Studies on ion movement in malpighian tubules of locusta migratoria l. with particular reference to electrical events

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STUDIES ON ION MOVEMENT IN MALPIGHIAN TUBULES OF *LOCUSTA MIGRATORIA* L. WITH PARTICULAR REFERENCE TO ELECTRICAL EVENTS

by

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DECLARATION

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

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Paul Baldrick
Durham
December 1987

To my parents

and "moineau"

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ABSTRACT

Intracellular microelectrodes have been used in conjunction with ion substitution, and agonists and inhibitors of known transport processes to investigate the mechanisms whereby ions cross the basal and apical cell membranes of the Malpighian tubules of Locusta. Values for basal, apical and transepithelial potentials in 'Normal' saline were -71.6 ± 0.3 mV, -82.6 ± 0.8 mV and +5.7 ± 1.0 mV (lumen positive) respectively. Ion substitution experiments, involving Na^+,K^+ and Cl^- in the bathing media, indicated that the basal membrane was more permeable to K^+ than Na^+ and Cl^-. Two different electrical responses to high $[K^+]_o$ saline (the Type A and Type B response) were noted and these probably reflect distinct physiological states of basal membrane permeability.

Experiments with ouabain and vanadate suggested that whilst Na^++K^+-ATPase activity, which has been demonstrated in microsomal preparations, was not significantly electrogenic, asymmetric ionic distribution across the basal membrane was partly maintained by this enzyme. Furthermore, 3-H ouabain-binding studies indicated that Na^++K^+ exchange 'pump' turnover was adequate to account for substantial K^+ entry and Na^+ exit across the basal membrane. The electrochemical gradient across the apical membrane suggests that K^+ exit from the cell must involve an active process with Cl^- following passively.

Data from ion substitution experiments and treatment with furosemide and bumetanide suggest that Cl^- entry across the basal membrane may be via cotransport with Na^+ and/or K^+. However, the differential electrical responses to Na^+ free and Cl^- free salines question the role of Na^+ in this process.

The effects of c AMP, Ca^{2+} substitution and various inhibitors on basal and apical membrane potentials, taken in conjunction with the results referred to above, are discussed and a hypothetical model proposed whereby changes in intracellular Ca^{2+} and c AMP effect control of ion movements across the two cell surfaces.

GLOSSARY

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin (Fraction V, Sigma)
Ca^{2+}-ATPase	magnesium dependent, calcium stimulated adenosine triphosphatase
c AMP	cyclic adenosine 3',5'-monophosphate
$Cl^{-}+HCO_3^{-}$-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
H^{+}-ATPase	magnesium dependent, hydrogen stimulated adenosine triphosphatase
HEPES	4-(2-hydroxylethyl)-1-piperazine-ethanesulphonic acid
5-HT	5-hydroxytryptamine
K^{+}-ATPase	magnesium dependent, potassium stimulated adenosine triphosphatase
$K^{+}+H^{+}$-ATPase	magnesium dependent, potassium-hydrogen stimulated adenosine triphosphatase
mM	millimolar
mV	millivolts
$Na^{+}+K^{+}$-ATPase	magnesium dependent, sodium-potassium stimulated adenosine triphosphatase
S.E.M.	standard error of mean
SITS	4-acetamide-4'-isothiocyano-2 2'-di-sulphonic acid stilbene
T.C.P.	transcellular potential
T.E.P.	transepithelial potential
Tris	tris [hydroxymethyl] aminomethane
V_A	apical membrane potential
V_B	basal membrane potential

CHAPTER 1

Introduction

It is generally accepted that fluid movement across a variety of epithelia, whether secretory or absorptive in nature, is a consequence of active ion transport with water movement being osmotically linked to ion movements (e.g. Ernst et al, 1980; Maddrell, 1971; 1977). In insects, Malpighian tubules form an important part of the excretory system being responsible for the formation of the primary urine and functioning to regulate and control the haemolymph composition and volume (Maddrell, 1980). The mechanism of ion and water transport across the Malpighian tubules of Carausius was first studied by Ramsay (1953; 1954) and has been the subject of many subsequent studies, e.g. Calliphora (Berridge, 1968; 1969), Rhodnius (Maddrell, 1969; 1971; 1977), Locusta (Anstee and Bell, 1975; 1978; Morgan and Mordue, 1981; 1983a) and Glossina (Gee, 1975; 1976).

At present the mechanism of solute-coupled water movements is not fully understood in most epithelia. To understand ion and water movements, it is necessary to point out that epithelia contain two major diffusional pathways - a transcellular route in which ions penetrate the apical and basal membranes and a paracellular pathway consisting mainly of "tight" junctions in series with the lateral spaces between epithelial cells. Initially, it was thought that such junctions presented a barrier to paracellular transport (DiBona, 1972). However, in the early 1970s, it was recognized that such epithelial "tight" junctions were not tight enough to preclude paracellular transepithelial transport (DiBona, 1972; Frömter and Diamond, 1972). Consequently, the term "tight" and "leaky" (Frömter and Diamond, 1972) became common descriptions in epithelial classification (Schneeberger and Lynch, 1984). In "leaky"

epithelia, transepithelial diffusion of ions occurs paracellularly through the tight junctions and lateral intercellular spaces (Frömter and Diamond, 1972). Examples are gallbladder of Necturus (Frömter, 1972), kidney proximal tubule of Necturus (Guggino et al., 1982) and intestine of fresh water prawns (Ahearn, 1980) and Aplysia (Gerencser, 1982). In contrast in tight epithelia, most ionic diffusion and water movements occur transcellularly because the combined resistances of apical and basal membranes is lower than that of the tight junctions and intercellular spaces. Examples of this second category where "tightness" has been quantified electrophysiologically include toad urinary bladder (Reuss and Finn, 1974), fundic gastric mucosa (Spenny et al., 1974), rabbit urinary bladder (Lewis et al., 1976) and locust rectum (Hanrahan et al., 1982).

It is now generally thought that epithelia which transport isotonic fluid possess cell junctions which are "leaky" (Staehelin, 1974; Lord and Di Bona 1976; Lane, 1979) and therefore allow a considerable flow of ions and water through the paracellular route (Sackin and Boulpaep, 1975; Gupta and Hall, 1979). Studies on the Malpighian tubules of insects indicate that whilst in some species a slight hypo-osmotic urine is secreted (Carausius, Ramsey, 1954; Dysdereus, Berridge, 1965) in the majority of species, including Locusta, the tubules produce a urine which is marginally but consistently hyperosmotic to the bathing medium over a wide range of osmotic concentrations (Berridge, 1968; Maddrell, 1969; Phillips, 1964; Anstee et al., 1979). On this basis one would have predicted that insect Malpighian tubules would be "leaky". However, recent studies by O'Donnell and Maddrell (1983) and O'Donnell et al. (1984) suggest that water probably moves through the cells during fluid secretion by the Malpighian tubules of Rhodnius and that water

movements are coupled to salt transport across the basal and apical cell membranes. These workers argue that water movements via a paracellular route are unlikely to represent a significant component of total transepithelial tubule secretion in this species. Similarly, from measurements on transepithelial concentration gradients and resistance, Williams and Beyenbach (1984) have stated that the Malpighian tubule of Aedes should be classified as a moderately "tight" epithelium. However, in other insect epithelia, there is good evidence that some of the fluid moves paracellularly and that solute/solvent coupling occurs in an extracellular compartment (O'Donnell and Maddrell, 1983). Indeed, paracellular fluid flow has been suggested in the rectal pads of Periplaneta and Calliphora (Wall et al, 1970; Gupta and Hall, 1983) and the salivary glands of Calliphora (Gupta et al., 1978).

The fact that the fluid secreted by nearly all Malpighian tubules is marginally but consistently hyperosmotic to the bathing fluid (Maddrell, 1977) shows that the produced rates of fluid flow are in fairly close inverse relationship to the osmotic concentration of the bathing solution. In other words, the rate of solute movement is approximately constant but water movements change so that the fluid produced is slightly hypertonic (Maddrell, 1977). Several theories have been proposed to explain how solute transport effects such iso- or near-isomotic secretion across epithelia. Diamond and Bossett (1967; 1968) proposed the so-called standing-gradient osmotic flow hypothesis for fluid transporting epithelia. This model is based on the functional geometry of the tissue and depends upon channels which are structurally or functionally closed at one end. Solute is pumped into the closed ends of the spaces from the adjacent cytoplasm, making the region hyperosmotic to the cell. Water moves into the

space from the adjacent cytoplasm so that towards the open end of the space, the fluid is isosmotic to the cytoplasm. Thus, a standing-gradient is established along the length of the channels at equilibrium. Difficulties arise when this model is applied to insect Malpighian tubule secretion because (a) the infolding and microvilli of Malpighian tubules are shorter than in the tissues for which the model was originally developed (Taylor, 1971; Maddrell, 1977), (b) biological membranes do not seem to have sufficiently high osmotic permeability to permit isotonic flow in epithelia to occur by local osmosis (Hill, 1975a;b) and (c) ion concentrations exist in the microvilli of Malpighian tubules which are unfavourable to a standing-gradient osmotic flow (Gupta et al., 1976; 1977).

Another theory which has been proposed to explain the mechanism of ion and water movements across epithelia is based on electro-osmosis (Hill, 1975b; 1977). Maddrell has discussed the implication of this electro-osmosic theory for insect Malpighian tubules. He suggests that electrogenic cation pumps, situated on the apical plasma membrane of tubules, produce an electrical potential difference across this membrane. The resulting electrochemical gradient would draw Cl^- out from the cell through the membrane, and in crossing the membrane Cl^- would functionally interact with water molecules and cause them also to move out of the cell. Therefore, this mechanism relies on the maintenance of a potential gradient across the cell membrane.

Finally, it may be that simple 'local osmosis' (Diamond, 1964) may be responsible for water movements across the tubule. This theory proposes that the cytoplasm is marginally hypertonic to the bathing medium as a result of solute pumping across the basal membrane, and similarly, the lumen becomes marginally hypertonic to the cytoplasm.

Water flows passively as a result of these small osmotic pressure differences, their magnitude being determined by the rate of solute transport and the osmotic permeability of the membrane. This theory has been favoured by Maddrell (1972) and Taylor (1971) for insect Malpighian tubules. They suggest that the foldings of the basal and apical membranes is primarily a device for increasing the effective passive permeability of the cells to solute and that the driving force for water movements is, in fact, the overall osmotic pressure difference between the lumen and the bathing solution.

Whatever the exact mechanism for coupling of ions and water movements, the various models agree, that fluid secretion by insect Malpighian tubules is a consequence of solute transport. Indeed, Ramsay (1953) first recognized the central role of potassium transport in generating fluid secretion in Malpighian tubules. Subsequent studies have confirmed that this cation is the 'prime mover' in generating fluid secretion in the Malpighian tubules of most insects (Berridge, 1968; Maddrell, 1969; Anstee and Bell, 1975). However, this is not the case for all insects which have been investigated. Exceptions include stimulated fluid secretion by the Malpighian tubules of Rhodnius (Maddrell, 1969; 1977) in which fluid secretion requires the presence of Na^+ and K^+ and Glossina (Gee, 1975; 1976) in which Na^+ is the "prime mover".

Whilst the active pumping of K^+ from the haemolymph to the lumen has been established for the Malpighian tubules of many insects (Ramsay, 1958; Berridge, 1968; Maddrell and Klunswan, 1973), the nature of the ion translocation mechanism has still to be fully elucidated. It is generally agreed 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). Although

mainly a potassium pump, this hypothetical cation pump appears to be relatively unspecific and will readily transport other cations under appropriate conditions (Berridge, 1968; Maddrell cited in Berridge et al., 1976; Maddrell, 1977). This lack of specificity suggests that the cation which is transported is determined by the major cation present within the intracellular compartment which, in turn, is determined by the permeability of the basal plasma membrane rather than by the affinity of the pump for a particular ion. Indeed, Maddrell (1977) and O'Donnell and Maddrell (1984) have used these suggestions in an attempt to explain how one basic mechanism can account for the secretion of Na^+ rather than K^+ in some species of insect (e.g. Rhodnius, Glossina and Aedes). Thus, in the Malpighian tubules of Rhodnius, these workers have suggested that the apical cation pump has a higher affinity for sodium than potassium and would act to maintain the intracellular level of Na^+ lower than that of K^+. The actual rate at which cations are pumped across the tubules from the bathing solution into the lumen by this pump depends not only on the affinity of the pump for the two cations but also how fast these cations enter the cell. This, in turn, depends partly on the electrochemical gradient across the basal cell membrane and on the permeability of this membrane to these ions. Thus, small changes in the relative permeability of the basal membrane to Na^+ and K^+ may cause large changes in the ionic composition of the fluid secreted by Malpighian tubules. The ability of tubules from Glossina to secrete a Na^+ rich fluid at a high rate (Gee, 1975), for example, might be simply explained by their having a high permeability to Na^+ than have other tubules; as a result, Na^+ enter faster than K^+ and as the apical pump has a higher affinity for Na^+ in any case, it is these ions which are transported. In contrast, in the K^+-secreting

tubules of the majority of insect species, K^+ enters the cell faster than Na^+, Na^+ being virtually excluded, and so it is K^+ which is transported. The extent to which such a model, for cation transport, however, might be applicable to all insect Malpighian tubules, remains to be established.

It is interesting to note that although active K^+ transport was first deduced from ion distribution against electrochemical gradients in the Malpighian tubules of several insects by Ramsay (1953), such movement has been confirmed by measurement of net ^{42}K fluxes in short-circuited lepidopteran midgut by Harvey and Nedergaard (1964). This led Anderson and Harvey (1966) to propose the presence of an electrogenic K^+ pump in the midgut of Manduca sexta which is located in the apical membrane of the goblet cells (Harvey et al., 1983). As well as Malpighian tubules and lepidopteran midgut, evidence exists to suggest the presence of an apical K^+ pump in the dipteran salivary glands (Prince and Berridge, 1972; Berridge et al., 1975; Gupta et al., 1978), the sensory sensilla of dipteran labella (Thurm and Küppers, 1980; Wieczorek, 1982) and lepidopteran labial glands (Halim and Kafatos, 1974). More recently, the occurrence of a potassium sensitive ATPase activity has been reported in preparations of Manduca sexta midgut (Wolfersberger, 1979; Wolfersberger et al., 1982; Deaton, 1984) and the labellum from the dipteran Protophormia (Wieczorek, 1982; Wieczorek et al., 1986). However, although this putative K^+-ATPase may be a candidate for the electrogenic apical potassium pump, as yet no biochemical evidence exists for K^+-ATPase activity in insect Malpighian tubules (see Chapter 3). Wieczorek et al. (1986) have pointed out that the midgut K^+-ATPase activity of Manduca midgut is not homologous to a K^++H^+-ATPase as suggested by Deaton (1984) and English and Cantley (1984) in the

same species. The electroneutral K^++H^+-ATPase has, since its discovery by Sachs et al. (1976), been found only in specialized cells of vertebrate stomach (Schuurmans Stekhoven and Bonting, 1981). In this tissue, this apical membrane enzyme transports K^+ into the cell in exchange for H^+ (Rabon et al., 1983). From studies on a Manduca sexta embryonic cell line CHE, English and Cantley (1984) and English et al. (1986) suggested that a similar enzyme may function in some insect cells, coregulating both cellular pH and K^+.

At this point it is important to point out that the transport of ions across cell membranes requires the presence of cell membrane proteins and these act in various ways (Scobie et al., 1986). Uniporters facilitate movement of an ion down its own concentration gradient. Symporters facilitate diffusion of an ion across a membrane down its concentration gradient in association with the movement of a second ion without a concentration gradient; this results in secondary active transport of the second ion. Antiporters link diffusion of one ion down its concentration gradient to movement of a second ion in the opposite direction. ATPases link hydrolysis of ATP to ionic movements while channels form a "hole" through a membrane, facilitating movement of ions down their concentration gradients. Indeed, the presence of ion channels is now well-established in epithelia and to date most have been found to be highly K^+ selective (Lewis and Hanrahan, 1986). K^+ channels are found in either apical or basolateral cell membranes from leaky as well as tight epithelia (Van Driessche and Zeiske, 1985). The fact that cellular Ca^{2+} entry leads to an increase in K^+ permeability - the Gardós effect - in red cells (Gardós, 1958) - has recently lead to the demonstration of the presence of specific basolateral Ca^{2+}-activated K^+ conductance channels in a

variety of cell types (Latorre and Miller, 1983; Van Driessche and Zeiske, 1985). To date, selective ion channels have been characterized in epithelia for Na^+, Ca^{2+} and Cl^-, as well as for K^+ (Van Driessche and Zeiske, 1985). Almost all ionic channels display the "gating" phenomenon, i.e. transitions between one or more open or closed conformations that are triggered by factors such as voltage, blocking the channel or inducing its opening by interaction with appropriate substances, or conformational fluctuations that are simply spontaneous (Van Driessche and Zeiske, 1985).

Despite the number of models for Malpighian tubule fluid secretion, much controversy exists to explain the mechanism for ion entry into the cell across the basal membrane. Berridge (1967) and Berridge and Oschman (1969) suggested that movement across the basal membrane occurs as a result of a coupled Na^++K^+-exchange pump. In the majority of tissues examined the enzyme responsible for this 'pump' is a Mg^{2+}-dependent Na^++K^+-stimulated ATPase (Skou, 1957). Since the early discoveries of Hodgkin and Keynes (1955), Skou (1957) and Caldwell et al. (1960), which lead to the description of the Na^++K^+-ATPase, this enzyme has been demonstrated in a great variety of animal cells and tissues (Bonting, 1970; Anstee and Bowler, 1984; Norris and Cary, 1981). Indeed, a Na^++K^+-ATPase has been exhibited in microsomal preparations from the Malpighian tubules of Locusta (Anstee and Bell, 1975; Anstee et al., 1979) and has many of the properties of Na^++K^+-ATPase from other species (Bonting, 1970; Anstee and Bowler, 1979; 1984). The Na^++K^+-ATPase has been reviewed by many authors (Glynn and Karlish, 1975; Robinson and Flashner, 1979; Skou, 1975; Schuurmans Stekhoven and Bonting, 1981) and has been shown to be electrogenic in nature, pumping 3 Na^+ out of the cell for every 2 K^+ it pumps in, for the majority of cells (Post and Jolly, 1957; Cantley,

1981). Several attempts at cytochemical and histochemical localization of this enzyme in insect tissue point to the enzyme being present in the basal and lateral plasma membrane (Anstee and Bowler, 1984), which is consistent with conclusions from studies on many vertebrate transporting epithelia (Di Bona and Mills, 1979; Ernst et al., 1980; Towle, 1984). Autoradiographic studies of [^{3}H] ouabain binding to larval dragonfly rectal cells by Komnick and Achenbach (1979) have demonstrated that the Na^++K^+-ATPase is restricted to the basolateral membranes in contact with interstitial fluid and blood. An ultracytochemical study of K^+-activated phosphatase in blue crab gill epithelium has shown the majority of this enzyme to be localized in basolateral membranes (Towle, 1984).

Despite the vast literature on the Na^++K^+-ATPase, controversy exists concerning its involvement in secretion of fluid by insect Malpighian tubules. The conflicting reports are mainly due to the failure of some investigators to demonstrate an effect on tubule function by the cardiac glycoside, ouabain, a specific inhibitor of Na^++K^+-ATPase (Skou, 1965; Albers et al., 1968; Wallick et al., 1980). A review by Anstee and Bowler (1979) offers some possible explanations for the causes of these conflicting reports (also see Chapter 3). In addition, it is of interest that the sensitivity of the Na^++K^+-ATPase to cardiac glycosides such as ouabain differs markedly from species to species (Wallick et al., 1980). Some researchers suggest that the Na^++K^+-ATPase from different sources bind ouabain at the same rate and that differences in sensitivity are determined by differences in the rates of dissociation of the glycoside from the enzyme (Wallick et al., 1980; Erdmann and Schoner, 1973). Rubin et al. (1981) report that the ouabain dissociation reaction is considerably faster with microsomal preparations from brain of Manduca sexta than with bovine brain, accounting for the decreased sensitivity of the insect

enzyme to inhibition by ouabain. Other workers suggest that the lower sensitivity of the Na^++K^+-ATPase from certain species cannot be explained solely on the basis of the dissociation rate (Wallick et al., 1981; Pitts et al., 1977). To date, with the notable exception of the study by Rubin et al. (1981) referred to above, few studies report the use of [^{3}H]ouabain in insect tissue preparations. Fristrom and Kelly (1976) and Jungreis and Vaughan (1977) studied [^{3}H]ouabain binding to imaginal discs of Drosophila melanogaster, and midgut and nerve of three lepidopteran species, respectively. Unfortunately, both these studies were carried out under conditions that were inappropriate for determining maximal binding, owing to the inclusion of K^+ in the incubation medium.

In order to overcome the problem of ouabain-insensitivity in the Malpighian tubules of Calliphora and Carausius, Maddrell (1971) proposed a model of active ion transport across both the basal and apical cell membranes in these insects which does not involve a Na^++K^+-exchange pump. This model suggests that K^+ is actively transported into the cell by an electrogenic 'pump' which is stimulated by Na^+ and is situated on the basal cell membrane, whereas Na^+ and Cl^- enter the cell passively. On the apical surface, Na^+ and K^+ are transported into the lumen by electrogenic 'pumps', whilst the transport of Cl is passive. At the same time, Maddrell (1971; 1980) suggested that basal membrane electrogenic Na^+ and K^+ pumps may be found in the Malpighian tubules of Rhodnius with Cl^- entering the cell passively. These ions would then enter the lumen via three electrogenic 'pumps' for Na^+, K^+ and Cl^-. A later model (mentioned earlier) by Maddrell (1977) proposed to suit transport through Malpighian tubules of any insect (regardless of whether K^+ or Na^+ is the prime mover) can be seen in Fig. 1A. This model suggests that K^+, Na^+ and Cl^- all enter the cell passively across the basal membrane. An electrogenic cation pump on the apical membrane is responsible for K^+

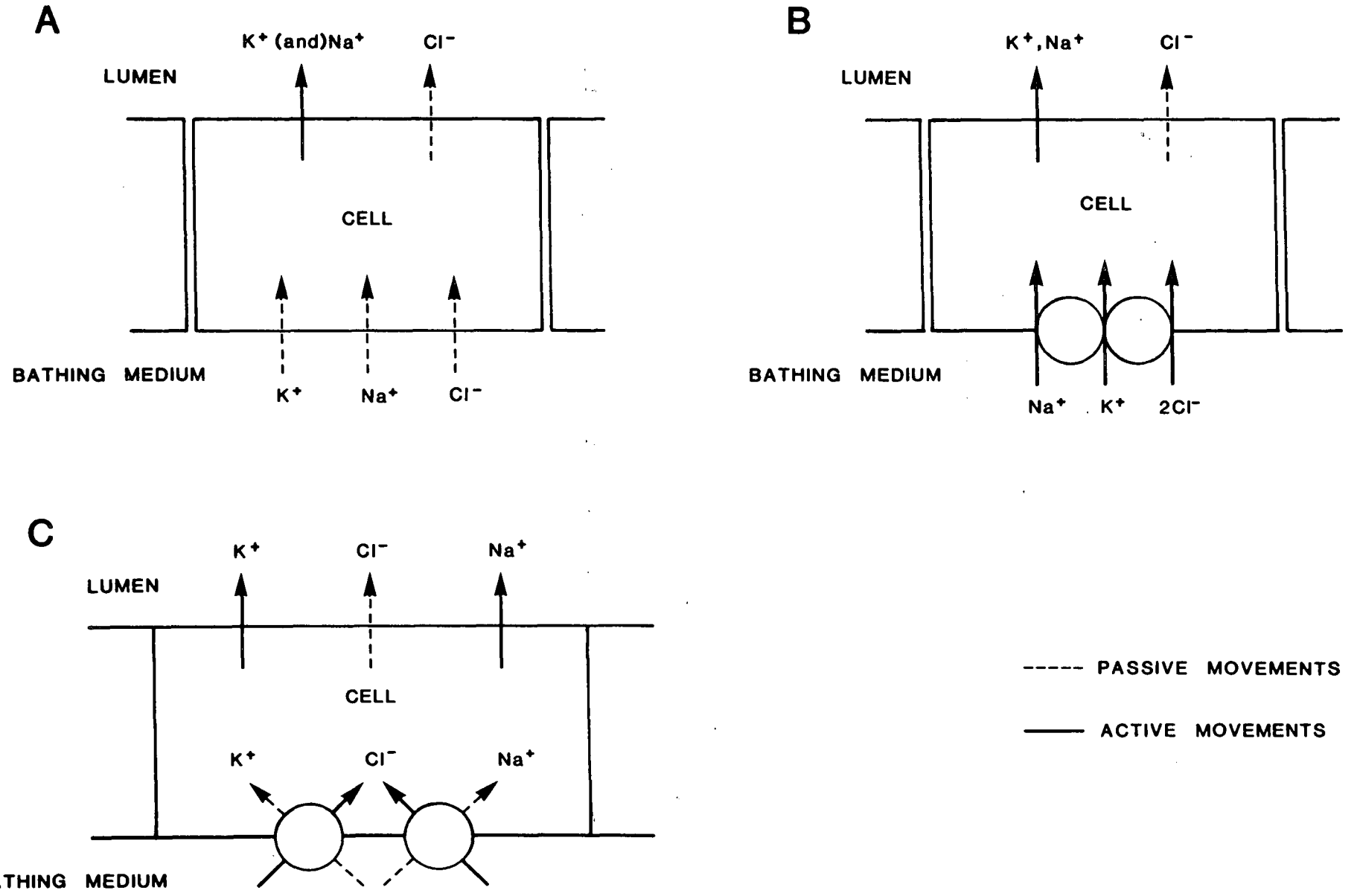
Fig. 1 A-C

Schematic diagrams of three models proposed to explain ion translocation across Malpighian tubules. In all cases, the basal membrane faces the bathing medium whilst the apical membrane faces the lumen.

Fig. 1A This general model has been described to explain ion transport across the Malpighian tubules in all insects (Maddrell, 1977). In this model, K^+,Na^+ and Cl^- all enter the cell passively across the basal membrane. Across the apical membrane, K^+ and/or Na^+ exit the cell by means of a cation pump, whilst Cl^- exits passively.

Fig. 1B In this model, which has been proposed for the Malpighian tubules of Rhodnius (O'Donnell and Maddrell, 1984), a basal membrane Na^+K^+ $2\,Cl^-$ cotransport is thought to move Na^+,K^+ and Cl^- into the cell. The exit of K^+ and/or Na^+ from the cell occurs, it is suggested, by means of a catholic apical pump, whilst Cl^- exits passively.

Fig. 1C This model has been proposed for the Malpighian tubules of Locusta by Morgan and Mordue (1983a). It is thought that a passive entry of K^+ and Na^+ into the cell occurs across the basal membrane, whilst Cl^- enters by an active process. In contrast, an active process is required for the apical membrane exit of K^+ and Na^+, whilst Cl^- exits the cells by a passive process.


A
K+ (and)Na+
Cl−
LUMEN
CELL
BATHING MEDIUM
K+
Na+
Cl−
B
K+,Na+
Cl−
LUMEN
CELL
BATHING MEDIUM
Na+
K+
2Cl−
C
K+
Cl−
Na+
LUMEN
CELL
K+
Cl−
Na+
BATHING MEDIUM
----- PASSIVE MOVEMENTS
—— ACTIVE MOVEMENTS

and/or Na^+ exit while Cl^- leaves the cell passively. More recently, O'Donnell and Maddrell (1984) have modified this model for the Malpighian tubules of Rhodnius upon finding that the basal membrane is largely impermeable to sodium and chloride and that furosemide and bumetanide, blockers of sodium chloride entry (Palfrey and Rao, 1983), have an inhibitory effect. Earlier, Maddrell (1971) had queried the feasibility for K^+ to move passively from low extracellular to high intracellular potassium concentrations across the basal membrane. As a result, O'Donnell and Maddrell (1984) have proposed that a basal membrane Na^+, K^+, 2 Cl^- cotransport step occurs to allow the entry of these ions into the cell (Fig. 1B). A catholic apical pump exists for the extrusion of K^+ and/or Na^+ out of the cell while chloride enters the lumen passively. Furthermore, Maddrell (personal communication) has said that a basal membrane Na^++K^+-ATPase occurs in Rhodnius tubules and acts to maintain the cellular gradients during non-stimulated fluid secretion. Similarly, Gee (1976) has suggested that such a pump may be necessary to maintain the intracellular ion concentration of resting Malpighian tubule cells of Glossina. From a study on the Malpighian tubules of Locusta, Morgan and Mordue (1983a) have proposed that K^+ and Na^+ can enter the cell across the basal membrane passively, but Cl^- must enter by an energy-dependent mechanism (Fig. 1C). An energy dependent mechanism, however, is required for K^+ and Na^+ exit across the apical membrane whilst Cl^- enters the lumen down a favourable electrical gradient.

Although K^+ and Na^+ have been considered to be the prime movers in fluid secretion, anions too play an important role in Malpighian tubule urine production. Nevertheless, for many years active cation transport across both vertebrate and invertebrate epithelia has been intensively studied with Cl^- and HCO_3^- assuming a secondary role as passive counter-ions. However, recently there has been an explosive interest in

transepithelial movement of both these anions, especially since Cl^- has been found to be moved actively in a wide range of species (Frizzell et al., 1979). Some of the proposed schemes for the transport of anions are shown in Fig. 2A-F. As previously mentioned, it was generally thought that Cl^- transport across the basal membrane of Malpighian tubule cells was passive and that Cl^- followed K^+ out of the cell on the luminal side (Maddrell, 1977). Indeed, Dalton and Windmill (1980) have suggested that Cl movement across the tubules of Musca is passive. However, the recent models of O'Donnell and Maddrell (1984) and Morgan and Mordue (1983a) for Rhodnius and Locusta tubules respectively (Fig. 1A-C), both propose that Cl^- enters the cell across the basal membrane by a non-diffusive mechanism. Furthermore, although chloride movement is thought to be passive across the salivary glands of Calliphora, Berridge et al. (1976) could not completely exclude the possibility of a pump on the basal membrane which transports chloride into the cell. Although they did not speculate, Williams and Beyenbach (1984) found evidence for coupled cation-Cl secretion during stimulation with head extract, in the Malpighian tubules of Aedes.

Intensive studies on several vertebrate epithelia have revealed various types of Cl^- transport including electrically silent Cl^--HCO_3^- exchange, Na^+-dependent co-transport of Cl^- (or Na^+, K^+, Cl^- cotransport) and Cl^- secretion which is often electrogenic (Phillips and Lewis, 1983; Frizzell et al., 1979; Gerencser and Lee, 1983; Reuss et al., 1983; Hanrahan and Phillips, 1984). The NaCl co-transport is active by nature and is thought to be effected through an electrically neutral Na^+-coupled carrier mechanism, which drives Cl^- uphill into epithelial cells via the inward flow of Na^+ down a favourable electrochemical potential gradient, i.e. the transport of Cl^- is secondarily active (Frizzell et al., 1979; Shorofsky et al., 1982). The Na^+ gradient across the cell is established

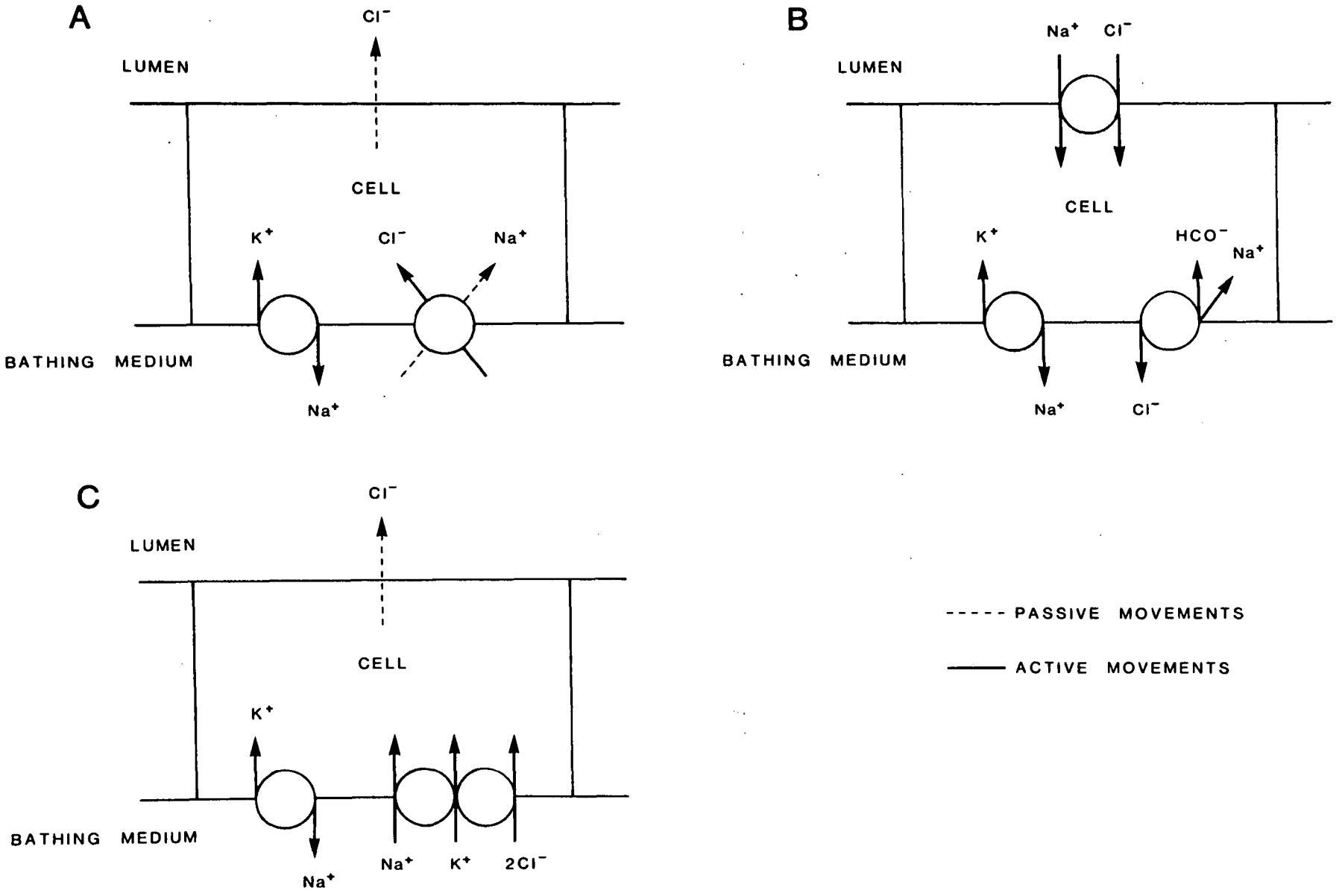
Fig. 2 A-F

Schematic diagrams of various models proposed to explain anion (and cation) transport across the cells of secretory and absorptive epithelium. In all cases, the basal (serosal) membrane faces the bathing medium whilst the apical (mucosal) membrane faces the lumen.

Fig. 2A This model has been described to explain Cl^- secretion in canine tracheal epithelium (Shorofsky et al., 1982) and the secretory shark renal proximal tubules (Sawyer and Beyenbach, 1985b). In this model, a secondary active entry of Cl^- into the cell across the basal membrane occurs as a consequence of an inward movement of Na^+ down its electrochemical gradient, by means of a Na^+Cl^- cotransporter. This transport is driven by the action of the Na^++K^+-ATPase. Cl^- leaves the cell across the apical membrane by a conductive pathway.

Fig. 2B In this model, proposed to explain the transport of Cl^- across the absorptive Necturus proximal tubule, Reuss et al. (1983) suggest that a Na^+Cl^- cotransporter which is independent of K^+, occurs on the apical membrane to allow the entry of Cl^- into the cell. This anion then leaves the cell across the basal membrane via a transport system that moves both Na^+ and HCO_3^- into the cell in exchange for Cl^-.

Fig. 2C This model has been described to explain NaCl secretion in the shark rectal gland tubules (Greger et al., 1984). Cl^- entry into the cell occurs by means of an electroneutral basal membrane Na^+,K^+,2 Cl cotransport step. The gradient for this transport is provided by the action of a Na^++K^+-ATPase. A conductive pathway allows the exit of Cl^- at the apical membrane. In a similar model, proposed for the absorptive diluting segment of mammalian nephron. Greger (1985) has suggested that an apical Na^+,K^+,2 Cl^- cotransporter allows the entry

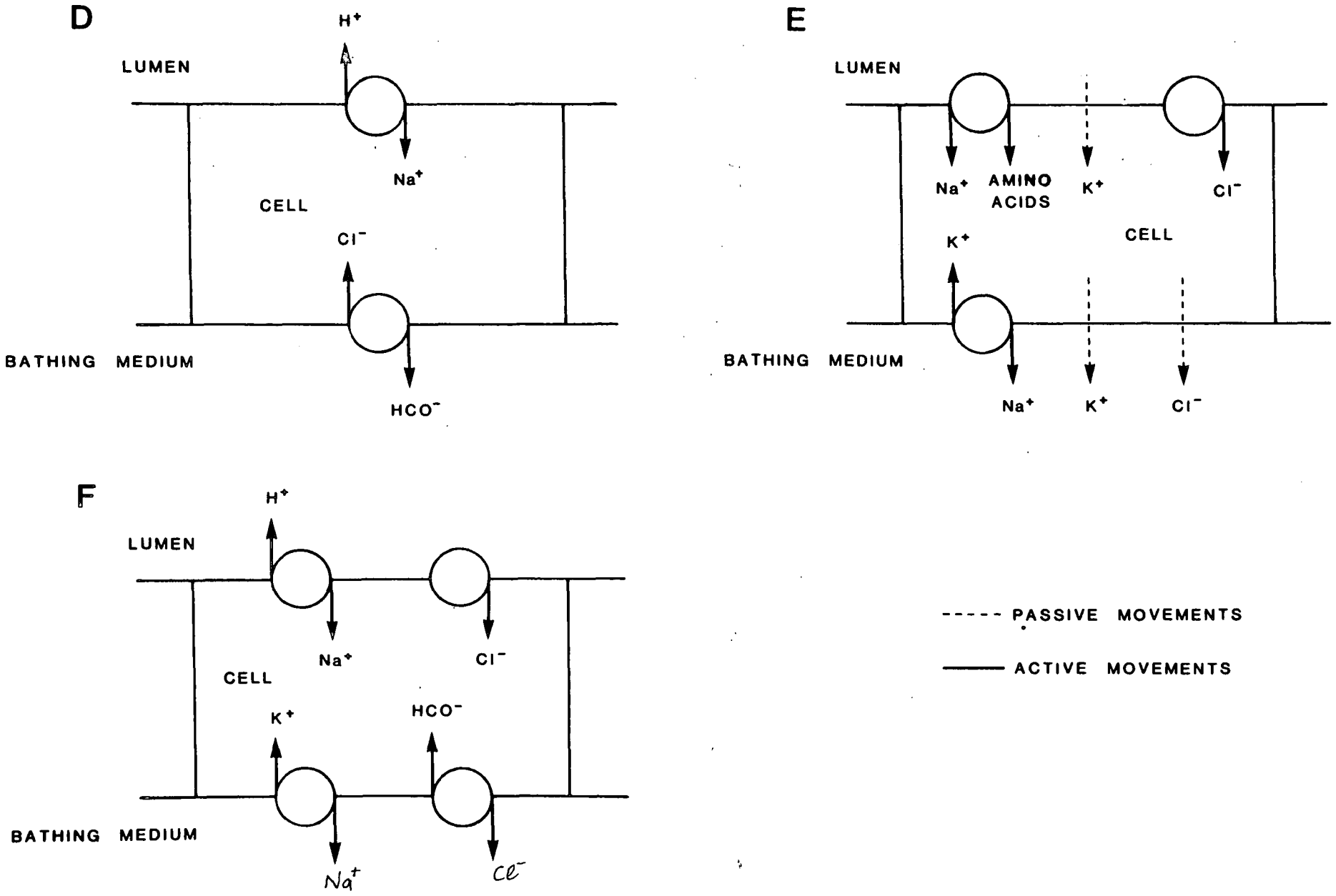

A
Cl⁻
LUMEN
CELL
K⁺
Cl⁻
Na⁺
BATHING MEDIUM
Na⁺
B
Na⁺ Cl⁻
LUMEN
CELL
K⁺
HCO⁻
Na⁺
BATHING MEDIUM
Na⁺
Cl⁻
C
Cl⁻
LUMEN
CELL
K⁺
BATHING MEDIUM
Na⁺
Na⁺
K⁺
2Cl⁻
----- PASSIVE MOVEMENTS
—— ACTIVE MOVEMENTS

of Cl^- into the cell, with this anion exiting either via a conductive pathway or by means of an electroneutral K^+Cl^- symport on the basolateral membrane.

Fig. 2D This model shows the exit of HCO_3^- from rabbit proximal tubule during acid secretion (Reuss et al., 1983). Acid secretion involves the movement of H^+ from the cell into the tubule lumen. The mechanism of HCO_3^- exit involves an electroneutral basolateral Cl^--HCO_3^- exchanger. Related models have shown that HCO_3^- may exit the cell via an electrogenic HCO_3^- pathway or an electrogenic transporter that mediates the coupled exit of HCO_3^- and Na^+ (Reuss et al., 1983).

Fig. 2E In a model to describe ion translocation in locust rectum, Hanrahan and Phillips (1984) have proposed that during transepithelial absorption, Cl^- enters the cell across the apical membrane by an active mechanism which is thought to be electrogenic and K^+-stimulated. The entry of K^+ occurs through a parallel conductive pathway whilst Na^+ and amino acids enter by means of a cotransporter. It is thought that Cl^- exits across the basal membrane via a conductive pathway, with K^+ probably leaving the cell by electrodiffusion. The whole cellular mechanism is maintained by the action of a Na^++K^+-ATPase.

Fig. 2F In this model described to explain Cl^- absorption in Amphiuma intestine, White (1980 and 1986) has proposed that an electrogenic Cl^- uptake mechanism occurs in the apical membrane with Cl^- leaving the cell by means of a basolateral Cl^--HCO_3^- exchange. This process, may be in part, controlled by an apical membrane Na^+-H^+ exchanger which affects the availability of HCO_3^- for the basolateral Cl^--HCO_3^- exchange.


D
H+
LUMEN
Na+
CELL
Cl−
BATHING MEDIUM
HCO−
E
LUMEN
Na+
AMINO ACIDS
K+
Cl−
CELL
K+
BATHING MEDIUM
Na+
K+
Cl−
F
H+
LUMEN
Na+
Cl−
CELL
K+
HCO−
BATHING MEDIUM
Na+
Cl−
----- PASSIVE MOVEMENTS
—— ACTIVE MOVEMENTS

by the basolateral Na^++K^+-ATPase pump (see Fig. 2A and B). Similarly, in Na^+, K^+, Cl^- cotransport the energy for Cl^- transport is apparently provided by the cation electrochemical gradient (Phillips and Hanrahan, 1984; Fig. 2C). The Na^+ (or Na^+,K^+) coupled secretion mechanism is located within the apical membrane if Cl^- is actively absorbed by the epithelium or is located within the basolateral membrane if Cl^- is actively secreted (Gerencser and Lee, 1983; Fig. 2A,B and C).

A broad range of vertebrate transporting epithelia have been shown to exemplify NaCl co-transport absorption and secretion, including frog cornea, canine tracheal epithelium and shark renal proximal tubules (Candia, 1986; Welsh, 1983; Sawyer and Beyenbach, 1985b; Frizzell et al., 1979; Gerencser and Lee, 1983). The more recently described Na^+, K^+, Cl^- cotransport has also been implicated in both transepithelial absorptive and secretory salt transport in a variety of species and cell types, notably epithelial cells such as shark rectal gland tubules and mammalian kidney nephron (Greger and Schlatter, 1984; Greger, 1985; Rugg et al., 1986). Indeed, it is this transporter which O'Donnell and Maddrell (1984) have proposed to occur in the basal membrane of tubule cells of Rhodnius (Fig. 1B). Recent reviews on the Na^+,K^+,Cl^- transporter (Palfrey and Rao, 1983; Geck and Heinz, 1986) have shown it to be electroneutral with a stoichiometry of 1 Na^+ : 1 K^+ : 2 Cl^-. Palfrey and Rao (1983) point out that it is as yet unclear whether NaCl and NaKCl cotransport mechanisms are distinguishable, or whether all NaCl cotransport systems involve K. However, some NaCl cotransport systems do not seem to involve K (Ericson and Spring, 1982).

The widely accepted epithelial Cl^- transport process involving Cl^--HCO_3^- countertransport or exchange has been found in a variety of tissues (Gerencser and Lee, 1983) including the rectal salt gland

epithelium of Aedes (Strange and Phillips, 1984; Fig. 2B). The energy source for this process is unknown but it has been suggested that uphill Cl^- transport is energized by a favourable downhill electrochemical potential gradient for HCO_3^- (Frizzell et al., 1979). In Aedes rectal gland, the actively coupled 1 : 1 Cl^--HCO_3^- exchange is thought to occur on the basolateral membrane (Strange and Phillips, 1984). Recent research has pointed to double exchange as opposed to direct cotransport as a possible mechanism by which the transport of Cl^- could be coupled to the flux of Na^+ in some epithelia (Warnock et al., 1983). This electroneutral process involves the parallel operations of Na^+-H^+ and Cl^--HCO_3^- (or Cl^--OH^-) exchanges coupled by circular proton movements. Such a mechanism has been described as the predominant or sole mechanism of apical membrane NaCl entry in Necturus gallbladder (Reuss, 1984, but see Spring in Warnock et al., 1983). It has been pointed out in the literature that although differences occur between the two mechanisms as to the effects of various transport inhibitors, an absolute, unambiguous test for distinguishing double exchange from direct cotransport does not occur for an intact epithelium (Warnock et al., 1983).

