared to be elicited through the stimulation of inositol phospholipid metabolism. These workers also reported that treatment of intact cells with A23187 led to inhibition of cellular glucagon-stimulated adenylate cyclase activity.

cAMP, in turn, may regulate Ca^{2+} levels within cells (Berridge, 1976). Enouf *et al.* (1987), recently concluded that a cAMP-dependent protein phosphorylation may be involved in the regulation of the Ins-1,4,5-P_3-induced Ca^{2+} release in human platelets. cAMP has also been found to modulate Ca^{2+} movement across the sarcoplasmic reticulum by stimulating the Ca^{2+}-ATPase which sequesters Ca^{2+} during cardiac muscle relaxation (Kirchberger *et al.*, 1972; Tada *et al.*, 1974, 1975). cAMP has also been implicated as a regulator of Ca^{2+} movement across mitochondria (Borle, 1974). Berridge (1976) suggested that the ability of cAMP to release Ca^{2+} stored within mitochondria could account for the increase in Ca^{2+} efflux which occurs during stimulation of rat liver (Friedmann and Park,

1968; Friedman, 1972), toad bladder (Thorn and Schwartz, 1965) rat α-cells (Brisson and Malaisse, 1973) salivary glands of *Calliphora erythrocephala* (Prince *et al.*, 1972) and mammalian salivary glands (Nielsen and Petersen, 1972). In contrast, cAMP seems to inhibit Ca^{2+} entry into lymphocytes (Freedman *et al.*, 1975) and mast cells (Foreman *et al.*, 1976).

Clearly, there are complex feedback interactions operating between cAMP, Ca^{2+}, Ins-1,4,5-P_3 and the DAG/PKC pathways which are of great importance in regulating cell activity (Prince and Berridge, 1972; Berridge, 1975, 1976). Further work involving the use of phorbol esters (PKC-stimulators), ionophore A23187, and the measurement of DAG production on stimulation with CC extract, may shed light on the various interactions occurring in the Malpighian tubule cells of *Locusta migratoria*.

RESULTS

Cytochemical localization of K^+-NNPase

The structure of the cells of the Malpighian tubules of *Locusta migratoria* has been well documented (Martoja, 1959, 1960; Peacock, 1975; Charnley, 1975; Bell, 1977; Bell and Anstee, 1977; Donkin, 1981). In the present study, electron microscopy confirms that tubules consist of cells of two types (Plate 3.1); primary (Type 1) cells and stellate (Type 2) cells (Charnley, 1982). Primary cells are considered to be mainly responsible for ion and water transport (Maddrell, 1971), representing approximately 90% of total tubule cell number (Baldrick, 1987), whilst stellate cells may be primary mucocytes (Charnley, 1982). The latter take up lead (Berridge and Oschman, 1969; Peacock, 1975), and have been demonstrated (Baldrick, 1987) by a lead staining method (see Berridge and Oschman, 1969). As in other secretory epithelia, locust Malpighian tubule cells, particularly primary cells, are characterized by extensive basal cell membrane infoldings and an apical microvillar brush border (Plate 3.1). The preservation of fine structure of the tubules during the present cytochemical localization of nitrophenyl phosphatase (NPPase) activity was rather unsatisfactory, as also reported by Komnick and Achenbach (1979). It should be noted that, in all cases, there were inconsistent precipitate distributions; heavily stained regions being found close to unstained regions.

All subsequent references are confined to the more abundant primary, type I cells.

Cytochemical localization of K^+-NNPase using the standard incubation medium

The following results were obtained from tubules incubated in the standard incubation medium, as described previously (Chapter 2). Plates 3.2a and 3.2b show the general distribution of reaction product along the basal and apical cell membranes, respectively. Reaction product appears to be almost exclusively restricted to the basal cell membrane (Plate 3.2a). The mitochondria, which are closely associated with the basal infoldings, and the basement membrane are free of deposits. The reaction product is located on the cytoplasmic side of the basal cell membrane infoldings, whilst the surrounding cytoplasm is free of deposits. Plate 3.2b shows local deposits of reaction product around the microvilli in the apical region, with no deposits being found in the lumen.

Experiments were then carried out under various kinetically defined conditions to determine the subcellular site of K^+-NPPase activity (Ernst, 1972a,b) by distinguishing this enzyme from unspecific phosphatase activity.

PLATE 3.1

Electron micrograph showing a typical section through a Malpighian tubule of *Locusta migratoria*. This confirms that the locust tubule consists of two cell types; primary (Type 1, T_1) cells and stellate (Type 2, T_2) cells. T_1 cells are considered to be mainly responsible for ion and water transport and represent about 90% of the total tubule cell number. T_2 cells may be primary mucocytes.

BI	Basal cell membrane infoldings
MV	Microvilli
N	Nucleus
L	Lumen

Scale: 5 μm.

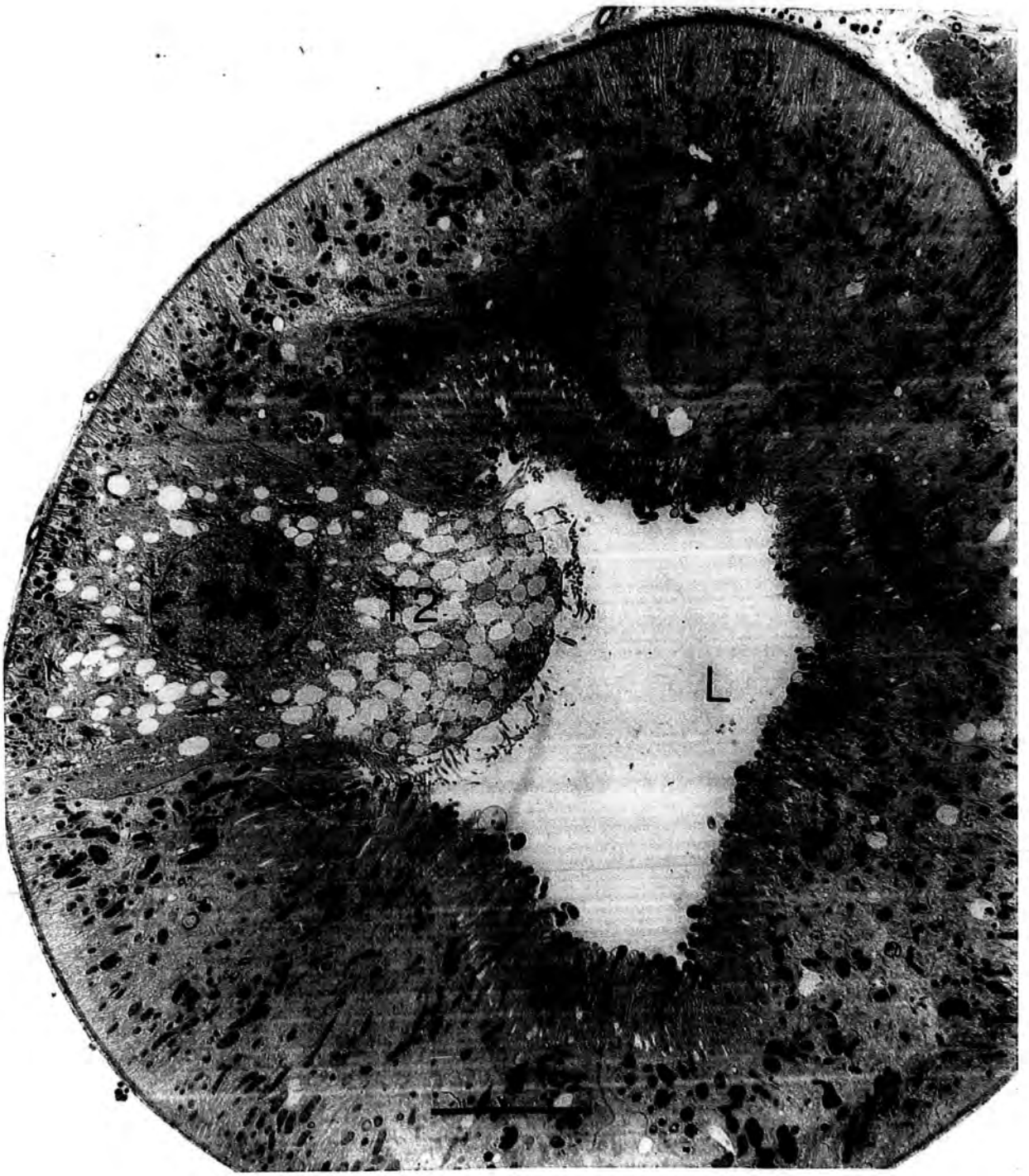

T2
L

PLATE 3.2a

This micrograph shows the general distribution of reaction product along the basal cell membrane infoldings (BI) of tubules incubated in the standard incubation medium. Reaction product is exclusively restricted to the cytoplasmic side of the basal membrane infoldings, whilst the surrounding cytoplasm is free of deposits. Mitochondria (M), which are closely associated with the basal infoldings, the basement membrane (BM) and nucleus (not shown) are also free of deposits.

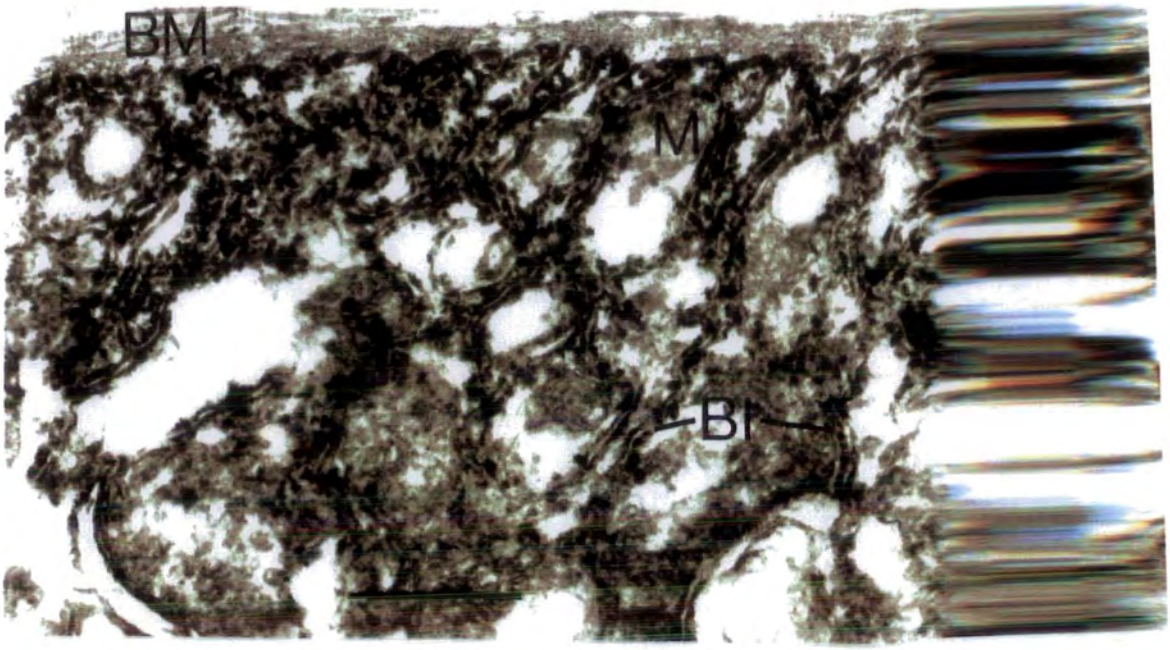
Scale: 0.5 μm.

PLATE 3.2b

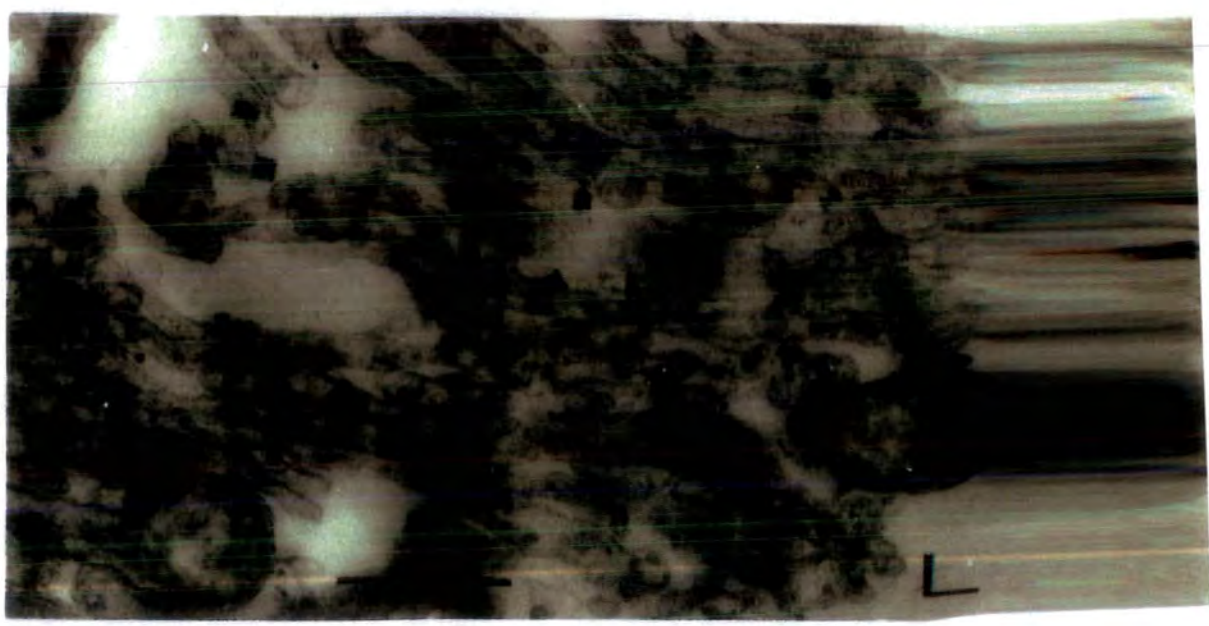
This micrograph shows the general distribution of reaction product around the microvilli (MV) in the apical region of tubules incubated in the standard incubation medium. No deposits were found in the lumen (L).