The exit of Cl^- from cytoplasm to lumen or blood is less well studied than Cl^- entry. Since there appears to be a favourable electrochemical gradient for Cl^- movement out of the cell in many tissues then Cl^- movement across the exit membrane may be downhill if the conductance is sufficiently high (White, 1986; Frizzell et al., 1979; Fig. 2A and C). Such an exit mechanism has been suggested for a wide variety of epithelia including 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 1981; Sansom et al., 1984). Guggino and Giebisch (in Reuss et al., 1983) have suggested that as well as a conductive pathway, Cl^- can leave the

cell by an non-electrodiffusive 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; Fig. 2B). This mechanism has recently been proposed by Kuijpers et al. (1984) to explain fluid secretion in rabbit pancreas and is thought to be driven by the Na^+ gradient in the basolateral membrane. Furthermore, in Amphiuma intestinal cells, Cl^- exit is thought to occur via a tentative $Na\text{-}Cl\text{-}HCO_3^-$ exchange (White et al., 1984; White, 1986). It appears that a Na^+-independent cotransport of K^+ and Cl^- in the form of a KCl symport may be responsible for Cl^- exit in various transporting epithelia including Necturus proximal tubule and gallbladder (Reuss et al., 1983; Corcia and Armstrong, 1983). Gupta and Hall (1983) have suggested that uphill Cl^- entry across the basal membrane of cockroach salivary gland cells may be driven by a large downhill gradient of Na^+. Although these authors have proposed a passive exit for Cl^-, they state that the transcellular movement of Cl^- (and the entire secretory mechanism) could be driven by a Cl^- pump at the apical membrane.

The ability to regulate internal H^+ ion concentration is a virtually universal attribute of living cells. It appears that acid production by cellular metabolism or H^+ permeation down an electrochemical gradient requires extrusion of protons (or absorption of alkali) to maintain a constant cellular pH (Ross and Boron, 1981). Thus, in many systems the downhill movement of Na^+ via a $Na^+\text{-}H^+$ exchange drives secondary active H^+ secretion out of the cell. Intracellular OH^- generated by H^+ secretion is buffered by CO_2 to form HCO_3^- (catalysed by the enzyme carbonic anhydrase). The exit of base from the cell may occur by passive diffusion of ionic HCO_3^- although evidence for coupled transport mechanisms such as $Cl^-\text{-}HCO_3^-$ exchange or electrogenic $Na^+\text{-}HCO_3^-$ cotransport have also

been shown (Aronson, 1983; Fig. 2D). It is of interest that both Na^+-H^+ exchange and carbonic anhydrase have important transport roles. Since the discovery of a directly coupled Na^+-H^+ exchange process (Murer et al., 1976), this electroneutral transporter has been found in a variety of renal tissue (Aronson, 1981; 1983). The Na^+-H^+ exchanger appears to occur largely on the apical membrane of absorptive tissue (Warnock et al., 1983), although research has suggested a basal location in some tissues (Boron and Boulpaep, 1983). The carbonic anhydrase enzyme has been found in insect ion transporting epithelia (Henry, 1984) and has been documented histochemically in Malpighian tubules of praying mantis (Polya and Wirtz, 1965). Henry (1984) points out that the distribution of carbonic anhydrase in arthropod tissues suggests that the enzyme is involved in ion transport processes. Its presence in ion transporting tissue such as insect midgut epithelium, Malpighian tubules and anal papillae, and crustacean gills is a strong indication that this enzyme plays a role in blood ion regulation (Henry, 1984; Strange and Phillips, 1984).

A considerable amount of Cl^- and HCO_3^- transport data has accumulated that does not conform to any of the well-established systems described above. This has led to the long-standing controversy as to the existence in animals of a primary Cl^- (and HCO_3^-) transport process (e.g. De Pont and Bonting, 1981; Gerenscer and Lee, 1983) involving an anion-ATPase in the plasma membrane (see Fig. 2E and F). Since its discovery by Kasbekar and Durbin (1965) anion-stimulated ATPase activity has been demonstrated in both microsomal and mitochondrial fractions of many tissues (De Pont and Bonting, 1981; Schuurmans, Stekhoven and Bonting, 1981; Gerenscer and Lee, 1983). Indeed, the presence of a Mg^{2+}-dependent anion-stimulated ATPase has been demonstrated in microsomal preparations from Malpighian tubules of Locusta (Anstee and Fathpour, 1979; 1981).

As the name of this enzyme implies, it is involved in the transport of anions, especially HCO_3^- and Cl^- and is variously referred to as a Mg^{2+}-dependent $Cl^- + HCO_3^-$-stimulated ATPase, a HCO_3^--stimulated ATPase or a Cl^--stimulated ATPase. Although considerable controversy surrounds the role of anion-stimulated ATPases in ion transport, it has been suggested that it has a function in the movement of ions and water across various epithelia (Kasbekar and Durbin, 1965; Simon and Thomas, 1972; Herrera et al., 1978; Jungreis, 1979). However, to assign a direct role of Cl^- or HCO_3^- transcellular transport to an ATPase, the enzyme should be shown to be an integral component of the plasma membrane (Gerenscer and Lee, 1983). De Pont and Bonting (1981) and Schuurmans Stekhoven and Bonting (1981) have stated that microsomal or plasma membrane localization of this enzyme is entirely due to mitochondrial contamination. However, various workers have demonstrated anion-stimulated ATPase activity in plasma membrane fractions either with virtually no mitochondrial contamination or a differentiated mitochondrial counterpart (Gerenscer and Lee, 1983; Komnick, 1978; Cole, 1979; Garner et al., 1983).

Despite the controversy, there is some evidence that anion ATPase activity is located in the plasma membrane of a variety of epithelia including locust rectal epithelia (Hanrahan and Phillips, 1983), Aplysia gut epithelia (Gerenscer, 1983), Amphiuma intestinal epithelia (White, 1980; 1986) and bullfrog intestinal epithelia (Armstrong et al., 1972) (see Fig. 2E and F). Other indirect evidence has occurred for dragonfly rectal epithelia (Komnick, 1978) and locust rectal epithelium (Herrera et al., 1978). Although Fathpour (1980) has hinted that a HCO_3^--ATPase may occur for transport of water and anions across the Malpighian tubules of Locusta, this worker was unable to ascertain with certainty whether the cellular location of this enzyme was mitochondrial

or non-mitochondrial in origin or both. In the rectum of Schistocerca, Hanrahan and Phillips (1983; 1984) have described an unusual chloride transport system which absorbs Cl^- from a KCl-rich Malpighian tubule fluid. This electrogenic Cl^- transport is activated and stimulated directly by K^+ and is independent of Na^+ and HCO_3^-. Herrera et al. (1977; 1978) have also found evidence of an active mechanism, probably on the basal side of the cells, for transporting Cl^- from the lumen to the haemolymph across the rectal wall in Schistocerca. Gerenscer (1983) has presented results that are consistent with an active Cl^- extension process which exists in the basolateral membrane of Aplysia intestinal epithelium. This mechanism is electrogenic and is independent of Na^+ and may be a Cl^-+HCO_3^--stimulated ATPase (Gerenscer and Lee, 1985).

The study of the divalent cation Ca^{2+} in transport processes, especially among insect epithelia, is limited in comparison to studies on monovalent ions. However, since Douglas' (1968) proposal of a central role for Ca^{2+} in secretory processes, it has become evident that this cation 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). Indeed, recent evidence indicates that calcium plays an important role in regulating net transport of ions and water in transporting epithelia (Windhager and Taylor, 1983; Taylor and Windhager, 1979). For a proper understanding of the role of Ca^{2+} in cellular function, it is crucial to have clear information regarding the mechanism by which free intracellular Ca^{2+} is regulated. It appears that in addition to the contribution of intracellular sequestering organelles in such control, voltage-dependent and receptor-operated calcium channels are also important (Spedding, 1985), although the function of such Ca^{2+} channels as a major pathway for Ca^{2+} influx (down the electrochemical gradient) is best charcterized in excitable

tissue (Reuter, 1983). Calcium as a conveyor of information is well known in the form of a second messenger in many tissues (Berridge, 1980). Indeed, second messengers feature significantly in conveying information between both surfaces of the cell in a number of transporting epithelia such as intestine, mammalian salivary glands, cornea and insect salivary gland (Berridge, 1980).

More importantly, the Ca^{2+}+Mg^{2+}-ATPase and Na^{+}-Ca^{2+} exchanger have been shown to be an integral part of intracellular Ca^{2+} regulation in many cells. Since its discovery by Schatzmann (1966), a Ca^{2+}-ATPase has been found in a large number of vertebrate tissues (Carafoli and Zurini, 1982) including the basolateral membrane of kidney proximal tubules (Gmaj et al., 1983; Doucet and Katz, 1982) and has been shown to be responsible for the extrusion of calcium ions across the cell membrane against a steep electrochemical Ca^{2+} gradient (Carafoli and Zurini, 1982). Similarly, since the original proposal by Blaustein (1974), the existence of a Na^{+}-Ca^{2+} antiporter, in basolateral membrane vesicles from a variety of transporting epithelia, has been documented (Gmaj et al., 1979; Chase and Al-Awqati, 1981; Chase, 1984). This exchanger operates in an electrogenic fashion with a proposed stoichiometry of 3-5 Na^{+} transported for every Ca^{2+} (Mullins, 1979). Although the physiological and functional importance of Na^{+}-Ca^{2+} exchange in transporting epithelia is still controversial (Snowdowne and Borle, 1985; Mandel and Murphy, 1984), recent research has suggested the exchanger functions by transporting calcium out of the cell in exchange for the movement of sodium ions across the basal membrane (Scoble et al., 1986; Taylor and Windhager, 1983). Thus, this exchange appears to have an important role in the maintenance and function of cytosolic calcium in renal transporting tissue (Scoble et al., 1986).

Remarkably little is known about calcium transport by Malpighian tubules. Ramsay (1956) could find no evidence for active transport of calcium in the tubules of Carausius and Maddrell (1971) has suggested that calcium transport across both the basal and apical membranes of the tubules of Carausius and Calliphora is by passive movements. However, Wright and Cook (1985) have suggested that the surprisingly large amounts of calmodulin (a calcium binding protein responsible for regulating the Ca^{2+}-ATPase - Villalobo et al., 1986) present in Malpighian tubules of the cockroach Leucophaea probably reflects some kind of involvement with the active transport mechanisms of fluid secretion. Among other transporting epithelia, Barkai and Williams (1983) have suggested that calcium pumps consisting of calcium-activated ATPases play an important role in transporting Ca^{2+} from the drinking fluid to the haemolymph and reabsorbing Ca^{2+} which has been excreted by the Malpighian tubules in the urine, in the larva of Aedes. In cockroach salivary gland, House and Ginsborg (1982) have postulated a Na^{+}-Ca^{2+} exchange mechanism on the basal membrane to maintain low levels of intracellular Ca^{2+} activity, with Na linked to feed the Na^{+}+K^{+}-ATPase on the basal side (Gupta and Hall, 1983). Also, Na^{+}-Ca^{2+} exchange appears to be an important mechanism of calcium efflux at the basolateral membrane during transcellular Ca^{2+} transport by the midgut of Calliphora (Taylor, 1984). A basolateral Ca^{2+}-ATPase may also be present in this tissue.

In many insects, fluid secretion and the related ionic processes have been shown to be under control of the putative insect neurosecretory hormone known as diuretic hormone (Phillips, 1981; 1982). Diuretic hormone (D.H.) is a family of neuropeptides present in at least 20 insect species, which probably regulates the passive permeability of

Malpighian tubules (Phillips, 1981; 1982). In the locusts Schistocerca and Locusta, D.H. appears to be a small peptide (Mordue and Goldsworthy, 1969) which is stored in and released from the storage lobes of the corpus cardiacum (Mordue, 1969; 1972). The hormone is released into the haemolymph in response to feeding (Maddrell, 1963; Mordue, 1972; Gee, 1977; Nijhout and Carrow, 1978) thereby reducing the water load incurred as a result of food intake. As noted by Maddrell et al. (1971), Morgan and Mordue (1984) and Gee (1975), all parts of the insect central nervous system may contain extractable diuretic activity. Recently, insect D.H. has been partially purified and characterized in several insects including Locusta, but progress has been limited by the instability of the purified form (Aston and Hughes, 1980; Morgan and Mordue, 1983b; Phillips, 1982).

Over recent years, it has been found that the biogenic amine 5-hydroxytryptamine (5-HT or serotonin) mimics the action of D.H. on the Malpighian tubules of some insects but not others (Phillips, 1981). Similarly, it has been found that external application of the cyclic nucleotide, c AMP, mimics the action of D.H. in many insects (Phillips, 1982) and D.H. stimulation causes an increase in tissue levels of c AMP in Rhodnius and Locusta (Aston, 1979; Rafaeli et al., 1984; Morgan and Mordue, 1984). Furthermore, 5-HT has been implicated in the activation of cyclic nucleotides in many invertebrate tissues (Walker, 1984) and it has been shown that this amine increases the intracellular concentration of c AMP in the salivary glands of Calliphora (Berridge and Patel, 1968). Morgan and Mordue (1984), however, have shown that 5-HT does not produce an elevation in intracellular c AMP in Locusta tubule cells nor stimulates adenylate cyclase activity in broken cell preparations.

Despite these facts, little is known about the means by which

hormonal regulation of ion and fluid secretion, by the Malpighian tubules of insects, occurs. Prince and Berridge (1973), Berridge (1980) and Berridge and Heslop (1982), however, have examined the receptor mechanisms mediating the action of 5-HT in the salivary glands of Calliphora. In this epithelia, it is thought that the normal information of the 5-HT molecule is decoded into second messengers in the form of c AMP and calcium through a specific interaction with a cellular receptor (Prince and Berridge, 1973). Thus, a 5-HT_1 receptor opens channels for calcium and a 5-HT_2 receptor activates adenylate cyclase to generate c AMP (Berridge and Heslop, 1982). The second messengers c AMP and Ca^{2+} are then responsible for mediating the ability of 5-HT to greatly accelerate the secretion of ions and water (Berridge, 1980). Recent research has shown that another second messenger, inositol 1,4,5-triphosphate (IP_3), is generated by the 5-HT_1 receptor (Berridge, 1986) and this appears to mobilize intracellular calcium (Berridge et al., 1984).

It is difficult to apply this model for hormone-stimulated fluid secretion by the salivary glands of Calliphora to Malpighian tubules as 5-HT is thought to be the physiological hormone in the former epithelia (Trimmer, 1985). Indeed, although Maddrell et al. (1971) found that the Malpighian tubules of Rhodnius and Carausius were sensitive to remarkably low concentrations of 5-HT, they were able to conclude that 5-HT was not the physiological hormone released in response to feeding. Despite this, Morgan and Mordue (1984) have proposed a hypothetical model for the hormonal control of fluid secretion by the tubules of Locusta. 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 c AMP synthesis and the other triggering a different secondary cellular event (R_2), possibly

increasing intracellular Ca^{2+} concentration. It is suggested that D.H. 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 at R_2. Two independent agonists may act at the two receptor sites, with maximal activity being achieved by stimulation via either receptor (Morgan and Mordue, 1984).

In view of the lack of information concerning the nature of the mechanisms of ion and water transport across the Malpighian tubules of insects, the present study has been carried out to examine the electrical events associated with ion movements across cells of the tubules of Locusta. From this, it is hoped to construct a model which describes the ionic fluxes which support fluid secretion by the Malpighian tubules of Locusta. In addition, a study was undertaken to characterize ouabain binding to the Na^++K^+-ATPase of Malpighian tubules of Locusta, to determine the sensitivity of the enzyme preparation to ouabain and to assess the likely contribution of the Na^++K^+-ATPase 'pump' to cation translocation in this tissue.

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 hours light : 12 hours dark. Circulation of air was provided by three electric fans and a continuous air exchange was maintained by a fan-driven ventilator (Xpelair). Humidity was controlled by three humidifiers (Lumatic, Humidifier Group, Bromley, Kent, England). Locusts were kept 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 which resulted in cage temperatures varying from 30° - 40°C depending on the distance from the bulb and the photoperiod. The humidity inside the cage also varied with the addition of fresh food and water. Locusts were fed daily on fresh grass, water and Bemax. Animals were reared at sufficiently high population density to prevent reversion to the solitaria phase (Joly and Joly, 1953).

Glassware and Reagents

Pyrex glassware was used throughout and prior to use was cleaned by soaking overnight in a 2% (w/v) solution of 'Quadralene' laboratory detergent followed by rinsing in tap water (four times). It was then dried in an oven at 100°C.

All chemicals and drugs were AnalaR grade or the purest commercially available and were largely supplied by Sigma Co., Poole, Dorset, England or B.D.H., Poole, Dorset, England. Sodium acetazolamide (Diamox) was obtained from Lederle (American Cyonamid Company, Pearl River, N.Y., U.S.A.) and Amiloride was a gift from Merck Sharp and Dolme Research

Laboratories (Heddesdon, Hertfordshire, England). Bumetanide was kindly supplied by Dr. S.H.P. Maddrell and was originally obtained from Leo Pharmaceutical Products, Denmark.

Solutions

The composition of the 'Normal' insect saline was as follows (mM): NaCl 100, KCl 8.6, $MgCl_2(6H_2O)$ 8.5, $CaCl_2$ 2, NaH_2PO_4 4, $NaHCO_3$ 4, Glucose 34, HEPES 25, NaOH 11, pH 7.2. In ion substitution experiments alternative salines were used (Table 1). Various potassium concentrations were obtained by mixing calculated amounts of high K^+ and zero K^+ saline (these had the following K^+ concentrations (mM): 128, 64, 32, 8.6, 4.3, 2.5, 1.25 and 0 K^+). All solutions were adjusted to pH 7.2.

In studies involving monensin, verapamil, A23187, SITS, furosemide and bumetanide it was necessary to dissolve each agent in spectroscopically pure absolute ethanol (Fison Scientific Apparatus, Loughborough, Leics., England) before adding it to the appropriate saline. In all experiments involving such agents, the same concentration of solvent (< 0.1%) was included in the control.

Oxygen Electrode

Measurements of oxygen consumption by Malpighian tubules were carried out using a polarographic electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A., YSI Model 53) which is of similar design to the Clark oxygen electrode (Clark, 1956). The former oxygen electrode has been described in detail by Fathpour (1980).

Adult female locust Malpighian tubules were dissected out under ice-cold 'Normal' saline, weighed after blotting dry and placed in the incubation chamber of the oxygen electrode which contained 3 mls of air saturated 'Normal' saline at 30 ± 0.1°C. Following a 10 min equilibation period, the rate of oxygen consumption was determined polarographically.

Table 1

Composition of different salines used in the study of *in vitro* Malpighian tubules of *Locusta*. All values are in mM.

*Note: The K^+ concentration in Na^+ free saline was 8 mM and not 8.6 mM as in 'Normal' saline.

Saline (Values in mM)

	'Normal'	High K^+ (Na^+ free)	K^+ free	Cl^- free	Na^+ free*	High K^+, Cl^- free	K^+ free Cl^- free	K^+ free, Na^+ free	High K^+, Low Na^+	Ca^{2+} free	High K^+, Ca^{2+} free
NaCl	100	-	108.6	-	-	-	-	-	10	100	-
KCl	8.6	108.6	-	-	-	-	-	-	98.6	8.6	108.6
$CaCl_2$	2	2	2	-	2	-	-	2	2	-	-
$MgCl_2.6H_2O$	8.5	8.5	8.5	-	8.5	-	-	8.5	8.5	8.5	8.5
NaH_2PO_4	4	-	4	4	-	-	4	-	-	4	-
KH_2PO_4	-	4	-	-	4	4	-	-	4	-	4
$NaHCO_3$	4	-	4	4	-	-	4	-	-	4	-
$KHCO_3$	-	4	-	-	4	4	-	-	4	-	4
Glucose	34	34	34	34	34	34	34	34	34	34	34
HEPES	25	25	25	25	25	25	25	25	25	25	25
NaOH	11	-	11	11	-	-	11	-	-	11	11
KOH	-	11	-	-	-	11	-	-	11	-	-
Na Gluconic Acid	-	-	-	100	-	-	108.6	-	-	-	-
K Gluconic Acid	-	-	-	8.6	-	108.6	-	-	-	-	-
Ca Gluconic Acid	-	-	-	2	-	2	2	-	-	-	-
Mg Gluconic Acid	-	-	-	8.5	-	8.5	8.5	-	-	-	-
Choline Chloride	-	-	-	-	108.6	-	-	108.6	-	-	-
Trizma Base	-	-	-	-	11	-	-	11	-	-	-
Choline Bicarbonate	-	-	-	-	-	-	-	4	-	-	-
Trizma Phosphate	-	-	-	-	-	-	-	4	-	-	-
EGTA	-	-	-	-	-	-	-	-	-	2	2

The consumption of oxygen was monitored with a Servoscribe pen recorder (Goerz Electro) and the data are expressed in µmoles oxygen consumed/g wet weight/min. The oxygen content of the saline in the incubation chamber was determined on the basis that the oxygen content of 1 ml of saline saturated with air at 30°C contained 0.223 µmoles oxygen (Chappell, 1964). Thus for the 3 mls of reaction medium used in this study, the oxygen content was 0.669 µmoles oxygen.

Determination of ATPase Activity

The methods of preparation of membrane microsomal fractions, measurement of ATPase activity and the estimation of microsomal protein are as described by Peacock et al. (1976) and Anstee and Bell (1975) (see also Anstee and Bowler, 1984).

Reagents (Final Concentration)

Homogenization medium (pH 7.2)

Histidine/HCl	40 mM
Mannitol	250 mM
EDTA	5 mM
Sodium deoxycholate	0.1 w/v

Sodium iodide extraction medium (pH 7.2)

$MgCl_2$	5 mM
NaI	4 mM
EDTA	10 mM

Washing Medium

NaCl	5 mM
EDTA	5 mM

Ionic reaction medium for total ATPase (pH 7.2)

$MgCl_2$	4 mM
NaCl	100 mM
KCl	20 mM
Histidine/HCl	50 mM

Ionic reaction medium for Mg^{2+} ATPase (pH 7.2)

$MgCl_2$	4 mM
Histidine/HCl	50 mM

Reaction stopping solution (Cirrasol solution) (Atkinson et al., 1973)

Mix equal volumes of 1% Cirrasol ALN-WF in deionized water with 1% ammonium molybdate in 0.9 M H_2SO_4.

The reaction was stopped by adding 4 mls of this mixture to each solution.

Substrate used

Tris ATP	3 mM

The disodium salt of ATP was converted into Tris ATP by a method described by Anstee and Bowler (1984). This involved suspending 20 g Dowex 50-X8 resin in 1 ℓ of 1M HCl and stirring for 30 mins. After the Dowex beads had been allowed to settle, the acid was poured off. The beads were then washed with deionized water until the washings were at pH 3-4. These washed beads were stored wet at 4°C until required.

A layer of charged Dowex was placed in a Buchner funnel lined with 2-3 layers of moistened filter paper. A known volume of disodium ATP solution was added and left to drain through slowly without suction. This procedure was repeated six times. The effluent was collected in a conical flask as ATP in an acid form. This was then converted to Tris salt by the addition of drops of 2M Tris until the pH was 7.2, made up to the required volume and stored at 20°C.

(a) Preparation of Microsomal Fraction

Forty adult female locusts were killed by twisting the head so as to break 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 Malpighian tubules and a small 'collar' of attached gut were quickly dissected free under ice-cold homogenizing medium. Tubules were pooled in 10 mls of fresh ice-cold homogenizing medium and homogenized. After this process, the resulting homogenate was extracted with an equal volume (10 mls) of ice-cold NaI extraction medium for 30 mins at 0-4°C (Nakao et al., 1965). The extract was then diluted to 50 mls with ice-cold deionized water and centrifuged at 50,000 g at 0-4°C for 30 mins. The resulting pellet was discarded and the supernatant centrifuged at 100,000 g for 60 mins. The supernatant was discarded and the resulting pellet was resuspended in approximately 10 mls of washing medium and centrifuged at 100,000 g for 45 mins. This washing procedure was repeated twice, centrifuging for 20 mins each time. The final microsomal pellet was suspended in an appropriate volume of ice-cold deionized water.

(b) ATPase Activity

Incubations were carried out at 30 ± 0.1°C for 20 or 30 mins. Pairs of boiling tubes containing 1 ml of the appropriate ionic medium and 0.5 mls, 12 mM Tris ATP were set up and equilibrated at 30°C for 10 mins. The reactions was started by the addition of 0.5 mls of the microsomal fraction and was stopped by the addition of 4 mls of freshly prepared Cirrasol solution (Atkinson et al., 1973).

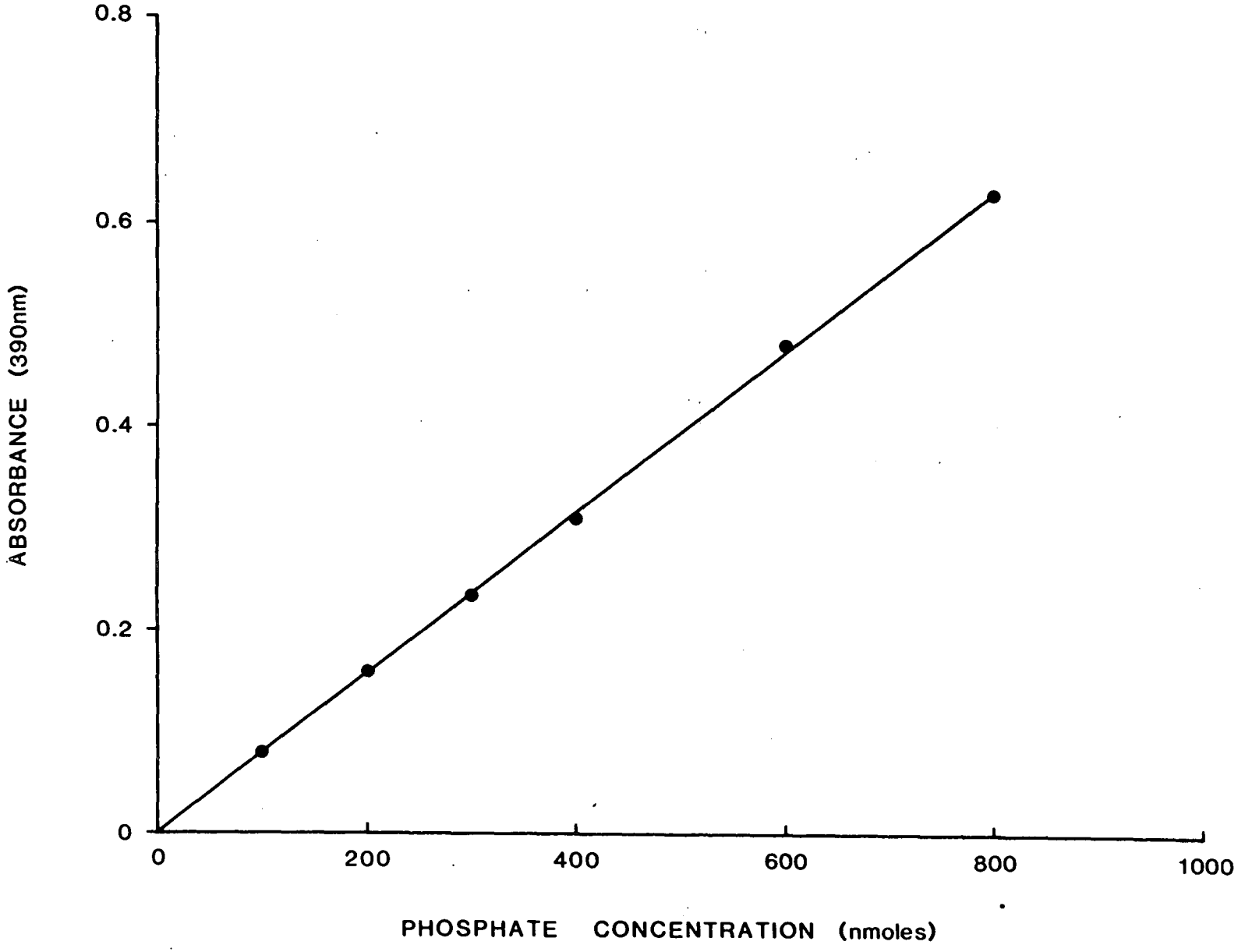
$Na^+ + K^+$-ATPase activity was obtained as the difference in inorganic phosphate (indicated by a yellow colour) released in media containing Na^+, K^+ and Mg^{2+} (with and without 1mM ouabain) and Mg^{2+} alone. Inorganic phosphate released was proportional to the absorbancy value measured at 390 nm in a Pye Unicam 1800 Dual Beam Spectrophotometer. The amount of phosphate released was determined by reference to a standard calibration curve (Fig. 3). This was prepared from the serial dilution of a stock phosphate solution containing 20 µg phosphorus (as KH_2PO_4/ml). Samples

Fig. 3

A standard calibration curve for determination of inorganic phosphate.

Ordinate: Absorbance 390 nm

Abscissa: n moles Inorganic Phosphate


0.8
0.6
0.4
0.2
0
ABSORBANCE (390nm)
0
200
400
600
800
1000
PHOSPHATE CONCENTRATION (nmoles)

of 20, 15, 10, 5, 2, 1 and 0 µg Pi/ml were used. To 2 mls of each sample, 4 mls of Cirrasol solution was added with the tubes being allowed to stand at room temperature for 10 mins before the absorbancy was measured. Enzyme activities were expressed as µmoles inorganic phosphate liberated/ mg protein hr^{-1}.

This method was modified slightly to determine the inhibition of $Na^{+}+K^{+}$-ATPase by ouabain (Anstee and Bowler, 1984). Microsomes were pre-incubated for 15 mins in the presence of Na^{+},Mg^{2+}, ATP and ouabain (0 to 10^{-3} M) before the reaction was started by the addition of K^{+} in a ouabain solution.

(c) Protein Estimation

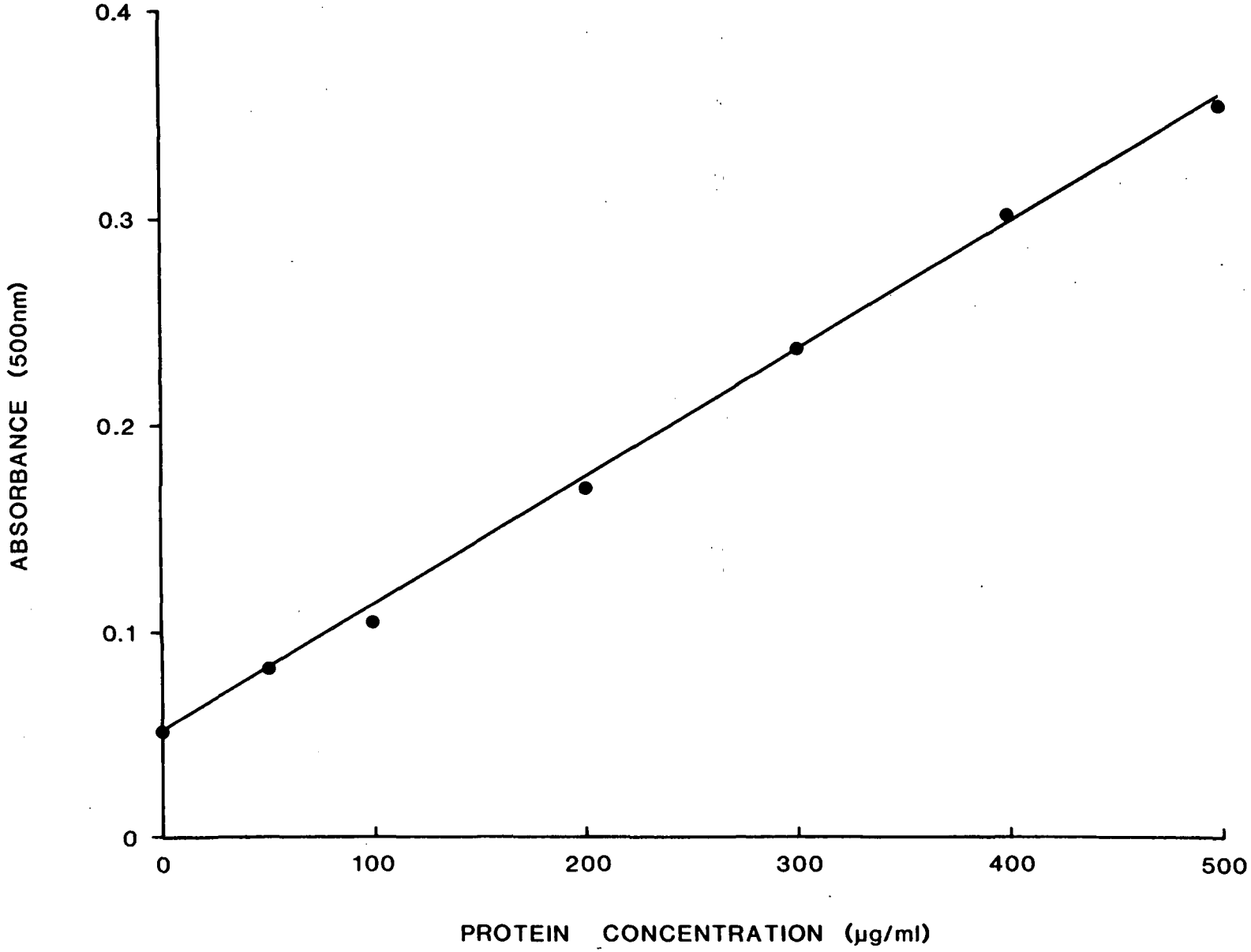
Protein estimations were made by the method of Lowry et al. (1951) using bovine serum albumen Fraction V (BSA) as a standard, and Folin Solution A and Folin Solution B. Folin Solution A was prepared by mixing equal volumes of 0.5% $CuSO_4$ and 1% KNa Tartrate and to each volume of this mixture adding 50 volumes of 2% Na_2CO_3. Folin Solution B was prepared by diluting 4 volumes of Folin Ciocalteaus phenol reagent with 6 volumes of deionized water. A 500 µg/ml stock solution of BSA was serially diluted to give samples with the following protein concentration: 500, 400, 300, 200, 100, 50 and 0 µg/ml. To calculate the protein concentration, 3 mls of Folin Solution A was added to 0.2 mls of protein solution and allowed to stand for 30 mins at room temperature. Then 0.3 mls of Folin Solution B was added and the resulting solutions were allowed to stand for a further 60 mins at room temperature before the absorbancy was read. A standard calibration curve (Fig. 4) relating absorbancy at 500 nm to protein concentration was constructed. The protein concentration was expressed in µg/ml.

Fig. 4

A standard calibration curve for determination of protein.

Ordinate: Absorbance at 500 nm

Abscissa: Concentration of Protein in µg/ml


0.4
0.3
0.2
0.1
0
ABSORBANCE (500nm)
0
100
200
300
400
500
PROTEIN CONCENTRATION (µg/ml)

Ouabain Binding

The binding of ouabain with Malpighian tubule membrane microsomal preparation from Locusta was determined by a rapid Millipore filtration procedure similar to that described elsewhere (Hansen, 1971; Harms and Wright, 1980; Rubin et al., 1981; Anstee and Bowler, 1984). Approximately 1 mg microsomal enzyme protein was incubated in 5 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl, 3 mM ATP in 20 mM imidazole/HCl (pH 7.2) with [3H]-ouabain at 30°C. At the end of the appropriate incubation period a sample of the medium (usually 100 µl) was removed and rapidly filtered through nitrocellulose filters (pore size 0.45 µm) on a Millipore sampling manifold by suction. Following washing with three separate 5 ml aliquots of cold (0-4°C) washing medium, whose composition was identical to that of the incubation medium but without ouabain or ATP, the filters were dissolved in Liquiscint scintillation cocktail (National Diagnostics) and the amount of labelled ouabain determined by liquid scintillation counting in a Packard TRI-CARB 300C liquid scintillation counter. Counting efficiency was approximately 30%. Quenching was monitored by the external standard channel ratio that was calibrated by internal standards (Kazazoglou et al., 1983). Non-specific ouabain-binding was determined by running a parallel set of incubations in which 3 mM unlabelled ouabain ('excess') was also present in the incubation medium. Specific binding of ouabain was obtained by substration of the ouabain bound non-specifically. Dissociation of ouabain from the enzyme preparation was determined by a chase method (Wallick et al., 1980). The amount of membrane suspension needed was allowed to bind [3H]-ouabain at 30°C, as described above, for a sufficient time (45 mins) for equilibrium to be attained. At this time an excess of unlabelled ouabain was added to a final concentration of 1 mM and at appropriate times aliquots were removed, filtered, washed and counted. [3H]-ouabain (1.55 TBq/mmol) was obtained from Amersham International plc., Amersham, England.

Determination of Malpighian Tubule Cell Numbers

The number of cells per Malpighian tubule was estimated by counting the number of nuclei in a series of known lengths of unfixed tubules. These segments of tubule were then weighed and the weight of 1 mm of tubule calculated. By relating this value to the number of cells in 1 mm of tubule it is possible to obtain an estimate for the weight of a tubule cell and from the weight of the total tubule mass in a locust, the total number of Malpighian tubule cells. This value may then be used in conjunction with determinations of protein levels in microsomal preparations, from known numbers of locusts, to provide an estimation of microsomal protein yield per cell.

Statistical Analysis

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

Electron Microscopy, Cell Size and Lead Staining

(a) Electron Microscopy

In Locusta, Malpighian tubules arise from the alimentary canal and either pass anteriorly to loosely attach to the gut caeca (anterior tubules) or pass posteriorly to loosely attach to the rectum (posterior tubules). For transmission electron microscopy adult female locusts were killed by decapitation and the anterior Malpighian tubules were quickly dissected out in ice cold Normal saline. The tubules were then fixed for 1-1½ hrs at 4°C in Karnovsky's fixative (Karnovsky, 1965) which was composed of:

Solution A	
paraformaldehyde	2 g
distilled water	40 mls
in NaOH	2-6 drops

Solution B	
25% gluteraldehyde	10 mls
0.2 M cacodylate buffer pH 7.3	50 mls

Solutions A and B are mixed in 1 : 1 ratio just before use. After fixing, the tissue was post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.5) for 1 hr before dehydration through a graded series of ethanols to propylene oxide, prior to embedding in Araldite epoxy resin. Silver/grey sections wre cut on a Reichert NK ultratome, post stained with uranyl acetate followed by lead citrate (Reynolds, 1963), prior to their examination in an AE1 801 electron microscope.

(b) Cell Size

Cell size was estimated from measurements made on 5 μm thick serial sections through Malpighian tubules as prepared for electron microscopy above.

(c) Lead Staining of Type 2 Cells

Although one cell type predominates throughout the length of the tubules (Peacock, 1975), two types of cell have been found in the Malpighian tubules of Locusta (Charnley, 1984) - Type 1 or primary cells and Type 2 or stellate cells. The latter are demonstrable by virtue of the fact that they take up lead (Berridge and Oscham, 1969; Peacock, 1975). The relative number of Type 2 cells was estimated using this lead staining method. Whole mounts of anterior Malpighian tubules were prepared by fixing them for 15 mins in 2.5% gluteraldehyde in 0.15 M sodium cacodylate buffer (pH 7.3) containing 0.15% lead nitrate. After washing in fresh

0.15 M sodium cacodylate buffer for 1 hr, the tubules were treated with ammonium sulphide, mounted on glass slides in fresh buffer, examined and photographed under a Nikon Optiphot microscope (method attributed to M. Locke and cited in Berridge and Oscham, 1969).

Rate of Fluid Secretion

In vitro measurements of the rates of fluid secretion by 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, 1981). The gut of an experimental animal with the head still attached was immersed in a small volume of 'Normal' saline in a hollow in a perspex dish. Up to ten tubules were drawn out of the saline bath into the liquid paraffin and looped around a small peg. Each tubule was then partially severed at a convenient point along its length and the rate of secretion determined by measuring the rate of increase in the diameter of the approximately spherical droplet secreted from the cut.

The secretion rate of each tubule was determined by measuring the diameter of the secreted droplet at 5 min intervals over a period of 30 mins after an initial equilibrium period of 15 mins. This gave Rate 1 and at the end of this time, the saline was replaced by a fresh solution which had either the same (the controls) or a different (the experimental) composition. The rate of secretion was re-determined, after an equilibrium period of 20 mins for a further 30 mins (giving Rate 2). The volume of fluid secreted was calculated and expressed in nl/min. The effect of the particular treatment was determined by comparing Rates 1 and 2. In this way each tubule acts as its own control. This is necessary as the rate of secretion varies considerably from tubule to tubule. The temperature throughout was maintained at 30 ± 0.5°C by placing the perspex dish inside a water heated temperature chamber.

Electrophysiology

Malpighian tubules were dissected out as described previously under Normal saline at 30°C. Before experimentation, tubules were placed under liquid paraffin to check for leaks (bubbles of 'urine' emerging from a hole in the tubule). Leaking tubules were discarded. For electrical measurements tubules were set up in a Perspex perfusion chamber which was based on the 'gap' system of Berridge and Prince (1972) (see Figs. 5 and 6). The chamber consisted of five connected baths (A-E) which were coated to a depth of about 2 mm with the silicon elastomer Sylgard 184 (Dow-Corning, Seneffe, Belgium). A section of Malpighian tubule (usually about 17 mm) was drawn through slits connecting baths A, B, C and D and "pinned" into the Sylgard of baths A and D. Microelectrode recordings took place in the experimental bath C which was perfused via a glass inlet tube F. The volume of this bath was 0.5 ml and the perfusion rate was approximately 4 mls/min. Thus, the contents changed every 7.5 s. Superfusion occurred from baths C to E and the perfusate was drawn away from the system via a glass outlet tube G by means of a peristaltic pump (Watson Marlow 502S). Fluid was generally passed on to a waste bottle. The system also had a recycling facility by means of a series of 3-way taps. Fluid entering the experimental chamber arrived from one of various perfusion bottles (P.B.). These bottles were jacketed to allow the contained saline to be thermo-equilibrated at 30°C and were connected to a single Perspex manifold (M) (Fig. 7). A single outlet (O) from this manifold was in connection with the glass inlet tube (F) of the experimental bath C. Tests were carried out from time to time in which amaranth dye was introduced into the system. This confirmed that no observable mixing of solutions occurred at manifold junctions.

All tubing used was P.V.C. (Gallenkamp) or silicon (Watson Marlow).

Fig. 5

A schematic diagram of the Perspex experimental chamber (PC) used for superfusing and the recording of membrane and transepithelial potentials of the Malpighian tubules of Locusta. The chamber consists of five connected baths (A-E), a glass inlet tube with support (F) and a glass outlet tube with support (G). During experimentation, a Malpighian tubule was drawn through slits between baths A to D and rested on a raised Sylgard block (H) in the experimental bath C. It was in this bath that microelectrode recordings from the tubule took place. Perfusion occurred from bath C to E; this two chamber perfusion arrangement ensured no electrical 'pickup' occurred. Areas I and J represent cut away regions in the Perspex chamber into which the Ag/AgCl electrodes were placed (see Fig. 6).