Scale: 0.5 μm.

a



b



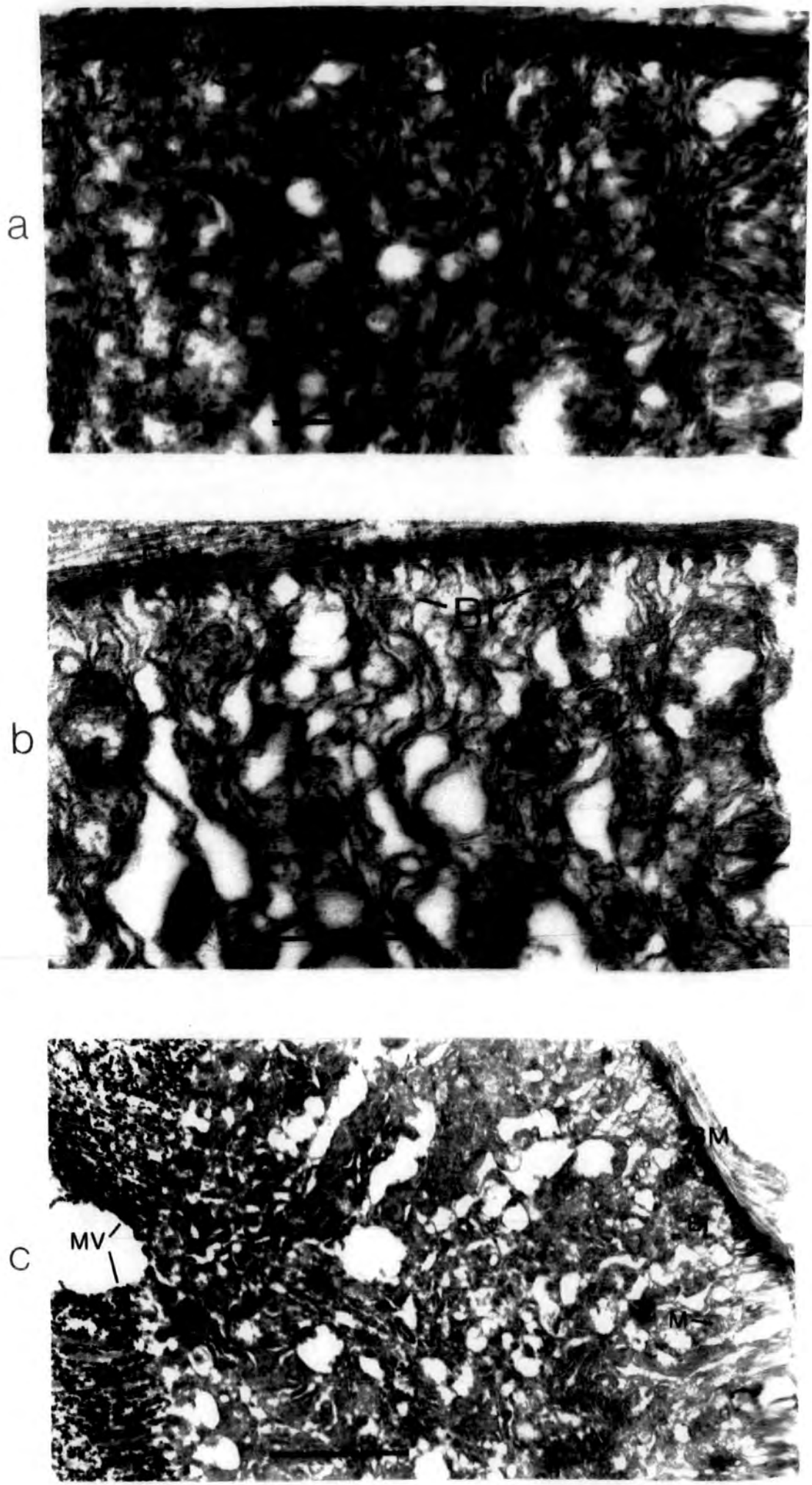
PLATES 3.3a-c

When tubules were incubated in a medium free of K+, there was a great reduction (PLATE 3.3a) or complete absence (PLATE 3.3b) of reaction product deposited along the basal infoldings (BI).

Scales: 0.5 μm.

In contrast, large deposits were observed lining the outer surface of the microvilli (MV) in the apical region, with the basement membrane (BM) and mitochondria (M) free of deposits (PLATE 3.3c).

Scale: 2.5 μm.


a
b
BL
c
MV
M
BL
M

PLATES 3.4a and b

Substitution of the substrate, nitrophenyl phosphate, with β-glcerophosphate in the incubation medium allowed selective demonstration of alkaline phosphatase activity. PLATE 3.4a shows the complete absence of reaction product along the basal infoldings (BI). PLATE 3.4b shows local deposits lining the microvilli (MV) in the apical region, representing alkaline phosphatase activity.

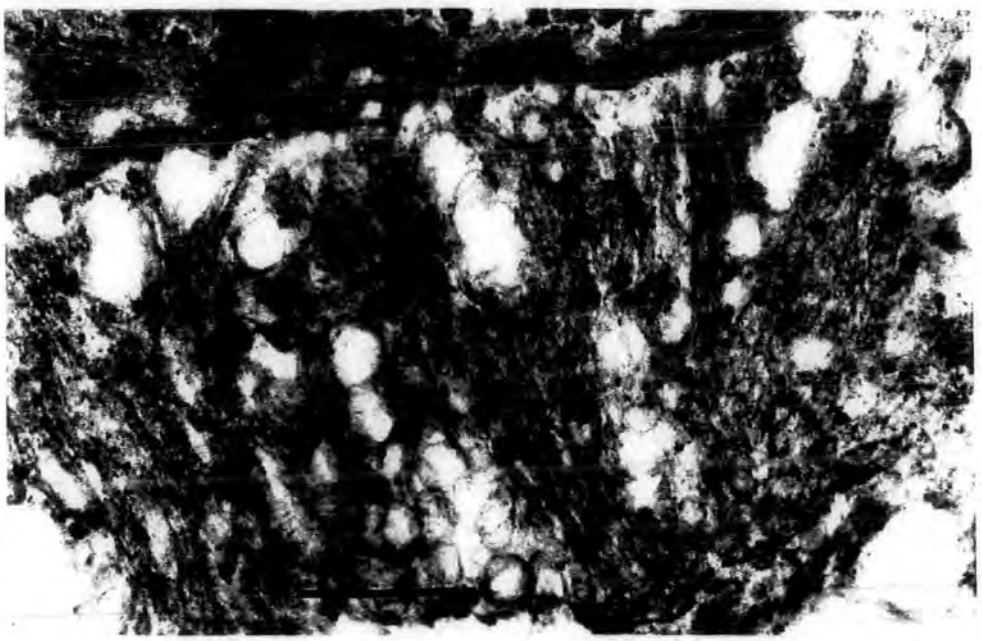
Scales: 0.5 μm.

PLATE 3.4c

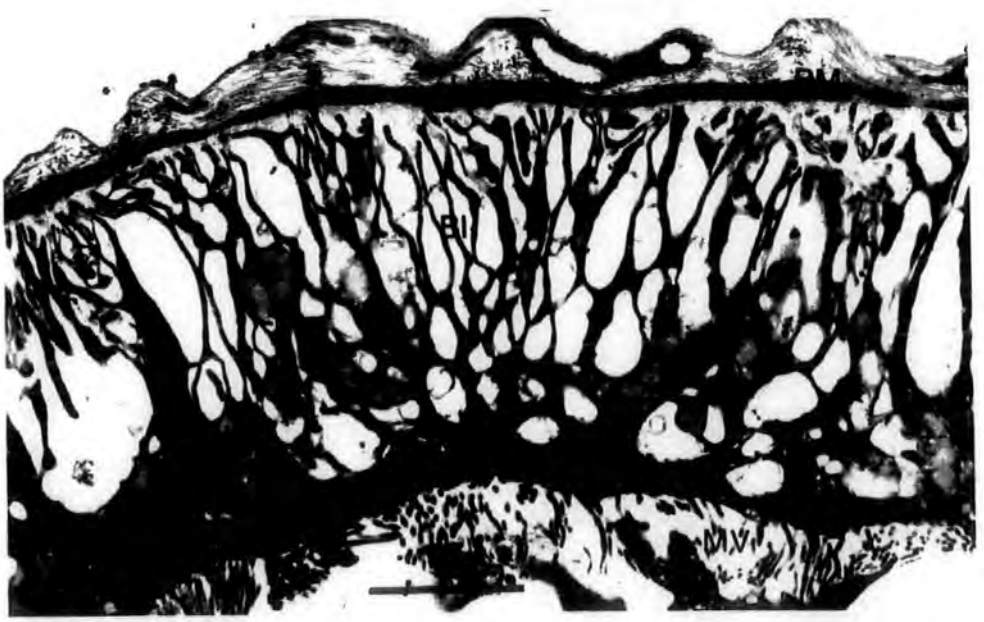
Micrograph showing the cellular disruption resulting from the inclusion of 10 mM ouabain in the standard incubation medium. There appears to be a complete absence of reaction product along both the basal infoldings (BI) and microvilli (MV).

Scale: 2.5 μm.

a



b



c

FIGURE 3.14

Comparative measurements of *p*-nitrophenyl phosphatase (NPPase) activity in crude homogenates of paraformaldehyde-fixed and unfixed Malpighian tubules of *Locusta migratoria*. Homogenates were incubated in various biochemical and histochemical reaction media at pH 7.2 or pH 9.0, as indicated.

ATPase activity was measured in the following biochemical reaction media which consisted of:

Standard incubation medium minus K^+ (KCl replaced by 5mM choline chloride).

Standard incubation medium; 100 mM Tris-HCl buffer, pH 7.2 or 9.0, 100mM sucrose, 10 mM $MgCl_2$, 10 mM KCl and 5 mM NPP (*p*-nitrophenyl phosphate).

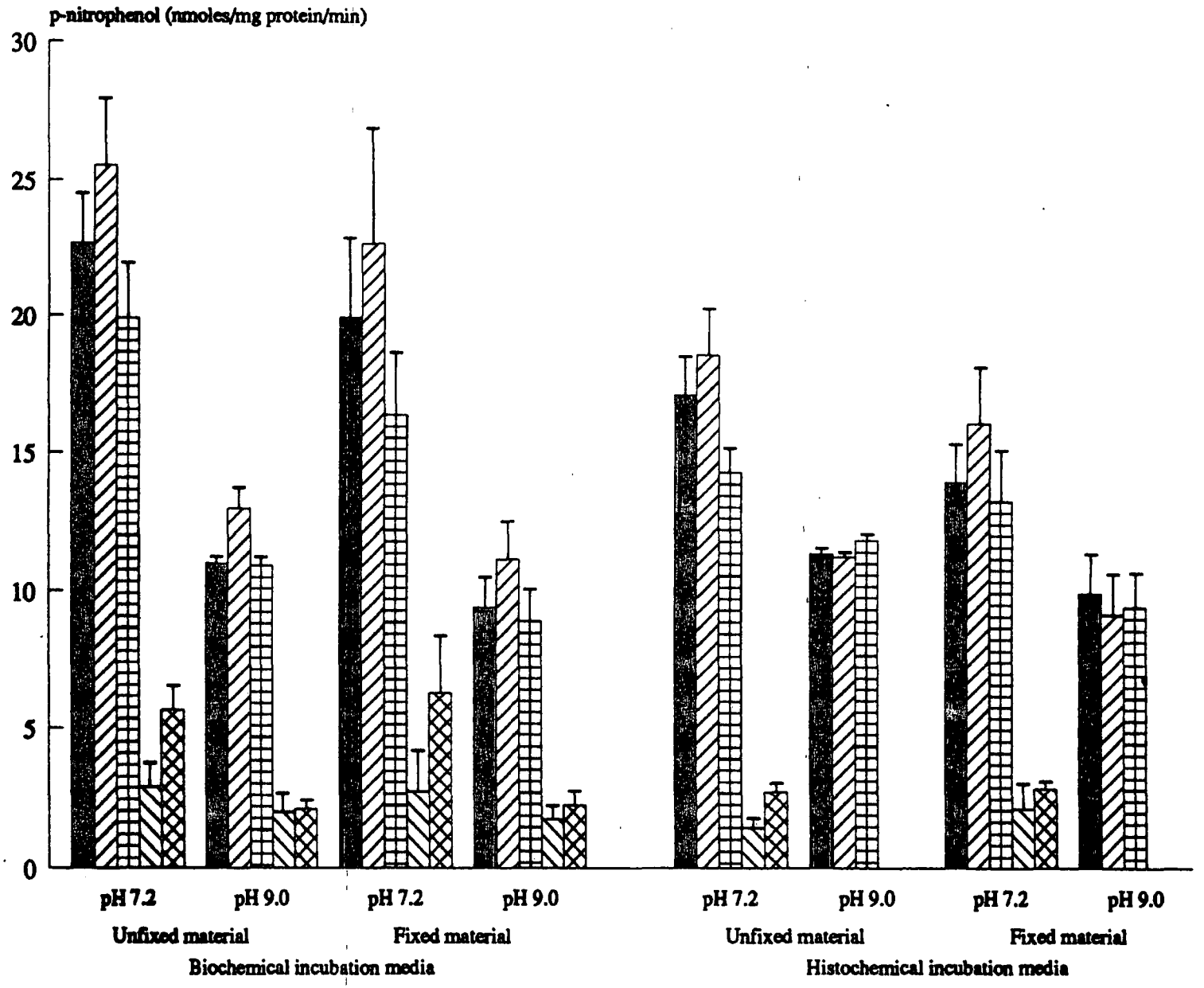
Standard incubation medium plus 5 mM ouabain.

The histochemical reaction were the same as the biochemical media plus 20 mM $SrCl_2$.

The following ATPase activities were calculated from the data observed in the various media (see text for details, Chapter 2).

K^+-stimulated ATPase activity.

Ouabain-sensitive ATPase activity.


p-nitrophenol (nmoles/mg protein/min)
30
25
20
15
10
5
0
pH 7.2
pH 9.0
pH 7.2
pH 9.0
pH 7.2
pH 9.0
pH 7.2
pH 9.0
Unfixed material
Fixed material
Unfixed material
Fixed material
Biochemical incubation media
Histochemical incubation media

Effect of K^+ substitution

Substitution of potassium chloride by choline chloride in the incubation medium resulted in a great reduction in the amount (Plates 3.3a), or complete absence (Plates 3.3b) of the reaction product deposited along the basal cell membrane infoldings. In contrast, large deposits were observed lining the outer surface of the microvilli in the apical region (Plate 3.3c). It should be noted that the basal infoldings, basement membrane and mitochondria are free of deposits in the same section (Plate 3.3c).

Effect of substrate substitution

Substitution of the substrate, nitrophenyl phosphate (NPP), with β-glycerophosphate in the incubation medium allowed the selective demonstration of alkaline phosphatase activity. This enzyme is an unspecific phosphatase, and will therefore utilize both NPP and β-glycerophosphate as substrates, whilst the latter is not a substrate for the K^+-dependent phosphatase (Ernst 1972b, 1975). Plate 3.4a shows the complete absence of reaction product along the basal cell membrane infoldings, whilst local deposits were found along the microvilli in the apical region (Plate 3.4b).

Effect of 10 mM ouabain

1 mM ouabain included in the incubation medium has been shown to give inconsistent results (Kalule-Sabiti, 1985), and as a result 10 mM ouabain was used in this study, with incubations carried out at 30°C as described previously (Chapter 2). Cell preservation was poor in the presence of 10 mM ouabain (Plate 3.4c), the ultrastructure being severely disrupted, and therefore the results obtained give unsatisfactory evidence for the presence of a ouabain-sensitive NPPase. There was a complete absence of reaction product along the basal infoldings, and along the microvilli in the apical region.

Comparative biochemical measurements of K^+-NPPase in fixed and unfixed Malpighian tubule homogenates of *Locusta migratoria* L.

Biochemical measurements of K^+-NPPase activity in fixed and unfixed tubule homogenates were determined in order to ascertain what proportion of enzyme activity was inhibited by the incubation and fixation processes involved in the cytochemical localization of the enzyme (previous section).

Figure 3.14 shows the mean NPPase activities of crude, unfixed and fixed Malpighian tubule homogenates incubated at pH 7.2 and 9.0 in various biochemical and

histochemical (+ 20 mM $SrCl_2$) incubation media. Unless otherwise stated, statistical analyses were carried out using the Student's *t*-test for single data.

Effect of K^+ on NPPase activity in unfixed tubule homogenates

Examination of data in Figure 3.14 shows that the addition of 10 mM K^+ to the biochemical incubation medium resulted in a significant increase ($P<0.05$) in NPPase activity in crude homogenates of unfixed tubules at both pH 7.2 and pH 9.0; the K^+-stimulated NPPase activity representing approximately 11% and 15% of total NPPase activity, respectively. Similarly, inclusion of 10 mM K^+ in the histochemical incubation medium (+ 20 mM $SrCl_2$) resulted in a significant increase in NPPase activity at pH 7.2 in unfixed material; K^+-stimulated NPPase activity representing about 8% of total NPPase activity. In contrast, at pH 9.0, no significant K^+-stimulated NPPase activity was measured.

Effect of ouabain on NPPase activity in unfixed tubule homogenates

Data in Figure 3.14 also shows that the addition of 5 mM ouabain to the biochemical incubation medium containing 10 mM K^+ effected a significant reduction in total NPPase activity at both pH 7.2 and pH 9.0, by approximately 22% and 16%, respectively. These data represent the ouabain-sensitive NPPase activity as a percentage of the total NPPase activity. Ouabain is considered to be a specific inhibitor of $(Na^+ + K^+)$-ATPase, of which the K^+-stimulated NPPase is a component. On this basis, the ouabain-sensitive component would be expected to be of similar magnitude to the K^+-stimulated component. However, it would appear that the former component is somewhat greater at both pH 7.2 and pH 9.0. This difference might be explained if K^+, from normal intracellular sources, were being introduced into K^+-free incubations with the crude tissue homogenates, resulting in an overestimation of the basal level of NPPase activity. On this basis, incubations carried out in the presence of ouabain probably represent more reliable measures of non-specific NPPase activity, and the ouabain-sensitive component is probably a better estimate of K^+-stimulated NPPase activity.

Inclusion of 5 mM ouabain in the histochemical incubation medium containing 10 mM K^+ resulted in a 23% reduction in NPPase activity in homogenates of unfixed Malpighian tubules at pH 7.2. Thus, the ouabain-sensitive NPPase activity is proportionally similar to that observed in the absence of strontium. However, at pH 9.0, no significant ouabain-sensitive NPPase activity was measured.

Effect of pH on NPPase activity in unfixed tubule homogenates

The effect of raising the pH of the biochemical incubation media from pH 7.2 to pH 9.0 was to significantly reduce ($P<0.01$) NPPase activity, in the absence and presence of 10 mM K^+, by about 50% (Fig. 3.14), suggesting that inhibition was of the basal NPPase activity alone. However, given that the ouabain-sensitive NPPase activity was reduced by about 63% and that this is probably a more reliable estimate of K^+-stimulated NPPase activity, it would seem that increasing the pH of the medium has indeed had a significant effect on reducing the latter activity.

Raising the pH of the histochemical incubation media from pH 7.2 to pH 9.0 not only resulted in a near-complete inhibition of K^+-stimulated NPPase activity and complete inhibition of ouabain-sensitive NPPase activity, but also resulted in a significant reduction ($P<0.01$) in non-specific NPPase activity by about 39%, to a value similar to that obtained in the biochemical medium (i.e. in the absence of strontium) (Fig. 3.14).

Effect of K^+ on NPPase activity in fixed tubule homogenates

Examination of data in Figure 3.14 shows that the inclusion of 10 mM K^+ in the biochemical incubation medium resulted in an increase in NPPase activity in crude homogenates of paraformaldehyde fixed tubules at both pH 7.2 and pH 9.0; the K^+-stimulated NPPase activity representing about 12% and 16% of the total NPPase activity, respectively. Thus, the K^+-stimulated NPPase activity observed in homogenates of the fixed material appears to constitute approximately the same proportion of the total NPPase activity as it does in the unfixed material, described previously.

The inclusion of 10 mM K^+ in the histochemical incubation medium resulted in an increase in NPPase activity in crude homogenates of paraformaldehyde fixed tubules at pH 7.2, the K^+-stimulated NPPase activity representing about 13% of the total NPPase activity. In contrast, there was no significant K^+-stimulated NPPase activity at pH 9.0.

Effect of ouabain on NPPase activity in fixed tubule homogenates

Figure 3.14 also shows that the addition of 5 mM ouabain to the biochemical incubation medium containing 10 mM K^+ effects a reduction in NPPase activity in fixed tubule homogenates at both pH 7.2 and pH 9.0 by about 28% and 21%, respectively.

5 mM ouabain included in the histochemical incubation medium containing K^+, resulted in a significant reduction ($P<0.02$) in total NPPase activity at pH 7.2 of about 17%, representing the ouabain-sensitive NPPase activity. In contrast, at pH 9.0 no significant ouabain-sensitive NPPase activity was measured. Since, as mentioned previously, the ouabain-sensitive component is likely to be the more appropriate estimate of K^+-sensitive NPPase activity, one must conclude that the latter activity is not demonstratable at pH 9.0 in the histochemical medium.

Effect of pH on NPPase activity in fixed tubule homogenates

The effects of raising the pH of the biochemical incubation media from pH 7.2 to pH 9.0 on NPPase activity, in fixed tubule homogenates, was essentially as observed with unfixed material (Fig. 3.14). Once again, there was a significant reduction (approximately 50%) in NPPase activity in the absence and presence of 10 mM K^+, and the ouabain-sensitive NPPase activity was significantly reduced by about 65%.

Increasing the pH of the histochemical incubation media from pH 7.2 to pH 9.0 resulted in a significant reduction ($P<0.05$) in total NPPase activity of about 43%, to a value similar to that obtained in the absence of strontium. Furthermore, this treatment effected complete inhibition of K^+-stimulated NPPase activity and near-complete inhibition of ouabain-sensitive NPPase activity.

Effect of pH

Three-way multivariate analysis indicates that the rise in pH of incubation media from pH 7.2 to pH 9.0 significantly reduces non-specific ($P<0.01$), K^+-stimulated ($P<0.05$) and ouabain-sensitive NPPase activity ($P<0.01$), and that this reduction is independent of the effect of strontium in the latter two cases. In the case of non-specific NPPase, the effects of pH and strontium do interact to cause a significant ($P<0.05$) reduction in activity. The effects of pH are independent of fixation in all cases.

Effect of strontium

Three-way multivariate analysis also indicates that the inclusion of strontium in the incubation medium significantly reduces non-specific ($P<0.05$), K^+-stimulated ($P<0.05$) and ouabain-sensitive NPPase activity ($P<0.01$). As mentioned previously, the effects of strontium and pH do not interact in the latter two cases, but do interact in the case of the non-specific NPPase. The effects of strontium are independent of fixation in all cases.

Effect of fixation

Three-way multivariate analysis, and the Student's *t*-test for single data, indicate that fixation of Malpighian tubules prior to homogenization has no significant effect on non-specific, K^+-stimulated or ouabain-sensitive NPPase activity. As mentioned previously, the effects of strontium or pH are independent of fixation in all cases.

Biochemical Studies on Membrane Fractions from the Malphighian Tubules of *Locusta migratoria*: An attempt to localize the Mg^{2+}-dependent, $(Na^+ + K^+)$- and HCO_3^--stimulated ATPases

An attempt was made to separate and partially purify the basal and apical cell membranes from the Malpighian tubules of *Locusta migratoria* using the modified technique of Rodriguez and Edelman (1979) described in Chapter 2. Biochemical studies were carried out, to determine $(Na^+ + K^+)$- and HCO_3^--stimulated ATPase activities within each pellet and supernatant obtained (summary of membrane separation, Fig, 3.15), and their subcellular localization with reference to the enzyme activity of apical cell membrane marker, alkaline phosphatase, and the mitochondrial membrane marker, succinate dehydrogenase (SDH).

Effect of K^+ and $(K^+ + Na^+)$ on ATPase activity

Figure 3.16 shows the distribution of K^+-stimulated ATPase, $(Na^+ + K^+)$-ATPase and alkaline phosphatase activities in crude homogenates of Malpighian tubules of *Locusta migratoria* and the various supernatant and membrane fractions derived from them. It can be seen that the K^+-stimulated, Mg^{2+}-dependent ATPase activity was largely confined to the membrane fractions P_2, P_4, P_7 and P_8, with maximal mean specific activity (175 nmoles P_i liberated/mg protein/min) being observed in the latter fraction. In all experiments on these pellets, there was an increase in Mg^{2+}-dependent ATPase activity on addition of K^+ to the incubation medium. However, no such activity was observed in supernatant S_1 from which these pellets originate. The explanation for this apparent anomaly is uncertain. The general increase in purity of the fractions through the membrane separation procedure for Mg^{2+}-dependent ATPase (Table 3.6) may account for the lack of K^+-stimulated ATPase activity in S_1, but caution must be taken when attributing subsequent pellets with K^+-stimulated ATPase activity under these circumstances. In fractions where no K^+-stimulated ATPase activity was observed, the presence of K^+ in the incubation medium actually led to a reduction in the activity of the Mg^{2+}-dependent ATPase (Appendix Table A.3). If such monovalent cation inhibition of the Mg^{2+}-ATPase were to occur in the pellets exhibiting

FIGURE 3.15

Flow diagram showing the sequence of different pellets (P) and supernatants (S) obtained during the isolation and partial purification of apical (P_5) and basolateral plasma membranes (P_7, P_8) from the Malpighian tubules of *Locusta migratoria*. For the complete summary, see Fig. 2.9, Chapter 2.

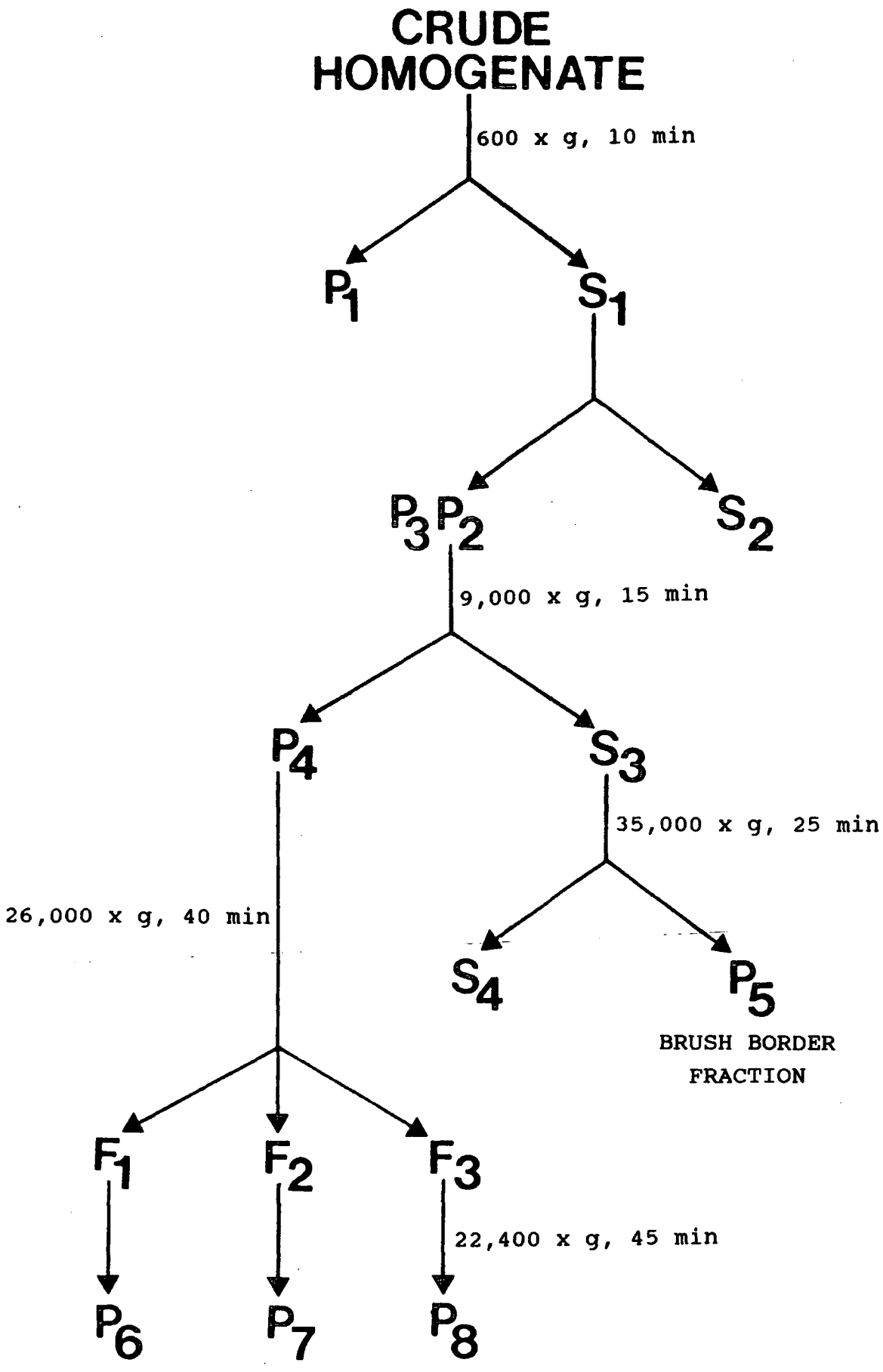

CRUDE HOMOGENATE
600 x g, 10 min
P1
S1
P3 P2
S2
9,000 x g, 15 min
P4
S3
35,000 x g, 25 min
26,000 x g, 40 min
S4
P5
BRUSH BORDER FRACTION
F1
F2
F3
22,400 x g, 45 min
P6
P7
P8
BASOLATERAL FRACTIONS

FIGURE 3.16

Comparative measurements of $(Na^+ + K^+)$-ATPase and alkaline phosphatase activity in the various membrane fractions obtained from the Malpighian tubules of *Locusta migratoria.*

Left ordinate: ATPase activity expressed as nmoles P_i liberated/mg protein/min.