EXPERIMENTAL CHAMBER USED FOR SUPERFUSING

LOCUSTA MALPIGHIAN TUBULES IN VITRO

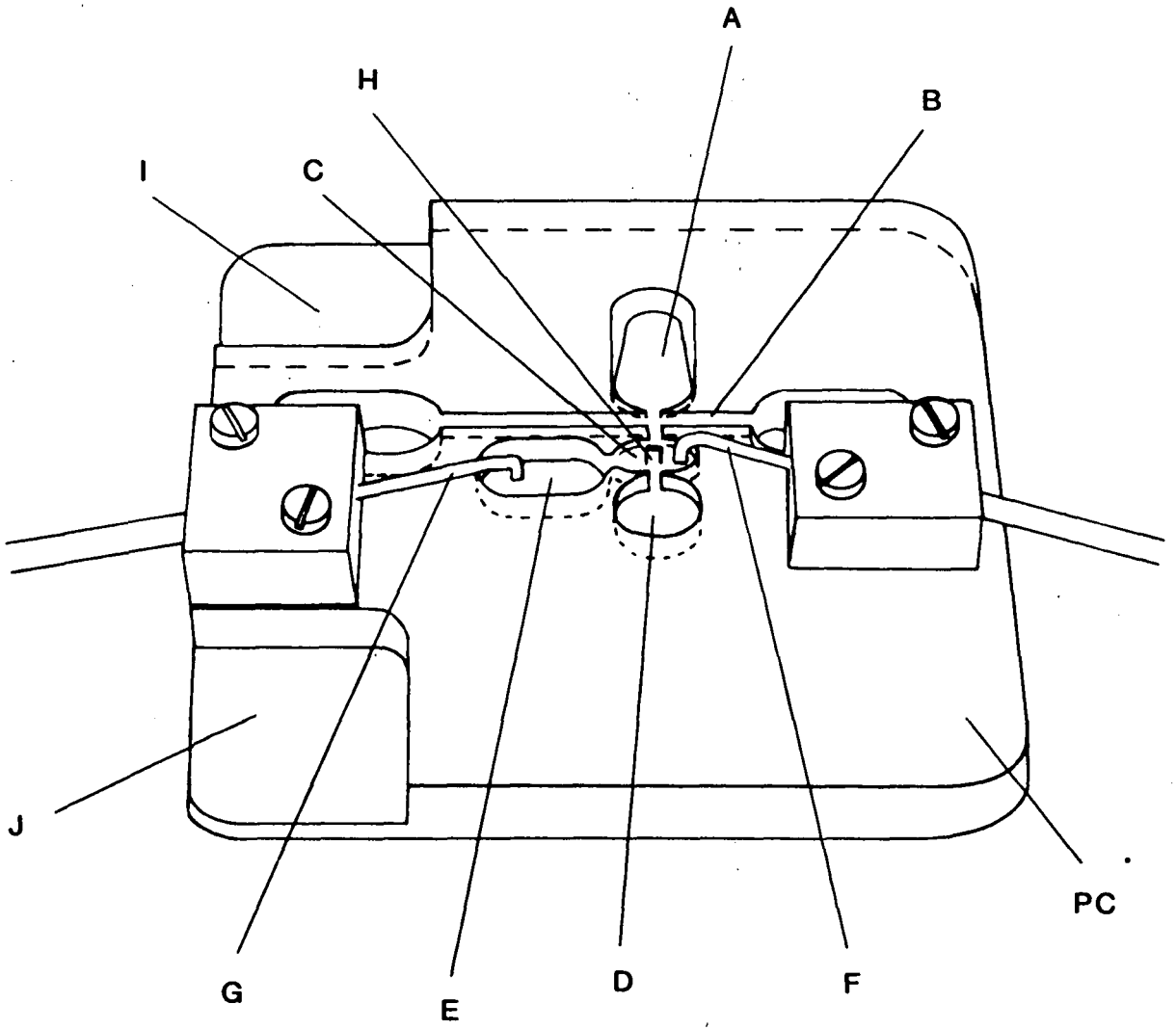


Fig. 6

This shows the position of a Malpighian tubule in the experimental chamber used for superfusing and recording of membrane potentials. Tubules of about 17 mm length were drawn taut from baths A to D and fixed into 'nicks' in the Sylgard bases of these baths to prevent movement. The tubule rested on a raised block of Sylgard (H) in bath C and this ensured that the tubule had a firm base for microelectrode penetration. The portion of tubule in bath A was partially severed using a tungsten needle; ensuring the lumen of the tubule was contiguous with the contents of the experimental bath A. Baths A and C contained saline whilst baths B and D were filled with liquid paraffin (LP) to provide insulation. Surface tension kept saline and liquid paraffin from mixing.

This figure also shows the circuit diagram of the system used for the simultaneous measurement of membrane and transepithelial potentials in the Malpighian tubules of Locusta. The transepithelial potential (T.E.P.) was measured by connecting baths A and C to Ag/AgCl electrodes in 2 M KCl (E) via agar bridges (AG). The Ag/AgCl electrodes were connected to an oscilloscope (CRO) and a pen-recorder via two components of a high input impedance amplifier (PE_1 and E_1). T.E.P. was measured by having one E/AG in bath A and the other in bath C. Membrane potentials were measured by connecting single barrelled microelectrodes (ME) via two components of an amplifier (PE_2 and E_2) to a second channel of the oscilloscope (CRO) and the pen recorder. The presence of a switch box (SB) and the fact that the two amplifier components PE_1 and PE_2 were connected allowed the measurement of the basal cell membrane potential (V_B) and T.E.P. with the perfusate as reference (bath C) and the apical cell membrane potential (V_A) and T.E.P. with the luminal fluid as reference (bath A).

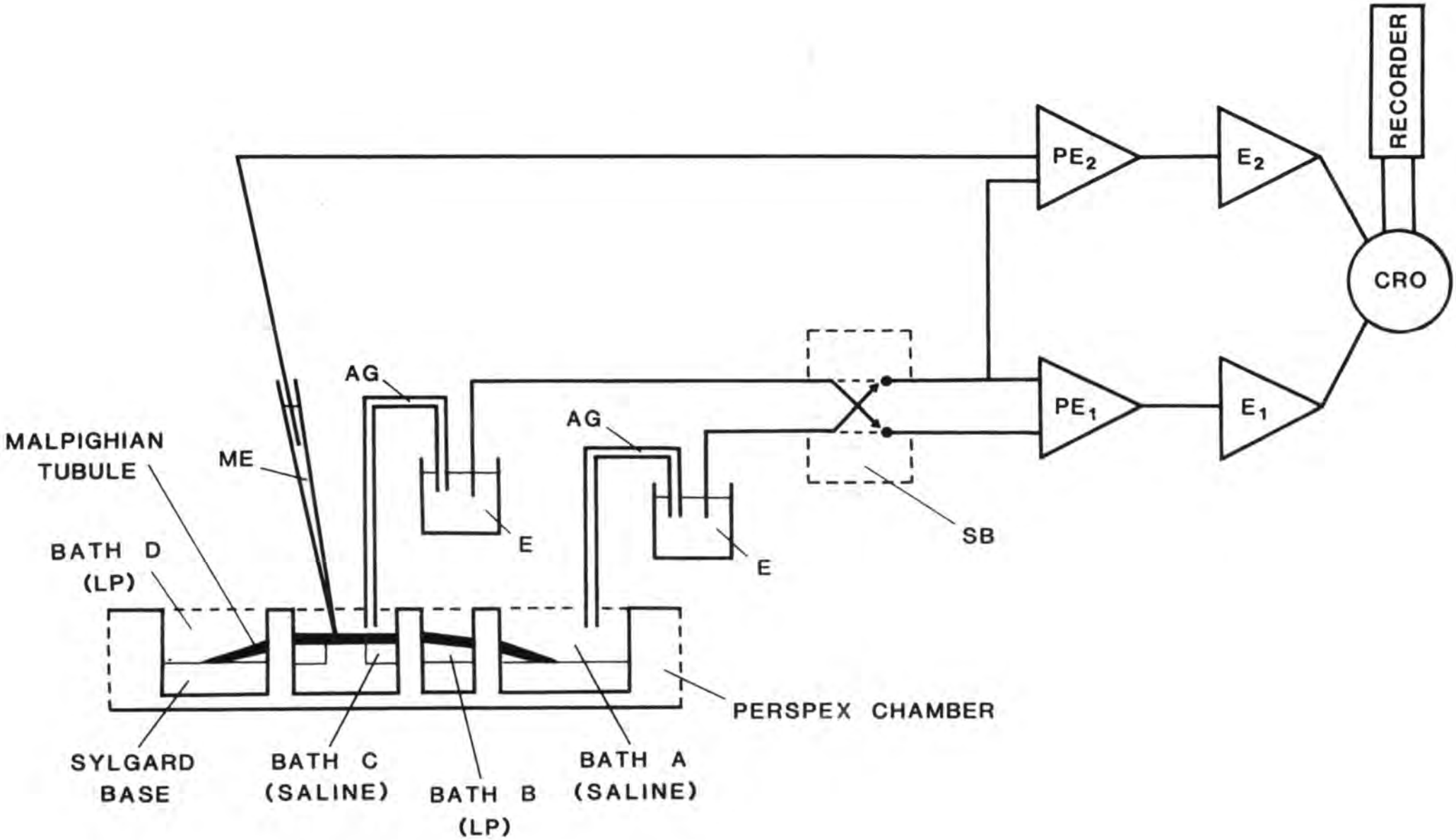
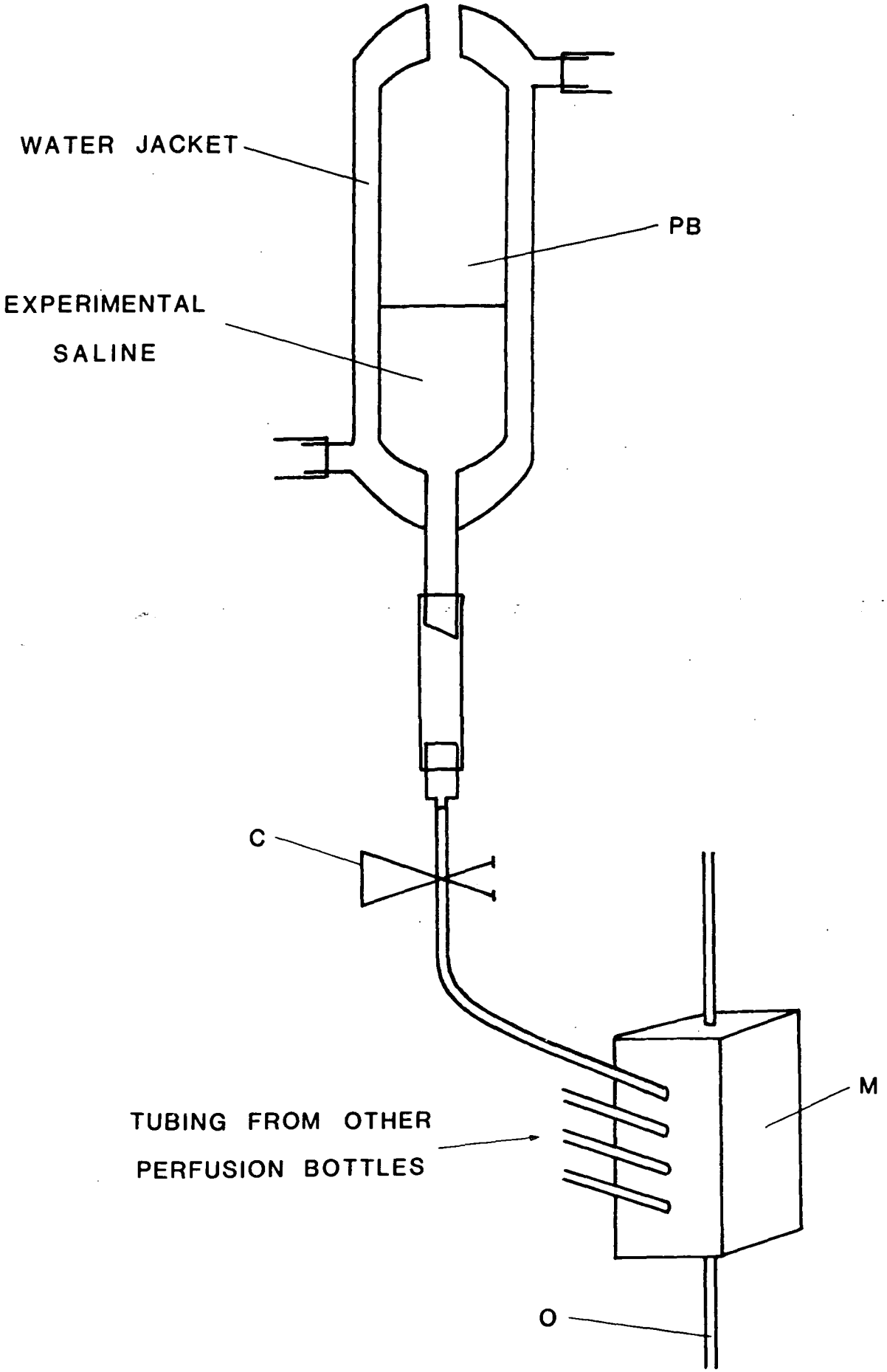

RECORDER
CRO
PE2
E2
PE1
E1
AG
AG
ME
MALPIGHIAN TUBULE
E
E
SB
BATH D (LP)
PERSPEX CHAMBER
SYLGARD BASE
BATH C (SALINE)
BATH B (LP)
BATH A (SALINE)

Fig. 7

A schematic diagram of a perfusion bottle (PB) and manifold (M) used in superfusing Malpighian tubules from Locusta. A number of water-jacketed perfusion bottles were connected to a single manifold whose single outlet (O) was in connection with the glass inlet tube (F) of the experimental chamber. Perfusing solutions were changed by clipping off the flow from one bottle and opening the clip (C) on the tubing of another bottle.


WATER JACKET
PB
EXPERIMENTAL
SALINE
C
TUBING FROM OTHER
PERFUSION BOTTLES
M
O

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 temperature place. A 'seal' between the chamber and the plate was achieved by means of liquid paraffin. Illumination was by a fibre optic system (Ealing Beck Ltd., Watford, Herts., England) arranged at one side of the chamber.

(a) Electrical Recording

The arrangement for recording electrical potentials was similar to that of Berridge and Prince (1972). Fig. 6 shows that the transepithelial potential (T.E.P.) was measured by connecting baths A and C of the experimental chamber to Ag/AgCl electrodes in 2 M KCl (E) via glass Normal saline/4% agar bridges (AG). Each Ag/AgCl electrode was connected to the head-stage (PE_1) of a high input impedance field effect amplifier E_1 (based on the design of Colburn and Schwartz, 1972). The output from this amplifier was displayed on an oscilloscope CRO (Telequipment Type D1010) and permanent records made on a vertical multi-channelled pen recorder (Servogor 460 - Metrawatt, Nürnberg, F.R.G.). Initial zeroing of the system was carried out by placing both agar bridges in bath C. Any deviation of potential from a standardized baseline was due to junction potentials between KCl and the Ag/AgCl electrodes or old agar bridges (Barry and Diamond, 1970). This was eliminated by scraping the Ag wires and coating 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 (ME) fabricated from 1 mm diameter thin-walled filamented glass capillary tubing (GC 200F-10, Clarke Electromedical, Reading, England). These were pulled on a horizontal electrode puller (Palmer Ltd., London) and

back-filled with 4 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 a microelectrode and used to connect it to the input stage (PE_2) of a high input impedance field-effect amplifier (E_2) whose output was displayed on a second channel of the oscilloscope CRO and recorded on the Servogor chart recorder.

The simultaneous recording of the tubule membrane potential and T.E.P. occurred as a result of the two amplifiers (E_1 and E_2) being connected at the input stages PE_1 and PE_2. The introduction of a relay switching mechanism (SB) connected to a foot pedal (RS 316-939) changed the reference from bath A (the luminal fluid) to bath C (the perfusate) so that the basal cell membrane potential (V_B) and T.E.P. was recorded with the perfusate as reference. Upon switching, the apical cell membrane potential (V_A) and T.E.P. was recorded with the luminal fluid as reference.

Microelectrodes were mounted on a Prior micromanipulator (Prior, England) and positioned in the perfusing fluid near to the Malpighian tubule. The tip resistances of the microelectrodes, inserted in the perfusing fluid, were 20-50 MΩ and the tip potentials (potential between the electrolyte in the microelectrode and the perfusate) were about 3 mV. Microelectrode tips were less than 1 μm; fine tips were needed to allow easy impalement. The resistances of microelectrodes were measured using a constant current generator system incorporated in the amplifiers (based on the design of Colburn and Schwartz, 1972). A 1 nA current was passed down a microelectrode resulting in a voltage deflection on the oscilloscope. Using Ohm's Law (V = IR) a value for microelectrode resistance was calculated. The constant current generator was used periodically to measure the membrane input resistance by passage of current (usually 1 nA) through the tip of the microelectrode while it

was in a cell (however, see later).

Before experimentation, a microelectrode was equilibrated in the perfusate and the resistance noted. After this period, the microelectrode was positioned very close to the tubule using the fine advance on the micromanipulator. Further changes with the manipulator adjusted the microelectrode to an angle of about 60° with respect to the tubule. Gentle tapping of the baseplate of the manipulator caused the microelectrode to rapidly penetrate a cell as signalled by the rapid registration of a resting potential on both the oscilloscope and the recorder. Occasionally, oscillatory voltages were passed across the microelectrode to facilitate penetrations of cells. This was achieved by momentarily overcompensating the negative capacity adjustment of the amplifier (PE_2). The criteria adopted as indicators of a successful microelectrode penetration were:

(i) the change in potential from baseline was abrupt

(ii) the intracellular voltage remained constant within 2 mV for 5-10 mins after impalement

(iii) 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 the voltage 'drift' was more than 10 mV over the period of 1 hr).

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

After a successful penetration, solution changes were carried out as described earlier. Experimentation on a tubule usually did not last longer than 1.5 hrs. After this time, the microelectrode was removed from a cell and the microelectrode resistance measured as a means of checking for electrode blocking. Blocking of the microelectrode tip sometimes caused a slight 'drift' of the recorder traces and this was

related to criterion (iii) described above as to whether an experiment was accepted or not. Changing to a new perfusing solution often lead to a liquid junction potential at the agar bridge in the experimental bath. Membrane potentials and T.E.P. measurements were corrected for these junction potentials whose magnitude (usually only a few mV) were calculated by changing solutions after experimentation with the microelectrode withdrawn from the tubule but remaining in the perfusate. The perfusion chamber was thoroughly washed out with de-ionized water and dried after each experiment. Setting up and penetration of tubules was viewed under a Zeiss microscope. Experiments were carried out in a Faraday cage to cut out electrical interference and a vibration damped bench was used.

This system allowed three voltage measurements, namely basal and apical cell membrane potentials and T.E.P. measurements to be recorded in the present study. (In addition, T.E.P. was also calculated from T.E.P. = $V_B - V_A$.) All potentials were measured in millivolts (mV).

(b) Intracellular Injection of Fluorescent Dye

The fluorescent dye Lucifer Yellow CH was injected intracellularly into Malpighian tubule cells by iontophoresis through the microelectrode (Stewart, 1978; Hanrahan and Phillips, 1984; Thomas and May, 1984). A 3 mm column of dye was placed into the tip of a microelectrode by immersing the blunt end of the electrode into a 5% solution of Lucifer Yellow CH in 1 M lithium chloride. The rest of the microelectrode was back-filled with 1 M LiCl. Cells were penetrated as described in section (a) above - microelectrode resistances being higher on average (50-60 MΩ). Dye was injected by passing a 10 nA hyperpolarizing current for 20 mins through the microelectrode using the constant current source described earlier. Continuous penetration during injection was ensured by switching off the current for a few seconds to observe the membrane potential. After

injecting, tubules were left for 15 mins in Normal saline, then fixed in 4% paraformaldehyde buffered with 0.1 M phosphate at pH 7.2. After 1 hr of fixation, tubules were dehydrated in ethanol (70, 95 and 100% for 5, 5 and 10 mins respectively) and then cleared for 5 mins in xylene. Tubules were mounted in Fluoromount mountant (BDH, Chemical Ltd., Poole, England) and viewed in whole mounts under a u.v. fluorescence microscope (Nikon). Controls in which tubules were immersed (and not injected) in a 5% Lucifer Yellow solution were carried out.

Note on Membrane Resistance Measurements

Results using Lucifer Yellow showed that the Malpighian tubules of Locusta rapidly excreted this dye. Indeed, no fluorescence was observed under the u.v. microscope. The lack of Lucifer Yellow staining was probably not due to a failure of the dye to come out of the microelectrode, and this was supported by the number of impalement experiments, along with the reliability of the technique as shown by other workers (Stewart, 1978; Hanrahan and Phillips, 1984; Thomas and May, 1984). Thus, it was impossible to determine whether Malpighian tubule cells were electrically coupled or not. For this reason, the measurement of the basal, apical and transepithelial resistance was not carried out in this study.

CHAPTER 3

Results

Section 1: Ouabain-binding Studies on the Na^++K^+-ATPase

The method of extraction of microsomal preparations from the Malpighian tubules of Locusta and the technique of ouabain-binding have been described earlier (see Materials and Methods, Chapter 2). In the present study twelve separate Na^++K^+-ATPase preparations were used. Each was obtained from homogenates of Malpighian tubules prepared from 40 locusts. The mean specific activity of the Mg^{2+}-ATPase and the Na^++K^+-ATPase was 1.56 ± 0.22 and 5.86 ± 0.82 µmol inorganic phosphate liberated/mg protein per h, respectively. Thus, Na^++K^+-ATPase activity accounted for ca. 75% of the total ATPase activity of these preparations.

Inhibition by Ouabain of Na^++K^+-ATPase Activity

Na^++K^+-ATPase activity was assayed in the presence of different concentrations of ouabain over the range 10^{-8}-10^{-3} M. The inhibition curve for ouabain obtained from this assay (Fig. 8) shows that as the concentration of ouabain increased so did the inhibition of the Na^++K^+-ATPase activity. The concentration of ouabain effecting 50% inhibition of activity (I_{50}) was 1.12 µM (95% confidence limits = 0.7-1.9 µM).

Ouabain Binding

Na^++K^+-ATPase binds ouabain specifically according to the mass-law equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI$$

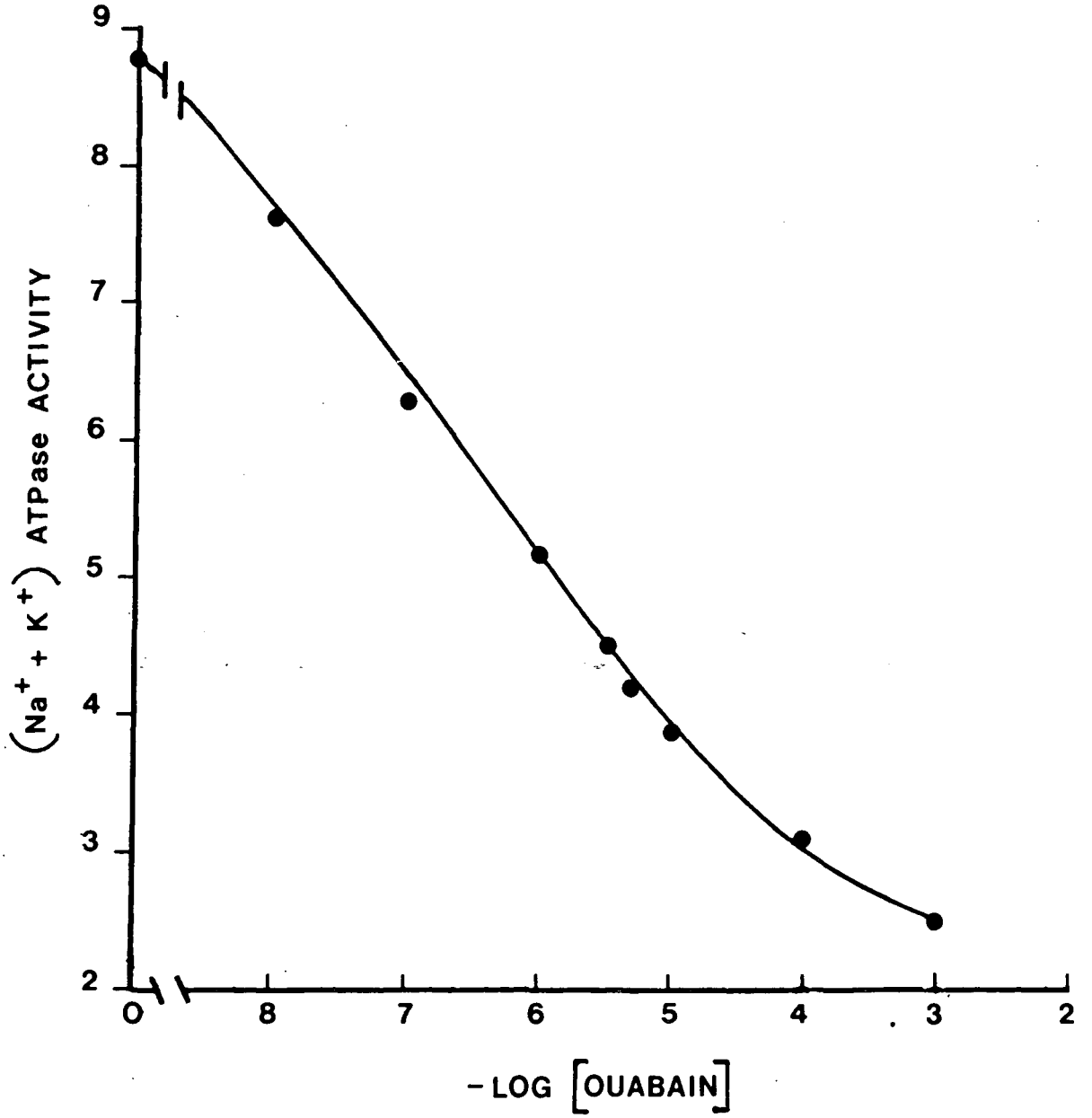
where E is the receptor concentration, I is the ouabain concentration, EI is the ouabain-receptor complex concentration, and k_1 and k_{-1} are

Fig. 8

Effect of different concentrations of ouabain (1×10^{-8} M to 1×10^{-3} M) at 30°C on $Na^+ + K^+$-ATPase activity. Typical experiment which is representative of four experiments.

Ordinate: Probits of Fraction of $Na^+ + K^+$-ATPase Activity Remaining

Abscissa: Negative Logarithm of Ouabain Concentration (M)


9
8
7
6
5
4
3
2
$(Na^+ + K^+)$ ATPase ACTIVITY
0
8
7
6
5
4
3
2
- LOG [OUABAIN]

the association and dissociation rate constants, respectively.

The formation of [^{3}H]ouabain-enzyme complex follows second-order kinetics (Erdmann and Hasse, 1975). Thus, both the initial receptor concentration and the initial ouabain concentration need to be known before the association rate constant (k_1) can be calculated. However, Wallick et al. (1980) suggest that if the concentration of ouabain is maintained in large excess of the receptor, the forward reaction becomes pseudo first-order (Hill, 1909). This greatly facilitates the determination of k_1 from the equation:

$$k_1 = (k_{obs} - k_{-1})/I$$

where k_{obs} is the observed first-order approach to equilibrium (Wallick et al., 1980).

Fig. 9 shows the time course of ouabain binding to a microsomal preparation from Malpighian tubules of Locusta. [^{3}H]Ouabain binding reached a maximum after 5-10 min and remained stable over a 1 h incubation period. Fig. 10 shows that the initial rate of binding follows pseudo first-order kinetics under the conditions used and is consistent with the findings of other researchers (Wallick et al., 1980). Such plots were used to determine k_{obs} and hence the association rate constant, k_1. The mean calculated k_1 was 1.5×10^3 $M^{-1} \cdot s^{-1}$ (Table 2).

In the present study, the dissociation of ouabain from the enzyme was determined following incubation in the presence of 3×10^{-6} M [^{3}H]ouabain at 30°C. The dissociation of the ouabain-receptor complex follows first-order kinetics and consequently the dissociation rate constant (k_{-1}) can be calculated from the exponential decay of ouabain binding (Erdmann and Hasse, 1975) (Fig. 11). The mean calculated k_{-1} was 3.7×10^{-3} s^{-1} (Table 2).

Fig. 9

Time course of specific ouabain-binding to a microsomal preparation from Malpighian tubules of Locusta at 3×10^{-6} M (▲) and 3×10^{-7} M (●) concentration. Typical experiment representative of three experiments.

Ordinate: Ouabain Bound (p mol/mg protein)

Abscissa: Time in Minutes

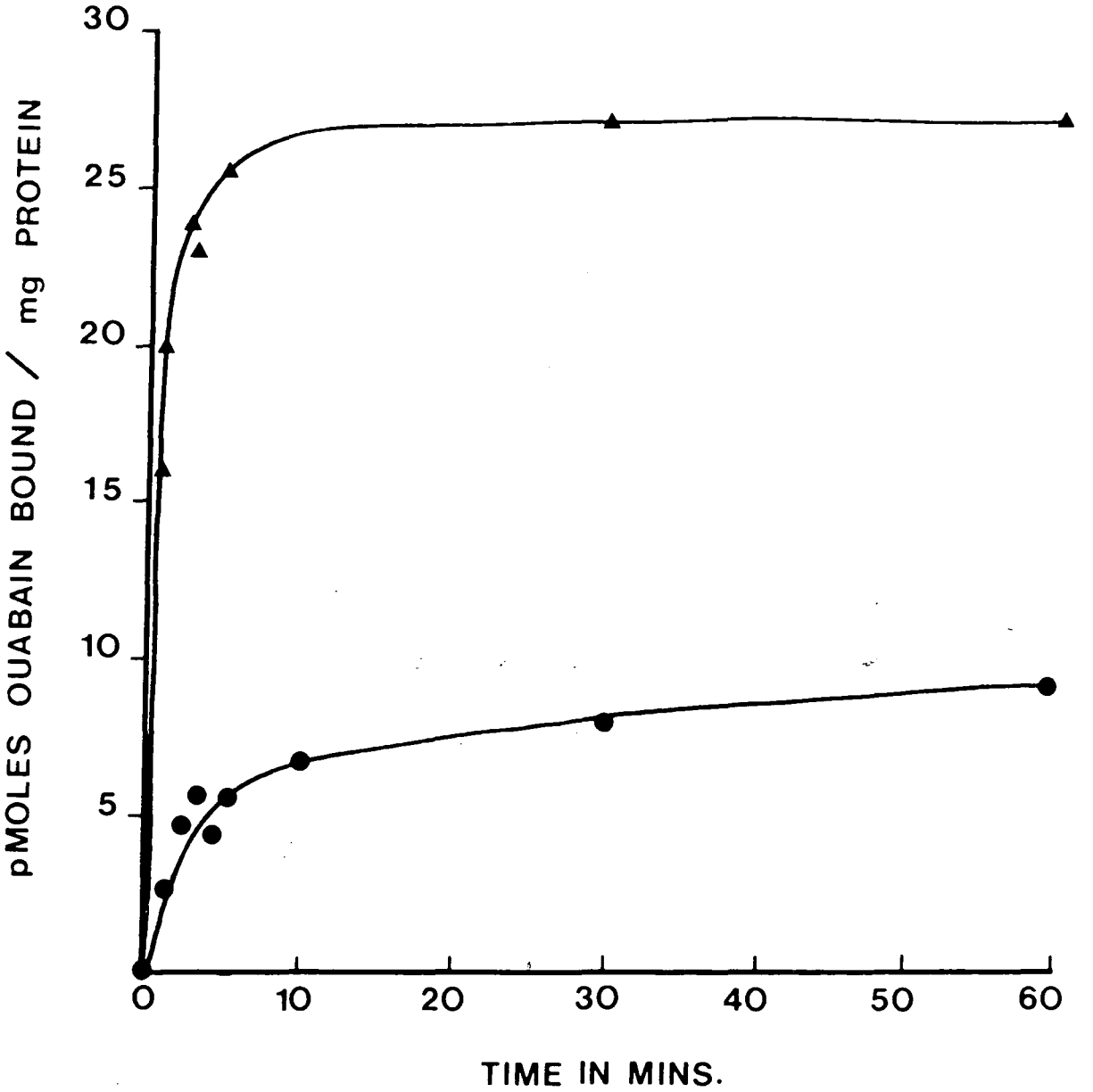

30
25
20
15
10
5
pMOLES OUABAIN BOUND / mg PROTEIN
0
10
20
30
40
50
60
TIME IN MINS.

Fig. 10

Pseudo first-order binding of ouabain to a microsomal preparation of Malpighian tubules of Locusta. Binding was carried out in the presence of 3×10^{-6} M ouabain. Typical experiment representative of three experiments.

Ordinate: $(Ae-A)/Ae$ ($\log_e$ scale) where Ae and A are Ouabain Bound at Equilibrium and at Time, t, Respectively

Abscissa: Time in Minutes

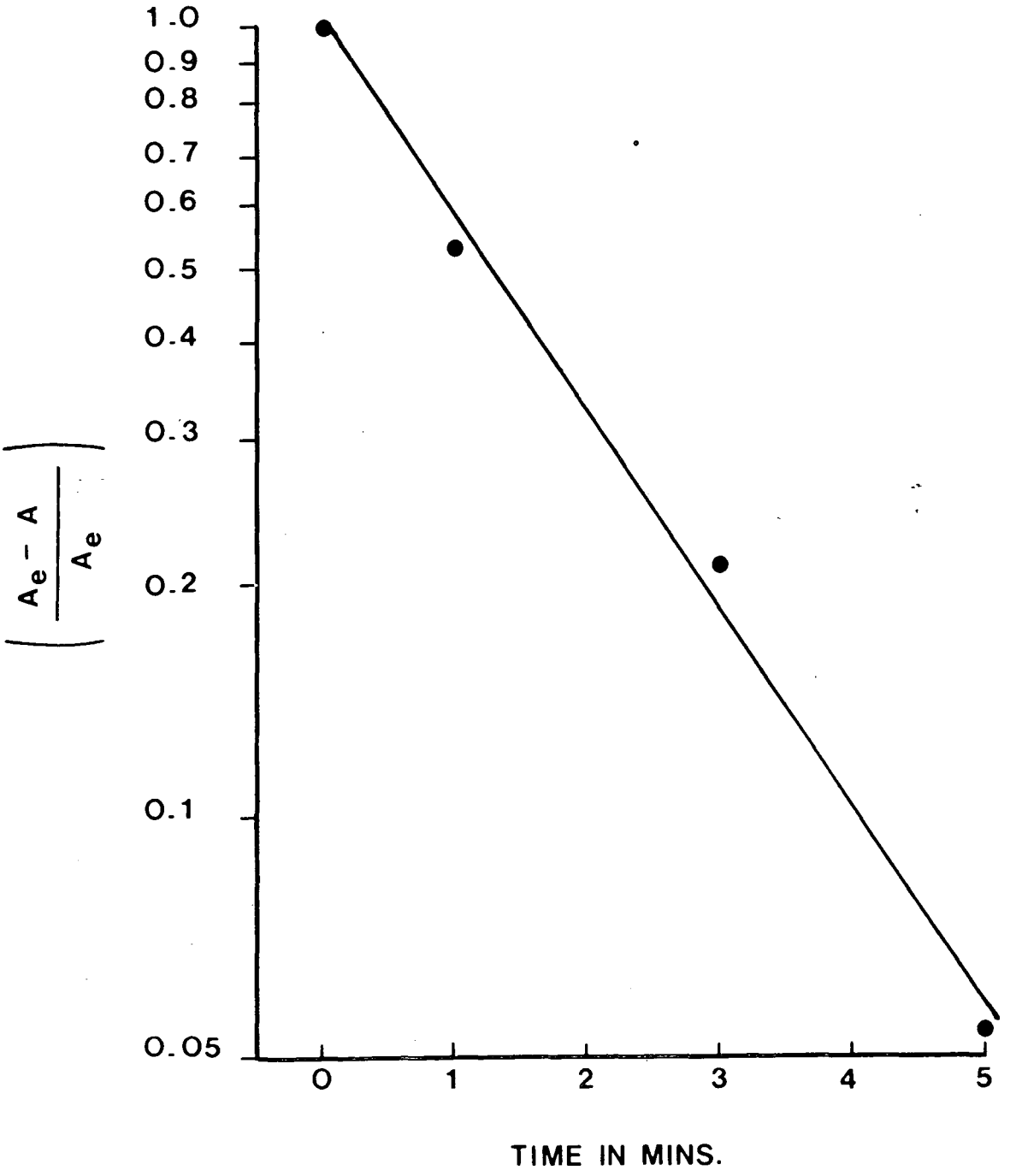

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
$\left(\frac{A_e - A}{A_e}\right)$
0
1
2
3
4
5
TIME IN MINS.

Table 2

Kinetic constants for ouabain-binding to microsomal preparations of Malpighian tubules of Locusta. n represents the number of independent experiments and a and b represent values for high- and low-affinity sites, respectively. K_d and B_{max} were calculated from Scatchard plots of data.

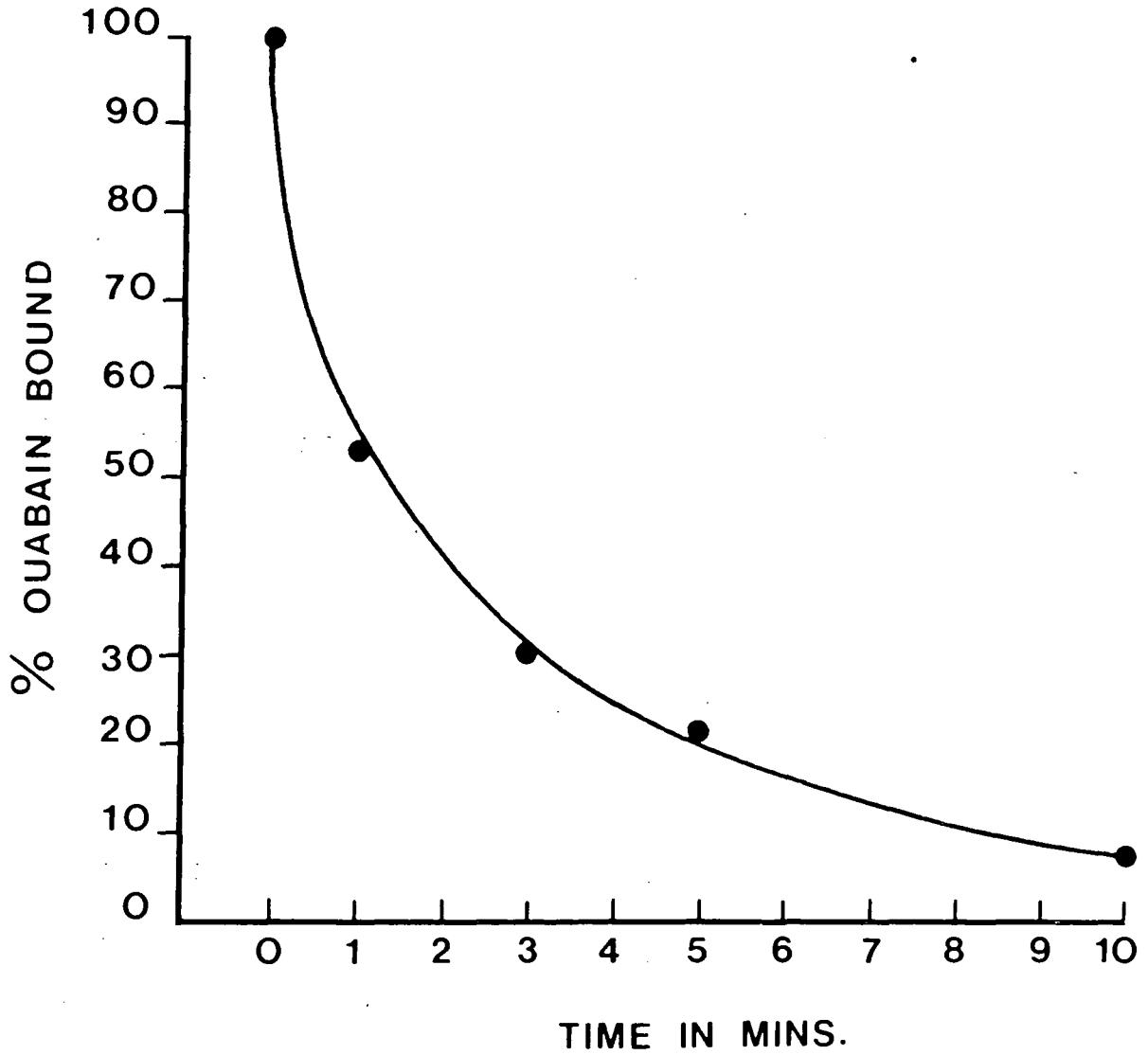
Parameter		Value ± S.E.M.	n
k_1 ($M^{-1} \times s^{-1}$)		$1.5 \times 10^3 \pm 3.5 \times 10^2$	3
k_{-1} (s^{-1})		$3.7 \times 10^{-3} \pm 0.6 \times 10^{-3}$	5
k_{-1}/k_1 (M)		2.5×10^{-6}	-
K_d (M)	a	$0.2 \times 10^{-6} \pm 0.1 \times 10^{-6}$	3
	b	$4.2 \times 10^{-6} \pm 1.3 \times 10^{-6}$	3
B_{max} (p mol/mg protein)	a	11.0 ± 1.2	-
	b	25.9 ± 2.5	-

Fig. 11

Time course of ouabain dissociation from $Na^+ + K^+$-ATPase preparation. The enzyme-ouabain complex was formed by incubating the tissue for 45 mins at 30°C in the presence of 3×10^{-6} M[^{3}H]ouabain. The dissociation reaction was started by the addition of unlabelled ouabain to a final concentration of 1×10^{-3} M (see Materials and Methods). Typical experiment representative of five experiments.

Ordinate: Present Ouabain Bound

Abscissa: Time in Minutes After Addition of Excess Unlabelled Ouabain


100
90
80
70
60
50
40
30
20
10
0
% OUABAIN BOUND
0
1
2
3
4
5
6
7
8
9
10
TIME IN MINS.

The Michaelis constant or the equilibrium dissociation constant (K_d) can be calculated by the equation:

$$K_d = \frac{k_{-1}}{k_1}$$

(see Table 2) or can be measured directly from the equilibrium binding of ouabain as a function of ouabain concentration. The binding of [^{3}H]ouabain to Malpighian tubule Na^++K^+-ATPase preparations was determined after 45 min incubation at different concentrations of ouabain and the data were plotted according to Scatchard (1949) (Fig. 12). It can be seen that the resulting plot is curvilinear, suggesting either that there is binding to multiple independent binding sites or that there is negative cooperativity between binding sites (Hansen, 1976; Wallick et al., 1979). More recently, Noel and Godfraind (1984) have concluded, from their studies on rat heart, that ouabain-specific binding occurs at two classes of independent sites. On this basis, the binding capacities and affinities for high- and low-affinity sites have been determined in the present study (Table 2). The results suggest that about 30% of binding sites displayed a high affinity for ouabain (K_d = 0.2 x 10^{-6} M), whereas 70% of binding sites were characterised by a lower affinity (K_d = 4.2 x 10^{-6} M) (Table 2).

Estimates of Cell Size and Numbers

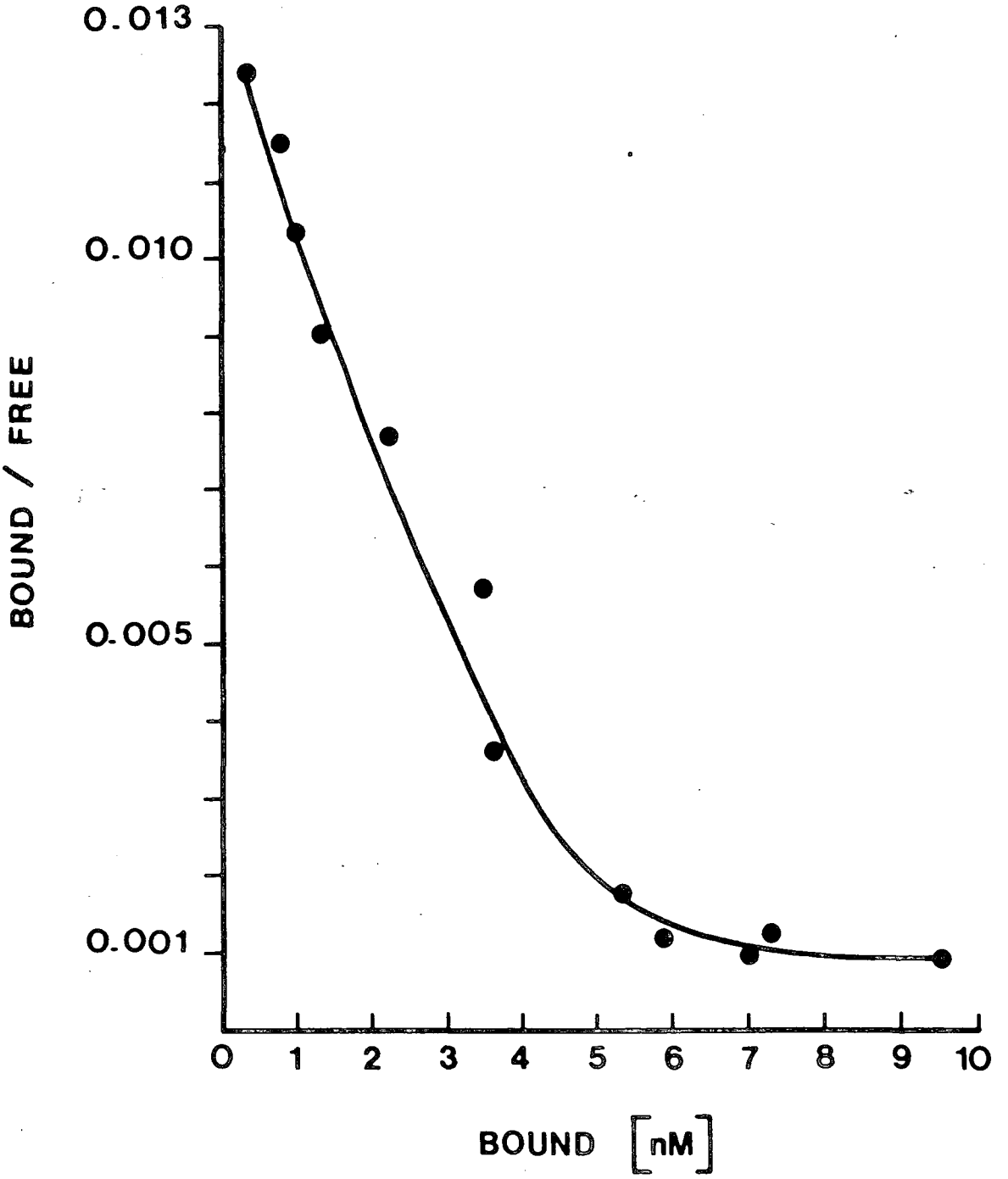
It was estimated that the total cell number for the Malpighian tubules of an adult locust was about 363000. Furthermore, approximately 189.4 ± 6.9 µg microsomal protein were extracted per insect from Malpighian tubules (n = 5 independent determinations, each involving 40 locusts). Thus approximately 5.2 x 10^{-7} mg of microsomal protein are derived from each cell.