Right ordinate : Alkaline phosphatase activity expressed as nmoles *p*-nitrophenol (NP) liberated/mg protein/min.

 K^+-stimulated ATPase activity.

 $(Na^+ + K^+)$-ATPase activity.

 Alkaline phosphatase activity.

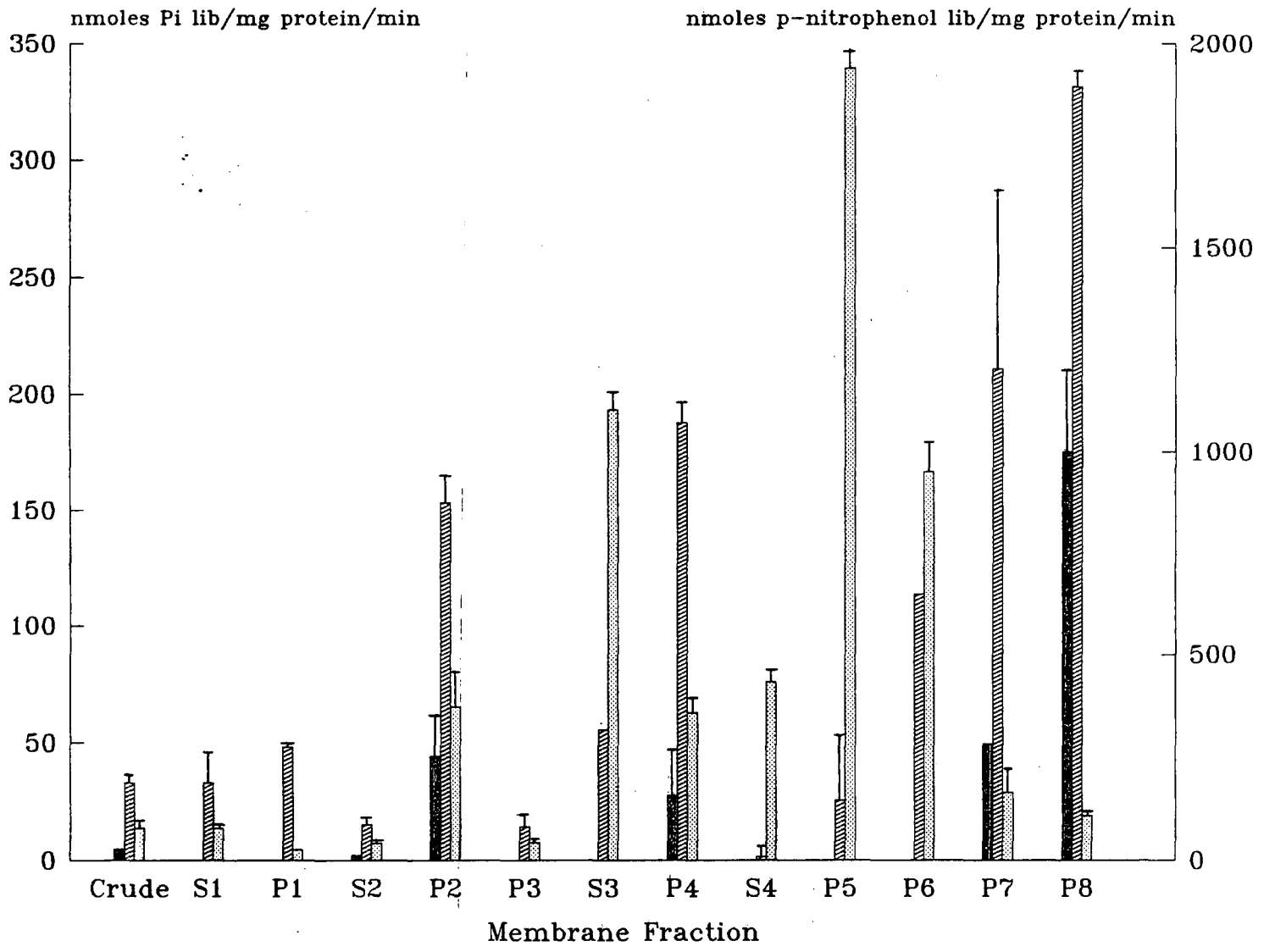

nmoles Pi lib/mg protein/min
nmoles p-nitrophenol lib/mg protein/min
350
300
250
200
150
100
50
0
2000
1500
1000
500
0
Crude
S1
P1
S2
P2
P3
S3
P4
S4
P5
P6
P7
P8
Membrane Fraction

FIGURE 3.17

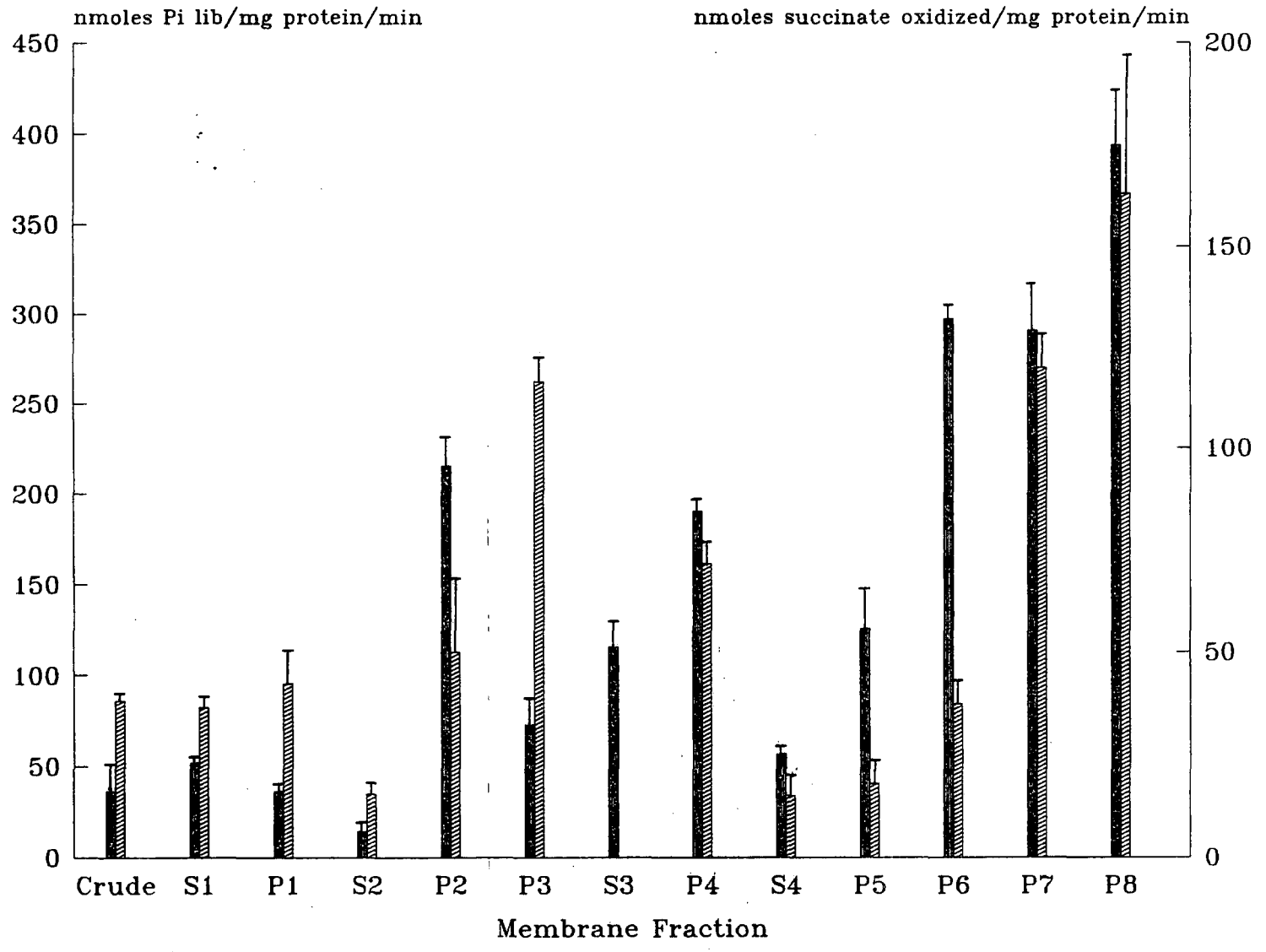
Comparative measurements of HCO_3^--stimulated ATPase and succinate dehydrogenase (SDH) activity in the various membrane fractions obtained from the Malpighian tubules of *Locusta migratoria*.

Left ordinate : HCO_3^--stimulated ATPase activity expressed as nmoles P_i liberated/mg protein/min.

Right ordinate : SDH activity expressed as nmoles succinate oxidized/mg protein/min.

 Anion-stimulated ATPase activity.

 SDH activity.


nmoles Pi lib/mg protein/min
nmoles succinate oxidized/mg protein/min
450
400
350
300
250
200
150
100
50
0
200
150
100
50
0
Crude
S1
P1
S2
P2
P3
S3
P4
S4
P5
P6
P7
P8
Membrane Fraction

K^+-stimulated ATPase activity, mentioned above, it is possible that some of this activity is masked, and therefore underestimated.

A classical Mg^{2+}-dependent (Na^++K^+)-stimulated ATPase, such as described previously in microsomal preparations of Malpighian tubules of *Locusta* (Anstee and Bell, 1975, 1978), was clearly demonstrated in most of the fractions studied (Fig. 3.16); the major specific activity being observed in pellets P_2, P_4, and P_6-P_8. In all experiments on these pellets there was an increase in Mg^{2+}-dependent ATPase activity on addition of K^+ and Na^+ to the incubation medium. This would suggest that the majority of the (Na^++K^+)-ATPase activity was precipitated in P_4 at the 9000 x g spin, with relatively low activity being observed in pellet P_5, and in S_3 from which it was derived. The distribution of alkaline phosphatase activity was in almost direct contrast to that of the (Na^++K^+)-ATPase; relatively large amounts of the former activity being seen in S_3 and P_5, but relatively small amounts in P_4, P_7 and P_8. Alkaline phosphatase is generally accepted as an apical cell membrane marker enzyme, and was certainly confined to the microvillar surface in the cytochemical localization studies described previously. Thus, the differential separation of (Na^++K^+)-ATPase and alkaline phosphatase at the 9000 x g spin indicates that the majority of the apical and basolateral cell membranes were separated into P_5 (via S_3) and P_4, respectively (Rodriguez and Edelman, 1979). The ratio of (Na^++K^+)-ATPase activity (expressed in nmoles P_i liberated/mg protein/min) to alkaline phosphatase activity (expressed in nmoles *p*-nitrophenol liberated/mg protein/min) was 1:0.8 and 1:0.3 in pellets P_7 and P_8, respectively, and 1:76.2 in pellet P_5. The presence of significant (Na^++K^+)-ATPase and alkaline phosphatase activities in P_6 suggests that this pellet contains a mixture of the two cell membrane fractions. Thus, despite some cross-contamination, the data show that the separation procedure of Rodriguez and Edelman (1979) has effected partial purification of the apical and basolateral cell membranes of locust Malpighian tubules.

Examination of data in Figure 3.17 reveals that there was relatively little mitochondrial contamination, as indicated by succinate dehydrogenase (SDH) activity, in the predominantly apical membrane fraction P_5. In contrast, the presence of significant levels of SDH activity in pellets P_7 and P_8 indicates that membranes originating from these organelles were present. This may be a consequence of the close spatial association reported between the basal cell membrane infoldings and mitochondria in the intact cell (see Plate 3.1) (Bell and Anstee, 1977), resulting in some co-separation. Any suggestion that the (Na^++K^+)-ATPase activity in P_7 and P_8 is mitochondrial in origin can be discounted on the grounds that the mitochondria-rich pellet P_3 (see Plate 3.5a) exhibited relatively low (Na^++K^+)-ATPase activity (Fig. 3.16, Appendix Table A.3); the activity ratio of (Na^++K^+)-ATPase to SDH being 1:8.2 for P_3 and 1:0.6 for both P_7 and P_8 (see Figs. 3.16 and 3.17 for units), which indicates that the enzymes were not co-separating.

Electron microscopy

Figures 3.5 a,b and 3.6a-c show a series of electron micrographs of pellets P_3, P_5 and P_6-P_8. It can be seen that, consistent with the distribution of SDH activity referred to above, P_3 consists largely of mitochondria. In contrast, P_5 consists mainly of circular membrane-bound vesicles of various sizes as do pellets P_6-P_8, although the latter pellets contain significantly more ribosomal particles indicating that they are not exclusively plasma membrane fractions.

Effect of HCO_3^- on ATPase Activity

Figure 3.17 shows the distribution of SDH and HCO_3^--stimulated ATPase activities in the various membrane and supernatant fractions. It can be seen that HCO_3^--stimulated ATPase activity was present in most of the membrane fractions derived from the Malpighian tubules of *Locusta migratoria*. Maximum mean specific ATPase and SDH activities were observed in pellet P_8. However, consideration of the HCO_3^--stimulated ATPase : SDH activity ratios for the different fractions indicates that the distribution of the HCO_3^--stimulated ATPase activity is not the same as that of the mitochondrial marker enzyme, SDH. Thus, for the mitochondria-rich fraction P_3 (Plate 3.5a), the above ratio was 1:1.6 (see Fig. 3.17 for units) whereas in pellet P_5, which is thought to be the apical cell membrane-rich fraction, and P_6, which contains both apical and basolateral membranes, a ratio of 1:0.1 was obtained. Using P_3 as a reference, it is estimated that the level of HCO_3^--stimulated ATPase activity in P_5 and P_6 which might arise from mitochondrial contamination is in the order of 9% of that observed. Similarly, the membrane fractions P_7 and P_8 show both HCO_3^-ATPase : SDH activity ratios of 1:0.4 suggesting that HCO_3^--stimulated ATPase activity in these fractions is not due to mitochondrial contamination alone; the estimated HCO_3^--stimulated ATPase activity derived from mitochondrial contamination representing about 26% of the total activity for each pellets. It appears, therefore, that HCO_3^--stimulated ATPase activity is not exclusively mitochondrial in origin and that significant levels of such activity is associated with both the apical cell membrane-rich fraction and those fractions containing the basolateral membranes.