Fig. 12

Scatchard plot of ouabain binding to a microsomal preparation from Malpighian tubules of Locusta. The enzyme-ouabain complex was formed by incubating the preparation for 45 mins at 30°C with 3×10^{-8}-10^{-5} M ouabain. Typical experiment representative of three experiments.

Ordinate: Rate of Bound/Free Ouabain

Abscissa: Concentration of Ouabain Bound (nM)


0.013
0.010
0.005
0.001
BOUND / FREE
0
1
2
3
4
5
6
7
8
9
10
BOUND [nM]

Measurements on serial sections through Malpighian tubules of Locusta indicate that the mean cell volume is 72430 ± 2355 μm^3 (n = 8) with approximate dimensions of 85 μm x 85 μm x 10 μm.

Cell Type and Distribution

Charnley (1984) has shown that two types of cell occur in the Malpighian tubules of Locusta, namely Type 1 (or primary) cells and Type 2 (or stellate) cells. The Type 1 cells are considered to be primary agents of ion and water transport (Maddrell, 1971) whilst the Type 2 cells may be primarily mucocytes (Charnley, 1984). Results from lead staining (Fig. 13) in the present study showed that 1 in 10 of the total cell number per tubule were Type 2 cells.

Discussion

The Na^++K^+-ATPase of Malpighian tubules of Locusta exhibits many of the properties of (Na^++K^+)-ATPases from other species (Anstee and Bowler, 1979; 1984). It is maximally activated at an ATP/Mg^{2+} ratio of 1 : 1.3 and at 100 mM Na^+/20 mM K^+ (Anstee and Bell, 1978) and is inhibited by ouabain (Anstee and Bell, 1975; 1978; Donkin and Anstee, 1980; Anstee and Bowler, 1984). In the present study, the concentration of ouabain which half-maximally inhibited enzymatic activity was 1.12 μM. This agrees well with I_{50} values reported for other insect preparations (Anstee and Bowler, 1984).

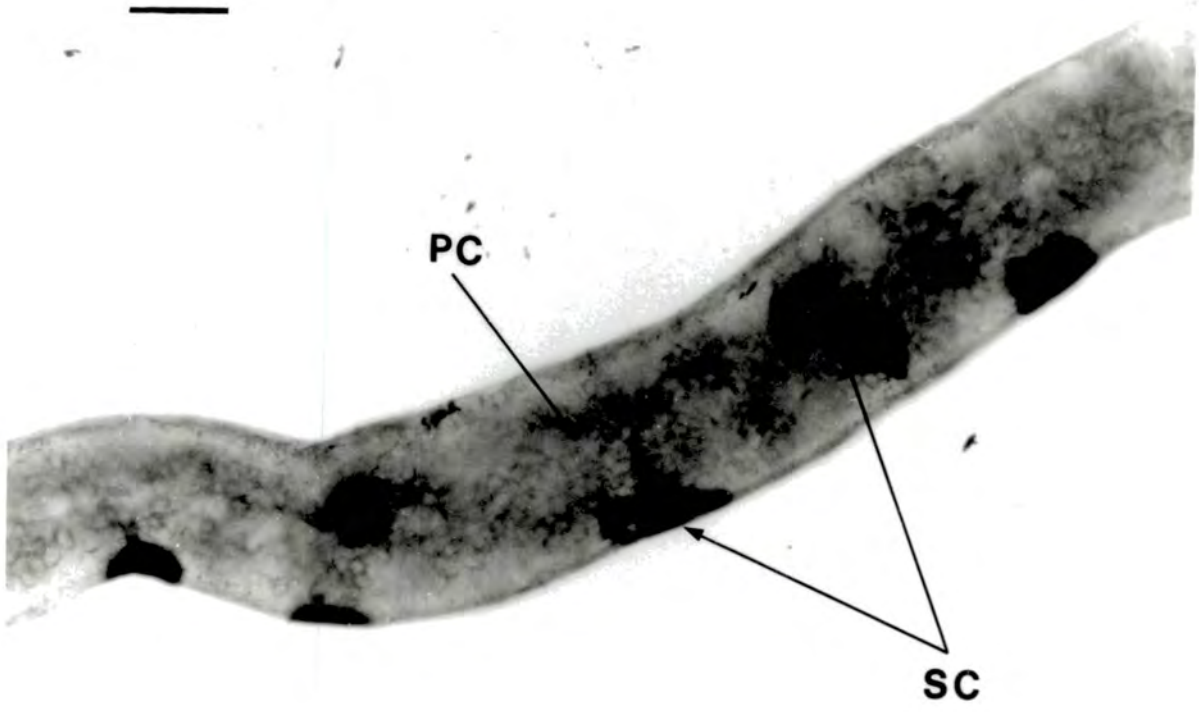
Many studies on the binding of cardiac glycosides to Na^++K^+-ATPase preparations result in saturable binding which involves a single class of binding site (Wallick et al., 1979; Erdmann, 1981). In the present study curvilinear Scatchard plots were obtained which suggests that microsomal preparations from Malpighian tubules of Locusta bind ouabain specifically with a dissociation constant of 0.2 x 10^{-6} M and

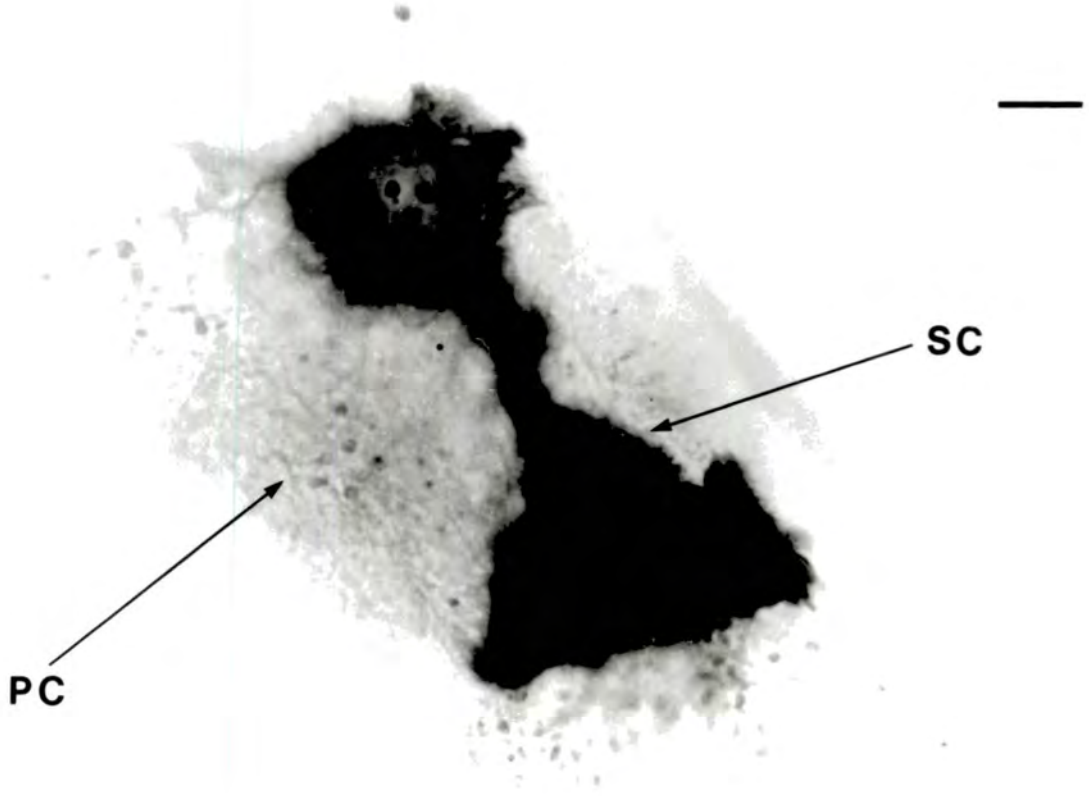
Fig. 13

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

Fig. 13a Primary and secondary cells at low magnification. PC, primary cell, SC, secondary cell. Scale 50 µm.

Fig. 13b Secondary cell at higher magnification. PC, primary cell, SC, secondary cell. Scale 25 µm.


PC
SC


SC
PC

4.2×10^{-6} M at high- and low-affinity sites, respectively. Other workers have reported the possible existence of at least two classes of binding sites of high and low affinities with different tissues from a variety of species (Hansen, 1976; Noel and Godfraind, 1984; Godfraind et al., 1980; Kazazoglou et al., 1983; Fricke and Klaus, 1977; Fricke, 1985). As pointed out earlier, such heterogeneity might be due to the presence of different $Na^+ + K^+$-ATPase conformations with different affinities for ouabain or the existence of independent binding sites. The existence of two classes of independent binding sites in rat heart has been proposed (Noel and Godfraind, 1984). Furthermore, it is reported that K^+ increased the proportion of the high-affinity sites in microsomal fractions from guinea-pig heart (Godfraind et al., 1980). However, the possibility that such curved plots are due to negative cooperativity cannot be ruled out. Noel and Godfraind (1984) reported that, in rat heart, the proportion of low- and high-affinity components observed in enzyme inhibition studies was similar to that measured in [^{3}H]ouabain binding experiments. They suggested that this might be explained on the basis of two isozymes being present. Furthermore, they suggest that, since ^{86}Rb uptake by cells in intact tissue was not affected by ouabain at concentrations less than 10^{-5} M but was almost completely abolished at 10^{-4}M (Erdmann et al., 1980), only low-affinity sites are inhibitory <u>in vivo</u>. Similarly, on the basis that K_d for low-affinity sites was a value close to ouabain I_{50}, it was suggested that the low-affinity sites could be inhibitory sites in guinea-pig heart (Godfraind et al., 1980). Giunta et al. (1985) have proposed a regulatory model in which it is suggested that at very low levels of cardiac glycoside, binding to the high-affinity sites causes conformational changes leading to enzyme activation, whereas at higher cardiac glycoside levels, binding to the low-affinity

site results in enzyme inhibition. These same workers (Guinta et al., 1985; 1984) suggest a model for activity modulation of Na^++K^+-ATPase activity, *in vivo*, involving ouabain-like compounds, such as have been reported in a variety of animal tissues (Flier et al., 1980; Lichtstein and Samuelov, 1980; Godfraind et al., 1982; 1983; Schwartz, 1983). Such compounds inhibit enzymatic activity in the same range of concentrations as ouabain. Thus it is proposed that under physiological conditions the level of circulating ouabain-like compounds can saturate the high-affinity site, promoting enzyme activation. An increase in the concentration of such digitalis-like compounds, it is argued, leads to saturation of the lower-affinity binding site also, resulting in enzyme inhibition. In the present investigation, the value for I_{50} was not significantly different from the dissociation constant (K_d) for the low-affinity site, suggesting that here also, the low-affinity site may be responsible for enzyme inhibition. The significance of the high-affinity sites and the application of the regulative model of Na^++K^+-ATPase activity, described above, to insect Malpighian tubules must await further investigation.

Table 3 compares the kinetic constants of ouabain-binding to Na^++K^+-ATPase in microsomal preparations of Malpighian tubules with those obtained elsewhere, from a variety of species. It can be seen that the association rate constant (k_1), the dissociation rate constant (k_{-1}) and K_d for *Locusta* are within the range of values quoted for a variety of tissues and different species.

There is disagreement concerning the ratio of ATP-binding and ouabain-binding sites to phosphorylation sites on the Na^++K^+-ATPase. Jørgensen (1974; 1977) has shown that there is one ouabain-binding site, one ATP-binding site and one phosphorylation site per 280000 molecular wieght unit. Similarly, a ratio of 1 : 1 for ATP sites to

Table 3

Comparison of mean kinetic constants for ouabain binding in preparations from different species. a and b represent values for high- and low-affinity sites, respectively.

Tissue	k_1 ($M^{-1} \times s^{-1}$)	k_{-1} (s^{-1})	k_{-1}/k_1 (μM)	K_d (μM)	Reference
Malpighian tubules of Locusta	1.5×10^3	3.7×10^{-3}	2.5	a 0.2 b 4.2	Present study
Rabbit kidney	0.5×10^2	2.4×10^{-5}		0.5	Shaver and Stirling, 1978.
Manduca sexta brain	1.9×10^4	2.2×10^{-3}		0.12	Rubin et al., 1981
Rat intestine	1.3×10^3	3.6×10^{-2}	29.0	15	Harms and Wright, 1980.
Cavia cobaya kidney				a 0.35 b 2.1	Giunta et al., 1985.
Chick cardiac cells	7.6×10^2	5.0×10^{-3}	6.6	a 0.03-0.05 b 2-6	Kazazoglou et al., 1983.
Rat heart				a 0.21 b 13	Noel and Godfraind, 1984.
Rabbit nephron	5.0×10^2	1.0×10^{-3}	2.0	1.8	El Mernissi and Doucet, 1984.
Rectal gland of Squalus				a 0.2 b 5.0	Silva et al., 1983.
Whole imaginal discs of Drosophila	4.7×10^2	8.6×10^{-5}		0.18	Fristrom and Kelly, 1976.

phosphorylation sites (Hegyvary and Post, 1971) and a 1 : 1 ratio for ouabain-binding sites to phosphorylation sites (Erdmann and Schoner, 1973; Albers et al., 1968) has been reported elsewhere. However, $Na^+ + K^+$-ATPase from guinea-pig kidney binds 4 mol ouabain/mol ^{32}P-labelled phosphoprotein formed (Erdmann and Schoner, 1973), whilst enzyme preparations from the electric eel organ bind only 1 ouabain per two phosphorylation sites (Albers et al., 1968).

In the Malpighian tubule preparations from Locusta maximum binding (B_{max}) was 11.0 pmol/mg protein at the high-affinity sites and 25.9 pmol/mg protein at the lower-affinity sites (see Table 2). These data, taken in conjunction with the mean $Na^+ + K^+$-ATPase activity measured, indicate a value of 6.3 pmol ouabain bound/μmol inorganic phosphate liberated at both the high- and the low-affinity sites. This yields an overall turnover of 2645/min, assuming that one ouabain is bound per pump site. This turnover number for the pump is compared with those reported for a number of other tissues and different species in Table 4.

It was estimated that each cell yielded approx. 5.2×10^{-7} mg membrane protein, a value which compared favourably with the 2.5×10^{-7} mg protein/cell quoted for vertebrate intestinal cells (Harms and Wright, 1980). However, in view of the fact that, in the present study, the yield was only a fraction of the total membrane protein per cell, this value is a substantial underestimate. Nevertheless, accepting its limitations we can use this value to make certain calculations on the basis of data obtained with Locusta. Thus, assuming 1 ouabain bound per enzyme site, it can be calculated that there are 3.4×10^6 high-affinity pump sites and 8.1×10^6 lower-affinity pump sites per tubule cell (i.e. a total of 11.5×10^6 pump sites/cell). This pump site density is compared with values reported for a variety of other cell types in Table 5. Harms and Wright (1980) calculated that there were approx.

Table 4

Comparison of turnover values for $Na^+ + K^+$-ATPase from various species.

Preparation	Turnover (ATP hydrolysed/ site per min)	Reference
Malpighian tubules of *Locusta*	2645	Present study
Red blood cell	1400	Joiner and Lauf, 1978.
Rabbit nephron	2000	El Mernissi and Doucet, 1984.
Bovine kidney	3430	Erdmann and Schoner, 1973.
Rat intestine	8300	Harms and Wright, 1980.
Guinea-pig kidney	11100	Erdmann and Schoner, 1973.
Bovine heart	8550	Erdmann and Schoner, 1973.
Bovine brain	11500	Erdmann and Schoner, 1973.

Table 5

Comparison of ouabain binding site density for various species.

a and b represent values for high- and low-affinity sites, respectively. * indicates value calculated from data given by Rubin et al. (1981) and assuming 5.2×10^{-7} mg protein per cell. + indicates values are likely to be underestimates.

Preparation	Number of Sites per Cell		Reference
Malpighian tubules of Locusta	+a	3.4×10^6	Present study.
	+b	8.1×10^6	
Chick heart membrane	a	1.5×10^5	Kazazoglou et al., 1983.
	b	1.4×10^6	
Rat intestine		1.5×10^6	Harms and Wright, 1980.
Rabbit renal tubule		4.1×10^6	Shaver and Stirling, 1978.
HeLa cells		8.2×10^5	Baker and Willis, 1972.
Cultured guinea-pig kidney		7.5×10^5	Baker and Willis, 1972.
Chloride cells of teleost gill		1.5×10^8	Karnaky et al., 1976.
Human erythrocyte		228	Erdmann and Hasse, 1975.
Manduca sexta brain		2.5×10^7 *	Rubin et al., 1981.
Whole imaginal discs of Drosophila melanogaster		1.8×10^4	Fristrom and Kelly, 1976.

$1.5 \cdot 10^5$ sites per cell (estimated from maximum phosphorylation studies) in rat intestine. However, their estimate was an order of magnitude larger when determined from maximum ouabain-binding data. The somewhat higher value reported for the salt-secreting chloride cells of teleost gill (viz. 1.5×10^8 sites/cell) (Karnaky et al., 1976) is thought to be related to their larger cell volume and the membrane magnification factor (Shaver and Stirling, 1978).

If the $Na^+ + K^+$-ATPase pump of Malpighian tubule cells of Locusta move Na^+ and K^+ with the stoichiometry of $3Na^+ : 2K^+$ per ATP hydrolysed at each site (see above) then, at the calculated turnover rate, 9.1×10^{10} Na^+ could be maximally pumped out of a tubule cell per min in exchange for 6.1×10^{10} K^+. If one assumes that the intracellular Na^+ concentration is approx. 13 mM as reported for Rhodnius (Gupta et al., 1976) and that cell volume is 72430 μm^3, then each cell contains ca. 5.7×10^{11} Na^+ (No. of intracellular Na^+ = Na^+ concentration (13 mM) x cell volume x Avogadro's number). Thus, at maximal pump rate, intracellular Na^+ would be depleted in about 9 min at 30°C. In addition, total ATP utilization, on the basis of 1 ATP hydrolysed per cycle of the pump, would be 3.0×10^{10} ATP/cell per min. Thus, 363000 tubule cells (i.e., estimated tubule cell per locust) would hydrolyse 1.1×10^{16} ATP per min and if 3 ATP are produced for each atom of oxygen consumed then, at maximum turnover, the total oxygen consumption necessary to sustain this pump rate would be 3.0×10^{-9} mol O_2 per min. This is equivalent to 0.12 µmol O_2/g wet weight per min. In the present study, the Malpighian tubules from Locusta consumed oxygen at a rate of 1.5 ± 0.2 µmol/g wet weight per min. Thus, approximately 8% of total metabolic activity would appear to be necessary to sustain maximal pump turnover at 30°C. This value compares favourably with the observation that 18% of tubule oxygen consumption is inhibited by 1 mM ouabain, bearing in mind the

various assumptions made in its calculation and given that the higher reported levels of inhibition (Anstee et al., 1979) would include secondary effects on metabolic rate due to the run-down of ion gradients.

Finally, it has been estimated that up to 1500 cells (approx. 75% of the estimated total cell number per tubule) may be responsible for the secretion of 'urine' at a mean rate of 3.4 nl/min per tubule, by in vitro preparation such as those used by Anstee et al. (Anstee et al., 1979; Donkin and Anstee, 1980). If all the K^+ transported into the cell by the Na^++K^+-ATPase pump were ultimately transferred to the lumen of the tubule by an apical electrogenic pump, the K^+ concentration of the secreted 'urine' could be as high as 45 mM. Indeed, at the basal secretion rate of 2.5 nl/min reported by Morgan and Mordue (1981), the K^+ concentration could be as high as 61 mM, a value which would represent a substantial component of the 140 mM K^+ concentration reported for locust 'urine' (Anstee et al, 1979; Morgan and Mordue, 1983). However, the exact values calculated in the present study should not be interpreted too precisely and whilst it is unlikely that the pump would be operating maximally at all times, it is nevertheless apparent that, since the number of 'pump' sites per cell has been underestimated, the measured turnover values are adequate to account for substantial K^+ transport in Malpighian tubules of Locusta migratoria.

Section 2: Electrophysiological Studies on V_B,V_A and T.E.P. Using K^+ free, Na^+ free and Cl^- free Salines

The electrophysiological recording apparatus and the method of the measurement of membrane potentials has been described earlier (see Materials and Methods, Chapter 2). The three electrical parameters measured in this study, namely the basal and apical cell membrane

potentials (V_B and V_A) and the transepithelial potential (T.E.P.) are all expressed in mV. Since V_B and V_A were found to be negative with respect to the reference electrode throughout this study (except under a few extreme experimental conditions described later) potentials are given a negative prefix. In contrast, T.E.P. varied in polarity and so is quoted with an accompanying positive or negative prefix. Finally, the microelectrode resistance in this study had a range of 14 to 55 MΩ with a mean value of 29.7 ± 0.5 MΩ.

Values for the mean recorded V_B,V_A and T.E.P. are shown in Table 6. Thus, the mean V_B for all cells examined was -71.6 ± 0.3 mV (n = 530), cytoplasm negative with respect to the bathing fluid and the mean V_A was -82.6 ± 0.8 mV (n = 372), cytoplasm negative with respect to the lumen. T.E.P. had a mean value of +5.7 ± 1.0 mV (n = 389), lumen positive and a range of -47 to +51 mV. From this range 64% of cells gave a positive T.E.P., 33% gave a negative T.E.P. and only 3% of cells gave a zero value for T.E.P. Thus, in just under two thirds of cells examined, V_A was more negative than V_B.

It can be seen from Table 6 that T.E.P. (+5.7 ± 1.0 mV) differed significantly ($p < 0.001$) from the transcellular potential, T.C.P. (V_B-V_A) of +11.0 ± 0.7 mV. This discrepancy can be explained by the fact that T.E.P. was obtained from the whole tubule whilst T.C.P. was the difference between V_B and V_A in only one cell. Differences between T.E.P. and T.C.P., therefore, may be explained on the basis of local coupling or short-circuiting between cells. In this study, all values reported are T.E.P. values.

Figs. 14, 15 and 16 show the distribution the individual potential components measured in Normal saline. It can be seen that V_B, V_A and T.E.P. were all Normally Distributed.

Cyclic oscillations of V_A occurred in Normal saline for 53% of

Table 6

The mean membrane potential parameters in Normal saline. V_B and V_A are the basal and apical cell membrane potentials whilst T.C.P. and T.E.P. are the transcellular (V_A-V_B) and transepithelial potentials, respectively. n represents the number of independent experiments, each involving separate tubule preparations.

Parameter	Treatment	$\bar{X}$ ± S.E.M. (mV)	Polarity (%)			n
			Positive	Zero	Negative	
V_B	Normal	-72.6 ± 0.3	-	-	-	530
V_A	Saline	-82.6 ± 0.8	-	-	-	372
T.C.P.		+11.0 ± 0.7	78	6	16	372
T.E.P.		+ 5.7 ± 1.0	64	3	33	389

Fig. 14

Distribution of the basal membrane potential (V_B) recorded from the Malpighian tubules of Locusta.

Ordinate: Number of Cells

Abscissa: V_B in mV

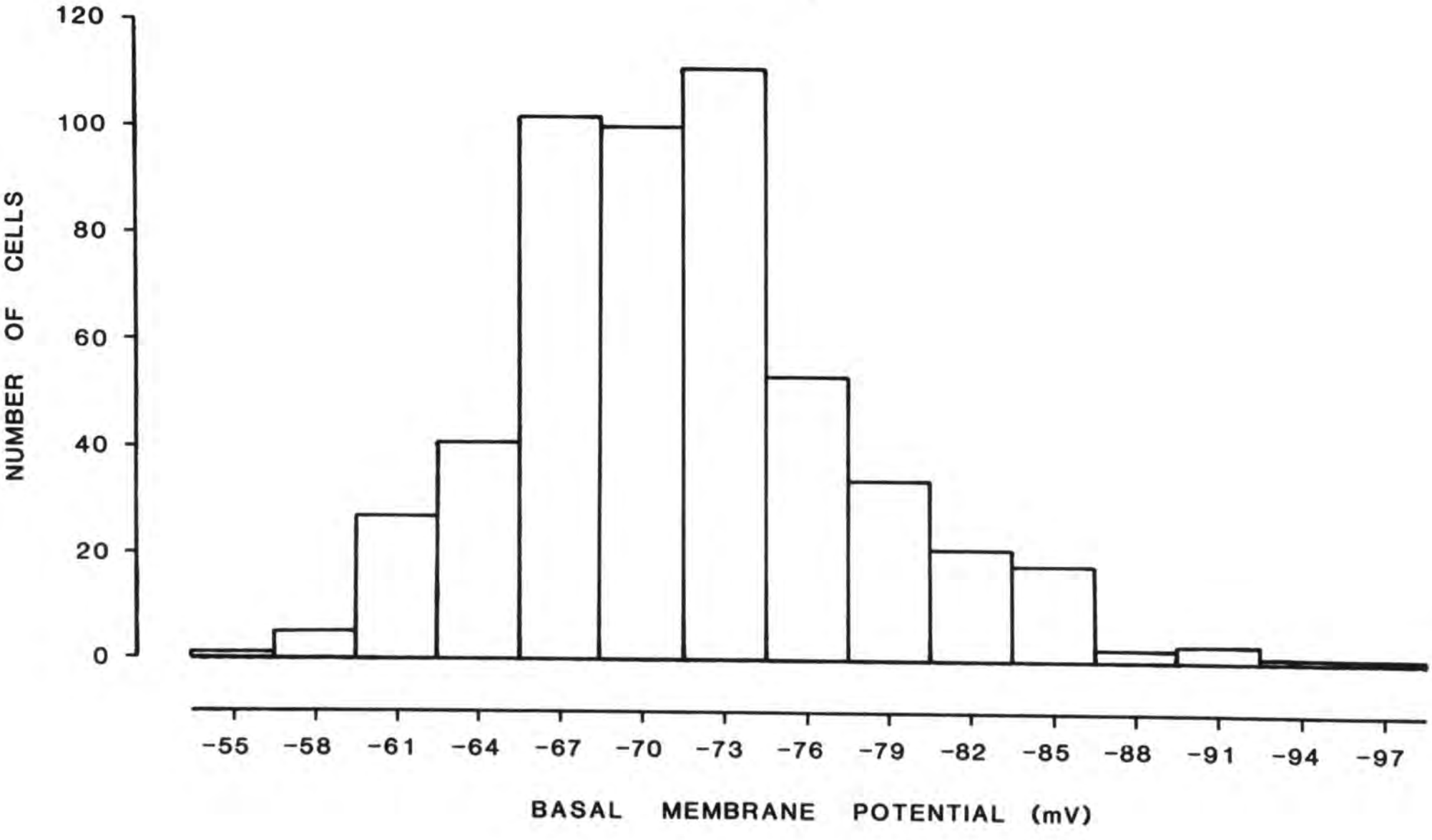

120
100
80
60
40
20
0
NUMBER OF CELLS
-55
-58
-61
-64
-67
-70
-73
-76
-79
-82
-85
-88
-91
-94
-97
BASAL MEMBRANE POTENTIAL (mV)

Fig. 15

Distribution of the apical membrane potential (V_A) recorded from the Malpighian tubules of Locusta.

Ordinate: Number of Cells

Abscissa: V_A in mV

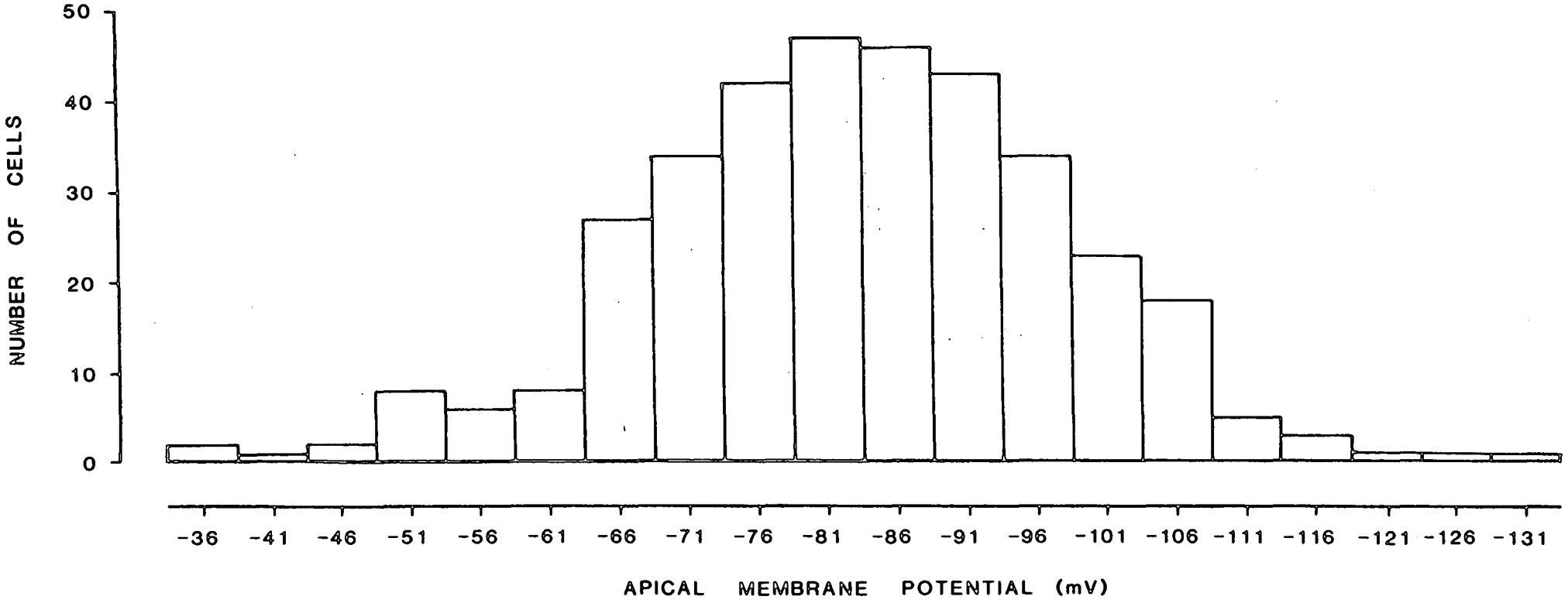
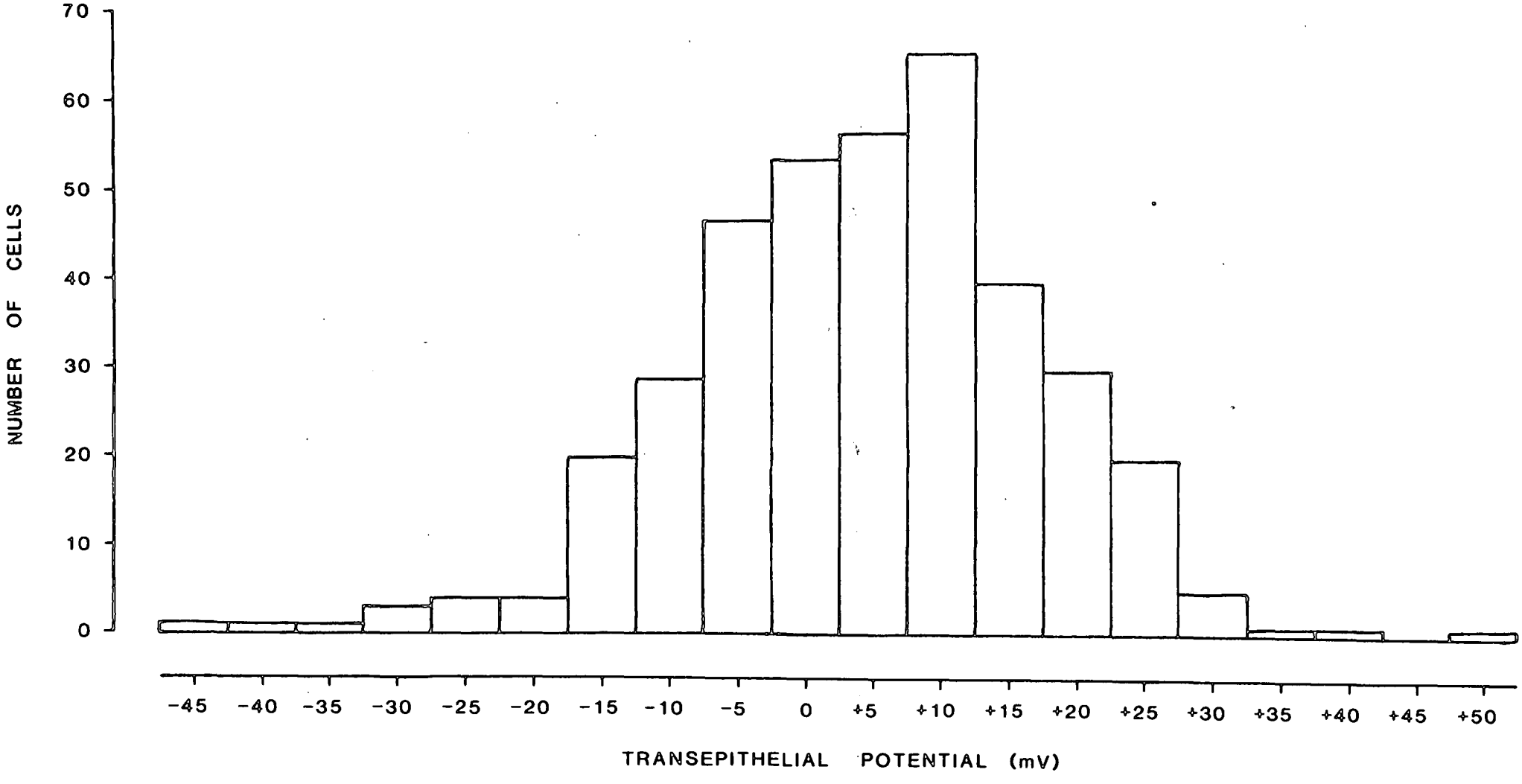

NUMBER OF CELLS
50
40
30
20
10
0
-36 -41 -46 -51 -56 -61 -66 -71 -76 -81 -86 -91 -96 -101 -106 -111 -116 -121 -126 -131
APICAL MEMBRANE POTENTIAL (mV)

Fig. 16

Distribution of transepithelial potential (T.E.P.) recorded from the Malpighian tubules of Locusta.

Ordinate: Number of Cells

Abscissa: T.E.P. in mV


NUMBER OF CELLS
70
60
50
40
30
20
10
0
-45
-40
-35
-30
-25
-20
-15
-10
-5
0
+5
+10
+15
+20
+25
+30
+35
+40
+45
+50
TRANSEPITHELIAL POTENTIAL (mV)

cells examined and coincident oscillations with the same time course were observed in T.E.P. (see Fig. 18). Furthermore, V_B did not show oscillations, the potential being steady within the noise of the recording system. The oscillations had a mean amplitude of 6.0 ± 0.3 mV measured from minimum to maximum and ranged up to 26 mV. Each oscillatory cycle took from 0.5 to 2 mins to complete.

Effects of Varying $[K^+]_o$ on Potentials

A study was carried out to determine the initial change in V_B as a result of varying the K^+ concentration of the bathing saline from 1.25 to 128 mM. Results obtained are shown in Table 7. It can be seen that, as the K^+ concentration of the perfusate was decreased below Normal saline values, V_B initially hyperpolarized whereas when $[K^+]_o$ was increased, V_B depolarized. The hyperpolarization of V_B in 1.25, 2.5 and 4.3 mM $[K^+]_o$ took between 1 and 2 mins and the new V_B was either maintained or the potential gradually became less hyperpolarized by 1 to 24 mV over the next 2 to 15 mins. The depolarization of V_B in 32, 64 and 128 mM $[K^+]_o$ took between 0.5 and 1.5 mins and the new V_B was maintained for at least 3 mins. The perfusate was returned to Normal saline after each change in external K^+ concentration and at least 5 mins elapsed before the next solution change. The original resting membrane potential was usually restored in 1-2 mins on return to 'normal' $[K^+]_o$ and was always re-established to within about 2 mV of the original V_B.

Fig. 17 shows the relationship between the mean V_B and $\log[K^+]_o$. It can be seen that V_B followed the Nernstian prediction of a 60.1 mV change in potential for a 10-fold increase in $[K^+]_o$ quite closely for external K^+ concentrations greater than the Normal saline (8.6 mM K^+). At $[K^+]_o$ lower than that of Normal saline, however, V_B started to

Table 7

Mean values for the initial V_B in various external concentrations of potassium. n represents the number of independent experiments, each involving separate tubule preparations.

$[K^+]_o$ (mM)	$\log[K^+]_o$ (mM)	New V_B ± S.E.M. (mV)	n
1.25	0.10	-107.7 ± 1.2	30
2.5	0.40	- 95.8 ± 1.1	29
4.3	0.63	- 87.5 ± 1.7	16
8.6	0.93	- 71.6 ± 0.3	530
32	1.51	- 39.6 ± 1.4	19
64	1.81	- 25.9 ± 1.1	31
128	2.11	- 6.6 ± 0.3	210

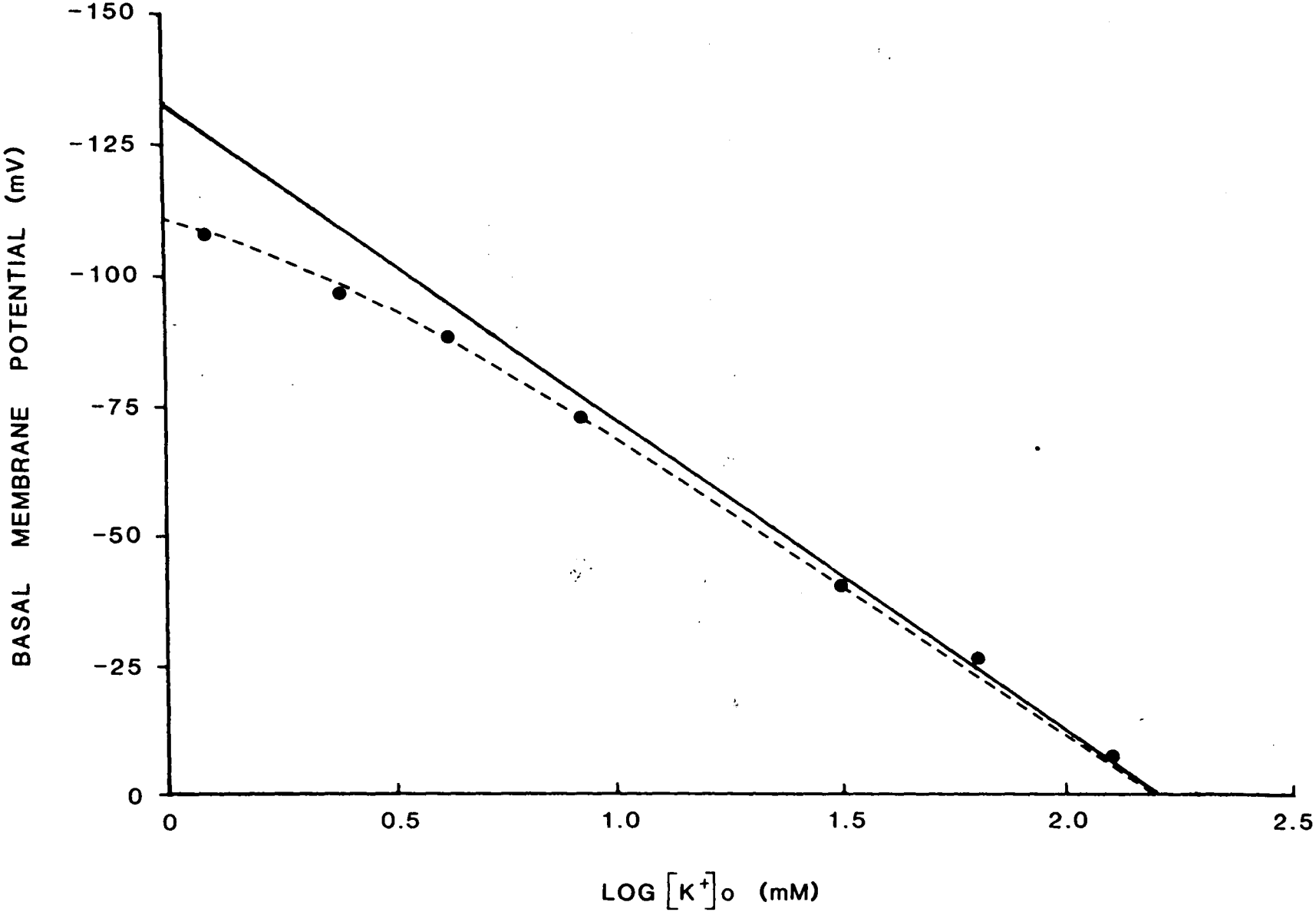
Fig. 17

Relationship between the basal membrane potential (V_B) and $\log_{10}$ concentration of external K^+.

(•) represents the mean measured V_B values.

(——) represents the Nernst prediction for V_B at 30°C.

(---) indicates the line calculated for V_B from the Goldman constant Field Equation based on the assumption that the basal membrane is 100 times more permeable to K^+ and Na^+ (see later).


-150
-125
-100
-75
-50
-25
0
BASAL MEMBRANE POTENTIAL (mV)
0
0.5
1.0
1.5
2.0
2.5
LOG [K+]o (mM)

deviate from the Nernstian prediction in a positive direction; the deviation getting larger as $[K^+]_o$ got smaller. This showed that at higher $[K^+]_o$, V_B was behaving as a potassium electrode whilst at lower $[K^+]_o$, other ions were becoming increasingly important in determining the membrane potential. For a K^+ electrode, $[K^+]_i$ is equal to $[K^+]_o$ when the membrane potential is zero. Thus, from Fig. 17, extrapolation of the Nernstian plot in the region where V_B acts as a K^+ electrode, gives a value of 157 mM for $[K^+]_i$ which compares favourably with the value of 165 mM expected for a perfect K^+ electrode.

Table 8 and Fig. 18 show the effect of changing the perfusate from Normal to 128 mM K^+ saline on V_B, V_A and T.E.P. It can be seen that V_B (as noted earlier) and V_A depolarized with the same time course to a new potential, over 0.5 to 1.5 mins, with the depolarization of V_A being 65% of the value found for V_B. The new depolarized V_B and V_A were stable during the maintained increase in $[K^+]_o$ for at least 3 mins. The depolarization of V_A was very variable with a range of 12 to 60 mV. Hence V_A, in 128 mM K^+ saline, varied from +16 to -89 mV (2% of cells actually depolarized to a positive potential with respect to the lumen). On the re-introduction of Normal saline, both membrane potential hyperpolarized to their original values, with the same time course, over 1 to 2 mins. In 128 mM $[K^+]_o$, T.E.P. followed the change in the differences between V_B and V_A; there being a significant increase in lumen positivity.

The responses of V_B, V_A and T.E.P. to 128 mM K^+ saline, described above, occurred in the majority of cells. However, the membrane potentials of a number of cells exhibited a different and unexpected behaviour in 128 mM K^+ saline. This atypical response to 128 mM $[K^+]_o$ will be described later. The more usual response, described in this section, will be, in future, designated the Type A Response.

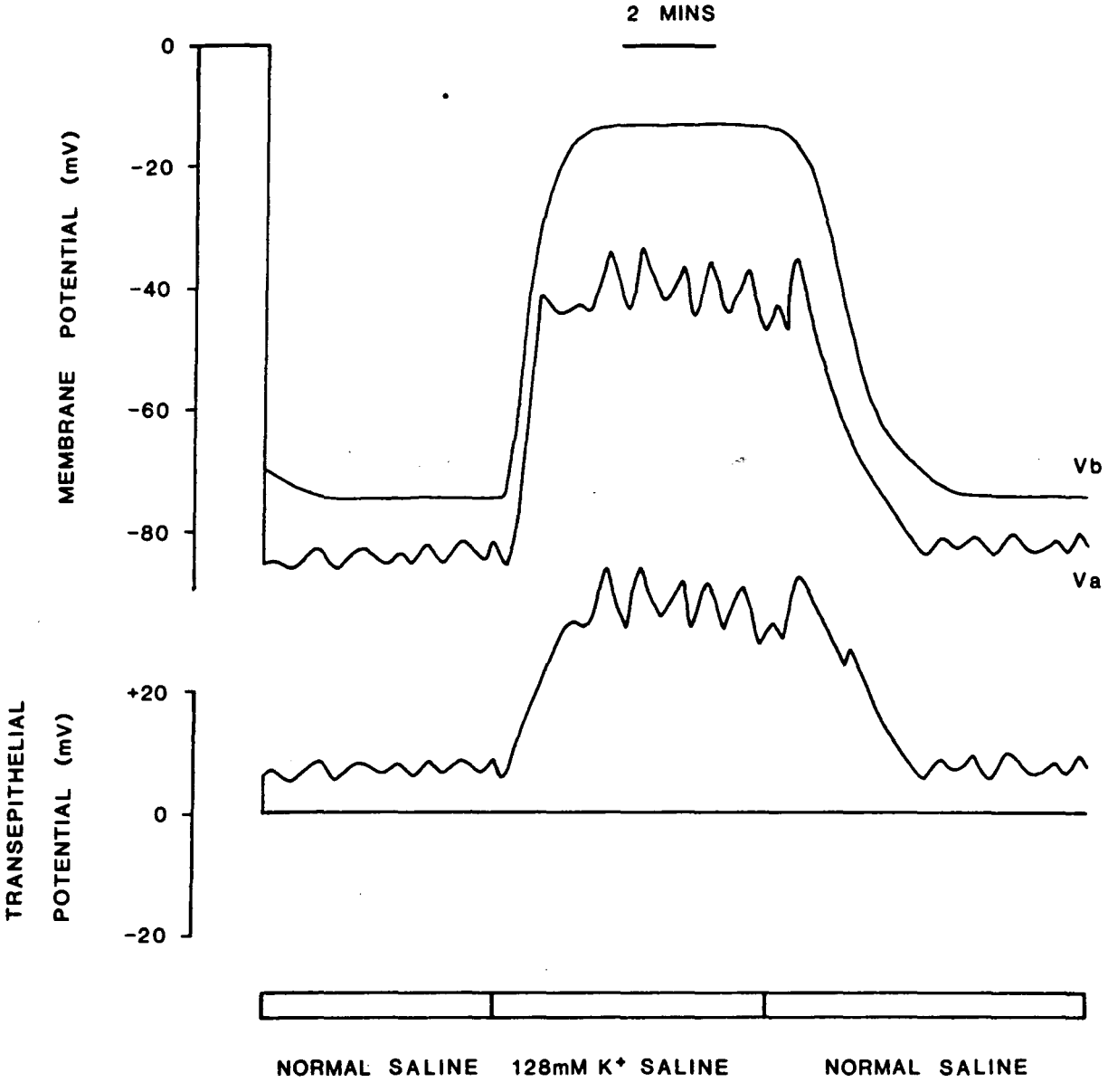
Table 8

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline (Type A response). n represents the number of independent experiments, each involving separate tubule preparations.

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	New P.D. Range (mV)	n
V_B	Normal	-71.5 ± 0.5	128 mM K^+	-6.6 ± 0.3	0 to -25	210
V_A	Saline	-83.1 ± 0.9	Saline	-41.1 ± 1.4	+16 to -89	166
T.E.P.		+ 5.6 ± 1.3		+33.6 ± 1.6	-43 to +84	171

Fig. 18

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline. Note that oscillations occurred in V_A and T.E.P. but not V_B.


2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
128mM K+ SALINE
NORMAL SALINE

Approximately 18% of all cells examined showed potential changes, following treatment with 128 mM $[K^+]_o$, that were not predictable on the basis of K^+ selectivity. These responses, referred to briefly above, are designated Type B Responses. Figure 19 shows an example of a typical Type B response by basal and apical membranes following a change from Normal to 128 mM K^+ saline. Thus, following the introduction of 128 mM $[K^+]_o$, there was an initial depolarization of both membrane potentials over 0.5 to 1 min. However, instead of levelling at the depolarized potentials, as in the Type A response, both V_B and V_A repolarized over the next 1 to 2 mins to new maintained values. Following the re-introduction of Normal saline, both membrane potentials depolarized over 0.5 mins before hyperpolarizing to the original resting potentials in 1 to 2 mins. Associated with these changes, T.E.P. increased in positivity over a 2 min period, in 128 mM K^+ saline, to a new maintained potential. With the re-introduction of Normal saline, T.E.P. returned to its original resting value over 1 to 2 mins.

Figure 20 is a diagrammatic form of Fig. 19 and describes the variety of range of potential changes seen in cells that were classified as showing the Type B response; these changes being divided into components. Table 9 shows the mean values for these components for V_B, V_A and T.E.P. No statistically significant difference could be found for V_B, V_A or T.E.P. between cells which exhibited Type B or Type A responses in Normal saline ($p = 0.3$-0.4, $p = 0.4$-0.5 and $p = 0.2$-0.3 respectively). However, examination of Table 9 shows that the mean initial depolarization (component b, Fig. 20) of V_B, despite being very variable (from 11 to 68 mV), was 23 mV less than the depolarization found in cells that showed the Type A response. As indicated above, the initial depolarization appeared somewhat quicker in cells exhibiting the Type B response; taking 0.5 to 1 min compared

Fig. 19

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline in cells which exhibited the Type B response. Note the unusual repolarization of V_B and V_A seen after the initial depolarization in 128 mM K^+ saline.

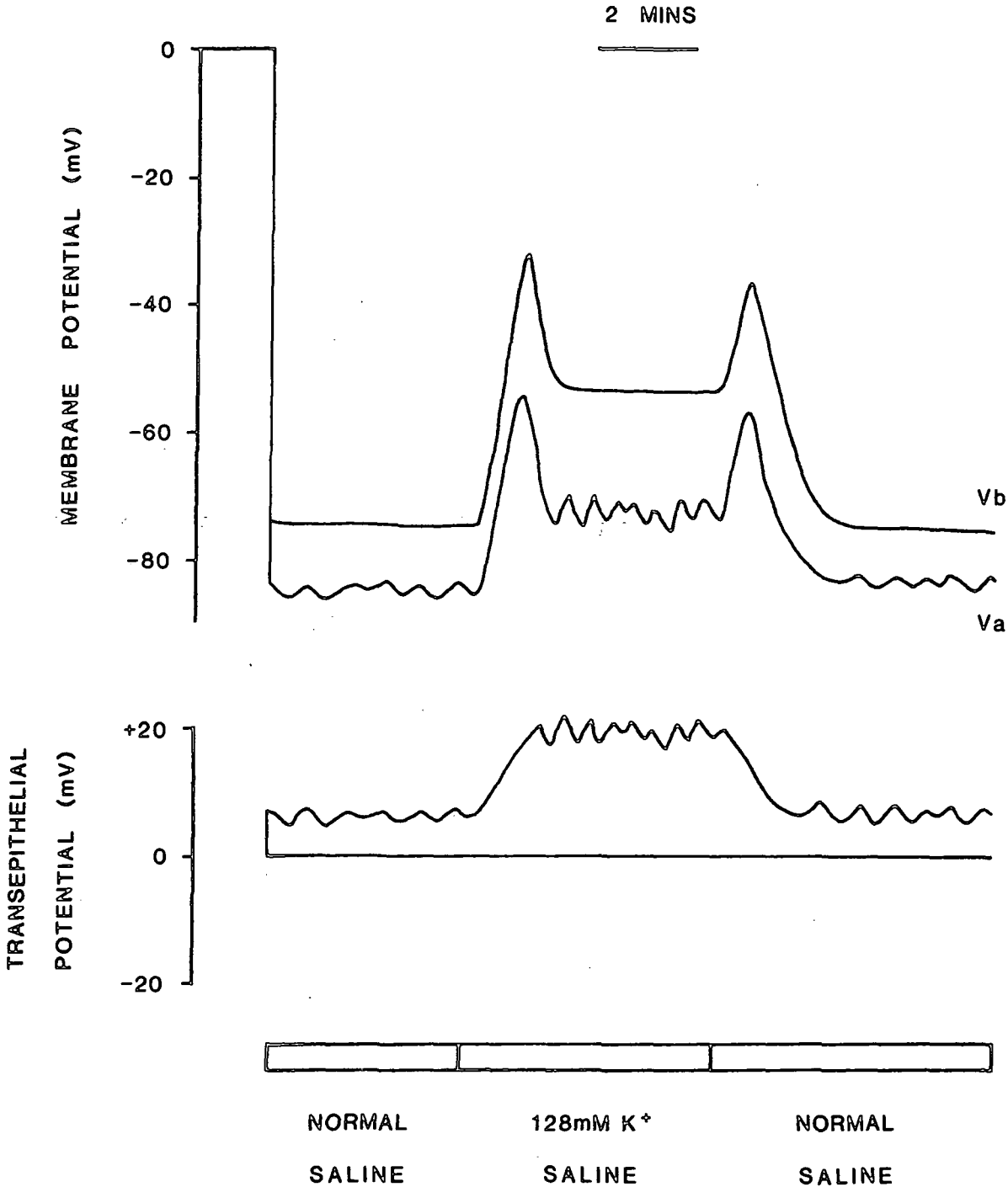

2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
128mM K+ SALINE
NORMAL SALINE

Fig. 20

The variety of membrane changes and resulting potentials in high K^+ (128 mM) saline during the 'typical' Type B response. Thus:

a = the membrane potential in Normal saline,

b = the initial change in potential in high K^+ saline,

c = the new potential,

d = the repolarizing change in high K^+ saline,

e = the repolarized potential,

f = the initial change in potential with the re-introduction of Normal saline,

g = the re-established resting potential in Normal saline.

Ordinate: Potential in mV

Abscissa: Time in Mins

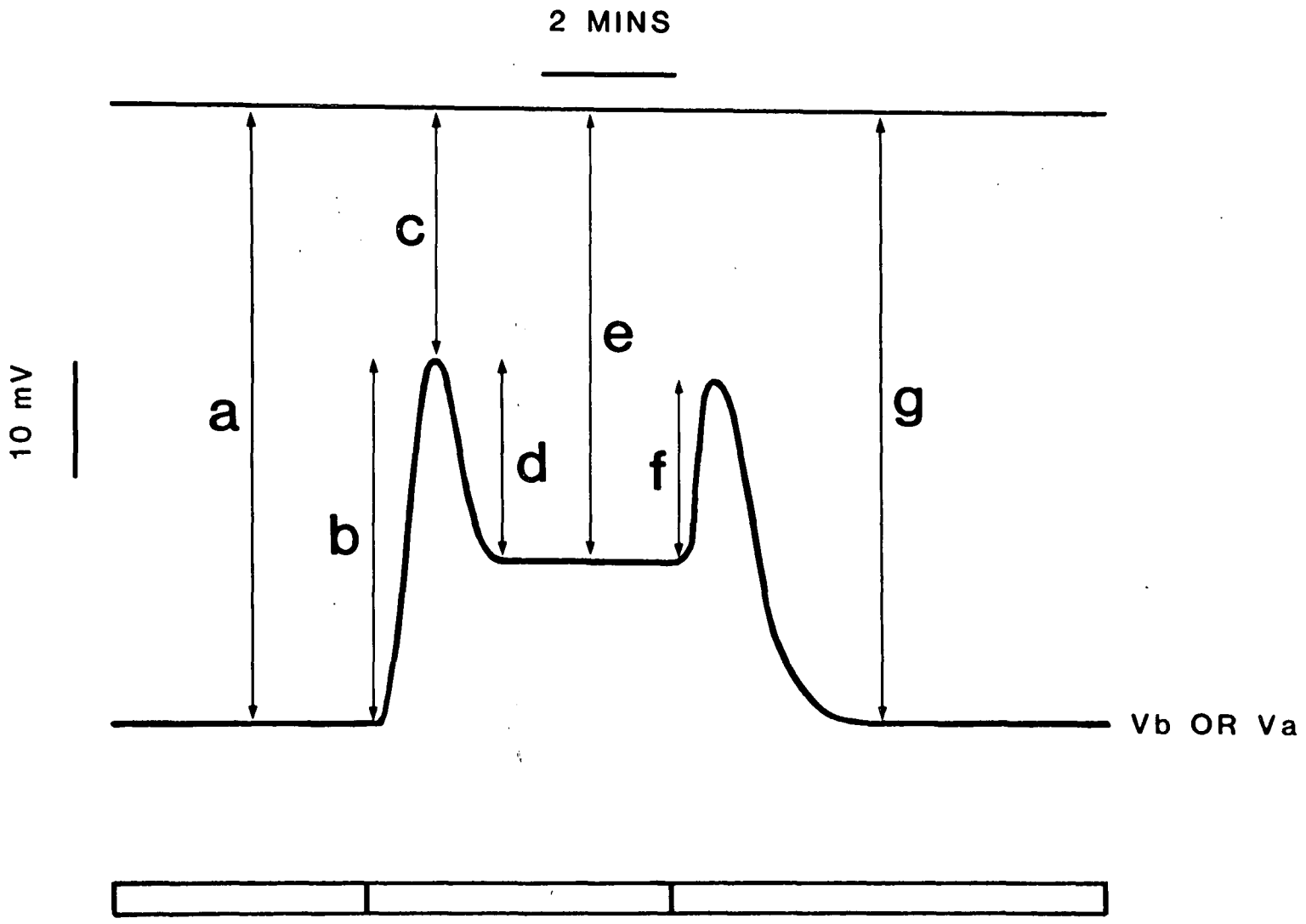

2 MINS
10 mV
a
b
c
d
e
f
g
Vb OR Va
NORMAL SALINE
128mM K+
SALINE
NORMAL SALINE

Table 9

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline in cells which exhibited the Type B response.

n represents the number of independent experiments, each involving separate tubule preparations.

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig. 20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig. 20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig. 20) (mV)	Treatment	Initial Δ P.D. ± S.E.M. (component f, Fig. 20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig. 20) (mV)	n
V_B	Normal	-73.4 ± 0.9	128 mM K^+	-30.9 ± 1.2	-54.3 ± 2.7	Normal	+20.2 ± 2.0	-74.2 ± 0.7	95
V_A	Saline	-84.2 ± 2.0	Saline	-54.0 ± 2.1	-76.1 ± 3.1	Saline	+20.0 ± 1.0	-83.1 ± 3.1	71
T.E.P.		+3.7 ± 1.4		+ 20.0 ± 1.7	+20.0 ± 1.7		-	+ 4.5 ± 0.9	70

with 1 to 2 mins in the Type A response. This difference cannot be attributed to differential perfusion rates; the latter remaining unchanged throughout.

Following the transient depolarization seen in 128 mM K^+ saline, a repolarizing change (component d, Fig. 20) of V_B occurred in approximately 69% of cells showing Type B responses and had a range of magnitude from 2 to 95 mV. This meant that in some cases V_B reached a value more negative than the membrane potential in Normal saline (-104 mV in the most extreme case). V_B was maintained at the repolarized potential (component e, Fig. 20), in about 65% of these cells, for at least 2 mins. On re-introduction of Normal saline, V_B showed an initial depolarization (component f, Fig. 20) in about 58% of cells, of between 5 and 50 mV, before hyperpolarizing to the original resting V_B (component g, Fig. 20) in 1 to 2 mins.

A degree of variation in the 'typical' Type B response, described above, was observed. Such variations are illustrated in Fig. 21. It must be emphasized, however, that despite variations, in all cases there was a marked deviation from the Nernstian prediction for K^+. The major differences from the 'typical' Type B response noted were as follows:

i) variation in the magnitude of the initial depolarization (component b, Fig. 20) and the repolarizing change (component d, Fig. 20) of V_B (Fig. 21, traces i),

ii) although nearly two thirds of cells showing Type B responses were maintained at the repolarized potential (component e, Fig. 20), about 35% of cells gradually became less negative by a mean of 4.7 ± 0.8 mV over 2 mins (Fig. 21, trace ii),

iii) whilst 69% of cells exhibited a repolarizing change (component d, Fig. 20) in 128 mM K^+ saline, approximately 25% of cells

Fig. 21

Examples of variations of V_B and V_A (i-v) found for the Type B response in high K^+ (128 mM) saline (see Fig. 19 for the 'typical' Type B response). $\longrightarrow$ indicates the change in perfusate from Normal to 128 mM K^+ saline.

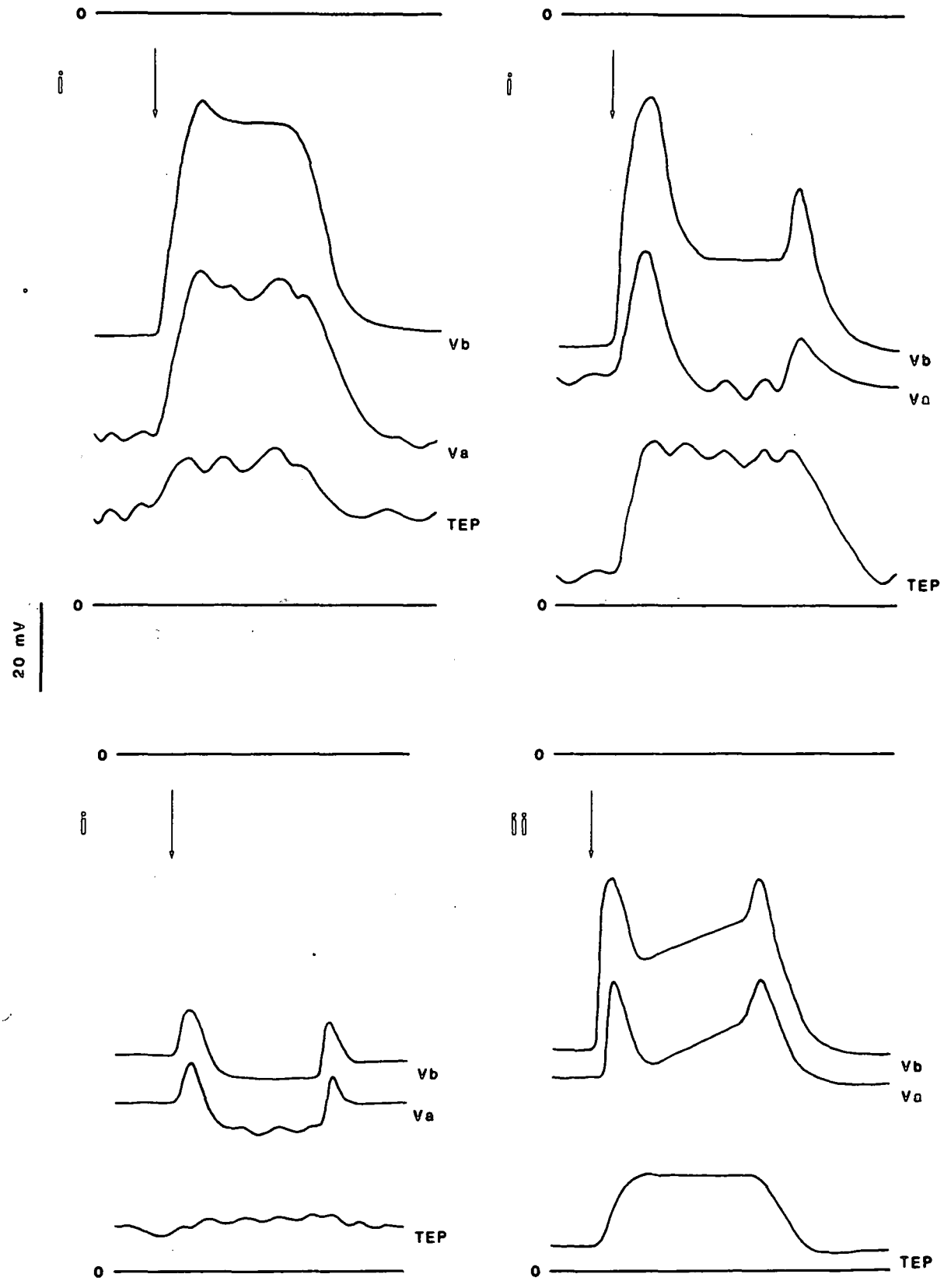
It can be seen that the variations for both membrane potentials occurred over the same time and voltage, resulting in T.E.P. increasing in positivity to a maintained value in high K^+ saline. Results show that a variety in the magnitude of the initial depolarization (component b, Fig. 20) and the repolarizing change (component d, Fig. 20) occurred for both membranes (i). Another variation included a lack of maintenence of the repolarized potential (component e, Fig. 20) in high K^+ saline (ii).

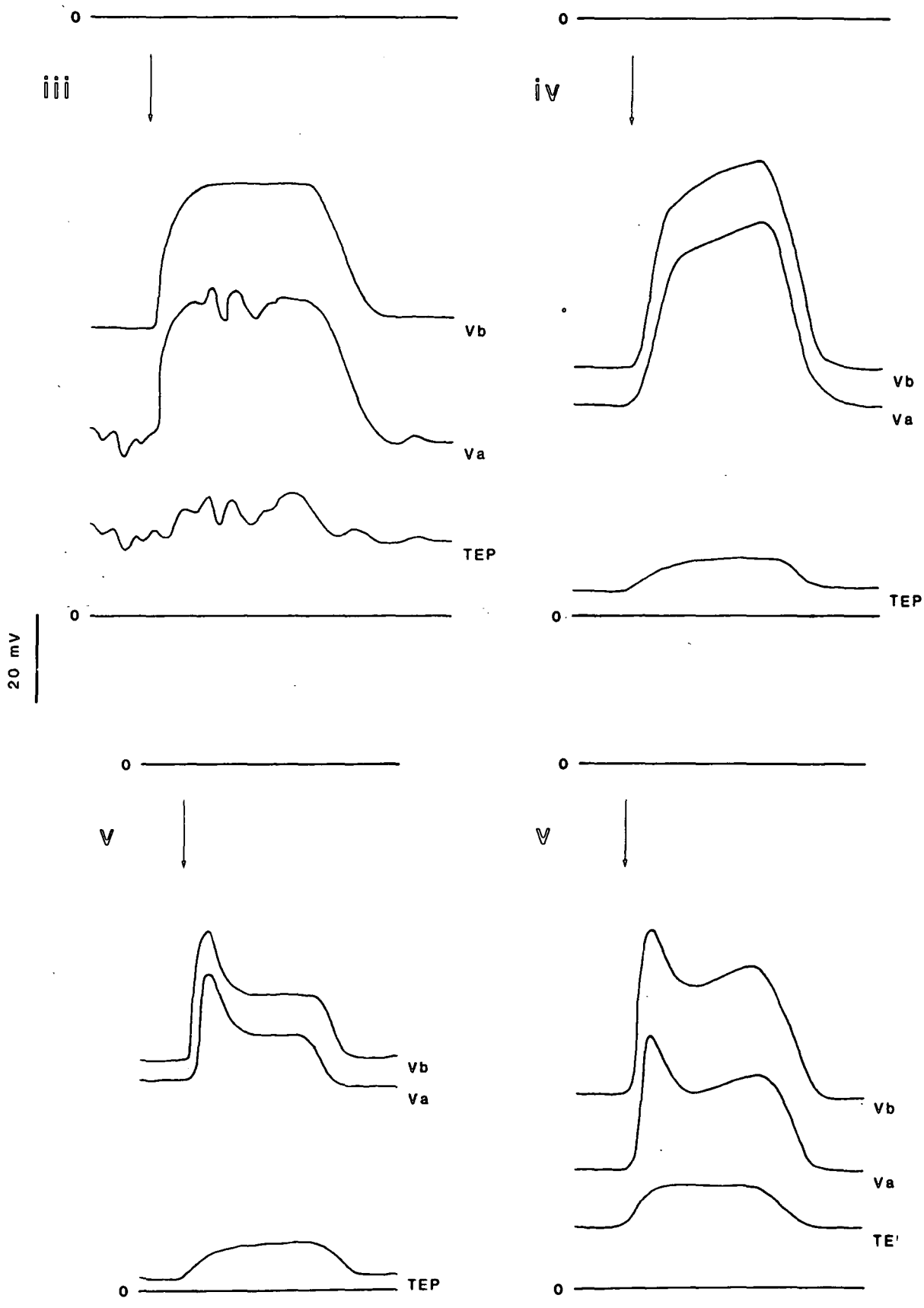
Further variations of the Type B response include a lack of a repolarizing change (component d, Fig. 20) in high K^+ saline and an initial depolarization in Normal saline (iii) and both the latter features plus a lack of maintenence of the depolarized potential (component c, Fig. 20) in 128 mM K^+ saline (iv). Finally, another variation included a lack of the initital depolarization (component f, Fig. 20) in Normal saline in cells which exhibited the repolarizing change (component d, Fig. 20) in high K^+ saline (v).

Actual values for these variations can be found in the text.

Ordinate: Potential in mV

Abscissa: Time in Mins


0
i
Vb
Va
TEP
0
20 mV
0
i
Vb
Va
TEP
0
0
i
Vb
Va
TEP
0
0
ii
Vb
Va
TEP
0
2 MINS


0
iii
Vb
Va
TEP
0
20 mV
0
iv
Vb
Va
TEP
0
0
v
Vb
Va
TEP
0
0
v
Vb
Va
TE'
0
2 MINS

studied did not. However, such cells showed a significant reduction in depolarization (component b, Fig. 20) compared with the Type A response, over 0.5 to 1.5 mins, in 128 mM $[K^+]_o$, giving a maintained potential (component c, Fig. 20) of -28.9 ± 2.7 mV which was stable for at least 2 mins (Fig. 21, trace iii),

iv) the remaining 6% of cells showed the reduced depolarization (component b, Fig. 20) of V_B, referred to in (iii) above, in 128 mM K^+ saline. However, the new V_B (component c, Fig. 20) then depolarized more slowly by 4.3 ± 0.6 mV over the next 2-3 mins (Fig. 21, trace iv),

v) the transient depolarization (component f, Fig. 20) of V_B, noted on re-introduction of Normal saline, did not occur in cells responding as described in (iii) and (iv) above, nor in approximately 21% of remaining cells exhibiting the more typical Type B behaviour (Fig. 21, traces v).

Table 9, along with Figs. 19 and 21, show that V_A showed qualitatively similar changes over the same time course as V_B, in the Type B response. Thus, the mean initial depolarization (component b, Fig. 20) of V_A, despite being very variable (from 7 to 51 mV) was 12 mV less than the value found in cells showing the Type A response. The mean depolarization of V_A was approximately 71% of the value found for V_B in these Type B responding cells - a value slightly higher than in the Type A response (65%). The repolarizing change (component d, Fig. 20) of V_A in 128 mM $[K^+]_o$ had a range of magnitude from 4 to 75 mV and this meant that, in some cases, V_A reached a value more negative than the potential in Normal saline (-128 mV in the most extreme case). On the re-introduction of Normal saline, V_A responsed with an initial depolarization (component f, Fig. 20) of between 4 and 45 mV before hyperpolarizing to a value

similar to the original resting V_A (component g, Fig. 20).

As with the Type B response for V_B, some variations in V_A were noted following exposure to 128 mM K^+ saline. These variations were almost identical to those described for V_B (see Fig. 21, traces I-V). Indeed, in all cases, the basal and apical membranes exhibited similar responses at the same time.

Table 9, along with Figs. 19 and 21, show the change in T.E.P. in 128 mM K^+ saline during the Type B response. It can be seen that T.E.P. gradually became more positive and levelled over 1.5 to 3 mins in 128 mM $[K^+]_o$ in most cells. The new T.E.P. was maintained for at least 2 mins and reduced over 1.5 to 2 mins to the original resting value when Normal saline was re-introduced. Some cells (about 21%) exhibited some small differences (ca. 3-4 mVs) between the relative changes of V_B and V_A such that T.E.P. changed with a pattern similar to that of the Type B response.

All membrane potentials measured in association with the Type B response, in 128 mM K^+ saline, were significantly more negative ($p < 0.001$) than those found in the Type A response. In addition, T.E.P. measured in association with the Type B response was significantly less positive ($p < 0.001$) in 128 mM $[K^+]_o$ than that measured during the Type A response due to V_B depolarizing less than V_A in the former situation. Furthermore, throughout this study, tubule cells from the same animal showed either Type A or Type B behaviour in 128 mM K^+ saline, suggesting that within a given insect all cells were in a similar physiological state. No seasonal distribution in the appearance of the Type B response was apparent.

Finally, the amplitude of the oscillations of V_A (and T.E.P.) in 128 mM k^+ saline were 11.4 ± 0.8 mV and 9.0 ± 1.0 mV respectively

in cells which exhibited the Type A and Type B response, with oscillations occurring in 61% and 50% of cells. These amplitude values were not significantly different ($p = 0.05-0.1$) from each other but were significantly greater ($p < 0.001$ and $p = 0.001-0.01$ respectively) than the value (6.0 ± 0.3 mV) found in Normal saline. However, although the mean amplitude of these oscillations increased in 128 mM K^+ saline, the maximum measured oscillation increased only slightly, from 29 mV in Normal saline to 33 mV in 128 mM $[K^+]_o$. Oscillations decreased to the original value when Normal saline was re-introduced.

Application of the Nernst Equation to Cells which Exhibited the Type B Response

Table 10 shows the response of V_B to a range of $[K^+]_o$, in cells which exhibited the Type B response. At $[K^+]_o$ less than Normal saline, V_B hyperpolarized as described earlier for the Type A response. At $[K^+]_o$ above Normal saline, the 'typical' Type B response was evident for 64 and 128 mM $[K^+]_o$. In 64 mM $[K^+]_o$, the mean initial depolarization (which took 1-1.5 mins) was 19 mV less than the value at the same concentration in the Type A response. Furthermore, a repolarizing change (component d, Fig. 20), in the range 4 to 20 mV, was observed in 64 mM K^+ saline, although an initial depolarization (component f, Fig. 20) on return to Normal saline was not observed. On changing to 32 mM $[K^+]_o$ from Normal saline, the mean initial depolarization was 12 mV less than in the Type A response and took 1.5 mins. At this $[K^+]_o$, no repolarizing change (component d, Fig. 20) occurred; V_B remaining at the new potential (component c, Fig. 20) for at least 2 mins. As in the Type B response in 128 mM K^+ saline, the established values of V_B, in 64 mM K^+ and 32 mM K^+ salines, were significantly more negative ($p < 0.001$) than the equivalent values during the Type A

Table 10

Mean values for V_B in various external concentrations of potassium in cells which exhibited the Type B response. n represents the number of independent experiments, each involving separate tubule preparations.

V_B ± S.E.M. (component a, Fig. 20) (mV)	$[K^+]_o$ (mM)	New V_B ± S.E.M. (component c, Fig. 20) (mV)	Repolarized P.D. ± S.E. (component e, Fig. 20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig. 20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig. 20) (mV)	n
	1.25	-105.0 ± 7.2	-		-		3
	2.5	- 94.1 ± 2.9	-		-		3
	4.3	- 85.4 ± 5.0	-	Normal	-		3
-73.4 ± 0.9	8.6	- 73.4 ± 0.9	-	Saline	-	-72.0 ± 2.3	95
	32	- 53.0 ± 5.2	-53.0 ± 5.2		-		3
	64	- 43.7 ± 3.2	-52.7 ± 2.3		-		3
	128	- 30.9 ± 1.5	-53.6 ± 2.7		+20.2 ± 2.0		95

response.

Figure 22 shows the relationship between the repolarized Type B potentials (component e, Fig. 20) and $\log[K^+]_o$. It can be seen clearly that at lower $[K^+]_o$, V_B deviates in a positive direction from Nernst as described earlier for Type A responses, whilst at higher $[K^+]_o$, a large deviation of V_B occurred in the negative direction from Nernst. The nature of this negative deviation from Nernst was investigated further.

Figure 22 shows that the mean established potentials in 32 mM, 64 mM and 128 mM $[K^+]_o$ during the Type B response were very similar (all about -53 mV). This suggests that the final membrane potential in $[K^+]_o$ greater than Normal saline, in cells which exhibit the Type B response, may be independent of $[K^+]_o$ above a certain value (i.e. 32 mM K^+ saline in this study).

It was noted that further additions of 128 mM K^+ saline, in cells which exhibited the Type B response, following at least 5 mins recovery of V_B in Normal saline, induced similar Type B behaviour. No significant differences ($p = 0.4$-0.5) in potential changes were noted between the first and subsequent Type B responses.

Finally, as a result of the 128 mM K^+ saline used in experiments described so far containing no Na^+, it may be argued that, despite its presence in 32 mM and 64 mM $[K^+]_o$, the Type B response was the direct result of a change in the Na^+ gradient across the cell membranes (see later). Thus, it was decided to examine the effect of some Na^+ in high K^+ saline treatments. Tables 11a and 11b show the effect of 118 mM K^+, 10 mM Na^+ saline on V_B, V_A and T.E.P. in cells which exhibited either the Type A or Type B response. Table 11a shows that although the depolarization of V_B and V_A in 118 mM K^+, 10 mM Na^+ saline were

Fig. 22

Relationship between the basal membrane potential and $\log_{10}$ concentration of external K^+ in cells which exhibited the Type B response.

(●) represents the mean measured V_B values in cells which exhibited the Type A response.

(o) represents a typical experiment representative of three experiments in cells which exhibited the Type B response.

(——) indicates the Nernst prediction for V_B at 30°C.

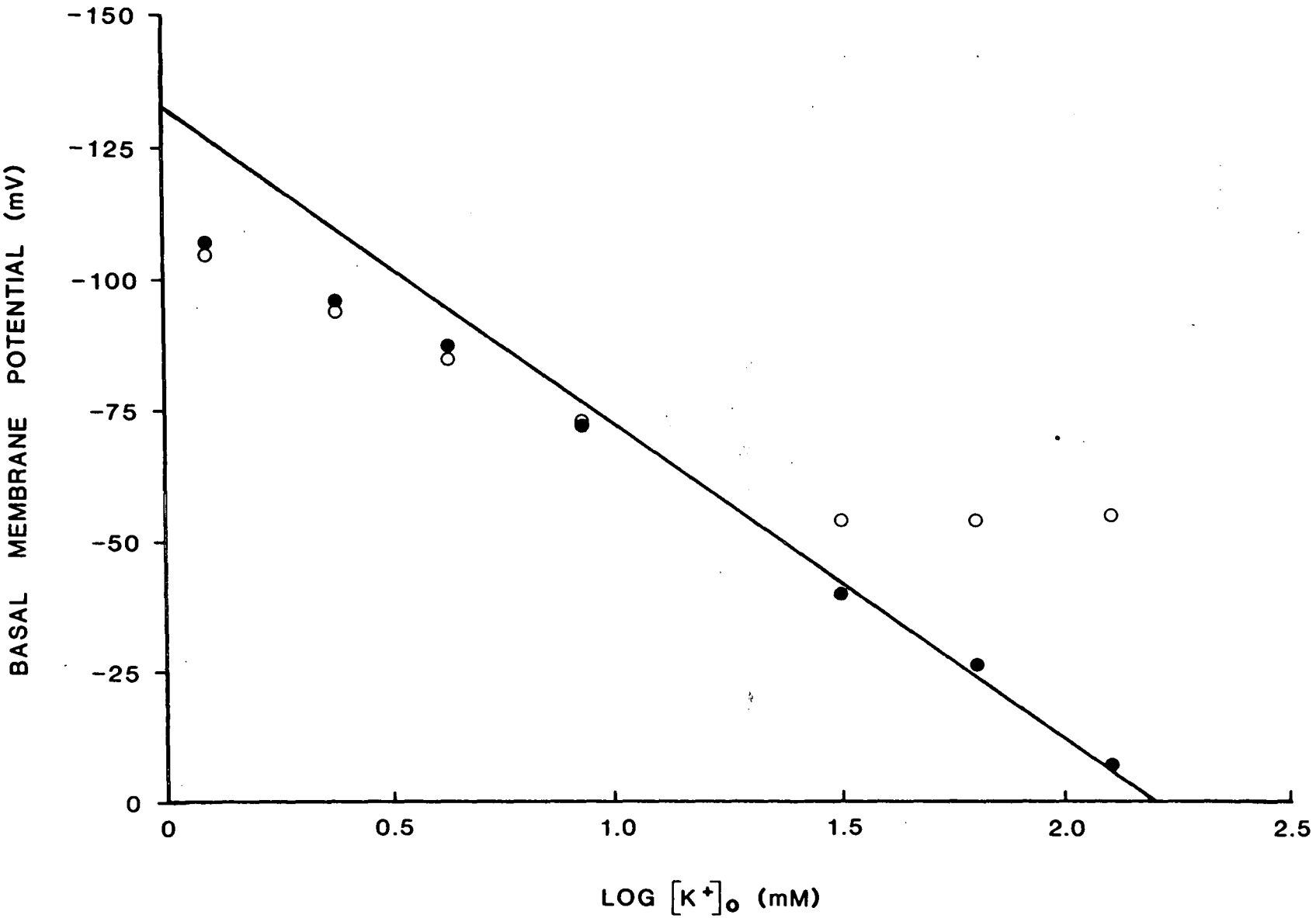

-150
-125
-100
-75
-50
-25
0
BASAL MEMBRANE POTENTIAL (mV)
0
0.5
1.0
1.5
2.0
2.5
LOG [K+]o (mM)

Table 11a

Effect of changing the perfusate from Normal to 118 mM K^+, 10 mM Na^+ saline in cells which exhibited the Type A response.

Table 11b

Effect of changing the perfusate from Normal to 118 mM K^+, 10 mM Na^+ saline in cells which exhibited the Type B response.

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

Table 11a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	n
V_B	Normal	-71.8 ± 2.8	118 mM K^+,	-11.5 ± 4.3	4
V_A	Saline	-84.5 ± 4.0	10 mM Na^+	-51.8 ± 11.1	4
T.E.P.		+ 9.0 ± 5.9	Saline	+34.5 ± 14.0	4

Table 11b

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig. 20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig. 20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig. 20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig. 20) (mV)	Initial P.D. ± S.E.M. (component g, (Fig. 20) (mV)	n
V_B	Normal	-75.0 ± 4.3	118 mM K^+,	-34.2 ± 6.4	-40.5 ± 4.8	Normal	-	-73.1 ± 5.1	5
V_A	Saline	-79.4 ± 5.5	10 mM Na^+	-52.2 ± 7.0	-61.5 ± 2.7	Saline	-	-80.3 ± 4.8	5
T.E.P.		+ 1.4 ± 5.2	Saline	+18.2 ± 4.1	-		-	+2.3 ± 4.2	5

slightly less than in 128 mM K^+ saline in cells which exhibited the Type A response, the new V_B and V_A (component c, Fig. 20) and T.E.P. were not significantly different ($p = 0.2$-0.3, $p = 0.3$-0.4 and $p > 0.9$ respectively). The slightly more negative value for V_B in 118 mM $[K^+]_o$ compared with 128 mM $[K^+]_o$ can be attributed to a Nernstian reduction in ΔV_B. This can be seen if the depolarized value for V_B in 118 mM $[K^+]_o$ is substituted into the Nernst plot of Fig. 17.

From Table 11b it can be seen that, in the presence of a small amount of Na^+, high K^+ saline still produced Type B behaviour in cells which exhibited the naturally-occurring Type B response. Indeed, the depolarized V_B and V_A (component c, Fig. 20) and T.E.P. were not significantly different ($p > 0.9$ for V_B, V_A and T.E.P.) from the equivalent values found in 128 mM K^+ saline. However, the small repolarizing changes (component d, Fig. 20) of V_B (from 5 to 7 mV) and V_A (from 4 to 17 mV) in 118 mM K^+, 10 mM Na^+ saline produced repolarized potentials (component e, Fig. 20) which were significantly less negative ($p = 0.02$-0.03 for V_B and $p < 0.001$ for V_A) than the equivalent potentials found in 128 mM K^+ saline. Furthermore, the initial depolarization (component f, Fig. 20) of V_B and V_A, which occurred in many cells on returning to Normal from 128 mM K^+ saline, did not occur in 118 mM K^+, 10 mM Na^+ saline. Thus, a small amount of Na^+ in high K^+ saline reduced the extent, but not the production, of the Type B response.

Effect of K^+ free Saline

Table 12 and Fig. 23 show that when the perfusate was changed from Normal saline to a K^+ free saline, in which K^+ was replaced by Na^+, both V_B and V_A hyperpolarized by 42.7 ± 0.8 mV and 23.6 ± 2.2 mV, respectively, within 1-2 mins. Thereafter, in the majority of cases, both membrane potentials depolarized. The extent of this depolarization

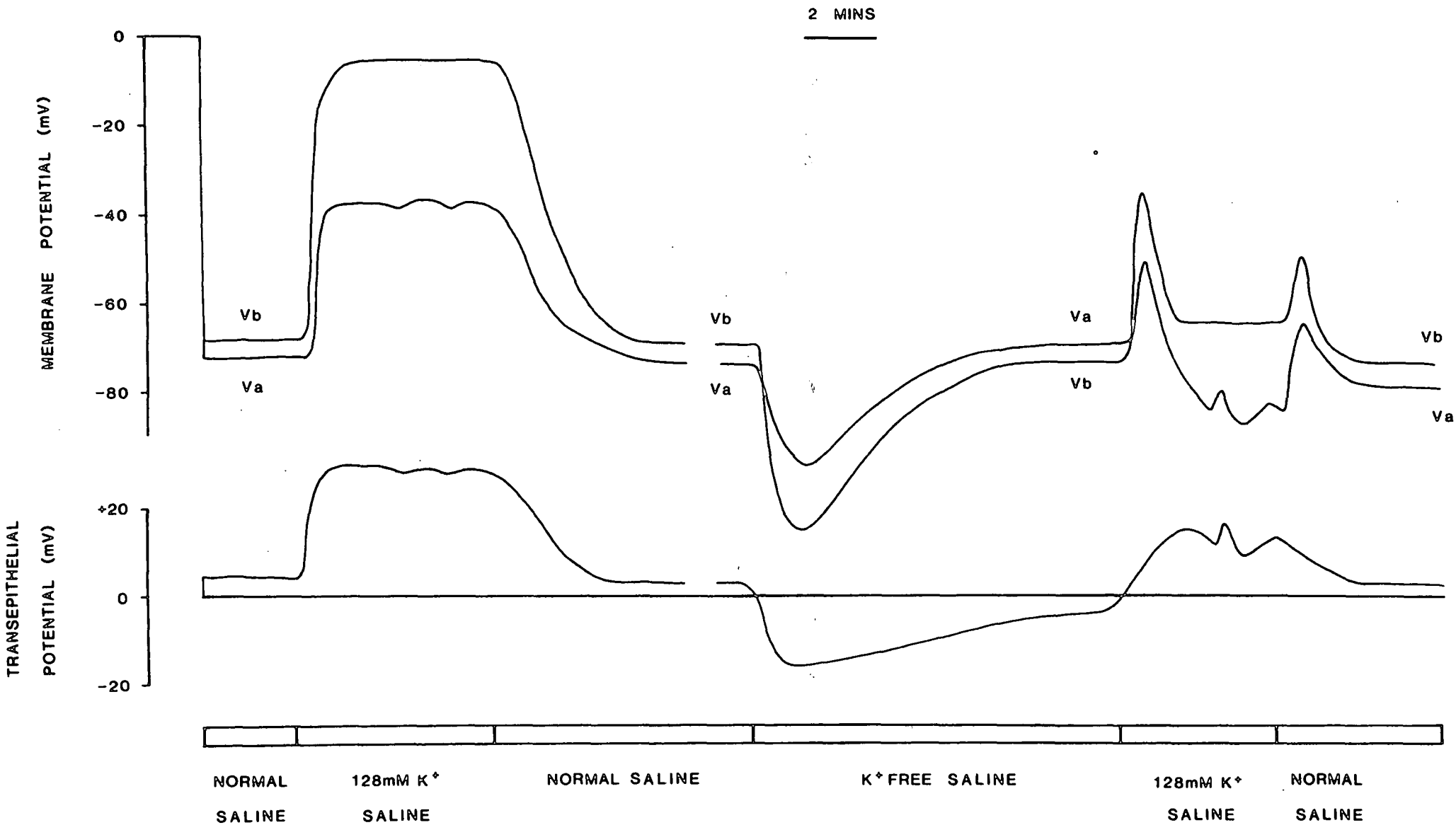
Table 12

Effect of V_B, V_A and T.E.P. of changing the perfusate from Normal saline to K^+ free or K^+ free, Na^+ free or K^+ free, Cl^- free saline. n represents the number of individual experiments, each involving separate tubule preparations.

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	n
V_B	Normal	-69.6 ± 0.9	K^+ free	-112.3 ± 1.2	42
V_A	Saline	-83.2 ± 3.1	Saline	-106.8 ± 3.2	19
T.E.P.		+ 8.3 ± 1.9		- 12.5 ± 2.3	19
V_B	Normal	-73.3 ± 1.8	K^+ free,	-107.9 ± 2.1	7
V_A	Saline	-78.0 ± 6.0	Na^+ free	-102.3 ± 4.4	7
T.E.P.		+ 2.4 ± 7.7	Saline	- 8.4 ± 4.8	7
V_B	Normal	-68.0 ± 4.4	K^+ free.	-114.3 ± 8.0	3
V_A	Saline	-79.0 ± 3.5	Cl^- free	-109.7 ± 10.2	3
T.E.P.		+ 8.3 ± 0.7	Saline	- 12.0 ± 14.2	3

Fig. 23

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from K^+ free to 128 mM K^+ saline. Note the induced Type B response which contrasts with the naturally occurring Type A response (as shown by an earlier addition of 128 mM K^+ saline).