PLATE 3.5a

Micrograph showing a section through the mitochondria (M)-rich pellet (P_3) obtained during the isolation and partial purification of the basolateral and apical cell membranes from the Malpighian tubules of *Locusta migratoria*.

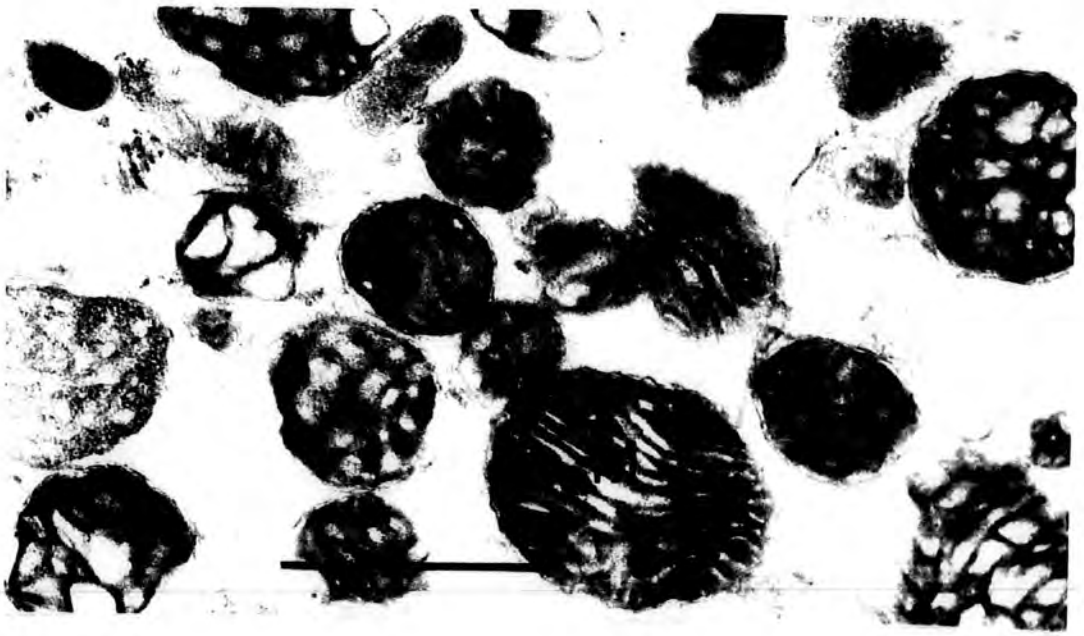
Scale: 1 μm.

PLATE 3.5b

Micrograph showing a section through pellet 5 (P_5) which, from biochemical studies, is believed to represent the apical membrane fraction.

Scale: 1 μm.

a



b

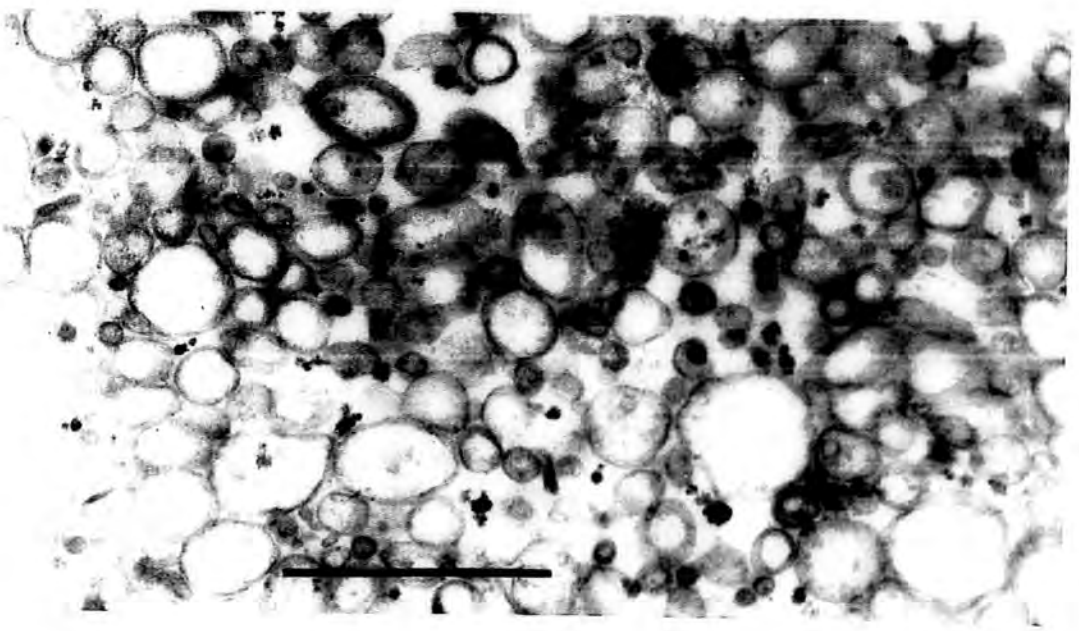


PLATE 3.6a

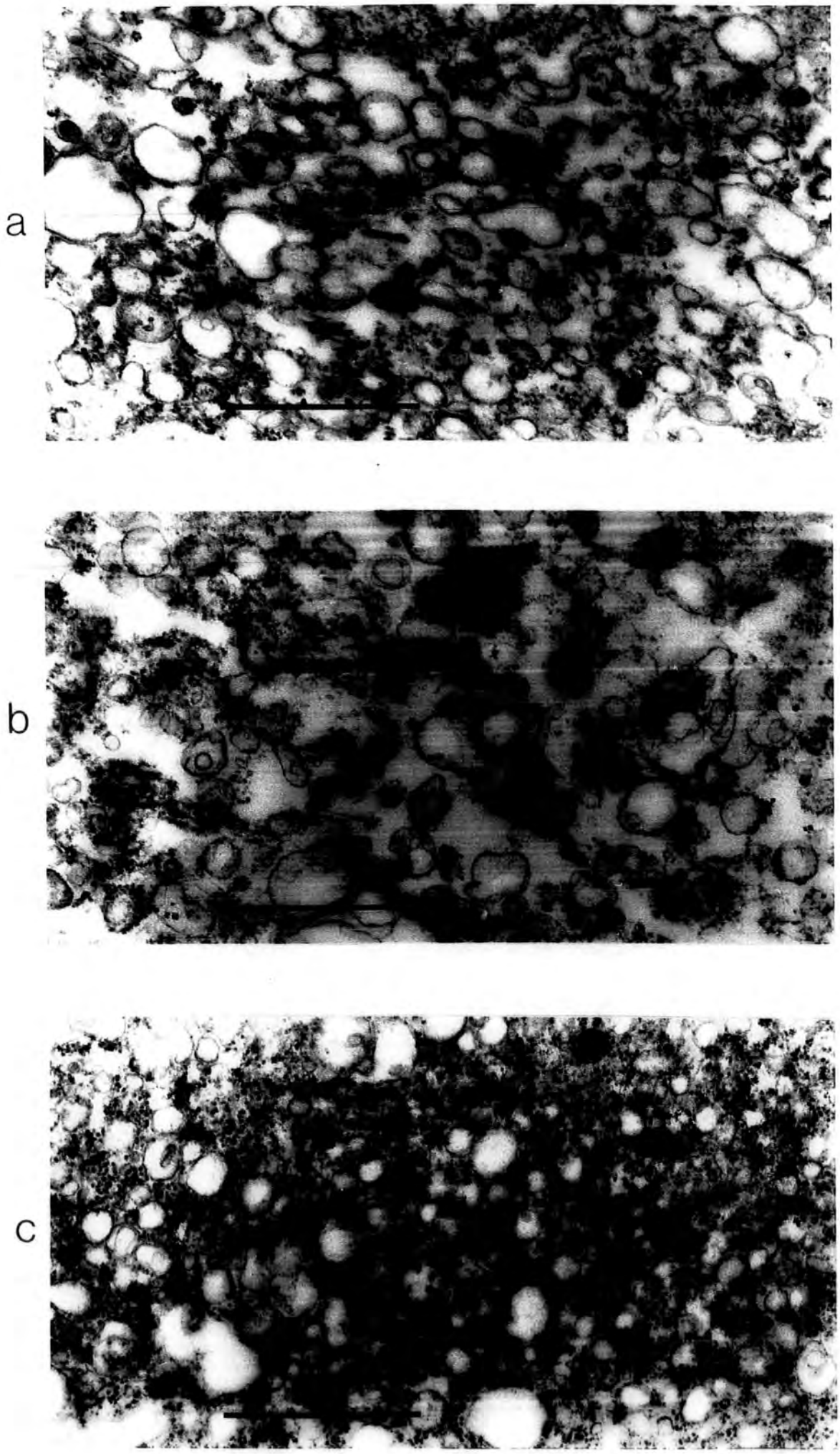
Micrograph showing a section through pellet 6 (P_6) which from biochemical studies, is believed to represent a mixture of both basolateral and apical cell membranes.

Scale: 1 μm.

PLATES 3.6b and c

Electron micrographs representing pellets 7 (P_7, PLATE 3.6b) and 8 (P_8, PLATE 3.6c) which from biochemical studies, are believed to represent the basolateral cell membrane fractions.

Scale: 1 μm.


a
b
c

DISCUSSION

Cytochemical localization and comparative biochemical measurements of K^+-NPPase in Malpighian tubules of *Locusta migratoria* L.

The cytological localization of the active transport step is important to any theory of epithelial fluid transport. As mentioned previously, (Na^++K^+)-ATPase activity has been demonstrated in microsomal preparations of *Locusta migratoria* (Anstee and Bell, 1975, 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986; present study). Demonstration of (Na^++K^+)-ATPase in the present study is based on the cytochemical localization of the K^+-dependent component of the transport ATPase complex (Ernst, 1972a,b; Schuurmans Stekhoven and Bonting, 1981). The specificity of the cytochemical reaction used in the present study was demonstrated by Ernst (1972a,b) and confirmed by subsequent studies (Firth, 1974; Mills and Ernst, 1975, 1977; Ellis and Goertemiller, 1976).

The advantages of the Ernst (1972a,b) technique over that of Wachstein and Meisel (1957) have been discussed previously (see Introduction, Chapter 1), and biochemical evidence (reviewed by Ernst, 1972a) supporting the former procedure has shown some correlation with other independent methods for localization of (Na^++K^+)-ATPase, such as ^{3}H-ouabain binding autoradiography (Stirling, 1972; Ernst and Mills, 1977, 1980) and immunocytochemistry (Kyte, 1976a,b). These methods have been compared by Komnick and Achenbach (1979) and Firth (1980).

In the present study, cytochemical localization of *p*-nitrophenyl phosphatase (NPPase) in the Malpighian tubules of *Locusta migratoria* was attempted by strontium precipitation of inorganic phosphate released at the site of *p*-nitrophenyl phosphate (NPP) hydrolysis in the K^+-dependent step (Ernst, 1972a,b). In all cases, there were inconsistent precipitate distributions; heavily stained regions being found close to unstained regions. However, results are discussed on the basis that localized distributions of precipitate reflected phosphatase activity.

K^+-dependent NPPase activity was distinguished from non-specific NPPase activity by substitution of NPP in the incubation medium with β-glycerophosphate; a substrate which is not hydrolysed by (Na^++K^+)-ATPase (Fujita *et al.*, 1966; Ernst, 1972a,b). On this basis, the complete absence of reaction product along the basal cell infoldings with

β-glycerophosphate as substrate, together with the marked reduction/absence of reaction product under K^+-free conditions with NPP as substrate, suggests that the basal cell membrane associated phosphatase activity is in part due to K^+-dependent NPPase and indicates the presence of $(Na^+ + K^+)$-ATPase activity (Ernst, 1972a,b). Similarly, Ernst (1972b) and Goertemiller and Ellis (1976) reported a great reduction in amount of precipitate along the basal cell membranes on deletion of K^+ from the incubation medium in avian salt gland and spiny dogfish rectum, respectively. In contrast, there was no significant reduction in the amount of precipitate associated with the apical microvilli suggesting that relatively little $(Na^+ + K^+)$-ATPase activity is associated with the apical cell membrane. These findings are consistent with the fact that, with the exception of frog choroid plexus (Quinton *et al.*, 1973), where $(Na^+ + K^+)$-ATPase was localized apically, localization of this enzyme has been reported to be almost exclusively associated with the cytoplasmic side of the lateral and basal cell membrane infoldings of secretory epithelia (e.g. avian salt gland, Ernst, 1972b; Ernst and Mills, 1977; teleost chloride cell, Karnaky *et al.*, 1976; alimentary tract of *Locusta migratoria*, Peacock, 1976; dragonfly larvae rectum, Komnick and Achenbach, 1979; *Nautilus* siphuncle, Greenwald *et al.*, 1984). Nevertheless, on the basis of the present cytochemical study it is not possible to discount the presence of some $(Na^+ + K^+)$-ATPase activity associated with the apical microvilli. However, the localized deposits of reaction product demonstrated in this apical region, with both substrates, suggest that the majority of this apical phosphatase activity is due to non-specific alkaline phosphatase activity.

Attempts to study the effects of ouabain on basal cell membrane K^+-dependent NPPase by electron microscopy were unsuccessful in the present study, for the reasons already given. Inconsistent inhibition with ouabain was observed in other studies (Komnick and Achenbach, 1979; Peacock, 1976; Kalule-Sabiti, 1985) although, complete inhibition of K^+-stimulated NPPase was reported with 10 mM ouabain in avian salt gland (Ernst and Mills, 1977). Ernst (1972,b) suggested that substantial amounts of K^+-dependent NPPase activity may be protected from ouabain inhibition by the presence of strontium in the incubation medium, thus requiring a higher ouabain concentration for complete K^+-NPPase inhibition, whilst Irvine and Phillips (1971) suggested that, when using intact tissues, a higher concentration of ouabain was required to overcome the comparatively long diffusion pathways. In the present study, incubations carried out at 30°C with 10 mM ouabain resulted in extremely unsatisfactory preservation of tubule fine structure, as also reported by Kalule-Sabiti (1985) and Komnick and Achenbach (1979). Thus, absence of reaction

product deposition in either the apical or basal regions of such tubule cells may be due to cellular disruption.

Whilst results in the present study, from cytochemical localization and membrane separation techniques used in conjunction with biochemical studies (see later), suggest the presence of $(Na^+ + K^+)$-ATPase on the basal cell membrane, in agreement with ^3H-ouabain binding autoradiographical studies on tubules of *Locusta migratoria* (Donkin, 1981), the possibility of an apically sited $(Na^+ + K^+)$-ATPase pump cannot be discounted. Furthermore, the partial or complete failure of control experiments and inconsistent precipitate distributions, also reported by Komnick and Achenbach (1979) and Peacock (1976), suggest that the Ernst (1972b) technique may not be a reliable method for the localization of $(Na^+ + K^+)$-ATPase in the Malpighian tubules of *Locusta migratoria*, or some other epithelia.

Biochemical experiments carried out in parallel to the cytochemical localization study, described previously, also call into question the validity of the Ernst (1972b) procedure for the localization of $(Na^+ + K^+)$-ATPase in this epithelium. It has been suggested that paraformaldehyde fixation preserves a large proportion of the K^+-activated NPPase activity under histochemical conditions (avian salt gland, Ernst, 1972a), whilst Komnick and Achenbach (1979) found fixation and strontium completely inhibited this activity. In the present study, fixation of the Malpighian tubules of *Locusta migratoria* had no significant effect on either K^+-stimulated, ouabain-sensitive or non-specific NPPase activity under biochemical or histochemical conditions at either pH 7.2 or pH 9.0. In contrast, strontium and the alkaline pH were found to inhibit K^+-stimulated, ouabain-sensitive NPPase activity in both fixed and unfixed tubule homogenates.

As mentioned previously (Chapter 1), Ernst (1972a) reported that the pH optimum for the enzyme in the absence of Sr^{2+} extended over a broad range (pH 8.0-9.0), with maximal activity at pH 8.5. However, on addition of 20 mM Sr^{2+} to the incubation medium, a shift of the optimum to pH 7.5 was observed. This would explain the present observation that the K^+-stimulated and ouabain-sensitive NPPase activities decreased on raising the pH of the histochemical incubation medium to pH 9.0, but not the finding that, in the absence of Sr^{2+}, such a change in pH did not result in increased K^+-stimulated and ouabain-sensitive NPPase activities. Indeed, the ouabain-sensitive component was significantly inhibited, in direct contrast to the findings of Ernst (1972a). Unfortunately, an alkaline pH, is prerequisite for the cytochemical localization of phosphatase activity, strontium phosphate being soluble at neutral pH (Ernst, 1972b). Consequently, at pH 9.0, there is likely to be approximately 43% or greater inhibition of total phosphatase activity in fixed material under histochemical conditions. Furthermore, whilst Ernst (1972a) reported that two-thirds of the total NPPase

activity in the presence of strontium was due to K^+-dependent NPPase activity in fixed avian salt gland at pH 9.0, in the present study, no significant K^+-dependent, ouabain-sensitive NPPase activity was demonstrated under these conditions in fixed tubule homogenates.

The Ernst (1972b) procedure, therefore, does not appear to allow consistent positive demonstration of K^+-activated, ouabain-sensitive NPPase activity in the Malpighian tubules of *Locusta migratoria*. This technique is probably only suitable where a tissue is rich in (Na^++K^+)-ATPase and where sufficient K^+-activated NPPase activity remains uninhibited after the histochemical procedure. However, it may be possible that, due to the structure of the intact locust Malpighian tubule, incubatory conditions may be conducive for some localized K^+-stimulated, ouabain-sensitive NPPase activity to remain uninhibited. Alternative methods, such as ^3H-ouabain binding autoradiography (Stirling, 1972; Komnick and Achenbach, 1979; Ernst and Mills, 1980; Donkin, 1981), immunoferritin localization of (Na^++K^+)-ATPase (Kyte, 1976a,b), and use of the modified Ernst technique of Guth and Albers (1974) (see Chapter 1) may give more promising results, although it is unlikely that the second technique would be used on insect tissue due to the difficulty in obtaining sufficient material for enzyme purification and subsequent antibody production (Anstee and Bowler, 1984).

Biochemical studies on membrane fractions from the Malphighian tubules of *Locusta migratoria*: An attempt to localize the Mg^{2+}-dependent, (Na^++K^+)- and HCO_3^--stimulated ATPases

The results presented previously confirm earlier reports of the presence of (Na^++K^+)-ATPase (Anstee and Bell, 1975, 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986; present study) and HCO_3^--stimulated ATPase (Anstee and Fathpour, 1979,1981) activities in microsomal preparations of *Locusta migratoria*. In addition, evidence was obtained for a ouabain-insensitive K^+-stimulated ATPase activity in this tissue. Electron microscopy and/or assays for alkaline phosphatase and SDH activities were used to identify the presence of apical cell membranes and mitochondrial membranes in various cell fractions. Differential separation of the apical cell membrane marker enzyme, alkaline phosphatase, and (Na^++K^+)-ATPase was observed at the 9000 x g spin and subsequent pellets, P_7 and P_8, contained relatively high specific (Na^++K^+)-ATPase activity, with little apical membrane contamination, reflected by the relatively low alkaline phosphatase activities. The mean specific alkaline phosphatase activity was greatest in pellet P_5, which

in turn exhibited relatively low (Na^++K^+)-ATPase activity. On this basis, it is clear that little, if any, (Na^++K^+)-ATPase activity is associated with the apical cell membranes of the Malpighian tubules of *Locusta migratoria*, although its total absence cannot be confirmed. The localization of (Na^++K^+)-ATPase activity in the basolateral membrane-rich fractions agrees well with the original membrane separation work of Rodriguez and Edelman (1979) on toad bladder epithelium, and with reports that the ouabain-sensitive Na^+ pump is restricted to this cell surface in a variety of different tissues from various animal species (Ernst *et al.*, 1980; Anstee and Bowler, 1984). Recently, Lechleitner and Phillips (1988) also provided biochemical and electron microscopic evidence for the concentration of (Na^++K^+)-ATPase in the basolateral membranes of locust rectal pad epithelium.

The current membrane separation technique also revealed a K^+-stimulated ATPase activity associated with the basolateral, but not the apical cell membrane-rich fractions. This enzyme is not stimulated by Na^+ or HCO_3^-, nor is it inhibited by ouabain or SCN^- (for review see Schuurmans Steckhoven and Bonting, 1981). From studies using Rb^+, it appears that the enzyme is responsible for the inward transport of H^+ in exchange for the outward transport of K^+ in the ratio of $4H^+$:$3.5Rb^+$ (Schuurmans Steckhoven and Bonting, 1981). As mentioned previously, K^+-stimulated ATPase activity has been demonstrated in larval midgut of *Manduca sexta* (Wolfersberger, 1979; Wolfersberger *et al.*, 1982; Deaton, 1984; Moffett and Koch, 1988a,b), and tentatively suggested in larval midgut of *Hyalophera cecropia* (Zerahn, 1985), the labellum of *Protophormia terraenovae* (Wieczorek, 1982; Wieczorek and Gnatzy, 1985; Wieczorek *et al.*, 1986) and was thought to be a candidate for the electrogenic K^+ pump. However, the absence of K^+-stimulated ATPase activity in the apical membrane-rich fraction would seem to argue that this enzyme is not a candidate for the apical cation pump in the Malpighian tubules of *Locusta migratoria*. Furthermore, given that the (Na^++K^+)-ATPase is a plasma membrane enzyme, and that the distribution of K^+-stimulated ATPase activity relative to (Na^++K^+)-ATPase activity is variable, one must question whether the K^+-stimulated ATPase is, in fact, plasma membrane in origin. The possibility that it is lysosomal in origin or associated with other cellular organelles cannot be discounted and the subcellular localization of this activity and its likely significance must await the outcome of further studies.

It would appear that considerable non-mitochondrial HCO_3^--stimulated ATPase activity is associated with both the apical and basolateral cell membrane-rich fractions of locust Malpighian tubules. As mentioned earlier (Chapter 1), previous studies have also revealed anion-stimulated ATPase activity in membrane fractions from the recta of

Schistocerca gregaria (Herrera *et al.*, 1978) and larval dragonfly (Komnick *et al.*, 1980), the midgut and integument of *Manduca sexta* (Deaton, 1984), the midgut of *Hyalophora cecropia* (Turbeck *et al.*, 1968), and in various other animal tissues (reviewed by Gerenscer and Lee, 1983). Linkage of such activity to active anion transport has been proposed (Komnick *et al.*, 1980; Bornancin *et al.*, 1980; Gerenscer and Lee, 1983, 1985). One problem encountered in determining the subcellular site of anion-stimulated ATPase activity has been the existence of a mitochondrial anion-stimulated ATPase and mitochondrial contamination of plasma membrane fractions. In the present study, the distribution of anion-stimulated ATPase activity was not equivalent to that of the mitochondrial marker enzyme, SDH. This may be taken as support for the suggestion that such activity is not exclusively mitochondrial in origin. However, the possibility that the differential separation of SDH and anion-stimulated ATPase activities may be a consequence of changes in substrate access to one or other enzyme or the presence of some inhibitory factor, cannot be excluded at this stage (Anstee and Fathpour, 1981).

Mg^{2+}-dependent HCO_3^--stimulated ATPase activity, which could be inhibited by SCN^- (Turbeck *et al.*, 1968; Wiebelhaus *et al.*, 1971; De Renzis and Bornancin, 1977; Van Amelsvoort, 1977), but not ouabain, was first discovered in microsomal preparations from frog gastric mucosa (Kasbekar *et al.*, 1965), and has since been reported in a variety of different tissues from different species (Blum *et al.*, 1971; Wiebelhaus *et al.*, 1971; De Pont *et al.*, 1972; Sachs *et al.*, 1972a; Izutsu and Siegel, 1975; Suzuki, 1978). The enzyme has been implicated in Cl^- (Hanrahan and Phillips, 1983; Lechleitner and Phillips, 1988) and HCO_3^- transport (Simon *et al.*, 1972a), Cl^-/HCO_3^- exchange (Herrera *et al.*, 1978)), H^+/HCO_3^- transport (Simon *et al.*, 1972a), Na^+/H^+ exchange (Kinne-Saffren and Kinne, 1974; Liang and Sacktor, 1976). However, the role of the Mg^{2+}-dependent anion-stimulated ATPase in ion transport is unclear.

Hanrahan and Phillips (1983) have suggested that active Cl^- transport is a primary transport process in locust rectum, and possibly involves an apical anion-stimulated ATPase. Indeed, Lechleitner and Phillips (1988) recently provided some evidence for an apical plasma membrane anion-stimulated ATPase, which may be responsible for active Cl^- transport in this tissue. These workers found such ATPase activity to be stimulated to the greatest extent by sulphite, followed by HCO_3^- and finally Cl^-, and strongly inhibited by thiocyanate. Similarly, Anstee and Fathpour (1981) also found the enzyme in the Malpighian tubules of *Locusta migratoria* to be stimulated to the greatest extent by sulphite, followed by selenite, borate, bicarbonate and bromide (see also, Blum *et al.*, 1971; Sachs *et*

al., 1972b; Simon *et al.*, 1972a,b; Ebel and Lardy, 1975; Liang and Sacktor, 1976). However, these workers found Cl^- to have no significant effect on enzyme activity, and nitrite inhibited ATP hydrolysis. The lack of specificity of the ATPase for stimulation or inhibition by different anions led to the more appropriate description of anion-sensitive ATPase (Van Amelsvoort *et al.*, 1977).

It would appear that the Malpighian tubules of *Locusta migratoria* possess a HCO_3^--stimulated ATPase in both the basolateral and apical cell membrane-rich fractions, with a higher specific activity associated with the former. Cl^-/HCO_3^- exchange at the basal surface may be responsible for Cl^- entry into the cell, with the $(Na^+ + K^+)$-ATPase responsible for K^+ entry. Parallel transport in the form of $(Na^+ + K^+)$-ATPase activity and Cl^-/HCO_3^- exchange at the basal surface would result in the transport of K^+ and Cl^- into the cell and is potentially more adaptable than the K^+-Cl^- cotransporter, with K^+ and Cl^- being transported independently, aided by intracellular pH. Cl^-/HCO_3^- exchange at the apical surface is unlikely as Cl^- exit is probably passive down its favourable electrical gradient.

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

Previous findings suggest that an increase in intracellular cAMP is responsible for the stimulation of an apical cation pump in CC extract-stimulated Malpighian tubules of *Locusta migratoria*. If this pump is an anion-stimulated ATPase, the hyperpolarization of V_A observed with dibutyryl cAMP would be abolished in the presence of SCN^-. In the present study, a hyperpolarization of V_A was observed in all three experiments after 5 min under these conditions, compared to control saline alone. This would suggest that cation extrusion across the apical cell membrane continues in the presence of SCN^- and that the pump responsible is not the anion-stimulated ATPase. However, the extent of the hyperpolarization was variable (4-19 mV), and so the change in potential (mean value *ca.* 10.8 mV) was not significant. Nevertheless, in view of the failure to demonstrate K^+-stimulated ATPase activity in association with the apical membrane fraction in the present study, it would appear that the anion-stimulated ATPase is a possible candidate for the

apical cation pump in the Malpighian tubules of *Locusta migratoria.* Further electrophysiological experiments which involve ion substitution and the use of SCN^- on CC extract-stimulated tubules may provide further information as to the role of anion-stimulated ATPase in ion transport and fluid secretion by this insect.

The roles of the various enzymes implicated in the functioning of the Malpighian tubules of *Locusta migratoria* are discussed further in Chapter 4.

CHAPTER 4

GENERAL DISCUSSION

The information obtained in the present study (Chapter 3) was used to construct a hypothetical model to describe the ion movements which support fluid secretion by the Malpighian tubules of *Locusta migratoria* and their endocrine control. The model (Fig. 4.1) is based mainly on the electrophysiological and biochemical evidence obtained. The merits of the model are discussed.

As mentioned earlier, $(Na^+ + K^+)$-ATPase activity has been reported in this tissue (Anstee and Bell, 1975, 1978; Donkin and Anstee, 1980; Anstee *et al.*, 1986) and confirmed in the present study. The association of this enzyme with the basal cell membrane of the Malpighian tubules of *Locusta migratoria* was inferred by cytochemical localization and membrane separation techniques in conjunction with biochemical studies and electron microscopy. Indeed, a basolateral location for this enzyme has been indicated in the majority of tissues studied (Ernst, 1972b; Karnaky *et al.*, 1976; Peacock, 1976). Ouabain-binding studies on microsomal preparations from this tissue showed that pump-site density and turnover activity of the $(Na^+ + K^+)$-ATPase is adequate to account for substantial K^+ transport in these tubules (Anstee *et al.*, 1986). Thus, it is proposed that a $(Na^+ + K^+)$-ATPase associated with the basal cell membrane (Fig. 4.1) is responsible for the transport of K^+ into the cell in exchange for Na^+, thereby maintaining the Na^+ gradient. Such a role for $(Na^+ + K^+)$-ATPase has previously been proposed in the Malpighian tubules of *Locusta migratoria* (Baldrick *et al.*, 1988), *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) and the salivary glands of *Calliphora erythrocephala* (Berridge, 1980).