2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
Vb
Va
Va
Vb
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
128mM K+ SALINE
NORMAL SALINE
K+ FREE SALINE
128mM K+ SALINE
NORMAL SALINE

was very variable and ranged from 2 to 90 mV, over 2 to 20 mins, for V_B and 2 to 48 mV, over 3 to 8 mins, for V_A. After depolarizing, a maintained V_B and V_A were established for at least 7 mins. A small proportion of cells studied (ca. 12%) maintained the potential after hyperpolarization. In view of this variation in the depolarization of both V_B and V_A in K^+ free saline, the final established potential could be either more negative or more positive than the original resting values in Normal saline. Associated with the above changes in V_B and V_A, T.E.P. decreased from +8.3 ± 1.9 mV to -12.5 ± 2.3 mV over 1 to 2 mins in K^+ free saline due to V_B hyperpolarizing more than V_A; V_A only hyperpolarized 55% of the value found for V_B. In one third of tubules studied, T.E.P. remained at this negative value. However, in the majority of tubules, the mean T.E.P. reduced by 8.4 ± 2.9 mV over 3 to 11 mins to a maintained T.E.P. which was then established for at least 5 mins. This reduction in T.E.P., to a less negative value, was due to V_B depolarizing faster than V_A before a maintained potential was achieved.

Only 27% of cells exhibited oscillations of V_A (and T.E.P.) in K^+ free saline and these had a mean amplitude of 4.0 ± 1.0 mV. Thus, although the frequency of oscillations were reduced in K^+ free saline, the amplitude was not affected ($p = 0.1-0.2$) compared with Normal saline.

In order to substantiate the earlier observation that the initial hyperpolarization of V_B and V_A in K^+ free saline was due largely to the lack of K^+, the effect of changing the perfusate from Normal saline to either K^+ free, Na^+ free or K^+ free, Cl^- free saline was examined (for composition of solutions, see Materials and Methods, Table 1.) Results are shown in Table 12. It was found that the hyperpolarization of V_B and V_A and the decrease of T.E.P. on introduction

of K^+ free, Na^+ free saline (which took 1-2 mins) produced potentials which were not significantly different ($p = 0.2-0.3$, $p = 0.8-0.9$ and $p = 0.8-0.9$ respectively) from the values found in K^+ free saline containing Na^+. After hyperpolarizing, V_B and V_A declined by 12 to 60 mV and by 8 to 62 mV, respectively over 6 to 13 mins in K^+ free, Na^+ free saline. Similarly, the hyperpolarization of V_B and V_A and the decrease of T.E.P. found with an absence of Cl^- in K^+ free saline, over 1-2 mins, produced potentials which were not significantly different ($p = 0.8-0.9$, $p = 0.7-0.8$ and $p > 0.9$ respectively) to values found in K^+ free saline. As with other K^+ free treatments, V_B and V_A declined by 12 to 54 mV and by 12 to 40 mV, respectively, over 6 to 11 mins in K^+ free, Cl^- free saline.

Table 13 shows the effect on V_B of adding Normal saline immediately after 10 to 20 mins K^+ free treatment. It can be seen that two types of response occurred and this was related to the negativity of V_B in K^+ free saline. Thus, for cells in which V_B had depolarized, in K^+ free saline, to a value similar or more negative than the original Normal saline potential, the introduction of Normal saline resulted in a depolarization of V_B between 12 and 54 mV, over 0.5 to 1 min, before a gradual hyperpolarization towards the original resting value. In contrast, cells in which V_B had depolarized, in K^+ free saline, to a value less negative than the original Normal saline potential, responded with an immediate gradual hyperpolarization in Normal saline; towards the original V_B. Hence, it would appear that once V_B had declined to a given value in K^+ free saline, no further depolarization was possible when Normal saline was introduced.

Effect of High K^+ (128 mM) Saline Directly After Treatment in K^+ Free Saline

It was found earlier that an unusual hyperpolarization of both

Table 13

Effect on V_B of changing the perfusate from K^+ free to Normal saline.

a represents cells in which V_B did not decrease greatly during K^+ free saline treatment.

b represents cells in which V_B decreased to a value less than the original resting potential, during K^+ free saline treatment.

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

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. After 0.5 min (mV)	New P.D. ± S.E.M. After 4 mins (mV)	n
V_B	Normal Saline	a -70.2 ± 0.9	K^+ free Saline	-74.4 ± 6.4	Normal Saline	-40.7 ± 3.8	-63.1 ± 4.9	10
		b -69.6 ± 1.1		-53.1 ± 2.7		-55.4 ± 2.9	-66.4 ± 3.3	10

V_B and V_A, the Type B response, was sometimes observed in the presence of 128 mM K^+ saline. In order to explore the possibility that the Type B response was related to an altered physiological condition in the cells involved, such as the state resulting from K^+ free saline, it was decided to introduce 128 mM K^+ saline directly after K^+ free treatment. In these experiments, cells showing the Type A response were selected and these were identified by changing the perfusate from Normal to 128 mM K^+ saline. Following a recovery period in Normal saline, the latter was substituted by K^+ free saline.

Table 14 and Fig. 23 show the effect of the introduction of 128 mM K^+ saline on V_B, V_A and T.E.P. after a period of 7 to 20 mins in K^+ free saline. It can be seen that this K^+ free treatment induced the Type B response by V_B and V_A in all cells studied. However, this was only achieved if tubules had been exposed to K^+ free saline for a minimum of 5 mins. Results show that the induced Type B response was similar in nature to the naturally occurring Type B response with V_B and V_A changing with exactly the same time course and with quantitatively similar potentials. Indeed, all the membrane changes and resulting V_B and V_A values in 128 mM K^+ saline after K^+ free treatment were not significantly different ($p > 0.9$) to the equivalent values found during the Type B response. Similarly, the mean T.E.P. in 128 mM K^+ saline was not significantly different ($p = 0.2$-0.3) from that found in tubules which exhibited the natural Type B response and was significantly smaller ($p < 0.001$) than that found in tubules which exhibited the Type A response. Again, as with natural Type B behaviour, this smaller T.E.P., compared with the Type A situation, was due to V_B depolarizing proportionally less than V_A. Thus, the initial depolarization (component b, Fig. 20) of V_A was ca. 70% of that for

Table 14

Effect on V_B alone or V_B, V_A and T.E.P. of changing the perfusate from K^+ free, 1.25 mM K^+ or 2.5 mM K^+ saline to 128 mM K^+ saline. n represents the number of individual experiments, each involving separate tubule preparations.

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20 (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20 (mV)	n
V_B	Normal	-70.4 ± 1.1	K^+ free	-75.0 ± 4.8	128mM K^+	-27.6 ± 3.4	-54.3 ± 3.4	Normal	+16.4 ± 3.0	-72.4 ± 1.3	24
V_A	Saline	-76.7 ± 3.8	Saline	-82.5 ± 5.6	Saline	-49.6 ± 4.1	-76.1 ± 4.9	Saline	+18.9 ± 3.8	-77.7 ± 3.5	15
T.E.P.		+ 5.2 ± 3.2		- 1.7 ± 3.7		+15.5 ± 3.5	-		-	+ 5.4 ± 2.9	18
V_B	Normal Saline	-71.0 ± 0.5	1.25mM K^+ Saline	-72.8 ± 4.1	128mM K^+ Saline	-21.8 ± 5.5	-41.6 ± 5.7	Normal Saline	+13.5 ± 1.7	-70.3 ± 1.0	5
V_B	Normal Saline	-71.0 ± 0.6	2.5mM K^+ Saline	-75.3 ± 0.7	128mM K^+ Saline	-12.7 ± 4.1	-	Normal Saline	-	-72.1 ± 0.8	3

V_B and compared favourably with the relationship found in cells which exhibited the Type B response naturally (ca. 71%). As for the latter response, some variations of V_B and V_A from the general pattern of induced Type B responses, were noted. These variations, however, were similar to those shown in Fig. 21.

It is clear that cells which normally exhibited Type A behaviour to 128 mM K^+ saline can be induced to give a Type B response. This supports the view expressed earlier that the differences between the Type A and Type B response are unrelated to penetration of different cell types.

Oscillations of V_A (and T.E.P.) were exhibited in 39% of cells and had a mean amplitude of 7.4 ± 0.6 mV; values were not significantly different ($p = 0.2-0.3$) from those found in cells exhibiting the naturally occurring Type B response in 128 mM K^+ saline.

As results show that a Type B response could be induced in 128 mM $[K^+]_o$ after K^+ free treatment, it was decided to investigate the effect of 1.25 mM and 2.5 mM K^+ saline pretreatment on the 128 mM K^+ saline response of V_B in cells which originally showed the Type A response. Results are shown in Table 14. It can be seen that a period of 8 to 10 mins in 1.25 mM K^+ saline resulted in an inducement of Type B behaviour in 128 mM K^+ saline. However, the magnitude of this Type B response was less than that found in both cells which exhibited the naturally occurring Type B response and cells which were induced to exhibit the Type B response with K^+ free saline treatment. Thus, the repolarized V_B (component e, Fig. 20), in 128 mM $[K^+]_o$ after 1.25 mM K^+ saline treatment, was significantly less negative ($p = 0.002-0.003$) than the equivalent potential of the Type B response but significantly more negative ($p < 0.001$) than the depolarized potential of the Type A response.

Results from Table 14 show that treating cells for up to 15 mins with 2.5 mM K^+ saline before the addition of 128 mM K^+ saline did not induce Type B behaviour. Thus, the depolarized V_B (component c, Fig. 20), after such treatment, was not significantly different ($p = 0.8-0.9$) from the equivalent value found in the Type A response.

A small number of cells ($n = 4$) were exposed to 64 mM K^+ saline immediately after treatment in K^+ free or 1.25 mM K^+ saline for 10 mins. In all cases, V_B still exhibited the Type A response. Thus, the maintained V_B of -23.0 ± 2.8 mV in 64 mM $[K^+]_o$ was not significantly different ($p = 0.4-0.5$) from that obtained in cells showing the Type A response in 64 mM K^+ saline.

It seems that in order to artificially induce the Type B response, $[K^+]_o$ has to be below a certain value (< 2.5 mM K^+) otherwise the original Type A response will still occur. Similarly, in order for the artificially induced Type B behaviour to be exposed, $[K^+]_o$ has to be above a certain value (> 64 mM K^+), otherwise the original Type A response will still occur. However, other factors besides $[K^+]_o$, including $[Na^+]_o$, may be important in these modified low and high K^+ salines.

Finally, it was decided to explore whether a Type B response could still be induced for V_B if an addition of Normal saline occurred between treatment with K^+ free and 128 mM K^+ salines ($n = 20$). It was found that a Type B response was still demonstrable in 128 mM $[K^+]_o$ after perfusing with Normal saline, following K^+ free treatment. Thus, the depolarized V_B (component c, Fig. 20) and repolarized V_B (component e, Fig. 20) in 128 mM K^+ saline were not significantly different ($p > 0.9$) from the equivalent values found during the naturally occurring Type B response.

Effect of Na^+ free Saline

Table 15a shows the effect on V_B, V_A and T.E.P. of changing the

Table 15a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to Na^+ free saline.

Table 15b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Na^+ free to 128 mM K^+ saline in cells which exhibited the Type A response.

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

Table 15a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	2	5	10	15	20	30	45
V_B	Normal	-68.5 ± 1.0	Na^+free	-62.5 ± 2.2	-70.3 ± 2.6	-62.6 ± 1.3	-55.9 ± 1.5	-49.2 ± 2.2	-46.6 ± 2.4	-43.3 ± 3.8	-44.3 ± 2.7
V_A		-76.9 ± 3.0		-70.9 ± 3.1	-76.1 ± 3.3	-71.2 ± 3.2	-64.7 ± 3.6	-57.2 ± 4.6	-54.8 ± 5.2	-50.0 ± 8.0	+52.0 ± 5.0
T.E.P.	Saline	+ 3.9 ± 2.9	Saline	-	-	+ 5.0 ± 2.7	+ 4.5 ± 2.8	+ 4.5 ± 3.2	+ 4.2 ± 3.6	+ 4.9 ± 4.8	+ 3.3 ± 9.2
n	-	18	-	9	9	18	17	14	12	7	3

Table 15b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.E. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-69.4 ± 1.1	Na^+free	-48.2 ± 3.6	128mM K^+	- 1.3 ± 2.8	-	Normal	-	-58.8 ± 3.8	10
V_A		-81.4 ± 3.5		-61.2 ± 4.6		-32.6 ± 4.1	-		-	-72.3 ± 3.8	10
T.E.P.	Saline	+ 6.4 ± 4.1	Saline	+ 6.6 ± 2.2	Saline	+28.5 ± 4.5	-	Saline	-	+ 8.2 ± 2.2	10

perfusate from Normal to Na^+ free saline (with choline replacing Na^+). Results show that V_B and V_A depolarized at similar rates in Na^+ free saline, gradually declining 21.9 ± 2.8 mV and 19.1 ± 2.9 mV respectively over the first 20 mins before levelling at the resulting potentials over the next 25 mins. As a consequence, little change occurred in T.E.P. throughout exposure to Na^+ free saline. However from Table 15a, it can be seen that in 50% of cells (n = 9), the membrane potentials did not depolarize immediately. Instead, V_B and V_A exhibited an initial depolarization between 2 and 18 mV over the first min of Na^+ free treatment followed by a hyperpolarization between 2 and 18 mV over the next min, before beginning to depolarize. No change in T.E.P. occurred as both membrane potentials changed by similar values.

Oscillations of 6.0 ± 2.0 mV in amplitude occurred in 44% of cells in Na^+ free saline. The frequency and amplitude of these oscillations of V_A (and T.E.P.) were not significantly different ($p > 0.9$) from the values found in Normal saline.

Effect of 128 mM K^+ Saline After Treatment in Na^+ Free Saline

It was observed earlier that exposure to K^+ free saline directly before the addition of high K^+ saline resulted in the inducement of a Type B response. It was decided to explore the possibility that a similar situation occurred from pretreatment in Na^+ free saline. Thus, the effect of 128 mM K^+ saline on V_B, V_A and T.E.P. was examined after a period of 10 to 45 mins in Na^+ free saline. Results are shown in Table 15b. It was found that membrane changes and resulting potentials to this Na^+ free / high K^+ treatment were the same whether the cells originally exhibited the Type A or Type B response (as found by an earlier addition of 128 mM K^+ saline). Thus, results from cells originally exhibiting Type A and Type B behaviour were

grouped together in Table 15b.

From Table 15b it can be seen that the introduction of 128 mM K^+ saline after Na^+ free treatment resulted in a 'reduced' Type A response for both V_B and V_A. Indeed, despite the fact that the mean V_B (component c, Fig. 20), after depolarization in 128 mM $[K^+]_o$ was negative, the range of maintained potential was +14 to -12 mV with two fifths of cells actually depolarizing to a positive V_B. However, although quite close to being significantly less negative than the equivalent values found in the Type A response, the depolarized V_B and V_A (component c, Fig. 20) in 128 mM K^+ saline were not different ($p = 0.05-0.1$ for both potentials). Furthermore, the initial depolarization of V_A (component b, Fig. 20) in high K^+ saline was 61% of that for V_B (c.f. 65% for the Type A response), producing a T.E.P. that was similar ($p = 0.2-0.3$) to the value found in the Type A response.

Although the depolarized V_B and V_A (component c, Fig. 20) were maintained for at least 3 mins in the majority of cells, a small percentage (30%) showed a small repolarizing change (component d, Fig. 20), of 12.3 ± 3.4 mV for V_B and 9.7 ± 1.2 mV for V_A, in 128 mM K^+ saline. This Type B response feature, which was not related to whether the cells originally exhibited Type A or Type B behaviour, however, only occurred in cells in which V_B had initially depolarized to a positive potential. Thus, this small hyperpolarization may simply be a change in the distribution of ions, after Na^+ free treatment, pushing the membrane potentials back towards the 'normal' Type A response values found in 128 mM K^+ saline.

On re-introduction of Normal saline, V_B and V_A only reached values 85% and 89% respectively of the final re-established potentials after 2 mins and took 6 to 28 mins to reach the original resting values.

Finally, it was noted that the introduction of high K^+ saline after treatment in Na^+ free saline produced a similar response whether cells originally exhibited the Type A or Type B response. This suggests that the alteration of ionic gradients during pretreatment in Na^+ free saline was sufficient to abolish Type B behaviour.

Effect of Cl^- free Saline

Table 16a and Fig. 24 show the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to Cl^- free saline (Cl^- was replaced by gluconic acid). It can be seen that whilst V_B did not change even after 40 mins exposure to Cl^- free saline, V_A hyperpolarized gradually over the first 5-10 mins in this modified solution before levelling after about 10 mins. The new maintained V_A was 10.7 ± 0.7 mV more negative than the original resting value. T.E.P. followed the change of V_A, becoming more positive in Cl^- free saline. Cells took between 3 and 8 mins to recover to the original resting V_A (and T.E.P.) on re-introduction of Normal saline.

Oscillations of 8.0 ± 1.9 mV amplitude occurred for V_A (and T.E.P.) in only 23% of cells in Cl^- free saline. This rate of oscillation was less but not significantly different in amplitude ($p = 0.3$-0.4) from the value found in Normal saline.

Effect of 128 mM K^+,Cl^- free Saline After Treatment in Normal Saline (with or without Cl^-)

A variety of experiments were carried out to examine the effect of Cl^- free saline on the response of V_B, V_A and T.E.P. in 128 mM K^+ saline. Table 16b and Fig. 24 show the effect on V_B, V_A and T.E.P. of introducing 128 mM K^+,Cl^- free saline, after a period of 5 to 20 mins exposure to Cl^- free saline, in cells which exhibited the Type A response. It can be seen that Cl^- free pretreatment followed by high K^+,Cl^-

Table 16a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to Cl^- free saline.

Table 16b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Cl^- free to 128 mM K^+,Cl^- free saline in cells which exhibited the Type A response.

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

Table 16a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10	15
V_B	Normal	-71.4 ± 0.9	Cl^-free	-	-	-	-
V_A	Saline	-78.8 ± 3.0	Saline	-81.5 ± 2.8	-86.4 ± 3.3	-90.5 ± 5.0	-91.0 ± 5.1
T.E.P.		+ 4.1 ± 3.0		+ 6.8 ± 2.9	+11.3 ± 3.4	+14.7 ± 5.1	+14.0 ± 5.3
n	-	22	-	22	22	13	3

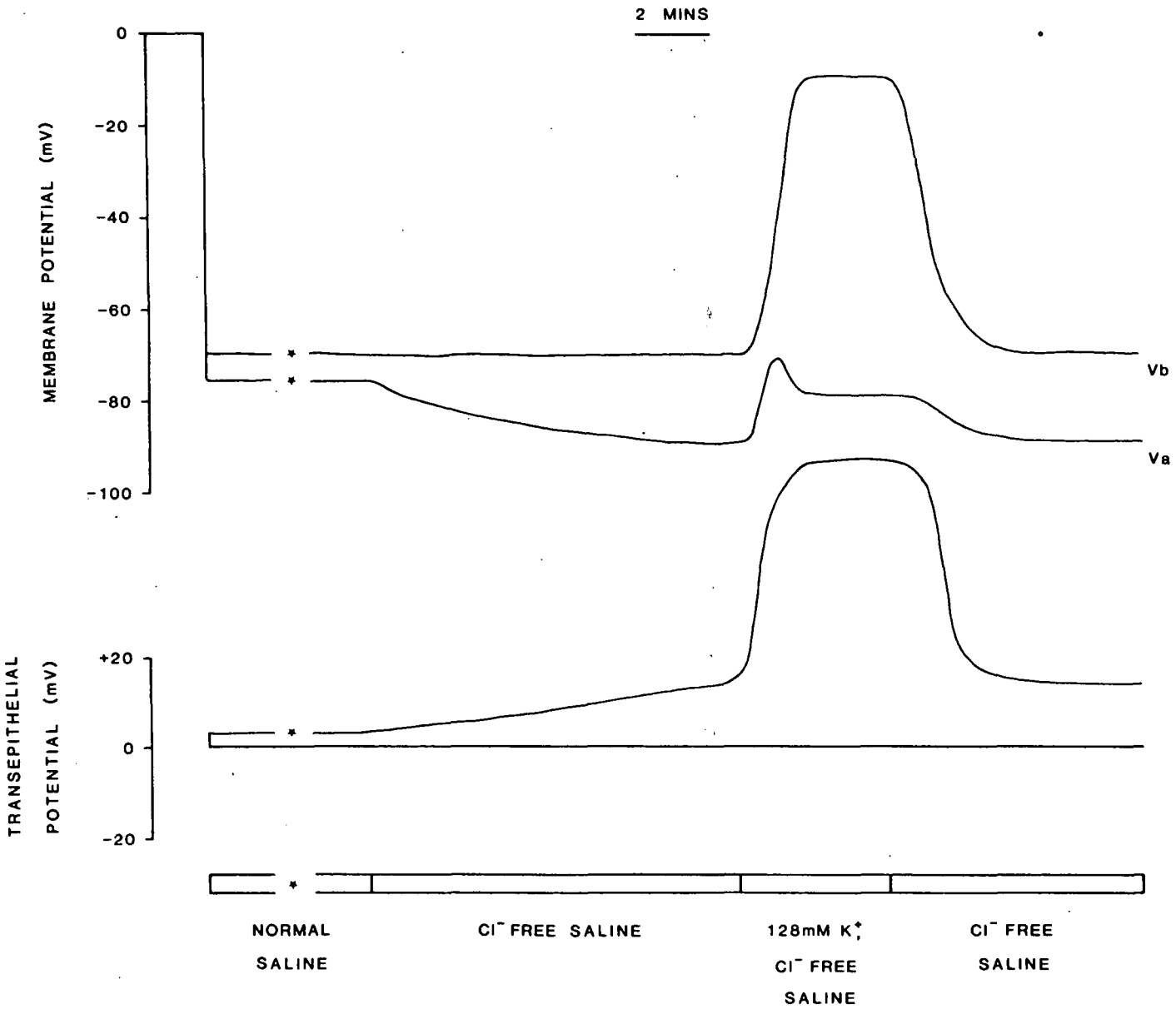
Table 16b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig. 20) (mV)	n
V_B	Normal	-74.0 ± 2.0	Cl^-free	-74.0 ± 2.0	128mM K^+,	-10.0 ± 2.1		Cl^-free	-	-72.3 ± 3.1	3
V_A	Saline	-77.7 ± 4.7	Saline	-86.7 ± 6.7	Cl^-free	-62.3 ± 8.8	-71.0 ± 7.9	Saline	-	-85.5 ± 7.1	3
T.E.P.		+ 3.7 ± 4.5		+ 8.7 ± 7.1	Saline	+62.7 ± 6.4			-	+ 9.1 ± 6.3	3

Fig. 24

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to Cl^- free saline before the subsequent addition of 128 mM K^+,Cl^- free saline, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -10 mV, V_A = -45 mV, T.E.P. = +31 mV).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0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
Cl⁻ FREE SALINE
128mM K⁺, Cl⁻ FREE SALINE
Cl⁻ FREE SALINE

free saline did not alter the Type A response of V_B. Thus, the maintained V_B in high K^+,Cl^- free saline was not significantly different ($p = 0.2$-0.3) from the equivalent Type A response potential found in 128 mM K^+ saline. Conversely, Cl^- free treatment and the subsequent addition of 128 mM K^+,Cl^- free saline resulted in V_A becoming more negative than in the Type A response by exhibiting membrane changes similar to those found in the Type B response. Hence, compared to the original Type A response, a reduced initial depolarization (component b, Fig. 20) of V_A occurred in 128 mM K^+,Cl^- free saline, followed by a repolarizing change (component d, Fig. 20) between 6 and 11 mV, over 1 min, to a maintained repolarized potential (component e, Fig. 20). The depolarized potentials (component c, Fig. 20) and repolarized potentials (component e, Fig. 20) for V_A in 128 mM K^+,Cl^- free saline were both significantly more negative ($p < 0.001$) than the depolarized V_A found in the Type A response, but were not significantly different ($p = 0.5$-0.6) from the equivalent values found in the Type B response. The initial depolarization (component b, Fig. 20) of V_A was only 38% of that for V_B (c.f. 65% and 71% found for the Type A and Type B response respectively). This was reflected in T.E.P. which was almost twice ($p < 0.001$) that found in the Type A response (+62.7 ± 6.4 mV compared with +33.6 ± 1.6 mV) during exposure to 128 mM K^+, Cl^- free saline.

In relation to the previous experiment, the effect of V_B, V_A and T.E.P. of introducing 128 mM K^+,Cl^- free saline, immediately after Normal saline, was examined in cells which exhibited the Type A response. Results are shown in Table 17a. It can be seen that this modified saline did not alter the Type A response of V_B; the depolarized V_B being similar ($p = 0.2$-0.3) to the value in 128 mM $[K^+]_o$. However, V_A did not exhibit the Type A response but gave a response qualitatively similar to that found in high K^+,Cl^- free saline after Cl^- free

Table 17a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+,Cl^- free saline in cells which exhibited the Type A response.

Table 17b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline before the subsequent addition of 128 mM K^+,Cl^- free saline in cells which exhibited the Type A response.

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

Table 17a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig. 20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-68.6 ± 1.1	128mM K^+, Cl^-free Saline	- 3.4 ± 2.9	-	Normal	-	-68.0 ± 1.8	5
V_A	Saline	-84.6 ± 6.7		-47.9 ± 7.5	-62.4 ± 7.8	Saline	-	-84.1 ± 6.0	5
T.E.P.		+ 9.5 ± 2.8		+54.8 ± 6.0	-		-	+10.1 ± 2.5	5

Table 17b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	Treatment	Re-established P.D. ± S.E.M. (mV)	n
V_B	Normal	-72.8 ± 2.0	128mM K^+	- 7.2 ± 2.6	128mM K^+, Cl^-free Saline	-	Normal	-72.4 ± 2.3	5
V_A	Saline	-78.6 ± 3.8	Saline	-38.0 ± 6.8		-47.8 ± 4.7	Saline	-78.2 ± 4.1	5
T.E.P.		+ 7.4 ± 2.2		+31.0 ± 5.0		+41.8 ± 2.3		+ 7.5 ± 2.4	5

pretreatment. As a result, the depolarized potentials (component c, Fig. 20) and repolarized potentials (component e, Fig. 20) of V_A in 128 mM K^+,Cl^- free saline were significantly more negative ($p < 0.001$) than the depolarized value in the Type A response but were not significantly different ($p = 0.4$-0.5) from the equivalent values found in the Type B response. The initial depolarization (component b, Fig. 20) of V_A in high K^+,Cl^- free saline was 56% of that for V_B and resulted in T.E.P. becoming significantly more positive ($p < 0.001$) than in the Type A response.

The ability of V_A to hyperpolarize in high K^+,Cl^- free saline in cells which exhibited the Type A response was examined further by changing the perfusate from Normal to 128 mM $[K^+]_o$ before the subsequent addition of 128 mM K^+,Cl^- free saline. In these experiments, 128 mM K^+,Cl^- free saline was added only when V_B, V_A and T.E.P. had demonstrated maintained values, in 128 mM $[K^+]_o$, similar ($p = 0.6$-0.7) to those found in the Type A response. Results are shown in Table 17b and Fig. 25. It was found that whilst the introduction of 128 mM K^+,Cl^- free saline did not change V_B, even after 4 mins exposure, V_A hyperpolarized 0 to 18 mV (20% of cells did not change), over 1 min, to a maintained value in this new saline. T.E.P. followed the change of V_A, becoming more positive over 1 min in 128 mM K^+,Cl^- free saline.

The effect of changing the perfusate from Normal to 128 mM K^+,Cl^- free saline on V_B,V_A and T.E.P. was examined for cells which exhibited the naturally occurring Type B response and is shown in Table 18a. It can be seen that the introduction of 128 mM K^+,Cl^- free saline did not qualitatively alter the sequence of potential changes for either membrane potential found in the natural Type B response. Thus, the depolarized potentials (component c, Fig. 20) and repolarized potentials (component e, Fig. 20) for both V_B and V_A, and thus T.E.P.,

Fig. 25

Typical example of the effect of changing the perfusate from Normal to 128 mM K^+ saline before the subsequent addition of 128 mM K^+,Cl^- free saline, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -8 mV, V_A = -39 mV, T.E.P. = +30 mV).

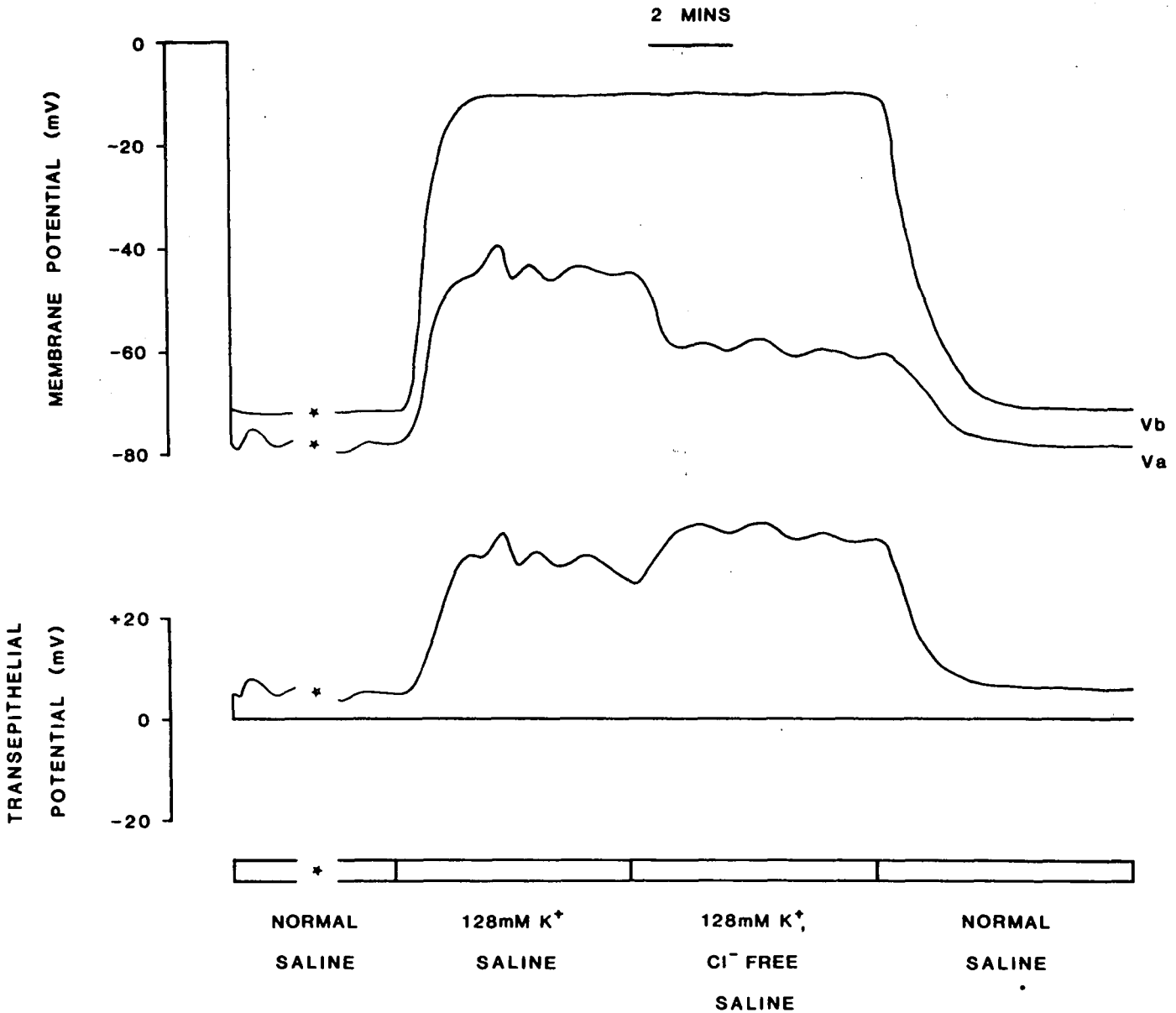

2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
128mM K+ SALINE
128mM K+, Cl- FREE SALINE
NORMAL SALINE

Table 18a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+,Cl^- free saline in cells which exhibited the Type B response.

Table 18b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline before the subsequent addition of 128 MM K^+,Cl^- free saline in cells which exhibited the Type B response.

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

Table 18a

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-74.0 ± 3.8	128mM K^+,	-29.5 ± 11.6	-51.5 ± 8.0	Normal	-	-76.1 ± 4.6	3
V_A	Saline	-77.3 ± 10.0	Cl^-free Saline	-48.5 ± 17.9	-73.5 ± 13.9	Saline	-	-80.3 ± 8.7	3
T.E.P.		+ 0.3 ± 5.2		+20.3 ± 12.5	-		-	+ 2.1 ± 4.0	3

Table 18b

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	New ± S.E.M. (mV)	Repolarized P.D. ± S.E.M. (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-74.3 ± 4.1	128mM K^+	-33.3 ± 5.4	-55.7 ± 10.9	128mM K^+	-37.0 ± 17.2	-48.3 ± 17.2	Normal	+ 9.0 ± 0	-73.8 ± 4.3	3
V_A	Saline	-81.0 ± 4.6	Saline	-54.3 ± 7.1	-77.3 ± 7.1	Cl^-free Saline	-92.0 ± 9.5	-83.3 ± 5.2	Saline	+13.0 ± 0	-82.0 ± 4.4	3
T.E.P.		+ 0.3 ± 7.7		+23.7 ± 4.7	-		+47.7 ± 7.1	+29.3 ± 4.4		-	+ 1.7 ± 6.6	3

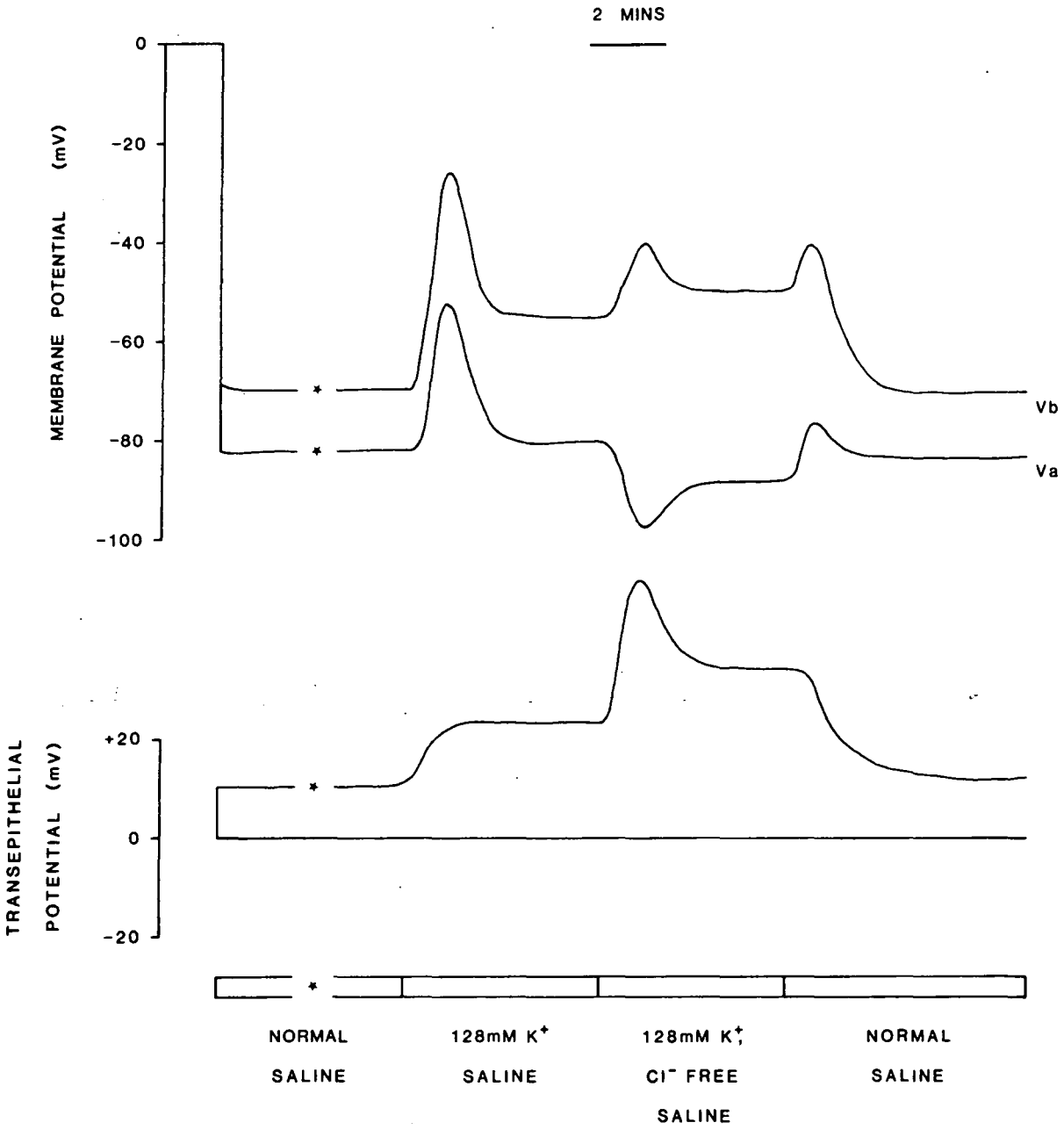
were not significantly different ($p > 0.9$, $p = 0.7$-0.8, and $p > 0.9$ respectively) from the values found in the Type B response when Cl^- was present. However, after reaching maximum repolarized potentials (component e, Fig. 20) over 1-2 mins during high K^+,Cl^- free exposure a gradual depolarization of both V_B and V_A occurred, with V_B declining by 11.3 ± 3.7 mV and V_A declining by 7.7 ± 3.1 mV over 2 mins. As a result of V_B depolarizing faster than V_A, T.E.P. steadily became more positive by 4.3 ± 2.3 mV over 2 mins. These results suggest that the maintenance of the Type B response may depend on the presence of external Cl^-.

To investigate the dependence of the Type B response on $[Cl^-]_o$, the effect of changing the perfusate from 128 mM K^+ to 128 mM K^+,Cl^- free saline was examined,in cells which exhibited the naturally occurring Type B response. In these experiments, 128 mM K^+,Cl^- free saline was added only when V_B and V_A (and T.E.P.) had exhibited maintained repolarized potentials (component e, Fig. 20), in 128 mM $[K^+]_o$, similar ($p = 0.8$-0.9) to those found during 'typical' Type B behaviour. Results are shown in Table 18b and Fig. 26. It can be seen that the absence of Cl^- in high K^+ saline affected both V_B and V_A; changing both potentials in opposite directions. Thus, with the introduction of 128 mM K^+,Cl^- free saline, directly after 128 mM $[K^+]_o$, V_B initially depolarized 18.7 ± 7.0 mV over 1 min, then hyperpolarized 11.3 ± 4.7 mV over the next min to a maintained value. In contrast, V_A hyperpolarized 14.7 ± 6.8 mV during the first min of 128 mM K^+,Cl^- free treatment, then depolarized 8.7 ± 4.4 mV over the next min to a maintained value. Following these changes, T.E.P. increased in positivity by 23.7 ± 9.4 mV over 1 min, then decreased 18.0 ± 6.4 mV over the next min to a maintained value during exposure to high K^+,Cl^- free saline. Hence, the introduction of 128 mM K^+,Cl^- free saline directly after 128 mM K^+ saline in cells

Fig. 26

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal to 128 mM K^+ saline before the subsequent addition of 128 mM K^+,Cl^- free saline in cells which exhibited the Type B response.

* indicates a period of 10 mins during which a Type B response to 128 mM K^+ saline was established (V_B = -53 mV [component e, Fig. 20], V_A = -77 mV [component e, Fig. 20], T.E.P. = +24 mV).


2 MINS
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
-100
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
128mM K+ SALINE
128mM K+, Cl- FREE SALINE
NORMAL SALINE

which exhibited the Type B response resulted in a transient depolarization of V_B and a transient hyperpolarization of V_A. Following these changes, maintained values for V_B and V_A, 8 mV less negative and 6 mV more negative respectively than the repolarized potentials (component e, Fig. 20) found in 128 mM $[K^+]_o$, were established.

Finally, it was found that cells artificially induced to exhibit the Type B response in high $[K^+]_o$ following K^+ free saline (n = 3), gave the same pattern of results when the perfusate was changed from 128 mM K^+,Cl^- free saline. Thus, V_B depolarized 18.0 ± 4.0 mV during the first min of high K^+,Cl^- free saline treatment, then hyperpolarized 7.0 ± 5.0 mV over the next min to a value 9 mV less negative than the repolarized potential (component e, Fig. 20) found in 128 mM K^+ saline. In contrast, V_A hyperpolarized 15.5 ± 5.5 mV over the first min of exposure, then depolarized 5.0 ± 3.0 mV over the next min to a value 11 mV more negative than the repolarized potential (component e, Fig. 20) found in high K^+ saline. As for the naturally occurring Type B response, T.E.P. increased in positivity, with the introduction of high K^+,Cl^- free saline, by 20.3 ± 5.2 mV over the first min of exposure, then decreased in value by 13.5 ± 8.5 mV over the next min.

Discussion

The basal and apical cell membrane potentials (V_B and V_A) and the transepithelial potential (T.E.P.) found in the present study are compared with values found in various invertebrate and vertebrate tissue in Table 19. Whilst the majority of tubules examined had a positive T.E.P., a considerable variation in potential occurred in the tubules of Locusta (from -47 to +51 mV) and 33% of tubules exhibited a negative T.E.P. value. A variation in T.E.P. from tubule to tubule has also been found in Locusta by Ramsay (1953; 1954) and Morgan and

Table 19

Membrane and transepithelial potentials reported for various tissues from different species.

V_B and T.E.P. are measured with respect to the bathing medium whilst V_A is measured with respect to the lumen.

Tissue		V_B (mV)	V_A (mV)	T.E.P. (mV)	Reference
Malpighian tubules of	Locusta	-71.6 ± 0.3	-82.7 ± 0.9	+ 5.7 ± 1.0	Present Study
	Locusta	-	-	+10.8 ± 2.1	Anstee et al., 1980
	Locusta	-	-	+ 8.7 ± 0.3	Fathpour et al., 1983
	Locusta	-39.4 ± 0.5	+44.0	+ 4.4 ± 0.5	Morgan and Mordue, 1983a
	Locusta	-	-	-16	Ramsay, 1953
	Schistocerca	-	-	+16.6 ± 3.1	Maddrell and Klunsuwan, 1973
	Rhodnius	-67.0 ± 2.0	-53.0 ± 4.0	- 8.0 ± 3.0	O'Donnell and Maddrell, 1984
	Aedes	-65.2 ± 1.5	-118	+53.7 ± 5.6	Sawyer and Beyenbach, 1985a
	Carausius	-	-	+21	Ramsay, 1953
	Calpodes	-	-	+25	Irvine, 1969
	Tipula	-	-	+32	Coast, 1969
Rectum of Schistocerca		-50.7 ± 0.3	-57.8 ± 0.5	+ 7.2 ± 0.3	Hanrahan and Phillips, 1984
Salivary glands of Calliphora		-61.1 ± 0.4	-	+ 4.0 ± 1.0	Berridge and Schlue, 1978
Midgut of Calliphora		-	-	-12.0 ± 1.0	O'Riordan, 1969
Proximal tubule of Squalus		-63.2 ± 3.5	-	+ 0.4 ± 0.4	Beyenbach and Fromter, 1985
Rectal gland tubule of Squalus		-68.0 ± 1.2	-	-12.0 ± 1.1	Greger and Schlatter, 1984
Cortical collecting duct of New Zealand white rabbits		-69.6 ± 3.0	-68.9 ± 3.1	- 0.7 ± 0.3	O'Neil and Sansom, 1984
Proximal straight tubules of Swiss mice		-62.0 ± 1.0	-	- 1.7 ± 0.1	Völkl et al., 1986

Mordue (1981; 1983a). Large differences in membrane potential values (both basal and apical) were recorded in the present study compared with those presented by Morgan and Mordue (1983a) for Locusta tubules. From a measured V_B value of -39.4 mV and a T.E.P. value of +4.4 mV in 20 mM K^+ Normal saline, Morgan and Mordue (1983a) erroneously calculated a value of +44 mV from T.E.P. = V_B-V_A for the potential across the apical membrane. This value differs greatly from the measured value of -82.6 mV in 8.6 mM K^+ saline in the present study. Furthermore, this difference cannot be accounted for on the basis of $[K^+]$ in the saline used. The value for V_B obtained in the present study is still 30% higher than that found by Morgan and Mordue (1983a) after extrapolation on a Nernst plot (see Fig. 17) to a value in 20 mM K^+ saline.

In the present study, treatment with modified salines resulted, in some cases, in V_B and V_A responding qualitatively similarly. This similarity was most obvious when comparing the changes which occurred in both membrane potentials in high K^+ saline and may be because the tubule of Locusta is a "leaky" epithelium. In insects, information on the "leakiness" of epithelia is somewhat limited. However, it appears that the Malpighian tubules of some species are "tight" with salt and water transport taking place largely by a transcellular route (O'Donnell and Maddrell, 1983; O'Donnell et al., 1984; Williams and Beyenbach, 1984), a process which also takes place in locust rectum (Hanrahan, 1984). In contrast, studies on other insect epithelia, including rectal pads of Periplaneta and Calliphora and salivary glands of Calliphora (Gupta et al., 1978; Gupta and Hall, 1981) indicate that some solute/solvent coupling occurs paracellularly. If the Malpighian tubules of Locusta are "leaky", electrical measurements across the cell would be short-circuited by the paracellular pathway with the result that one would observe similar potential changes across both

membranes. The similarity or dissimilarity, therefore, between the electrical responses of V_B and V_A could then be explained by the leakiness of the tissue, e.g. if V_A does not change in potential in high K^+ saline then it can be assumed that the tissue is "tight"; alternatively, if both V_B and V_A change by exact amounts in high K^+ saline, then it can be assumed that the tissue is totally "leaky". As neither of these extremes were found during experimentation, it may be that the tissue exhibits variable leakiness, through "leaky" tight junctions. Evidence for the latter has been found in many vertebrate systems (Di Bona, 1985).