The presence of a large negative apical membrane potential (*ca.* 75 mV) indicates a favourable electrical gradient for Cl^- exit, but an adverse electrical gradient for K^+ and Na^+ exit across this membrane. It would appear, therefore, that some form of apical pump is required for active transport of such cations into the lumen. Hence, an apical electrogenic K^+ pump is proposed (Fig. 4.1) as suggested elsewhere (Maddrell, 1977; Berridge, 1980; Morgan and Mordue, 1983; O'Donnell and Maddrell, 1984; Baldrick *et al.*, 1988). The pump is thought to be relatively unspecific, transporting K^+ and Na^+ out of cells (Maddrell, 1977; O'Donnell and Maddrell, 1984). However, in *Locusta migratoria* (Anstee and Bell, 1975), and the majority of insect species studied (Maddrell, 1977), K^+ is the main ion transported by this pump, forming the basis of the K^+-rich urine, with Cl^- following passively, as proposed for *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) and *Locusta migratoria* (Morgan and Mordue, 1983; Baldrick *et al.*, 1988). However, in the present

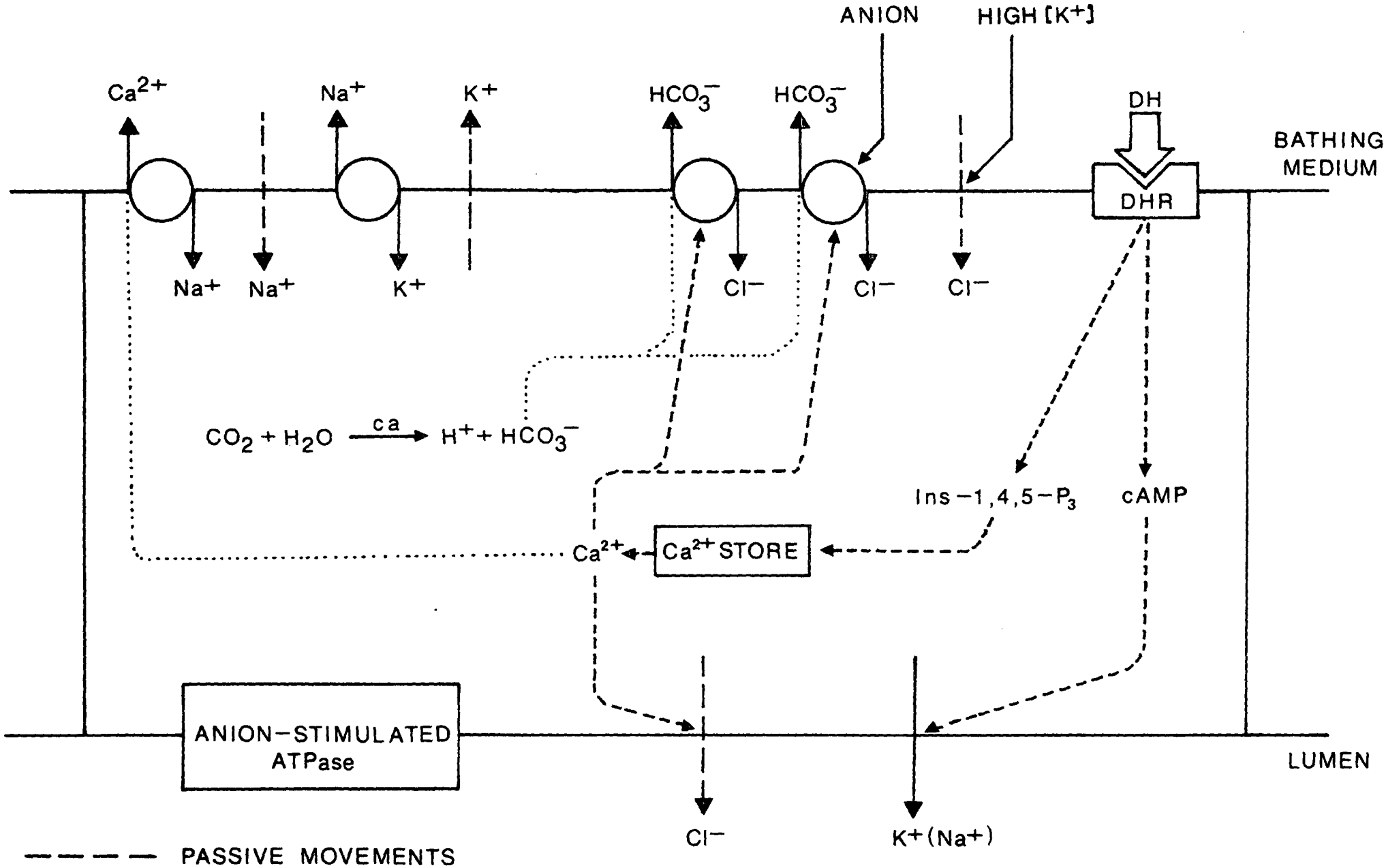
FIGURE 4.1

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

In this model, it is proposed that a basal (Na^++K^+)-ATPase maintains the cellular gradients. Intracellular Ca^{2+} may be controlled by the action of a basal Na^+/Ca^{2+} exchanger. It is proposed that passive exit of K^+, with its concentration gradient, and passive entry of Na^+, with its concentration and electrical gradient, occur across the basal membrane. It would appear that passive Cl^- entry, with its concentration and electrical gradients, only occurs on elevation of external [K^+]. K^+ entry across the basal membrane seems to be by the action of the (Na^++K^+)-ATPase. Cl^- entry may be by Cl^-/HCO_3^- exchange and by the action of a (Cl^-+ HCO_3^-)-ATPase, both aided by increases in intracellular pH.

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

c.a. - carbonic anhydrase.


ANION
HIGH [K+]
Ca2+
Na+
K+
HCO3−
HCO3−
DH
DHR
BATHING MEDIUM
Na+
Na+
K+
Cl−
Cl−
Cl−
CO2 + H2O
ca
H+ + HCO3−
Ins−1,4,5−P3
cAMP
Ca2+
Ca2+ STORE
ANION−STIMULATED ATPase
LUMEN
Cl−
K+(Na+)
PASSIVE MOVEMENTS
ACTIVE MOVEMENTS

study, there was no evidence for this pump being the K^+-stimulated ATPase observed in other insect tissues (Wolfersberger *et al*, 1982; Deaton, 1984; Zeiske *et al*, 1986; Wieczorek *et al.*, 1986).

Ion substitution experiments indicate that the basal membrane of the Malpighian tubules of *Locusta migratoria* is largely permeable to K^+, but relatively impermeable to both Na^+ and Cl^- (Baldrick *et al.*, 1988; present study). Morgan and Mordue (1983) suggested that Cl^- entry may be Na^+ and/or K^+ dependent. O'Donnell and Maddrell (1984) also reported the basal cell membrane of the tubules of *Rhodnius prolixus* to be largely impermeable to Na^+ and Cl^- and suggested that these ions may enter tubule cells via a Na^+-K^+-$2Cl^-$ cotransporter. Recently, Baldrick *et al.* (1988) tentatively suggested that Cl^- entry into tubule cells of *Locusta migratoria* may be via such a cotransporter, but that this was not necessarily dependent on Na^+. Cotransport with K^+ under Na^+-free conditions was not discounted. Indeed, O'Donnell and Maddrell (1984) suggested that the Na^+-K^+-$2Cl^-$ cotransporter will accept other stoichiometries, such as $2K^+$:$2Cl^-$ or $2Na^+$:$2Cl^-$, in the absence of either cation. However, there is no firm evidence for the presence of Na^+-K^+-$2Cl^-$ cotransport in tubules of *Locusta migratoria* on the basis that the effects of furosemide and bumetanide on this tissue (Baldrick *et al.*, 1988) were not as pronounced as those on tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984).

In the present study, net transepithelial flux of Na^+ from bathing medium to lumen was unaffected by the absence of Cl^- from the bathing medium, whilst net Cl^- flux was increased by *ca.* 95% under high K^+, Na^+-free conditions and reduced by *ca.* 50% on treatment with 1 mM furosemide. These results suggest that Cl^- entry into the cell may be dependent on $[K^+]_o$ and confirm that it is not dependent on Na^+. K^+-Cl^- cotransport is reported to be relatively insensitive to loop diuretics, such as furosemide and bumetanide, compared with the Na^+-K^+-$2Cl^-$ cotransport system (for review see O'Grady *et al.*, 1987). The lack of similarity between the electrophysiological responses of tubules from *Locusta migratoria* (Baldrick *et al.*, 1988) and *Rhodnius prolixus* (O'Donnell and Maddrell, 1984) to furosemide and bumetanide could therefore be explained on the basis of K^+-Cl^- cotransport in the former insect. However, for tubules bathed in control saline, the electrochemical gradients for both K^+ and Cl^- will be unfavourable for K^+-Cl^- co-entry by such a transport process. Therefore, Cl^- entry into the Malpighian tubules of *Locusta migratoria* would appear to be by some other mechanism(s), which may be furosemide-sensitive. Evidence for electroneutral Cl^- entry comes form the observation that V_B does not change on the removal of Cl^- from control bathing medium, whereas V_A shows a significant hyperpolarization consistent with continued cation extrusion across the apical membrane in the absence of Cl^- as counter-anion. Some Cl^- entry may occur in exchange for HCO_3^-, as proposed for the rectal gland of *Aedes dorsalis* (Strange and Phillips, 1984), or by the

activity of a (Cl^-+HCO_3^-)-ATPase, as proposed for the intestinal epithelium of *Aplysia* (Gerenscer and Lee, 1985). Previous ion substitution experiments using Cl^--free saline and HCO_3^--free saline containing acetazolamide gave similar electrophysiological results, suggesting that Cl^- and HCO_3^- transport were related (Baldrick, 1987). Furthermore, furosemide has been reported as being non-specific in its inhibition of transport processes; Cl^-/HCO_3^- exchange being inhibited with a $K_{0.5}$ of 2 x 10^{-4} M in human erythrocytes (Palfrey *et al.*, 1980; Chipperfield, 1986). In addition, HCO_3^--stimulated ATPase activity, associated with the basal cell membrane, was demonstrated in the present study. Such anion-stimulated ATPase activity, previously demonstrated in microsomal preparations of this tissue (Anstee and Fathpour, 1979, 1981; Fathpour and Anstee, 1981), was also found to be associated with the apical membrane. This enzyme has previously been implicated in Cl^-/HCO_3^- exchange (Herrera *et al.*, 1978), H^+/HCO_3^- transport (Simon *et al.*, 1972a), HCO_3^- transport and Na^+/H^+ exchange (Liang and Sacktor, 1976), and active Cl^- transport (Lechleitner and Phillips, 1988). Thus it is proposed that Cl^- entry into the Malpighian tubules of *Locusta migratoria* is by Cl^-/HCO_3^- exchange and the activity of a (Cl^-+HCO_3^-)-ATPase. It is proposed that both these Cl^- entry mechanisms are assisted by the increase in intracellular pH (Fig. 4.1). The presence of two Cl^- entry mechanisms in the basal membrane could explain the failure of SITS (Baldrick, 1987) or NaSCN (present study) to have any major effect on V_B or V_A, with one Cl^- entry mechanism compensating for the inhibition of the other.

Yet another mechanism for Cl^- entry across the basal membrane of the Malpighian tubules of *Locusta migratoria* cannot be discounted. K^+-stimulated Cl^- movement has been proposed by Baldrick *et al.* (1988) and observed in the present study, as mentioned previously, whilst K^+-stimulated electrogenic Cl^- transport has been reported in locust rectum (Hanrahan and Phillips, 1983). Indirect evidence for Cl^- entry via a K^+-dependent mechanism in the present study comes from the observations that the depolarization of V_B in high K^+, Na^+-free saline was increased in the absence of Cl^-, whilst no change in V_B was observed on removal of Cl^- from control saline. In addition, net Cl^- flux into the lumen was increased under the former conditions, as mentioned earlier. It has been suggested that the depolarization of V_A observed in high K^+, Na^+-free saline may be a consequence of increased availability of Cl^- partly shunting the apical electrogenic cation pump (Baldrick *et al.*, 1988), and this was reduced in the absence of Cl^- (Baldrick *et al.*, 1988; present study). It is possible that elevation of $[K^+]_o$ results in increased basal membrane permeability to Cl^- with passive influx of Cl^- down its now favourable electrochemical gradient. Thus, it is proposed that under conditions of high $[K^+]_o$, passive Cl^- entry is increased (Fig. 4.1).

In view of the failure to demonstrate K^+-stimulated ATPase activity associated with the apical membrane in the present study, the anion-stimulated ATPase would appear to be a

possible candidate for the cation pump. Electrogenic exchange would be required. Treatment of tubules with NaSCN resulted in little change in V_B, as expected on inhibition of an electroneutral (Cl^-/HCO_3^-)-ATPase. However, a transient depolarization of V_A was observed, followed by a repolarization. The effect on V_A is difficult to explain on the basis of reduced Cl^- entry and inhibition of an apical cation pump. In view of the lack of supporting evidence as to the nature of anion-stimulated ATPase in the Malpighian tubules of *Locusta migratoria*, its role in ion transport across this epithelium remains unclear at this time. The possibility that these enzymes are simply involved in the regulation of intracellular pH cannot be discounted.

Unlike the model explaining ion transport in the Malpighian tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984; Maddrell and Overton, 1988), the lack of firm evidence for linked NaCl entry, mentioned previously, makes it necessary to explain how Na^+ enters the tubule cells of *Locusta migratoria*. Although the basal cell membrane is relatively impermeable to this cation, it will probably leak into the cell down its considerable electrochemical gradient (Fig. 4.1). Some Na^+ may also enter through exchange with Ca^{2+} (Fig. 4.1), as suggested by Baldrick (1987). Such a Na^+-Ca^{2+} exchange mechanism will also serve to regulate intracellular levels of Ca^{2+}.

The question now arises as to how the basal and apical membrane permeabilities might be coupled in the Malpighian tubules of *Locusta migratoria*. A number of workers (Prince and Berridge, 1972; Berridge and Prince, 1972; Berridge *et al.*, 1975a,b; Maddrell, 1980) have proposed models for the endocrine control of ion translocation by epithelial cells in insects in which cAMP acts as an intracellular second messenger. It has been shown in *Rhodnius prolixus* that there is an increase in intracellular levels of cAMP following treatment with that insect's DH (Aston, 1975). Similarly, in *Aedes aegypti*, mosquito natriuretic factors increase intracellular levels of cAMP *in vitro* and *in vivo* (Petzel *et al.*, 1987). Indeed, the application of a crude corpus cardiacum (CC) preparation with diuretic hormone activity to the Malpighian tubules of *Locusta migratoria* resulted in a significant increase in intracellular levels of both cAMP and Inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) (present study; see Fogg *et al.*, 1990). The latter is an important second messenger involved in the mobilization of Ca^{2+} from intracellular stores (Berridge and Irvine, 1984; Abdel-Latif, 1986; Berridge, 1987). Ca^{2+} and cAMP are well known intracellular second messengers which mediate the effects of hormones between the basolateral and apical surfaces of cells (Berridge, 1980).