Although it is possible that the Malpighian tubules of Locusta possess "leaky" tight junctions, other evidence suggests that the tubules are "tight". This evidence includes the observations that a large T.E.P. was observed in both Normal and high K^+ saline, in many cases, and that the potential changes of the basal and apical membranes to modified saline was different in some cases. A large T.E.P. is not a characteristic of a leaky epithelium (Hanrahan, 1984). Thus, the depolarization of V_B in high K^+ saline, for example, may be the result of a Nernstian effect (see later) whilst the depolarization of V_A in the same saline may be a secondary effect, resulting from the decrease of V_B, possibly involving Cl^-. Indeed, results showed that the depolarization of V_A in high K^+ saline was reduced during the absence of Cl^-. Such an observation would be expected if the tubules of Locusta were "tight" as a short-circuiting effect, producing similar changes in V_B and V_A, whether Cl^- was present or absent in high K^+ saline, could not occur.

At this point, it is convenient to review the distribution of ions in the Malpighian tubules of Locusta (see Fig. 27). Although a basolateral location for the Na^++K^+-ATPase, which has already been

Fig. 27

Schematic diagram to show the distribution of potential and ions across the Malpighian tubule cell of Locusta (or other insects where pertinent).

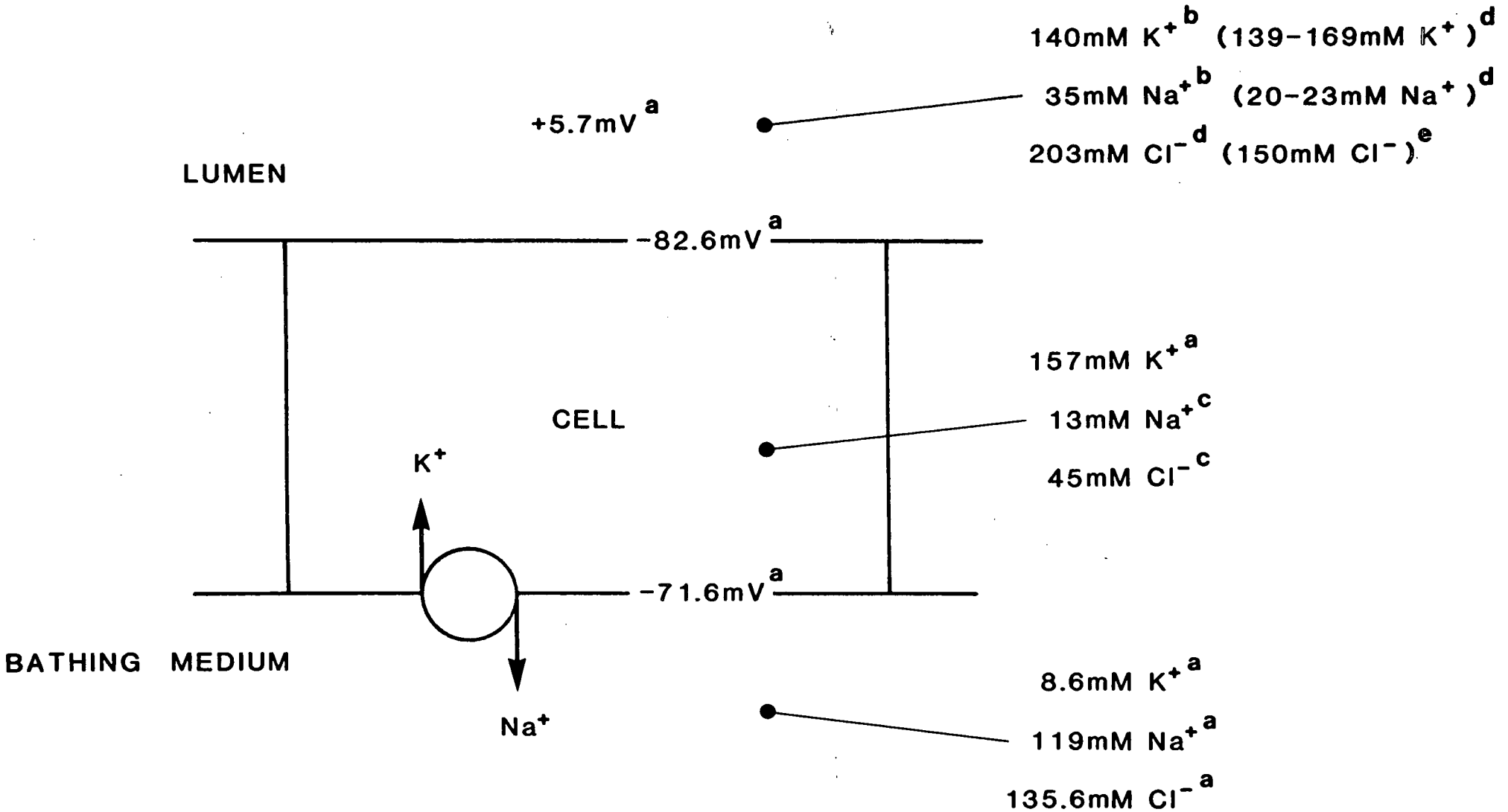
a indicates V_B (-71.6 mV), V_A (-82.6 mV), T.E.P. (+5.7 mV), the bathing medium concentration of K^+, Na^+ and Cl^- and the intracellular concentration of K^+ found in Locusta (Present Study)

b indicates the luminal concentration of K^+ and Na^+ reported in Locusta (Anstee et al., 1979)

c indicates the intracellular concentration of Na^+ and Cl^- reported in Rhodnius (Gupta et al., 1976)

d indicates the luminal concentration of Cl^-, K^+ and Na^+ reported in Locusta (Morgan and Mordue, 1983a)

e indicates the luminal concentration of Cl^- reported in Schistocerca (Maddrell and Klunsuwan, 1973).


140mM K+ b (139-169mM K+)d
35mM Na+ b (20-23mM Na+)d
203mM Cl- d (150mM Cl-)e
+5.7mV a
LUMEN
-82.6mV a
157mM K+ a
CELL
13mM Na+ c
45mM Cl- c
K+
-71.6mV a
BATHING MEDIUM
Na+
8.6mM K+ a
119mM Na+ a
135.6mM Cl- a

examined using [^{3}H] ouabain-binding studies, has been assumed in Fig. 27, this position has not been definitely proven. However, consistent with studies on vertebrate tissue, various data support an exclusively basolateral location for this enzyme in arthropod transporting epithelia (see Introduction, Komnick and Achenbach, 1979; Anstee and Bowler, 1984).

The studies of Anstee et al. (1979) have shown the concentration of K^+ and Na^+ in the "primary urine" secreted into the lumen of the tubules of Locusta. The values for $[K^+]$ and $[Na^+]$ quoted by these workers (see Fig. 27) are similar to those reported subsequently by Morgan and Mordue (1983a) in Locusta tubules and by Maddrell and Klunsuwan (1973) in Schistocerca tubules. Morgan and Mordue (1983a) quote a value of 203 mM luminal Cl^- and this is the only available figure for $[Cl^-]$ in the lumen of Locusta tubules. This urine $[Cl^-]$, however, is similar but somewhat higher than the value of 150 mM quoted by Maddrell and Klunsuwan (1973) in Schistocerca tubules. From a Nernst plot in the present study (Fig. 17), a value of 157 mM was calculated for intracellular K^+. This agrees fairly well with values found in Locusta skeletal muscle (140 mM, Leech, 1986), lepidopteran midgut (134 mM, Dow et al., 1984 and 140 mM, Zerahn, 1977), Calliphora salivary gland (170 mM, Berridge and Schlue, 1978) and Schistocerca Malpighian tubules (135 mM, Maddrell and Klunsuwan, 1973) but is higher than the 95 mM $[K^+]_i$ reported by Morgan and Mordue (1983a) in Locusta tubule cells and the 103 mM K^+ determined by X-ray microanalysis of the main cell cytoplasm of unstimulated Rhodnius tubules (Gupta et al., 1976). Morgan and Mordue (1983a) found $[Cl^-]_i$ in Locusta tubules to be 51 mM which is slightly higher than the Cl^- level (45 mM) in the main cell cytoplasm of Rhodnius tubules (Gupta et al., 1976). $[Na^+]_i$ has not been measured in Locusta tubules but electron-probe X-ray microanalysis

by Gupta et al. (1976) has shown that the levels of intracellular Na^+ in Rhodnius tubules are low (13 mM). This is in agreement with other transporting epithelia which, in general, have low Na^+ levels (Diez de los Rios et al., 1981; Wang et al., 1984).

The data above give an indication of the ionic concentration and electrical gradients across the basal and apical membranes. Thus, it would appear likely that, for a tubule bathed in Normal saline, entry across the basal membrane involves K^+ moving against its concentration but with its electrical gradient whilst entry of Na^+ involves this cation moving down its concentration and with its electrical gradient. In contrast, Cl^- entry suggests movement down its concentration gradient but against the electrical gradient. Presumably, the Na^++K^+-ATPase in the basal membrane maintains intracellularly high levels of K^+ and low levels of Na^+ to help create these concentration and electrical gradients.

Ion movements across the apical membrane from cytoplasm to lumen require that K^+ move against an electrical gradient, although there is little or no concentration gradient. In contrast, Na^+ exit from the cell requires movement against a concentration and electrical gradient whilst a similarly directed movement of Cl^- would be against its concentration gradient but along the electrical gradient. In the present study, the mean apical potential was -82.6 mV and it is difficult to explain this large negative V_A on the basis of a purely passive ionic permeability of the apical membrane. This provides indirect evidence in favour of an apical active transport mechanism as has been proposed elsewhere. Indeed, an apical cation pump has been proposed in the Malpighian tubules of Calliphora (Berridge, 1968), Carausius (Maddrell, 1977) and Rhodnius (O'Donnell and Maddrell, 1984) and for K^+ in the salivary glands of Calliphora (Prince and Berridge,

1972; Berridge et al., 1975), the labella of Protophormia (Thurm and Küppers, 1980; Wieczorek, 1982) and the labial glands (Hakim and Kafatos, 1974) and the midgut of Manduca sexta (Harvey et al., 1983).

Recent studies have revealed the presence of a potassium-sensitive ATPase in the K^+-transporting midgut of Manduca sexta (Wolfersberger et al., 1982; Deaton, 1984) and the labellum of Protophormia (Wieczorek, 1982; Wieczorek et al., 1986). It is suggested (Wieczorek et al., 1986) that this may represent the electrogenic apical K^+ pump. However, as was stated earlier, no biochemical evidence exists for K^+-ATPase activity in insect Malpighian tubules. The following table shows data recalculated from Anstee and Bell (1975) regarding the activity of the Na^++K^+-ATPase in the tubules of Locusta:

Na^+K^+-stimulated ATPase activity

Species	Tissue	Complete	$-Na^+$	$-K^+$	$-\frac{Na^+}{K^+}$	+ Ouabain (mM)
Locusta migratoria	Malpighian tubules	229.8 ± 27.3	38.9	28.8	29.3 ± 4.9	24.5 ± 5.9

Harvey et al. (1983) have incorrectly re-analysed this Na^++K^+-ATPase activity data such that it appears that a K^+-ATPase is present in Locusta tubules. Unfortunately, the significant K^+ stimulated ATPase activity Harvey et al. (1983) refer to is the result of an arithmetic error on their part. There is, therefore, no evidence or data for an ATPase activity in the tubules of Locusta.

K^+ Substitution

The substitution of K^+ by Na^+ in the bathing fluid in the present study showed that the basal membrane acts essentially as a potassium electrode. Many examples of a similar behaviour occur in a variety

of tissues (e.g. salivary gland of Calliphora, Prince and Berridge, 1972; Malpighian tubules of Rhodnius, O'Donnell and Maddrell, 1984; locust oocytes, Wollberg and Cocos, 1981 and locust skeletal muscle, Usherwood, 1978). The fact that the V_B measured deviated from the Nernst prediction in a positive direction in $[K^+]_o$ less than 8.6 mM makes it reasonable to assume that V_B is increasingly influenced by the Na^+ gradient. Furthermore, the deviation of V_B can be formally described by the Goldman constant field equation in which the concentration of each ion is multiplied by a permeability constant, P (Goldman, 1943). Thus:

$$V_B = 60.1 \log \frac{PK[K^+]_i + PNa[Na^+]_i}{PK[K^+]_o + PNa[Na^+]_o} .$$

Using values of 157 mM for $[K^+]_i$ (obtained from the Nernst plot in the present study) and 13 mM for $[Na^+]_i$ (Gupta et al., 1976) and assuming PK = 1 and PNa = 0.01, it is possible to apply this equation to the tubules of Locusta. Results can be seen in Table 20. It was found that calculated V_B values for a permeability ratio of 1 : 0.01 (K^+ : Na^+) gave values very similar to experimental results. These values were plotted in a graph of membrane potential versus $\log[K^+]_o$ (see Fig. 17). Thus, application of the Goldman constant field equation for a membrane permeable to both Na^+ and K^+ suggest that if the positive deviation from Nernst was due to Na^+, then the basal membrane must be approximately 100 times more permeable to potassium than sodium. This relationship is consistent with that reported for various other epithelia, e.g. Aplysia neurones (Sato et al., 1968), but is rather lower than in other cases, e.g. the Malpighian tubules of Aedes (Williams and Beyenbach, 1984). In the latter tissue, the basolateral membrane is reported to be permeable to both K^+ and Na^+ with membrane conductances for each ion being of

Table 20

Comparison of the measured V_B and V_B calculated from the Goldman Constant Field Equation (based on the assumption that the basal membrane is 100 times more permeable to K^+ than Na^+) in varying external concentrations of potassium. Also see Fig. 17.

$[K^+]_o$ (mM)	V_B Calculated From Goldman Constant Field Equation (mV)	Actual V_B (mV)
1.25	-107.8	-107.3
2.5	- 97.3	- 95.8
4.3	- 87.2	- 87.5
8.6	- 72.3	- 71.6
32	- 40.7	- 39.6
64	- 23.2	- 25.9
128	- 5.2	- 6.6

similar magnitude under unstimulated conditions (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985a).

T.E.P. measurements, in the present study, exhibited a maintained increase in positivity similar to values found by Fathpour et al. (1983) in Locusta tubules, for $[K^+]_o$ greater than 8.6 mM. Berridge et al. (1975) found that T.E.P. in the salivary glands of Calliphora became more positive in high K^+, low Na^+ saline, although a gradual drift back towards the resting potential also occurred. Similarly, O'Donnell and Maddrell (1984) found that treatment with K^+-rich, Na^+-free saline caused an increase in T.E.P. positivity in the tubules of Rhodnius (unstimulated by the secretagogue 5-HT). However, these workers found that the apical potential was insensitive to transient changes in bathing fluid K^+ concentration under resting conditions.

The introduction of high K^+ (128 mM), Na^+ free saline probably leads to a reduction in the K^+ gradient in the tubule cells of Locusta as $[K^+]_o$ becomes similar to $[K^+]_i$ (estimated at 157 mM). Under these conditions, the activity of the Na^++K^+ pump, which normally functions to maintain high $[K^+]_i$ across a large K^+ gradient (see Fig. 27), may be reduced. Although $[Na^+]_i$ may rise through this ion not being extruded across the basal membrane by the Na^++K^+ pump, the absence of Na^+ in the high K^+ perfusate may lead to a reduced $[Na^+]_i$ as sodium entry mechanisms will halt. Sodium may also leak out of the cell.

The depolarization of V_A in high K^+ saline does not seem to be due to an activation of an apical K^+ pump as this would lead to an increase in V_A as positive ions left the cell. Furthermore, it would be expected that the activity of an apical K^+ pump would not be affected as little change in $[K^+]_i$ probably occurs in high K^+ saline. An alternative explanation is that elevated $[K^+]_o$ indirectly increases the exit of Cl^- across the apical membrane, reducing V_A over the same time

course as V_B. This idea is supported by the observation that the absence of Cl^- in high K^+ saline reduced the depolarization of V_A. This would occur as Cl^- were not available to leave the cell across the apical membrane.

Other experiments with altered $[K^+]_o$ showed that the introduction of K^+ free saline caused a large and rapid hyperpolarization of both membrane potentials with T.E.P. becoming negative. The latter observation agrees well with the studies of Fathpour et al. (1983) for the tubules of Locusta and the response of V_B and T.E.P. was similar to that found by Berridge et al. (1976) and Berridge and Schlue (1978) for the salivary glands of Calliphora.

The introduction of K^+-free saline would probably lead to an inhibition of the Na^++K^+-ATPase in the tubules of Locusta as K^+ was not available to be pumped into the cell. This would, in turn, lead to a cellular accumulation of Na^+ and a reduction in the Na^+ gradient due to a reduction of the Na^+ extrusion mechanism. Indeed, Na^+ accumulation has been demonstrated in lobster ganglion following treatment in K^+ free saline (Livengood and Kusano, 1972). In addition, $[K^+]_i$ and the K^+ gradient may also be reduced as K^+ exits the cell across the basal membrane. As mentioned earlier, the initial hyperpolarization of V_B in K^+ free saline can be largely related to Nernst. The wide range of responses in this saline after the initial potential increase (from a maintained potential to a rapid depolarization), however, can be related to the permeability of the basal membrane to K^+ and the initial $[K^+]_i$. Berridge and Schlue (1978) have shown that $[K^+]_i$ fell from 170 mM to approximately 5 mM within 10 min of changing from 10 mM to 0.2 mM $[K^+]_o$ in the salivary glands of Calliphora. The hyperpolarization of V_A in K^+ free saline, observed in the present study, was probably not related to a reduction in the activity of an apical K^+ pump (which

would lead to a depolarization of V_A) but, as will be discussed later, may be due to an inhibition of apical Cl^- exit.

As a result of the postulated reduction of $[K^+]_i$ and the K^+ gradient in tubule cells of Locusta during exposure to K^+ free saline, it can be inferred that an initial depolarization of V_B would occur with the introduction of Normal saline directly after K^+ free saline due to the basal membrane acting as a K^+ electrode. This would be followed by a return to the original resting potential as the cellular gradients were re-established. This was not observed in the present study, with the addition of Normal saline after K^+ free saline resulting in V_B either depolarizing over a short period before hyperpolarizing or hyperpolarizing immediately. This result differs from that found in the salivary glands of Calliphora in which the initial depolarization upon changing from low K^+ to Normal saline was so large that the inside of the cell actually became positive with respect to the bathing medium (Berridge and Schlue, 1978). Thus, in the tubules of Locusta, a large initial depolarization due to the presence of Normal saline may be totally or partially masked by a hyperpolarized component. This 'component' may be due to an electrogenic Na^++K^+-ATPase "switching on" until the original intracellular K^+ levels were recovered. The reactivation of the Na^++K^+ exchange pump following the re-introduction of K^+ in the bathing medium has been shown by Livengood and Kusano (1972). However, studies with ouabain and vanadate (see later) suggest that the Na^++K^+-ATPase is not electrogenic under normal resting conditions. The lack of a large initial depolarization in Normal saline could also be explained by the fact that $[K^+]_i$ had not decreased enough to produce a K^+ electrode effect when Normal saline was introduced. Another explanation for the lack of a substantial depolarization of V_B in Normal saline after K^+ free treatment may be that such a change was masked

by a K^+-stimulated Cl^- entry, causing a large influx of Cl^- into the cell.

Na^+ Substitution

Experiments in which Na^+ was replaced by choline resulted in both membrane potentials gradually depolarizing at similar rates with no change in T.E.P. In 50% of cells examined both V_B and V_A gave an initial small depolarization followed by a small hyperpolarization before depolarizing. The latter effect may simply reflect a redistribution of ions caused by a change in ion flux across the epithelium. The fact that V_B showed a slow depolarization as opposed to a rapid change in potential in Na^+ free saline, confirms the observations made in K^+ substitution experiments viz that this membrane was not very permeable to sodium. Furthermore, Morgan and Mordue (1981) found that the reduction of Na^+ concentration in the bathing saline from 142 to 70 mM produced a negligible change in V_B in tubules from Locusta. The results from the present study differ from those reported by Williams and Beyenbach (1984) in the tubules of Aedes. They found that lowering the bathing Na^+ concentration caused a prompt reduction in the positivity of T.E.P. Sawyer and Beyenbach (1985a) found that a five-fold reduction of the bath $[Na^+]$ produced reversible hyperpolarizations of V_B under control conditions in the same tissue.

The gradual depolarization of both membrane potentials in Na^+ free saline may be largely attributed to an inhibition of the Na^++K^+-ATPase. The exclusion of Na^+ from the bathing saline may result in a reduction in $[Na^+]_i$ and a reversal and gradual reduction in the Na^+ gradient as this ion no longer entered the cell across the basal membrane. Indeed, a lowering of cell Na^+ has been shown in the electric organ of Electrophorus when Na^+ was replaced in the ambient medium by choline

(Albers et al., 1968). The reduction in $[Na^+]_i$ would be expected to reduce the functioning of the $Na^+ + K^+$ pump, the activity of which is dependent on the internal Na concentration in many tissues (Grantham, 1980). With the $Na^+ + K^+$ pump rate reduced, $[K^+]_i$ and the K gradient will probably reduce and this would produce a slow depolarization of the basal membrane. The reduction in $[K^+]_i$ may decrease the transport of this ion across the apical membrane, perhaps leading to a slow depolarization of V_A.

Cl^- Substitution

The introduction of Cl^- free saline (with Cl^- replaced by gluconic acid) resulted in no change in V_B, although V_A hyperpolarized (and T.E.P. increased in positivity) over 5-10 mins to a new maintained potential. The lowering of bath Cl concentration also increased the positivity of T.E.P. in most Malpighian tubules of Aedes examined by Williams and Beyenbach (1984) and in the salivary glands of Calliphora (Berridge, 1980). In contrast, O'Donnell and Maddrell (1984) found that T.E.P. in Rhodnius tubules (unstimulated by 5-HT) was relatively insensitive to $[Cl^-]_o$. In stimulated tubules, however, Cl^- free saline produced a large decrease in T.E.P. which was matched by an apical membrane hyperpolarization. V_B changed only very slightly in Cl^- free saline and these authors remark that the basal membrane, as found for Locusta, is not freely permeable to Cl^-.

In many vertebrate tissues, including mammalian nephron (Greger and Schlatter, 1983) and rabbit cortical collecting duct (Sansom et al., 1984), a reduction in basolateral $[Cl^-]$ resulted in a depolarization of V_B and this was consistent with the presence of a Cl^- conductance in the basolateral membrane. In the latter tissue, little change occurred in V_A. Similar to the present study, however, reduced (or free) basolateral

Cl^- solutions lead to only small V_B changes in rabbit and Necturus proximal tubules (Cardinal et al., 1984; Guggino et al., 1982, 1983) and Necturus gallbladder (Reuss, 1984).

Results suggest that under normal resting conditions a Cl^- conductance does not play an important role in Cl^- entry across the basal membrane of the Malpighian tubules of Locusta. Also, although Na^+-H^+ and Na^+-Ca^{2+} exchange may contribute to $[Na^+]_i$ (see later) there is the question of Na^+ entry into the cell across a membrane largely impermeable to this cation. As mentioned in the introduction, 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., 1979; Phillips and Lewis, 1983). Indeed, O'Donnell and Maddrell (1984) have suggested that an electroneutral $Na^+K^+Cl^-$ transport occurs across the basal membrane of the Malpighian tubules of Rhodnius. Morgan and Mordue (1983a) have also 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 the inward movement of Cl^- in the tubules of Locusta. As seen in the present study, the absence of a Na^+ and Cl^- conductance lead to the proposal of $Na^+K^+Cl^-$ cotransport in Rhodnius tubules.

Results from K^+, Na^+ and Cl^- substitution experiments do not support or reject the possibility of a NaKCl mechanism in the tubules of Locusta. It may be that under K^+ free or Na^+ free conditions, such a cotransporter still functions to allow Cl^- entry across the basal membrane. Indeed, O'Donnell and Maddrell (1984) propose that when the bathing saline contains Na^+ or K^+, but not both, Cl^- entry may be possible in the tubules of Rhodnius, if the cotransport mechanism accepts, though less readily, stoichiometries such as $2\,Na^+ : 2\,Cl^-$ or $2\,K^+ : 2\,Cl^-$. If Na^+ and Cl^- entry are linked in Locusta tubules, however, it seems odd that V_B gradually depolarized in Na^+ free saline but did not change

in Cl^- free saline. It may be that Cl^- entry involves other transport mechanisms, such as K^+-stimulated Cl^- entry, Cl^--HCO_3^- exchange or an anion Cl^-+HCO_3^- -stimulated ATPase (see later).

The hyperpolarization of V_A observed in the absence of Cl^- may be related to the functioning of an apical K^+ pump. Thus, in Cl^- free saline, Cl^- would become unavailable to act as a counterion across the apical membrane. The continued pumping of K^+ into the lumen would lead to a hyperpolarization of V_A as positive ions moved out of the cell. Such a mechanism has been described from the 5-HT stimulated Malpighian tubules of Rhodnius (O'Donnell and Maddrell, 1984) and salivary glands of Calliphora (Berridge, 1980) to explain the apical membrane hyperpolarization during treatment with Cl^- free saline.

Type A and Type B Response

Results showed that both membrane potentials depolarized to maintained potentials folowing treatment with high K^+ (128 mM) saline in most cells examined. The depolarization of V_B, as mentioned earlier, produced a potential very similar to that predicted by Nernst and this was called the Type A response. This Type A response of V_B to high K^+ saline occurred in approximately 80% of cells studied with the remaining 20% showing an atypical response which appears not to have been reported previously. The latter response has been referred to as the Type B response. This Type B response was characterized by depolarized apical and basal cell membrane potentials significantly more negative compared with the Type A response when the tubule was bathed in high K^+ saline.

Before further discussion it was necessary to examine the nature of this naturally occurring and permanent Type B behaviour. Leaving V_A aside for the present, it can be assumed that the introduction of high K^+ saline results in V_B starting to depolarize in a Nernstian

manner during the Type B response. It may be that as the membrane depolarizes, however, a hyperpolarized component "switches on" pushing V_B away from the value predicted by Nernst. This "switch on" reaches its maximum value after 1-2 mins and results in a maintained potential. The introduction of Normal saline may result in a depolarizing "switching off" before V_B hyperpolarizes in a Nernstian fashion to the original resting value. This idea that the Type B response was not a series of ionic events but the simple "switching on" of a hyperpolarized component is illustrated in Fig. 28. A subtraction of the hyperpolarized component showed that the Type B response may, in fact,be a Type A response which was overlaid and masked by a maintained hyperpolarization of V_B in high K^+ saline. The rate at which the hyperpolarized component was switched on and off and its ability to be maintained can be used to explain the variations of the 'typical' Type B response described elsewhere. Some of these explanations are shown in Fig. 28. Thus, the fact that some cells exhibiting the Type B response do not hyperpolarize after depolarizing in high K^+ saline nor depolarize before hyperpolarizing in Normal saline may suggest that the hyperpolarized component was switching on during the initial high K^+ depolarization. Similarly, a reduction of V_B after the hyperpolarized component has switched on in high K^+ saline may be explained by a simple depolarization of this component. As V_A also exhibited the Type B response it may be that a similar set of events, with a hyperpolarized component "switching on" and "off", was observed across the apical membrane.

What is the Type B response? Results showed that this unusual behaviour was not directly due to the enhanced $[K^+]_o$ as it only occurred spontaneously in about one fifth of cells examined in high K^+ saline. As high K^+ saline contained no Na^+, it could be argued that the Type B response was the direct result of a Nernstian Na^+ gradient. Thus, if

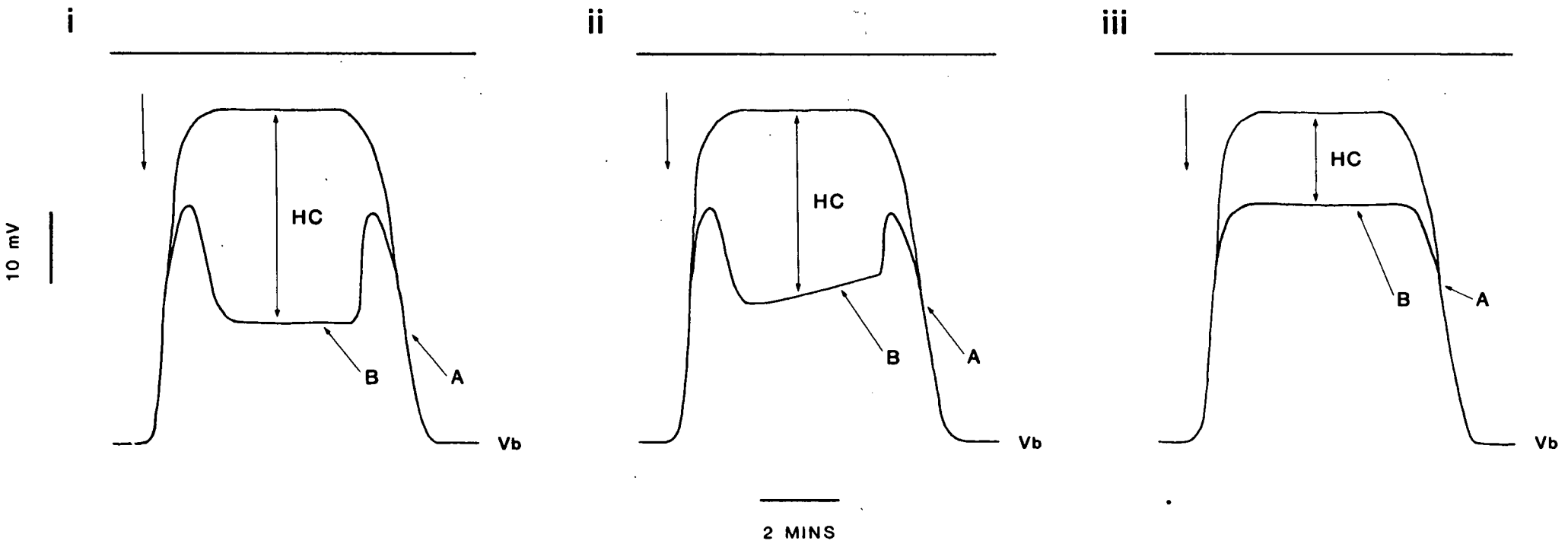
Fig. 28

This shows a schematic representation of three variations (i,ii,iii) of the basal membrane potential, during the Type B response (B) in 128 mM K^+ saline, with respect to the more usual Type A response (A). It can be suggested that the hyperpolarized component (H.C.) of the Type B response in all three cases may be the result of a "switching on" of some unknown ionic process in 128 mM K^+ saline followed by a "switching off" in Normal saline. As a result of the "switching on" in high K^+ saline, V_B becomes much more negative in the Type B response than predicted by Nernst. The different variations of the Type B response (Fig. 21, i-v) may be explained by the speed at which the hyperpolarized component is "switched on" and "off" when 128 mM K^+ and Normal saline are introduced (see text for details). A similar set of events, with a hyperpolarized component "switching on" and "off", may occur across the apical membrane.

→ indicates the change in perfusate from Normal to 128 mM K^+ saline

Ordinate: Potential in mV

Abscissa: Time in Mins


i
ii
iii
10 mV
HC
HC
HC
B
A
B
A
B
A
Vb
Vb
Vb
2 MINS

one assumes that $[Na^+]_i$ is approximately 13 mM as reported for the tubules of Rhodnius (Gupta et al., 1976) and that $[Na^+]_o$ is low (< 1 mM), then a potential of around 60 mVs could be generated across the basal membrane, if the membrane changed from being largely K^+ to Na^+ permeable in 128 mM K^+ saline. However, a small amount of Na^+ (10 mM) in the high K^+ (118 mM) saline showed that the Type B response was not due to an alteration in the Na^+ permeability.

The Type B response was observed for both membrane potentials if cells were introduced to 128 mM K^+ saline directly after at least 5 min treatment in K^+ free saline. Thus, K^+ free saline pretreatment was a means of inducing Type B behaviour in cells which originally exhibited the Type A response to high K^+ saline. The absence of external K^+, however, was not a pre-requisite for the induction of this response. Thus, pretreatment with 1.25 mM K^+ saline for at least 5 mins also induced the Type B response, although 2.5 mM K^+ saline pretreatment did not. The concentration of K^+ in the saline directly after the low K^+ treatment was also important as 64 mM K^+ saline did not reveal the Type B response after either 1.25 mM K^+ or K^+ free treatments.

Results from K^+ free (and 1.25 mM K^+) treatments suggest that the Type B response may be related to changes in $[Na^+]_i$ for, as mentioned earlier, this condition probably lead to an increase in intracellular Na^+. This is supported by the fact that treatment in Na^+ free saline which, unlike K^+ free saline, probably lead to a reduction in $[Na^+]_i$ did not induce the Type B response. The introduction of high K^+,Cl^- free saline after Cl^- free treatment also did not reveal the Type B response in cells which naturally exhibited the Type A response. However, a Type B response still occurred after such Cl^- substitution experiments in cells which exhibited this response naturally. This latter observation showed that the Type B response was probably not the result of an

electrogenic basal membrane Cl^- pump being "switched on", and that the absence of Cl^- did not prevent Type B behaviour from occurring.

As mentioned earlier, the depolarization of V_A during the Type A response may be due to high K^+ saline-induced intracellular changes leading to an increase in apical Cl^- exit. This was supported by the fact that the depolarization of V_A during the Type A response was very much reduced in high K^+,Cl^- free saline (V_B was not affected). So, although the raised $[K^+]_o$ may still have the ability of indirectly increasing the apical membrane Cl^- exit, the unavailability of Cl^- prevents a marked reduction in V_A.

Despite the appearance of Type B behaviour in high K^+,Cl^- free saline after Cl^- free treatment in cells which exhibited the naturally-occurring Type B response, results show that this was not maintained across both membranes indicating a dependence upon the presence of $[Cl^-]_o$. Also, the introduction of high K^+,Cl^- free saline after high K^+ saline exposed a Cl^--dependent potential in V_B and a Cl^--free activated potential in V_A during the naturally occurring Type B response. Thus, it appears that the Type B response may be related to an increase in $[Na^+]_i$ and exhibits a partial dependence on Cl^-.

Section 3: Electrophysiological Studies on V_B,V_A and T.E.P. Using Ouabain-, Vanadate-, Amiloride-, Monensin- and Ba^{2+}-Containing Salines

Effect of 1 mM Ouabain

Table 21a and Fig. 29 show the effect of Normal saline containing 1 mM ouabain on V_B, V_A and T.E.P. Results show that both membrane potentials gradually depolarized at similar rates during exposure to this inhibitor, producing little change in T.E.P. It was found that

Table 21a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM ouabain.

n^a represents the number of individual experiments for V_B, each involving separate tubule preparations;

n^b represents the number of individual experiments for V_A and T.E.P., each involving separate tubule preparations.

Table 21b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM ouabain to 128 mM K^+ saline in cells which exhibited the Type A response.

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

Table 21a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 5	10	15	20	30	45	60
V_B	Normal Saline	-68.9 ± 1.2	Normal Saline + 1 mM Ouabain	-66.1 ± 0.9	-	-62.1 ± 1.0	-60.3 ± 1.1	-55.6 ± 1.5	-50.3 ± 1.5	-46.4 ± 1.5
V_A		-88.7 ± 3.0		-85.0 ± 2.7	-83.2 ± 2.7	-79.9 ± 3.4	-79.2 ± 4.7	-75.0 ± 6.2	-	-
T.E.P.		+11.5 ± 3.3		+11.9 ± 3.1	+11.5 ± 3.2	+12.0 ± 3.5	+12.6 ± 4.3	+13.3 ± 7.3	-	-
n^a	-	34	-	34	31	31	27	25	21	20
n^b	-	11	-	11	11	9	6	4	-	-

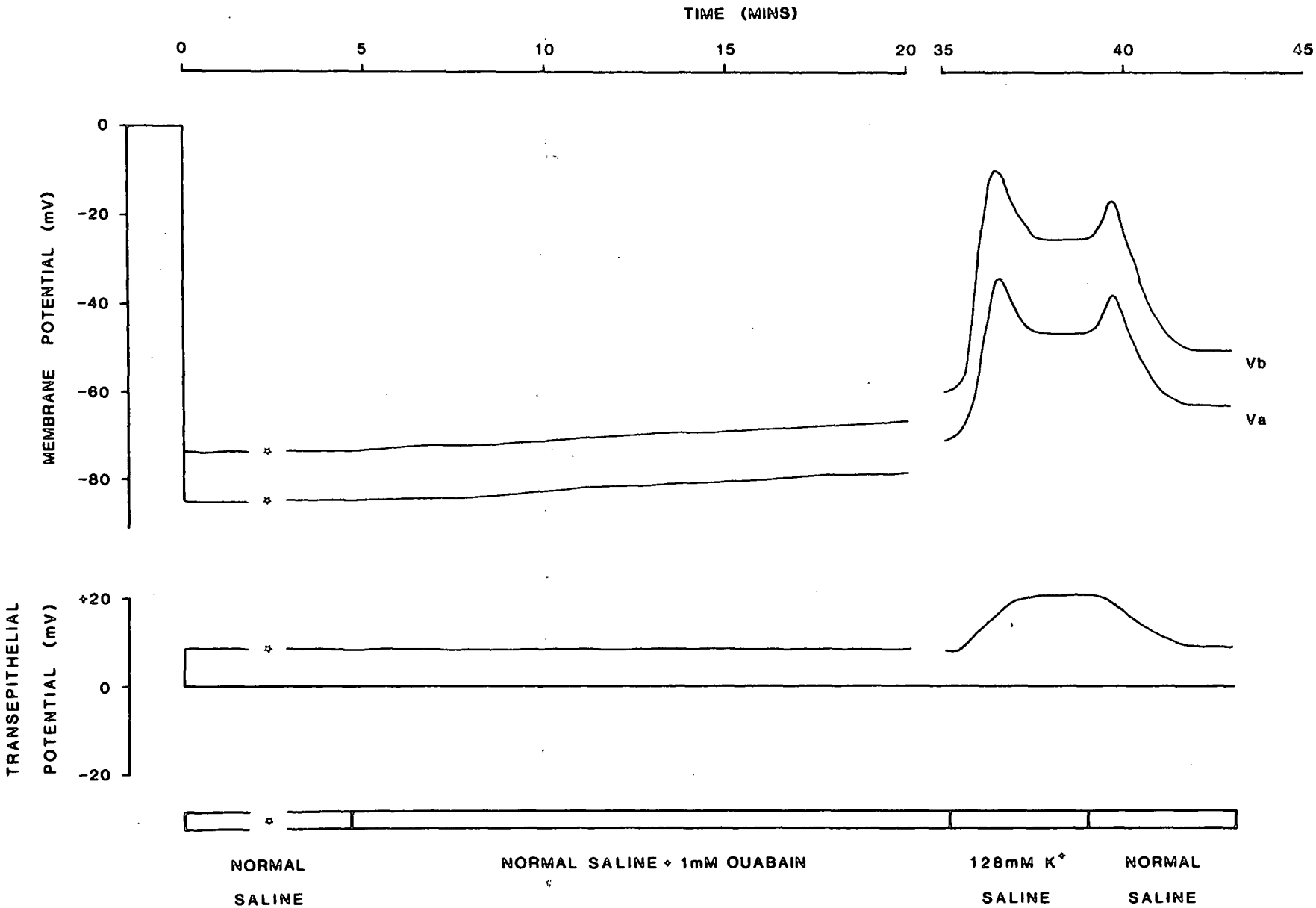
Table 21b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal Saline	-69.0 ± 1.4	Normal Saline + 1 mM Ouabain	-47.5 ± 1.8	128mM K^+ Saline	- 4.0 ± 1.8	-22.6 ± 3.7	Normal Saline	+8.9 ± 2.7	-41.0 ± 2.7	22
V_A		-85.7 ± 3.7		-64.0 ± 3.5		-31.2 ± 8.2	-36.2 ± 8.2		+8.0 ± 2.5	-61.0 ± 4.1	6
T.E.P.		+12.0 ± 3.2		+12.0 ± 5.5		+26.8 ± 8.2	-		-	+ 8.0 ± 5.3	6

Fig. 29

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM ouabain before the subsequent addition of 128 mM K^+ saline, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -8 mV, V_A = -43 mV, T.E.P. = +30 mV).


TIME (MINS)
0
5
10
15
20
35
40
45
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
NORMAL SALINE + 1mM OUABAIN
128mM K+ SALINE
NORMAL SALINE

V_B depolarized by one third of the original resting value after 1 hr. The fact that both V_B and V_A had only depolarized by 4% of the original resting values after 5 mins of ouabain treatment and did not exhibit an initial rapid depolarization suggests that the ouabain-sensitive Na^++K^+-ATPase was not strongly electrogenic in this tissue (see later).

The amplitude of the oscillations of V_A (and T.E.P.) increased significantly ($p = 0.001-0.01$) from 6.0 ± 0.3 mV in Normal saline to 13.1 ± 2.5 mV in Normal saline containing ouabain, with oscillations occurring in 64% of cells.

Effect of 128 mM K^+ Saline After Treatment in Normal Saline + 1 mM Ouabain

The effect of the introduction of 128 mM K^+ saline on V_B, V_A and T.E.P. after a period of 20 to 60 mins treatment in Normal saline + 1 mM ouabain, in cells which exhibited the Type A response, can be seen in Table 21b and Fig. 29. Results show that ouabain pretreatment induced a 'reduced' Type B response, in high K^+ saline, for both V_B and V_A. However, the initial depolarization (component b, Fig. 20) of V_B and V_A in 128 mM $[K^+]_o$ produced potentials (component c, Fig. 20) less negative but not significantly different ($p = 0.1-0.2$ and $p = 0.05-0.2$ respectively) from the equivalent values found during the Type A response. Furthermore, V_B depolarized to a positive value (up to +13 mV in the most extreme case) in just under one third of cells exposed to high K^+ saline after ouabain treatment. This response of V_B and V_A was similar to that found earlier when 128 mM K^+ saline followed Na^+ free treatment. However, unlike results from Na^+ free experiments, both V_B and V_A exhibited a ouabain-induced hyperpolarization following the initial depolarization in high K^+ saline to maintained potentials (component e, Fig. 20). This repolarizing change (component d, Fig. 20) was between 2 and 71 mV ($n = 22$) for V_B and 2 and 8 mV ($n = 6$) for V_A.

The variation in the repolarizing change in V_B meant that some cells exhibited a value for V_B, in high K^+ saline, more negative than the potential seen in the immediately preceeding Normal saline + ouabain treatment. The repolarized V_B and V_A (component e, Fig. 20) in 128 mM K^+ saline were significantly more negative ($p < 0.001$) and not different ($p = 0.5-0.6$) respectively compared with the maintained potentials of the Type A response. The repolarized V_B (component e, Fig. 20), however, was significantly less negative ($p < 0.001$) than the equivalent value in the Type B response. This 'reduced' Type B behaviour was reflected in T.E.P. which reached a value (+26.8 ± 8.2 mV) in 128 mM $[K^+]_o$ similar ($p = 0.4-0.5$) to that found in both the Type A and Type B response.

On re-introduction of Normal saline, V_B and V_A only reached values 86% and 95% respectively, of the potentials found before the addition of high K^+ saline, after 2-3 mins, and did not recover further. This suggests that the gradual depolarization of the membrane potentials by ouabain was irreversible.

Finally, it was found that the introduction of high K^+ saline + 1 mM ouabain, to cells which exhibited the Type A response and had no previous exposure to this inhibitor, did not induce Type B behaviour ($n = 3$). Thus, the depolarized potentials in cells so-treated were similar ($p = 0.3-0.4$) to those found in the Type A response. This suggests that pretreatment is needed in ouabain saline in order to induce Type B behaviour.

Effect of 1 mM Vanadate

Table 22a shows the effect of Normal saline containing 1 mM vanadate on V_B, V_A and T.E.P. It was found that both membranes exhibited a biphasic decrease in potential, with a more rapid depolarization of

Table 22a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM vanadate.

Table 22b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM vanadate to 128 mM K^+ saline + 1 mM vanadate in cells which exhibited the Type A response.