In the present study, the introduction of CC extract into the bathing medium surrounding the tubules resulted a small hyperpolarization of V_B of about 6.6 mV after about 5 min. Such a small response is perhaps surprising in view of the level of stimulation

of tubular fluid secretion (an increase of about 150%) that this extract promotes. Similar results were reported by Morgan and Mordue (1983) for *Locusta migratoria*, and Nicolson and Isaacson (1987) for *Onymacris plana*. The results obtained in this study indicate that the major effect of CC extract on potentials across the tubule cells of *Locusta migratoria* is a depolarization of V_A. Such an effect of DH on tubules of *Onymacris plana* was deduced by measurements of TEP and V_B (Nicolson and Isaacson, 1987). Similarly, O'Donnell and Maddrell (1984) showed that, in *Rhodnius prolixus*, the potential changes due to the action of 5-HT represented events at the apical membrane. In the present study, the application of dibutyryl cAMP in control saline effected a small hyperpolarization of V_B similar to that seen with CC extract, whereas the effect on V_A was different with the two treatments. Thus, V_A depolarized with CC extract, but hyperpolarized with dibutyryl cAMP. The suggestion that this hyperpolarization was a consequence of increased intracellular cAMP was supported by the similar response of V_A to treatment with the phosphodiesterase inhibitor IBMX. The difference in response by V_A to CC extracts and dibutyryl cAMP in the present study implies that cAMP alone cannot mediate the full effects of DH in Malpighian tubules of *Locusta migratoria*.

In salivary glands of *Calliphora erythrocephala*, cAMP is thought to stimulate an apical cation pump, whereas Ca^{2+} increases anion conductance across both the basal and apical cell membranes (Berridge and Prince, 1972; Berridge *et al.*, 1975a,b; Berridge, 1980), whilst in *Rhodnius prolixus* it is thought to increase apical Cl^- conductance (Phillips, 1982). As mentioned previously, treatment with dibutyryl cAMP and the phosphodiesterase inhibitor IBMX result in a hyperpolarization of V_A in control saline, which is consistent with cAMP-stimulation of an apical cation pump. Furthermore, the observation of essentially the same effects of cAMP and CC extract in Cl^--free media, viz. a hyperpolarization of V_A, is consistent with cAMP- and CC extract-stimulation of an apical electrogenic cation pump in the absence of Cl^- as counter-anion. A similar explanation has been suggested for the effect of 5-HT on V_A in tubules of *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). It has previously been proposed that the depolarization of V_A following superfusion with high K^+ salines is dependent on K^+-dependent Cl^- entry across the basal membrane (Baldrick *et al.*, 1988; present study). The observation that CC extract applied in high K^+, Na^+-free saline effected an enhanced depolarization of V_A, together with its effect in Cl^--free saline, supports the hypothesis that some component of the CC extract is modifying Cl^- movement across both the basal and apical cell membranes. Indeed, net transepithelial flux of Cl^- from bathing medium to lumen is significantly increased by CC extract. Thus, in the presence of Cl^-, the depolarization of V_A effected by CC extract is explained, in part, on the basis of increased anion movements with consequent short-circuiting of the apical cation pump. If this is so, then it implies that such increased

anion conductance must be independent of cAMP. The lack of any major effect of CC extract applied in control saline on V_B would suggest that increased Cl^- entry is by stimulation of some electroneutral transport process, possibly the Cl^-/HCO_3^- exchanger and/or the anion-stimulated ATPase (Fig. 4.1), and not an increase in basal membrane permeability to this anion. Indeed, the latter would favour Cl^- efflux.

As mentioned previously, CC extract effects an increase in Na^+ flux from bathing medium to lumen. It is unlikely that the extract modifies basal membrane permeability to Na^+ on the grounds that V_B remains unchanged with such treatment. Na^+ influx in the form of CC extract-stimulated Na^+/Ca^{2+} exchange cannot be discounted with the demonstration of increased intracellular levels of the Ca^{2+} mobilizing second messenger, Ins-1,4,5-P_3. Thus, it is proposed (Fig. 4.1) that DH acts by promoting Cl^- and Na^+ entry across the basal membrane by stimulating Cl^-/HCO_3^- and Na^+/Ca^{2+} exchange, respectively, and K^+ entry by increasing the activity of the basal (Na^++K^+)-pump. Meanwhile, at the apical surface, the hormone promotes active $K^+(Na^+)$ transport into the lumen, with Cl^- following passively down its favourable electrochemical gradient.

Hence, it is proposed that DH action on the Malpighian tubule cells of *Locusta migratoria* (Fig. 4.1) is mediated by the intracellular second messenger cAMP and Ca^{2+} (mobilized by Ins-1,4,5-P_3). cAMP acts by stimulating the apical cation pump, whilst Ca^{2+} effects an increase in Cl^- conductance across both the basal and apical cell membranes. As mentioned in Chapter 1, receptor stimulation, which triggers the hydrolytic cleavage of membrane phosphoinositol 4,5-bisphosphate by phospholipase C, yields two second messenger molecules, Ins-1,4,5-P_3 and *sn*-1,2-diacylglycerol (DAG) (Berridge, 1987). The latter is reported as an activator of Ca^{2+}-dependent protein kinase C (Nishizuka, 1984), and once activated, this enzyme proceeds to phosphorylate specific proteins which may contribute to the final response (Berridge, 1984, 1986, 1987). It is suggested that future experimentation on Malpighian tubules of *Locusta migratoria* should involve measurement of DAG levels in response to CC extract stimulation. In addition, the role of protein kinase C in ion transport by these cells might be studied by application of phorbol esters; known activators of protein kinase C.

In conclusion, the results obtained in the present study, and represented in the hypothetical model (Fig. 4.1), indicate that both cAMP and Ca^{2+} play a central role in controlling ion movement across the basal and apical cell membranes of the Malpighian tubule cells of *Locusta migratoria* in response to DH.

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APPENDIX

TABLE A.1 Comparison of Transepithelial (TEP) and Transcellular Potential Difference (TCP= V_B-V_A), Luminal Values with Respect to the Bathing Media

Saline	TEP (mV)	TCP (mV)	*t*	*P*	*n*
Control	+5.5 ± 1.3	+5.7 ± 1.4	0.556	ns	73
Na^+-free	+9.9 ± 6.2	+10.2 ± 6.0	1.105	ns	7
Cl^--free	+21.2 ± 3.3	+20.7 ± 3.7	0.446	ns	6
High K^+/Na^+-free	+45.5 ± 4.5	+47.8 ± 4.6	7.409	< 0.001	8
High K^+/Na^+, Cl^--free	+62.2 ± 7.3	+62.0 ± 7.7	0.480	ns	4
Ca^{2+}-free	+1.6 ± 2.5	+1.3 ± 2.6	1.287	ns	7

TABLE A.2 Comparison of Transepithelial Potential Measurements Between Reference Electrodes (TEP_1) and by Direct Penetration of the Tubule Lumen (TEP_2), Luminal Values with Respect to the Bathing Media

Saline	TEP_1	TEP_2	*t*	*P*	*n*
Control	+11.9 ± 3.0	+11.8 ± 3.0	0.390	ns	12
High K^+/Na^+-free	+13.6 ± 2.0	+13.5 ± 1.7	0.086	ns	6
High K^+/Na^+, Cl^--free	+20.4 ± 2.1	+20.8 ± 2.3	0.899	ns	7
K^+-free*	-7.7 ± 1.8	-7.7 ± 1.7	1.5×10^{-9}	ns	9

* K^+-free saline composition (mM): NaCl 108.6, $MgCl_2$ 8.5, $CaCl_2$ 2, NaH_2PO_4 4, Glucose 34, HEPES 25, NaOH 11, pH 7.2.

TABLE A.3 ATPase Activity of Various Membrane Fractions from Malpighian Tubules of *Locusta migratoria*

Composition of	Enzyme activity in the various membrane fractions (nmoles Pi liberated/mg protein/min)												
reaction media (mM)	Crude	S_1	P_1	S_2	P_2	P_3	S_3	P_4	S_4	P_5	P_6	P_7	P_8
pH 7.5													
(a) 4 Mg^{2+}	102.1 ± 14.1	102.1 ± 7.1	59.7 ± 9.3	53.6 ± 6.5	607.0 ± 60.4	113.3 ± 33.5	363.2 ± 73.7	544.0 ± 59.2	225.7 ± 5.4	410.9 ± 10.1	733.6 ± 3.9	684.7 ± 79.2	859.4 ± 21.7
(b) a + 100 Cl^-	107.4 ± 17.7	103.1 ± 5.5	73.8 ± 9.4	67.5 ± 7.9	569.9 ± 55.3	121.7 ± 6.1	304.1 ± 49.9	553.0 ± 63.7	205.5 ± 9.7	392.0 ± 7.5	685.0 ± 3.5	657.9 ± 97.9	814.7 ± 6.6
(c) a + 100 HCO_3^-	144.0 ± 25.9	155.1 ± 3.0	109.9 ± 15.4	81.7 ± 13.2	785.0 ± 71.5	194.1 ± 9.2	419.3 ± 65.2	743.0 ± 71.9	262.0 ± 6.3	517.2 ± 16.2	982.1 ± 3.6	948.6 ± 124.8	1208.4 ± 23.7
(b) minus (a)	5.3 ± 4.1	1.0 ± 2.8	6.6 ± 5.5	13.9 ± 1.4	-37.1 ± 5.3	-19.1 ± 6.2	-60.1 ± 23.8	8.9 ± 4.5	-20.2 ± 4.3	-18.9 ± 14.4	-47.6 ± 0.6	-26.8 ± 18.8	-44.7 ± 27.7
(c) minus (b)	36.6 ± 12.9	51.9 ± 3.2	36.1 ± 6.2	14.3 ± 5.5	215.1 ± 16.6	72.4 ± 14.2	115.2 ± 15.3	190.0 ± 8.2	56.5 ± 3.4	125.2 ± 21.8	297.0 ± 7.1	290.7 ± 27.0	393.7 ± 28.4
pH 7.2													
(d) 4 Mg^{2+}	97.9 ± 13.2	89.7 ± 7.6	61.7 ± 3.8	53.6 ± 7.2	537.4 ± 52.0	120.4 ± 9.0	285.7 ± 49.7	481.2 ± 25.8	178.7 ± 1.9	354.4 ± 10.9	641.7 ± 2.7	592.4 ± 63.8	642.9 ± 22.8
(e) d + 1 ouabain	97.2 ± 13.0	89.4 ± 7.4	61.5 ± 4.0	53.3 ± 7.2	530.3 ± 50.1	119.6 ± 9.4	285.7 ± 49.7	493.3 ± 39.6	176.0 ± 4.6	353.5 ± 10.3	641.4 ± 1.9	589.4 ± 62.0	638.4 ± 16.2
(f) e + 20 K^+	102.0 ± 11.6	88.8 ± 6.8	55.3 ± 3.5	55.5 ± 7.5	574.2 ± 55.6	116.3 ± 8.2	262.8 ± 54.0	500.5 ± 39.0	166.7 ± 4.7	346.7 ± 3.4	629.7 ± 2.7	638.6 ± 61.4	813.6 ± 36.3
(g) d + 20 K^+, 100 Na^+	146.3 ± 15.8	128.9 ± 8.4	118.5 ± 11.5	67.6 ± 10 1	680.7 ± 77.2	154.0 ± 3.9	290.4 ± 54.3	665.6 ± 26.6	145.2 ± 4.4	407.7 ± 15.0	680.1 ± 2.1	768.2 ± 136.1	988.7 ± 17.2
(h) g + 1 ouabain	113.3 ± 16.7	96.1 ± 10.2	70.4 ± 9.7	52.4 ± 7.2	527.7 ± 71.2	139.7 ± 1.4	235.2 ± 53.6	478.1 ± 32.9	143.9 ± 8.4	382.3 ± 42.7	566.6 ± 1.1	557.5 ± 59.3	657.4 ± 15.3
(f) minus (e)	4.8 ± 1.6	-0.6 ± 2.7	-6.2 ± 0.5	2.2 ± 1.4	44.0 ± 18.2	-3.3 ± 3.1	-22.9 ± 4.3	27.2 ± 19.5	-9.3 ± 9.3	-6.8 ± 7.0	-11.7 ± 4.6	49.2 ± 0.6	175.2 ± 50.0
(g) minus (h)	32.9 ± 2.4	32.9 ± 12.2	48.0 ± 2.0	15.2 ± 2.9	153.0 ± 12.3	14.3 ± 5.3	55.2 ± 0.7	187.4 ± 9.4	1.3 ± 4.0	25.4 ± 28.3	113.5 ± 0.9	210.7 ± 77.6	331.3 ± 7.1
n	4	4	4	4	4	4	2	3	2	3	2	3	3

(b) minus (a) represents the ATPase activity sensitive to the presence of 100mM NaCl in the incubation medium.
(c) minus (b) represents the HCO_3^--stimulated ATPase activity.
(f) minus (e) represents the ATPase activity sensitive to the presence of 20mM KCl in the incubation medium; positive values represent K^+-stimulated ATPase activity, whilst negative values represent an inhibition of the Mg^{2+}-ATPase.
(g) minus (h) represents the $(Na^+ + K^+)$-ATPase activity.
n represents the number of independent determinations.

TABLE A.4 Purity of ATPase, Succinate Dehydrogenase (SDH) and Alkaline Phosphatase Enzymes from the Various Membrane Fractions Obtained from Malpighian Tubules of *Locusta migratoria*

	Enzyme purities of the various membrane fractions												
	Crude	S_1	P_1	S_2	P_2	P_3	S_3	P_4	S_4	P_5	P_6	P_7	P_8
$(Na^+ + K^+)$-ATPase	100.0	99.8	145.7	46.2	464.6	43.4	167.6	569.2	3.9	77.2	344.5	639.8	1006.0
K^+-ATPase	100.0	0.0	0.0	0.0	915.4	0.0	0.0	149.8	0.0	0.0	0.0	1024.0	3649.8
HCO_3^--stim. ATPase	100.0	141.9	98.7	38.9	587.7	197.8	314.6	519.1	154.3	342.0	811.6	794.2	1075.7
SDH	100.0	95.6	110.9	40.2	130.8	305.3	0.0	187.7	39.6	47.1	97.8	314.1	426.6
Alkaline Phosphatase	100.0	97.9	31.7	52.7	473.7	55.0	1410.0	454.9	550.0	2473.1	1214.1	208.6	138.3