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

Table 22a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 5	10	15	20	30
V_B	Normal	-70.7 ± 2.6	Normal	-56.2 ± 5.2	-50.4 ± 6.4	-46.2 ± 3.6	-41.2 ± 2.9	-32.0 ± 7.0
V_A	Saline	-88.0 ± 3.8	Saline + 1 mM	-66.8 ± 5.0	-63.3 ± 5.4	-59.2 ± 4.9	-55.7 ± 4.9	-49.5 ± 10.5
T.E.P.		+12.1 ± 2.2	Vanadate	+ 4.3 ± 2.7	+ 3.6 ± 3.3	+ 4.0 ± 4.4	+ 4.2 ± 4.4	+ 8.0 ± 9.2
n	-	10	-	10	8	6	6	2

Table 22b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B		-70.7 ± 2.6	Normal	-30.5 ± 3.8	128mM K^+	+ 0.7 ± 2.3	-		-	-21.9 ± 5.2	10
V_A	Normal	-88.0 ± 3.8	Saline + 1 mM	-44.5 ± 4.9	Saline + 1 mM	-21.2 ± 3.9	-	Normal	-	-38.6 ± 6.1	10
T.E.P.	Saline	+12.1 ± 2.2	Vanadate	+ 7.8 ± 3.1	Vanadate	+17.7 ± 3.1	-	Saline	-	+10.4 ± 3.7	10

V_B and V_A over the first 5 mins exposure to vanadate saline, followed by a slower change over the next 25 mins to values 56% and 43% respectively less negative than the original resting potentials. In one extreme case, the introduction of Normal saline containing vanadate resulted in V_B and V_A depolarizing from -73 mV and -83 mV to -30 mV and -38 mV respectively in the first 5 mins; after a further 25 mins, V_B had decreased to -24 mV, whilst no further change occurred in V_A. As a result of V_A depolarizing quicker than V_B over the first 5 mins in vanadate saline, T.E.P. reduced in positivity to +4.3 ± 2.7 mV. However, this value was maintained throughout the remaining exposure to vanadate saline as both membrane potentials declined at similar rates.

After a period of 20 to 30 mins in vanadate treatment, 30% of cells were returned to Normal saline. None of these cells showed any sign of recovery over the next 3-4 mins. It would appear that the depolarization caused by vanadate was irreversible.

Oscillations of V_A (and T.E.P.) occurred in 30% of cells during exposure to 1 mM vanadate and were 4.0 ± 1.2 mV in amplitude. Although the frequency was slightly less than in Normal saline, the amplitdue of these oscillations was not significantly different ($p = 0.1$-0.2).

In addition to examining the electrophysiological response of V_B, V_A and T.E.P. during vanadate treatment, the effect of this inhibitor on fluid secretion by the Malpighian tubules of Locusta was also examined (see Appendix, Table 1). It was found that Normal saline containing 1 mM vanadate, significantly reduced ($p < 0.001$) fluid secretion by 82% ($n = 15$). This result compares favourably with the significant 77% reduction of fluid secretion ($n = 30$) found by Kalule-Sabiti (1985) in the same tissue.

Effect of 128 mM K^+ Saline + 1 mM Vanadate After Treatment in Normal Saline + 1 mM Vanadate

The effect of 128 mM K^+ saline + 1 mM vanadate on V_B, V_A and T.E.P. was examined after a period of 10 to 30 mins treatment in Normal saline + 1 mM vanadate. It was found that membrane changes and resulting potentials to this vanadate/high K^+ treatment were the same whether the cells originally exhibited the Type A or Type B response. Thus, results from cells originally exhibiting either Type A or Type B behaviour have been grouped together in Table 22b. It was found that vanadate pretreatment resulted in the production of a 'reduced' Type A response for V_B and V_A on introduction of 128 mM K^+ saline + vanadate. Indeed, the depolarized V_B (component c, Fig. 20) which had a range of +14 mV to -11 mV (with 60% of cells depolarizing to a positive V_B) had a positive mean value. Furthermore, the maintained V_B and V_A (component c, Fig. 20) were both significantly less negative ($p < 0.001$) than those predicted by Nernst. It was of interest, however, that the initial depolarization (component b, Fig. 20) of V_A in high K^+ saline + vanadate was 75% of that for V_B (c.f. 71% and 65% found in the Type B and Type A responses respectively) and this resulted in a T.E.P. value not significantly different ($p = 0.8$-0.9) from that found in the Type B response.

On re-introduction of Normal saline, V_B and V_A only reached values 72% and 87%, respectively, of the potentials found before the addition of 128 mM K^+ saline + vanadate, after 2 mins, and did not recover further.

It was noted that the introduction of high K^+ saline + vanadate, after vanadate treatment, produced a similar response whether cells originally exhibited the Type A or Type B response. This suggests that the alteration of the ionic gradients during pretreatment in Normal saline + vanadate was sufficient to abolish Type B behaviour.

Results in Table 23 and Fig. 20 show that the introduction of high K^+ saline + vanadate, to cells which exhibited the Type A response and had no previous exposure to this inhibitor, induced Type B behaviour for V_B and V_A. It can be seen, however, that this induced Type B response did not develop strongly. Thus, the novel introduction of vanadate in high K^+ saline produced depolarized potentials (component c, Fig. 20) that were not significantly different ($p = 0.05-0.1$ for V_B and $p > 0.9$ for V_A) from those found during the Type A response. After depolarizing, V_B and V_A exhibited repolarizing changes (component d, Fig. 20) between 3 and 6 mV and 3 and 11 mV respectively. The resulting repolarized V_B and V_A (component e, Fig. 20) were not maintained, but decreased 8.8 ± 3.2 mV and 15.3 ± 4.4 mV respectively, over 2 mins, after reaching a maximum value. Furthermore, during this 'weak' inducement of Type B behaviour, V_B depolarized less than V_A. Indeed, the initial depolarization of V_A in 128 mM K^+ saline was 81% of that for V_B (cf. 71% and 65% during the Type B and Type A response respectively) and this produced a T.E.P. which was not significantly different ($p = 0.7-0.8$) from that found in the Type B response.

Finally, it was found that the presence of 1 mM vanadate in 128 mM K^+ saline did not qualitatively alter the Type B behaviour of either membrane potential when compared with results in the absence of vanadate ($n = 4$). Thus, the novel introduction of 1 mM vanadate in high K^+ saline, in cells which originally exhibited Type B behaviour, had no significant effect ($p = 0.4-0.5$) on the Type B response values for V_B, V_A and T.E.P.

Effect of 1 mM Amiloride

Table 24a shows the effect of changing the perfusate from Normal saline to Normal saline containing 1 mM amiloride on V_B, V_A and T.E.P. Results show

Table 23

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to 128 mM K^+ saline + 1 mM vanadate in cells which exhibited the Type A response (i.e. a novel addition of vanadate in high K^+ saline).

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

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D.± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal Saline	-68.8 ± 1.8	128mM K^+ Saline + 1 mM Vanadate	-17.5 ± 5.5	-22.3 ± 5.8	Normal Saline	-	-56.8 ± 2.9	4
V_A		-84.3 ± 7.1		-42.5 ± 11.2	-48.3 ± 10.4		-	-70.3 ± 3.6	4
T.E.P.		+ 6.3 ± 3.9		+18.3 ± 5.1	-		-	+ 6.0 ± 2.9	4

Fig. 30

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to 128 mM K^+ saline + 1 mM vanadate in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -9 mV, V_A = -48 mV, T.E.P. = +36 mV).

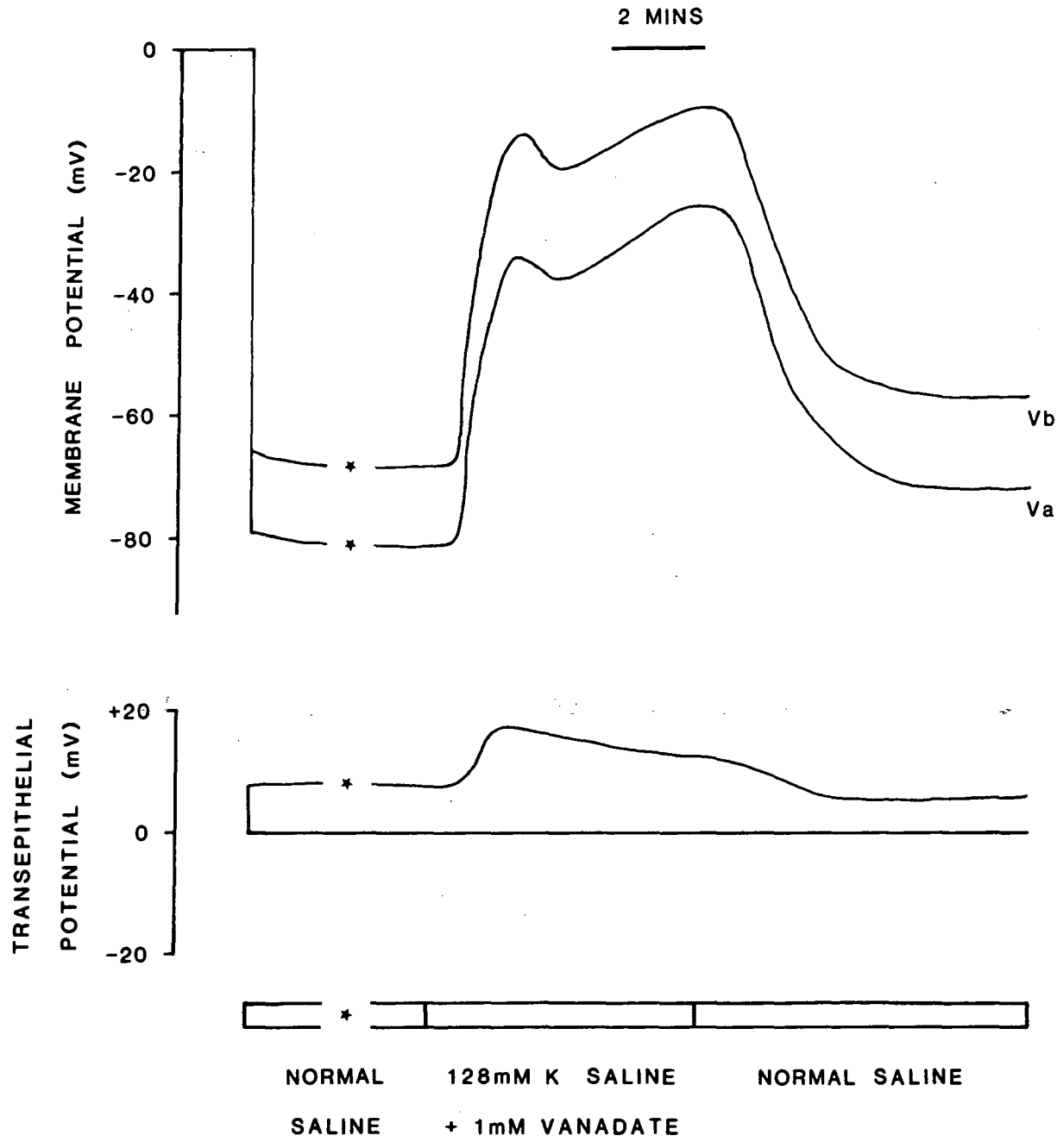

2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
128mM K SALINE + 1mM VANADATE
NORMAL SALINE

Table 24a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM amiloride.

Table 24b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM amiloride to 128 mM K^+ saline + 1 mM amiloride in cells which exhibited the Type A response.

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

Table 24a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10	15	20
V_B	Normal	-70.2 ± 1.3	Normal	-	-	-	-	-
V_A	Saline	-81.1 ± 4.4	Saline + 1 mM	-77.0 ± 4.4	-74.9 ± 4.4	-73.7 ± 4.4	-72.6 ± 4.9	-72.8 ± 5.9
T.E.P.		+ 6.9 ± 3.6	Amiloride	+ 2.8 ± 3.5	+1.9 ± 3.5	+ 0.6 ± 3.7	+ 0.5 ± 3.9	+ 0.7 ± 4.8
n	-	10	-	10	10	9	8	6

Table 24b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-70.2 ± 1.3	Normal	-70.2 ± 1.3	128mM K^+	- 7.7 ± 1.1	-	Normal	-	-68.2 ± 0.5	6
V_A	Saline	-74.7 ± 5.5	Saline + 1 mM	-72.2 ± 5.7	Saline + 1 mM	-25.2 ± 9.3	-	Saline	-	-76.0 ± 4.1	6
T.E.P.		+ 1.0 ± 5.5	Amiloride	- 1.8 ± 5.4	Amiloride	+14.2 ± 8.6	-		-	+ 3.0 ± 4.3	6

that whilst V_B did not change even after 60 mins of amiloride treatment, a gradual depolarization of V_A was observed to a value that was 7.3 ± 2.3 mV less negative than the original resting potential after 30 mins exposure. T.E.P. gradually decreased in positivity, towards zero, as the difference in potential between the two membranes decreased.

Oscillations of 5.3 ± 2.4 mV amplitude occurred for V_A (and T.E.P.) in 30% of cells in Normal saline + 1 mM amiloride. This rate of oscillation was less but not significantly different in amplitude ($p = 0.7-0.8$) from the value found in Normal saline.

It was found that Normal saline containing 1 mM amiloride significantly reduced ($p < 0.001$) fluid secretion by the Malpighian tubules of Locusta by 88% ($n = 14$) (see Appendix, Table 1). This result agrees favourably with the significant 94% reduction in fluid secretion ($n = 22$) found by Fathpour (1980) in the same tissue.

Effect of 128 mM K^+ Saline + 1 mM Amiloride After Treatment in Normal Saline + 1 mM Amiloride

The effect of the introduction of 128 mM K^+ saline + 1 mM amiloride, on V_B, V_A and T.E.P., directly after a period of 15 to 40 mins in Normal saline + 1 mM amiloride, on cells which originally exhibited the Type A response, can be seen in Table 24b. It was found that treatment in amiloride saline did not alter the Type A response of either V_B or V_A. Thus, the depolarized values (component c, Fig. 20) of V_B and V_A were not significantly different ($p = 0.3-0.4$ and $p = 0.05-0.1$ respectively) from the equivalent values found during the Type A response. However, although not significantly different, the depolarized V_A (component c, Fig. 20) in high K^+ saline + amiloride was 16 mV less negative than in the Type A response. This arose as a result of V_A initially depolarizing 75% of that for V_B (cf. 65% found during the

Type A response) and resulted in a T.E.P. value, in 128 mM K^+ saline + amiloride, which was significantly less positive ($p = 0.02$-0.03) than T.E.P. found in the Type A response.

Effect of 10^{-5} M or 10^{-4} M Monensin

It was found that V_B, V_A and T.E.P. did not change even after 30 mins exposure to Normal saline + 10^{-5} M monensin ($n = 3$). In contrast, changing the perfusate from Normal saline to Normal saline + 10^{-4} M monensin altered V_B, V_A and T.E.P. and results are shown in Table 25a and Fig. 31. It can be seen that V_B and V_A depolarized 10.3 ± 2.1 mV and 7.6 ± 2.3 mV respectively over the first 1 to 4 mins exposure to 10^{-4} M monensin saline, then hyperpolarized 23.1 ± 2.9 mV and 20.2 ± 2.9 mV respectively over the next 2 to 7 mins. These changes were then followed by a decline of V_B and V_A, 6.3 ± 1.1 mV and 8.0 ± 2.1 mV respectively, over the next 3 to 7 mins. The extent of this depolarization was not followed. Due to V_B and V_A changing by similar values (with V_A depolarizing and hyperpolarizing 74% and 87% respectively of the values found for V_B), T.E.P. changed little during Normal saline + 10^{-4} M monensin treatment. One third of the cells exposed to monensin were returned to Normal saline; V_B and V_A in these cells depolarized to the original resting values in about 4 mins.

A variety of experiments (the purpose of which will be explained later) were carried out to explore this ability of monensin to induce an initial depolarization, followed by a secondary hyperpolarization, of both membrane potentials. Therefore, the effect of changing the perfusate from Normal saline to Normal saline + 1 mM ouabain on V_B, V_A and T.E.P. before the subsequent addition of Normal saline + 10^{-4} M monensin + 1 mM ouabain was examined ($n = 5$). Cells were exposed to Normal saline + ouabain for 10 to 20 mins. It was found that the

Table 25a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10^{-4} M monensin.

Table 25b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 10^{-4} M monensin to 128 mM K^+ saline + 10^{-4} M monensin in cells which exhibited the Type A response.

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

Table 25a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	Repolarized P.D. ± S.E.M. (mV)	n
V_B	Normal	-71.5 ± 1.3	Normal	-61.2 ± 2.9	-84.3 ± 2.3	11
V_A	Saline	-89.3 ± 2.2	Saline + 10^{-4}M Monensin	-81.7 ± 2.9	-101.9 ± 3.4	11
T.E.P.		+10.0 ± 1.7		+12.7 ± 2.7	+ 9.0 ± 2.8	11

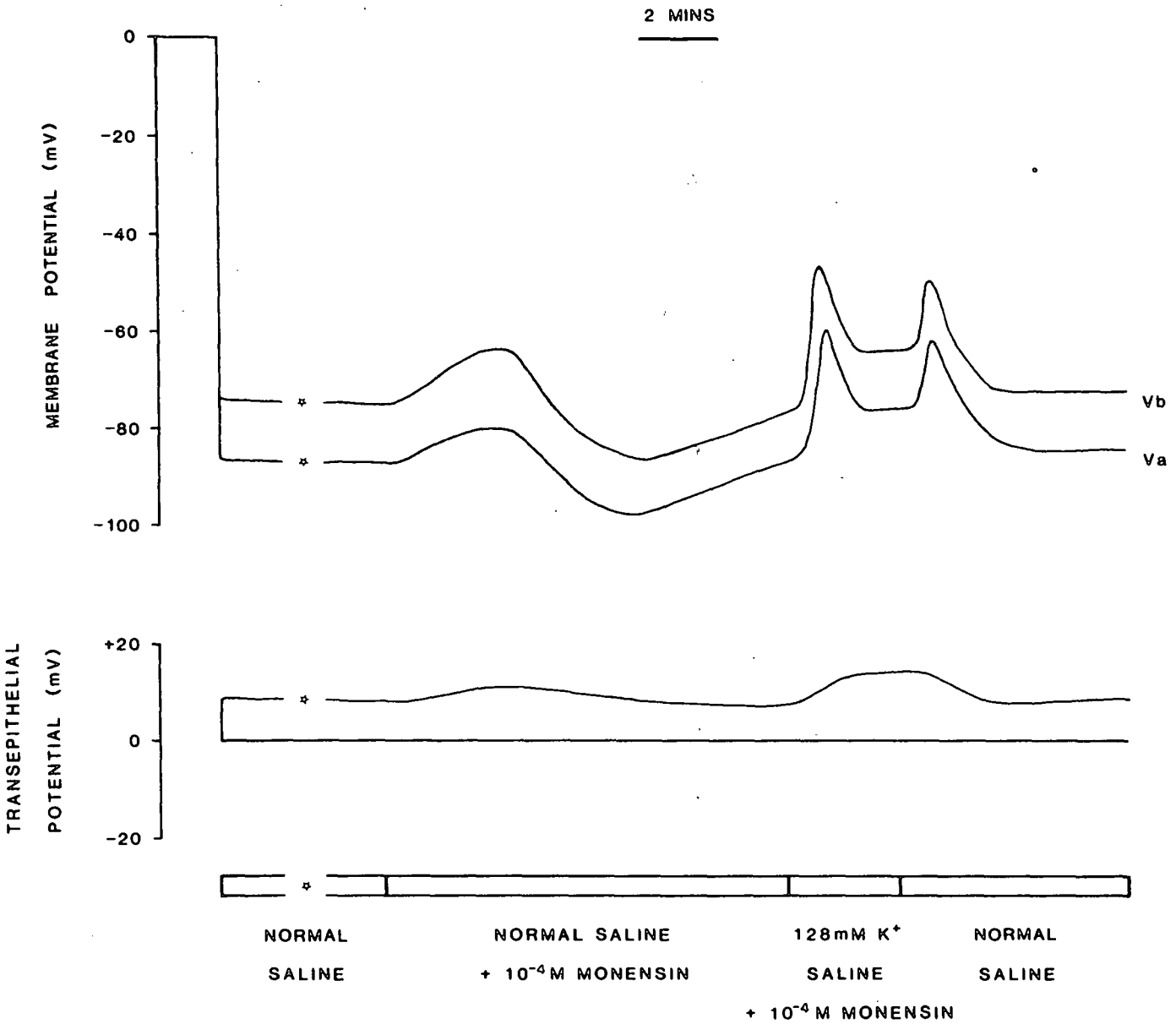
Table 25b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-72.0 ± 1.2	Normal	-73.9 ± 4.1	128mM K^+	-39.1 ± 8.4	-59.9 ± 7.7	Normal	+15.0 ± 6.3	-71.2 ± 2.1	8
V_A	Saline	-87.5 ± 5.6	Saline + 10^{-4} M Monensin	-90.0 ± 6.0	Saline + 10^{-4} M Monensin	-62.3 ± 10.4	-78.8 ± 7.1	Saline	+13.0 ± 6.7	-85.6 ± 4.7	8
T.E.P.		+10.8 ± 1.8		+11.9 ± 3.4		+19.3 ± 4.1	-		-	+ 9.9 ± 2.3	8

Fig. 31

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10^{-4} M monensin before the subsequent addition of 128 mM K^+ saline + 10^{-4} M monensin, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -5 mV , V_A = -38 mV, T.E.P. = +32 mV).


2 MINS
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
-100
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
NORMAL SALINE + 10^{-4} M MONENSIN
128mM K^+ SALINE + 10^{-4} M MONENSIN
NORMAL SALINE

introduction of Normal saline containing monensin and ouabain abolished the initial depolarization and reduced the secondary hyperpolarization of V_B and V_A. Thus, V_B and V_A hyperpolarized immediately by 6.8 ± 1.6 mV and 4.6 ± 2.4 mV respectively, over 2 to 3 mins, in this modified saline (cf. Table 25a). The new hyperpolarized V_B and V_A (-60.8 ± 2.1 mV and -82.8 ± 4.6 mV respectively) were maintained for 2 to 5 mins. Due to the similar changes of V_B and V_A, T.E.P. did not alter in saline containing monensin and ouabain. A period of ouabain pretreatment was needed to produce these effects as the introduction of Normal saline + monensin + ouabain directly after Normal saline resulted in the 'typical' monensin-induced depolarization and hyperpolarization phases described in Table 25a.

It was found that changing the perfusate from Normal saline to Na^+ free saline + 10^{-4} M monensin also abolished the initial monensin-induced depolarization of both membrane potentials found when this ion was present (n = 3). Instead, as seen in Normal saline containing monensin and ouabain, an immediate hyperpolarization of V_B and V_A (by 28.7 ± 8.0 mV and 18.0 ± 2.5 mV respectively) occurred over 1 to 5 mins to produce values of -103.3 ± 2.4 mV and -107.0 ± 6.6 mV respectively. These new values were not maintained but declined 32.3 ± 9.6 mV and 28.7 ± 8.1 mV respectively over the next 1 to 4 mins. Reflecting the changes seen in V_B and V_A, T.E.P. decreased in positivity by 8.3 ± 4.6 mV to +1.0 ± 5.0 mV over the first 1 to 5 mins in Na^+ free saline + monensin, then increased in positivity by 2.3 ± 2.2 mV over the next 1 to 4 mins.

As with Normal saline containing monensin, changing the perfusate from Normal saline to Cl^- free saline + 10^{-4} M monensin resulted in a depolarization of both membrane potentials; however, the new values were maintained and did not exhibit a secondary hyperpolarization (n = 5). Thus, V_B and V_A depolarized 16.6 ± 4.4 mV and 7.6 ± 2.2 mV respectively

over 2 to 5 mins (cf. Table 25a). The new potentials (-54 ± 3.7 mV and -71.4 ± 2.9 mV respectively) were maintained for 2 to 10 mins. T.E.P. increased in positivity by 11.2 ± 2.7 mV over 2 to 5 mins in Cl^- free saline + monensin to a maintained value of +16.0 ± 2.8 mV.

Finally, changing the perfusate from Normal saline to Ca^{2+} free saline + 10^{-4} M monensin resulted in no change of either V_B or V_A even after 15 mins exposure to this new solution (n = 6). This suggests that the initial depolarization, followed by the secondary hyperpolarization of both membrane potentials, found in Normal saline + 10^{-4} M monensin, was Ca^{2+}-dependent.

Oscillations of V_A (and T.E.P.) occurred in 36% of cells in Normal saline + 10^{-4} M monensin and were 6.0 ± 1.6 mV in amplitude. Although the frequency was less, the amplitude of these oscillations was not significantly different ($p > 0.9$) from the value found in Normal saline.

Effect of 128 mM K^+ Saline + 10^{-4} M Monensin After Treatment in Normal Saline + 10^{-4} M Monensin

The effect of the introduction of 128 mM K^+ saline + 10^{-4} M monensin on V_B, V_A and T.E.P. after a period of 5 to 20 mins treatment in Normal saline + 10^{-4} M monensin, in cells which exhibited the Type A response, can be seen in Table 25b and Fig. 31. It can be seen that monensin pretreatment induced the Type B response for both V_B and V_A. Thus, the initial depolarization (component b, Fig. 20) of V_B and V_A, which was quite variable (between 4 and 48 mV, and 2 and 49 mV respectively) produced depolarized values (component c, Fig. 20) that were not significantly different ($p = 0.3$-0.4 and $p = 0.4$-0.5 respectively) from the equivalent values found in the naturally occurring Type B response. After depolarizing approximately 50% of cells were maintained at this potential. However, V_B and V_A in the remaining cells repolarized

(component d, Fig. 20) by 20.8 ± 6.0 mV and 16.5 ± 5.7 mV respectively in high K^+ saline + monensin. The resulting repolarized potentials (component e, Fig. 20) were not significantly different ($p > 0.9$ for both V_B and V_A) from the equivalent values found during the naturally occurring Type B behaviour. Following the changes of V_B and V_A, T.E.P. increased in positivity, in 128 mM K^+ saline + monensin, to a value similar ($p = 0.8$-0.9) to that found in the Type B response.

Effect of 1 mM $BaCl_2$

The pH of all solutions in which $BaCl_2$ was used was 7.0, instead of the 'normal' pH 7.2. This occurred to avoid precipitation in solutions of the higher pH. Experiments involving solutions of pH 7.0 did not alter V_B, V_A and T.E.P. when compared with results using solutions of pH 7.2.

Table 26a and Fig. 32 show the effect of Normal saline containing 1 mM $BaCl_2$ on V_B, V_A and T.E.P. It can be seen that V_B and V_A initially hyperpolarized between 3 and 34 mV, and 2 and 22 mV respectively over 0.5 min and were maintained at the new potentials for up to 22 mins. The mean hyperpolarization of V_A was just over half that for V_B and resulted in V_B becoming more negative than V_A. This was reflected in T.E.P. which changed from +5.4 ± 1.9 mV to -5.1 ± 1.9 mV. On re-introduction of Normal saline, V_B, V_A and T.E.P. returned to the original resting values over 0.5 min.

A variety of experiments (the purpose of which will be explained later) were carried out to explore the ability of V_B and V_A to hyperpolarize in Ba^{2+} saline. Therefore, the effect of changing the perfusate from Normal saline to Normal saline + 1 mM Ba^{2+} on V_B, V_A and T.E.P. before the subsequent addition of Cl^- free saline + 1 mM Ba^{2+} was examined. Although the latter saline contained 1 mM $BaCl_2$, $[Cl^-]_o$

Table 26a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM Ba^{2+}.

Table 26b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM Ba^{2+} to Cl^- free saline + 1 mM Ba^{2+}.

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

Table 26a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	n
V_B	Normal	-72.9 ± 1.0	Normal	-93.4 ± 2.0	Normal	-72.7 ± 1.5	23
V_A	Saline	-79.9 ± 2.0	Saline + 1 mM	-90.7 ± 2.6	Saline	-79.5 ± 2.3	23
T.E.P.		+ 5.4 ± 1.9	Ba^{2+}	- 5.1 ± 1.9		+ 5.4 ± 2.8	23

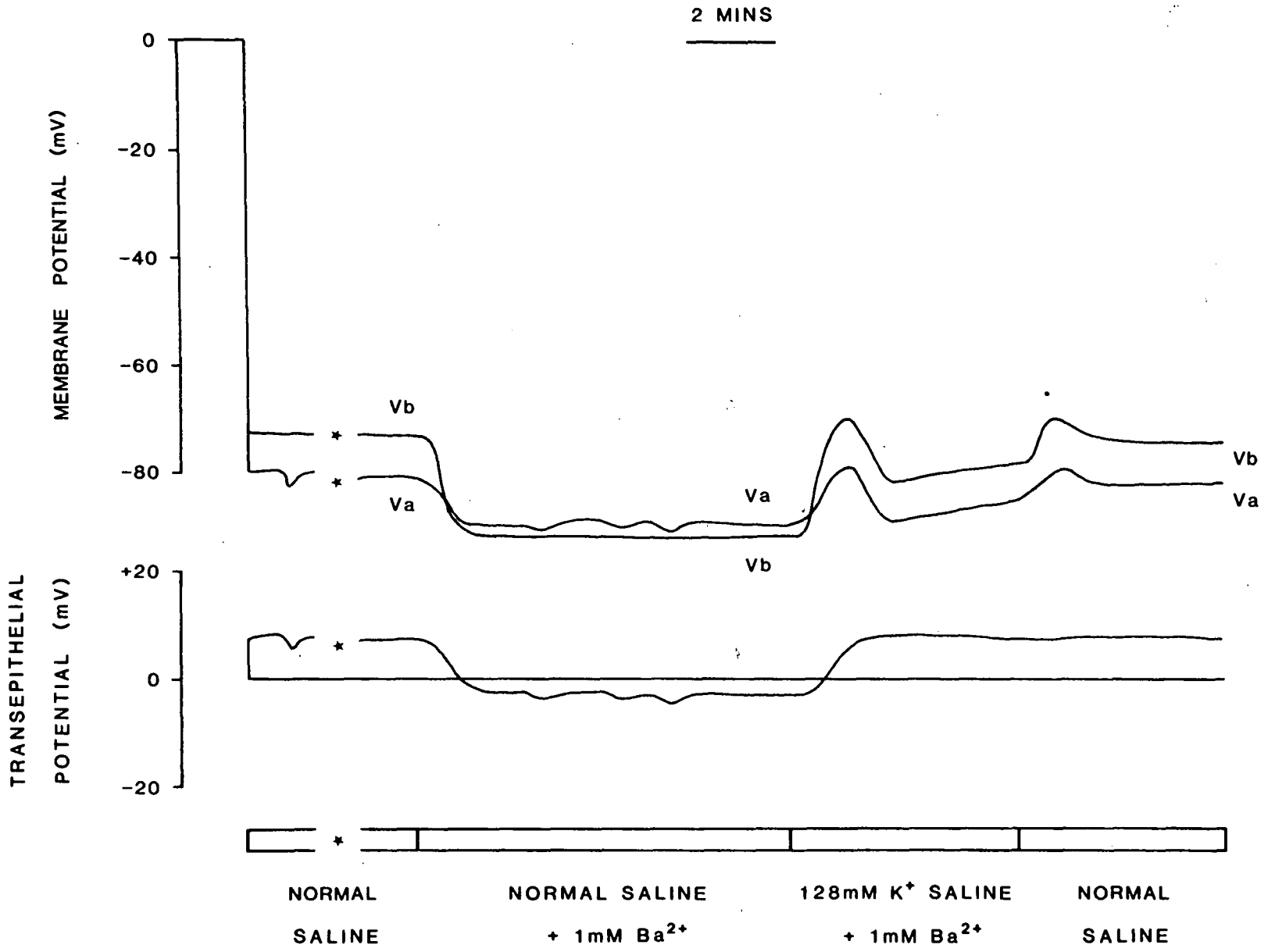
Table 26b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	Treatment	New P.D. ± S.E.M. (mV)	n
V_B	Normal	-72.2 ± 1.4	Normal	-93.6 ± 5.2	Cl^- free	-70.4 ± 9.1	5
V_A	Saline	-83.2 ± 3.2	Saline + 1 mM	-93.0 ± 4.6	Saline + 1 mM	-83.2 ± 5.3	5
T.E.P.		+ 8.0 ± 4.0	Ba^{2+}	- 2.8 ± 2.7	Ba^{2+}	+ 6.8 ± 8.4	5

Fig. 32

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM Ba^{2+} before the subsequent addition of 128 mM K^+ saline + 1 mM Ba^{2+}, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -8 mV, V_A = -43 mV, T.E.P. = +32 mV).


2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
Va
Vb
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
NORMAL SALINE + 1mM Ba2+
128mM K+ SALINE + 1mM Ba2+
NORMAL SALINE

was so low, that the solution was deemed to be 'Cl^--free'. In these experiments, Cl^- free saline + Ba^{2+} was added only when V_B, V_A and T.E.P. had demonstrated maintained values, in Normal saline + Ba^{2+}, similar ($p = 0.8-0.9$) to those found described above (see Table 26a). Results are shown in Table 26b. It was found that on introduction of Cl^- free saline, containing Ba^{2+}, both V_B and V_A depolarized, over 1 min, to new values that were maintained for at least 5 mins. These new potentials were very similar to the original resting values before Ba^{2+} treatment (with $p > 0.9$ for V_B, and V_A being identical to the original potential). T.E.P. increased in positivity, over 1 min, in Cl^- free saline + Ba^{2+}, to a value similar ($p > 0.9$) to that found originally. These results indicate that the hyperpolarization of V_B and V_A in Normal saline + Ba^{2+} was a Cl^--dependent event.

The Cl^--dependence of the Ba^{2+}-induced hyperpolarization of V_B and V_A was further investigated by changing the perfusate from Normal saline to Cl^- free saline + Ba^{2+} before the subsequent addition of Normal saline + Ba^{2+} ($n = 3$). It was found that both membrane potentials did not change, over 4 to 6 mins, on introduction of Cl^- free saline + Ba^{2+}, but hyperpolarized, over 0.5 min, in the ensuing Normal saline + Ba^{2+} treatment. The new V_B and V_A, which were maintained for up to 15 mins, were not significantly different ($p > 0.9$ and $p = 0.2-0.3$ respectively) from the values found in Normal saline + Ba^{2+} described earlier (cf. Table 26a).

The effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline containing both 1 mM Ba^{2+} and 1mM ouabain was examined ($n = 3$). It was found that such treatment reduced the Ba^{2+}-induced hyperpolarization and increased the rate of the ouabain-induced depolarization of both membrane potentials. Thus, the initial hyperpolarization of V_B and V_A in Normal saline containing Ba^{2+} and ouabain,

took 0.5 min, and was only 7.0 ± 3.5 mV (34%) and 2.3 ± 2.3 mV (21%) respectively compared to the values found when Normal saline + Ba^{2+} was introduced. V_B did not become more negative than V_A, resulting in the new mean T.E.P. remaining lumen positive (+1.0 ± 2.4 mV). After hyperpolarizing, V_B and V_A depolarized 8.7 ± 2.3 mV and 12.0 ± 8.0 mV respectively over 5 to 15 mins in Normal saline + Ba^{2+} + ouabain. This rate of decrease was approximately twice as fast as that found with Normal saline + 1 mM ouabain (1 mV/min compared with 0.5 mV/min for V_B and 1.4 mV/min compared with 0.6 mV/min for V_A). T.E.P. did not change during this decline of V_B and V_A.

The Ba^{2+}-induced decline of V_B and V_A in ouabain saline was further investigated by changing the perfusate from Normal saline to Normal saline + Ba^{2+} before the subsequent addition of Normal saline + Ba^{2+} + ouabain (n = 2). In these experiments, the latter saline was added only when V_B, V_A and T.E.P. had demonstrated maintained values, in Normal saline + Ba^{2+}, similar (p = 0.05-0.1) to those described in Table 26a. It was found that on introduction of Normal saline containing Ba^{2+} and ouabain, V_B and V_A depolarized 31.5 ± 16.7 mV and 32.0 ± 20.2 mV respectively over 10 to 25 mins. This decline of membrane potentials was approximately four times faster than the rate found in Normal saline + ouabain (1.8 mV/min compared with 0.4 mV/min for V_B and 1.8 mV/min compared with 0.5 mV/min for V_A). T.E.P. did not change during the decline of V_B and V_A in Normal saline + Ba^{2+} + ouabain.

Oscillations of 5.5 ± 0.8 mV in amplitude occurred for V_A (and T.E.P.) in 48% of cells exposed to Normal saline + 1 mM $BaCl_2$. These results were not significantly different (p = 0.4-0.5) from those found in Normal saline.

Finally, it was found that Normal saline containing 1 mM Ba^{2+} significantly reduced (p < 0.001) fluid secretion by the Malpighian

tubules of Locusta by 63% (n = 34) (see Appendix, Table 1).

Effect of 128 mM K^+ Saline + 1 mM $BaCl_2$ After Treatment in Normal Saline + 1 mM $BaCl_2$

Table 27 and Fig. 32 show the effect on V_B, V_A and T.E.P. of changing the perfusate to 128 mM K^+ saline + 1 mM $BaCl_2$ after a period of 5 to 20 mins in Normal saline + 1 mM $BaCl_2$, in cells which exhibited the Type A response. It can be seen that Ba^{2+} treatment induced an 'enhanced' Type B response for both membrane potentials which depolarized and repolarized by similar values. Thus, on introduction of high K^+ saline + Ba^{2+}, V_B and V_A depolarized (component b, Fig. 20) between 3 and 50 mV, and 0 and 42 mV respectively (V_A did not change in 10% of cells) and repolarized (component d, Fig. 20) between 3 and 46 mV, and 2 and 54 mV. As a result, the depolarized potentials (component c, Fig. 20) and repolarized potentials (component e, Fig. 20) for V_B and V_A were significantly more negative ($p < 0.001$) than the equivalent values found in the naturally occurring Type B response. Indeed, the repolarized V_B and V_A (component e, Fig. 20) were similar to the results found in Normal saline + Ba^{2+} before the addition of 128 mM K^+ saline + Ba^{2+}. Furthermore, during this 'enhanced' inducement of the Type B response, V_A depolarized less than V_B. Indeed, the initial depolarization (component b, Fig. 20) of V_A in 128 mM K^+ saline + Ba^{2+} was only 56% of that for V_B (cf. 71% during the Type B response). This produced a T.E.P. which was only +4.8 ± 2.7 mV, and significantly different ($p < 0.001$) from the +20.0 ± 1.7 mV value found in the naturally occurring Type B response.

Table 27

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM Ba^{2+} to 128 mM K^+ saline + 1 mM Ba^{2+}, in cells which exhibited the Type A response.

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

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-72.1 ± 1.1	Normal	-90.1 ± 2.3	128mM K^+	-70.9 ± 4.4	-85.9 ± 5.6	Normal	+18.6 ± 4.4	-73.4 ± 0.8	21
V_A	Saline	-79.6 ± 1.9	Saline + 1 MM Ba^{2+}	-88.6 ± 3.0	Saline + 1 mM Ba^{2+}	-77.8 ± 4.2	-97.4 ± 5.2	Saline	+20.7 ± 3.6	-79.4 ± 1.7	21
T.E.P.		+ 3.8 ± 1.9		- 5.3 ± 2.1		+ 4.8 ± 2.7	-		-	+ 4.0 ± 1.6	21

Discussion

Ouabain

Following the discovery by Skou (1957) of the involvement of Na^++K^+-ATPases in alkali metal transport across epithelia, Glynn (1957) noted that ouabain and other cardiac glycosides would inhibit this enzyme. Since, then, cardiac glycosides have been shown to inhibit ion transport across cell membranes and their specific effect on the Na^++K^+-ATPase is well established (Albers et al., 1968; Wallick et al., 1980; Kazazoglou et al., 1983).

In the present study, 1 mM ouabain caused an irreversible and gradual depolarization of both membrane potentials. As a consequence T.E.P. did not change during ouabain exposure. This result agrees with studies by Pilcher (1970) who reported that T.E.P. across the Malpighian tubules of Carausius was unaffected by 10^{-4} M ouabain. Also, T.E.P. was not affected by ouabain in Hyalophora midgut (Harvey and Zehran, 1971) and Calliphora salivary gland (Berridge et al., 1976). The lack of change of T.E.P., however, is in variance with the results of Anstee et al. (1980) and Fathpour et al. (1983), who found that the presence of 1 mM ouabain caused a gradual decrease in potential in the tubules of Locusta, with a new stable potential reached after approximately 30 mins. Ouabain has been shown to decrease T.E.P. in other tissues including cockroach intestine (O'Riordan, 1969; Datta, 1971), locust oocytes and rectum (Wollberg and Cocos, 1981; Irvine and Phillips, 1971) and midgut of larval Sarcophaga (Prusch, 1978).

The slow depolarization of V_B by ouabain in the tubules of Locusta was similar to that found in many tissues including the gallbladder epithelium of Necturus (Giraldez, 1984), proximal tubules from mouse kidney (Völkl et al., 1986) and the rectal gland tubules from Squalus (Greger and Schlatter, 1984) and is indicative of a

ouabain-sensitive Na^++K^+-ATPase. Berridge and Schlue (1978) reported that 10^{-4} M ouabain inhibited V_B in Calliphora salivary glands. Thus, in the present study, ouabain would stop the functioning of the Na^++K^+-ATPase probably causing $[Na^+]_i$ to rise as Na^+ would not be pumped out of the cell, resulting in a reduction in the Na^+ gradient. A rise in $[Na^+]_i$ following the inhibition of the Na^++K^+-ATPase is well documented (Cohen, 1983). Indeed, Giraldez (1984) showed that the intracellular activity of Na rose from 14.5 mM to 47.3 mM after 1 hr in the presence of 1 mM ouabain in Necturus gallbladder epithelium. Furthermore, an inhibition of the Na^++K^+-ATPase may result in a reduction in both $[K^+]_i$ and the K^+ gradient as this ion exits the cell across the permeable basal membrane, leading to a slow depolarization of V_B. Indeed, the presence of ouabain has been shown to cause significant decreases of cellular K^+ in frog skin (Cox and Helman, 1986). Similarly, a reduction of V_A may occur as a result of less K^+ being available to move across the apical membrane.

If an apical K^+ pump occurs in the Malpighian tubules of Locusta, it is unlikely to be affected by ouabain. It has been shown that the K^+ pump of lepidopteran midgut and dipteran labellum is ouabain-insensitive (Harvey et al., 1983; Wieczorek and Gnatzy, 1983; Wieczorek, 1986).

The effect of ouabain on V_B indicates that the Na^++K^+-ATPase does not contribute any significant electrogenic component to the membrane potential under 'normal' conditions in Locusta Malpighian tubules. If it did, the application of ouabain might be expected to produce an initial rapid depolarization of V_B to be followed by slow reduction. Such an electrogenic nature to the Na^++K^+-ATPase has been shown in Locusta oocytes (Wollberg and Cocos, 1981) and in Necturus gallbladder (Giraldez, 1984), the latter being indicated by

a fast depolarization of 4 mV which took place in less than 2 mins. However, in mouse neuroblastoma-gliomia hybrid cells, ouabain treatment indicates that the $Na^+ + K^+$ pump does not make a significant contribution to the resting potential (Lichtshtein et al., 1979).

As mentioned earlier, the $Na^+ + K^+$-ATPase seems to have an important role in ion and water transport by the Malpighian tubules of Locusta (Anstee et al., 1979) and this is supported by the fact that fluid secretion is inhibited after 20-30 mins exposure to 1 mM ouabain (Anstee and Bell, 1975; Anstee et al., 1979; Donkin, 1981). Other studies on the tubules of Locusta (Anstee et al., 1979; Mordue and Rafaeli-Bernstein, 1978), Drosophila (Weber-von Grotthuss et al., 1974) and pill millipede (Farquharson, 1974) have also shown Malpighian tubule function to be ouabain-sensitive. However, it must be noted, that fluid production by Malpighian tubules has also been reported to be ouabain-insensitive in several insect species (Calliphora, Berridge, 1968; Rhodnius, Maddrell, 1969; Carausius, Pilcher, 1970; Glossina, Gee, 1976; Zonocerus, Rafaeli-Bernstein and Mordue, 1978; Musca, Dalton and Windmill, 1980).

Apart from Malpighian tubules, ouabain sensitivity has been found in a wide range of insect tissues. These include inhibition of labial fluid secretion in Antherea (Kafatos, 1968), inhibition of Na^+ and K^+ fluxes across Periplaneta midgut (O'Riordan, 1969), Schistocerca midgut caecum (Dow, 1981) and insect nerve (Treherne, 1966), a reduction in water absorption from locust rectum (Goh and Phillips, 1978) and fluid absorption by Rhodnius midgut (Farmer et al., 1981) and a decrease in intracellular K^+ level in Calliphora salivary glands (Berridge and Schlue, 1978). Ouabain has also been shown to inhibit the ATPase activity in homogenates of several gut fractions of Glossina and Sarcophaga (Peacock, 1981; 1982), in homogenates of Malpighian tubules

and hindgut from Homorocoryphus (Peacock et al., 1976) and plasma membrane fractions from antenna of Periplaneta (Norris and Cary, 1981).

On the other hand, Jungreis (1977) and Jungreis and Vaughan (1977) failed to find any ouabain sensitivity in the midgut epithelium of three phytophyagous lepidoptera. A large short-circuit current found by Dow (cited in Harvey et al., 1983) demonstrated in isolated Manduca midgut was not affected by 1 mM ouabain. Also Wolfersberger (cited in Harvey et al., 1983) found no ouabain inhibition of ATPase activity in the same tissue. However, Anstee and Bowler (1979) have discussed the lack of agreement in the literature concerning ouabain-sensitivity of insect tissue and suggest that methodological differences account for much of the conflict in reported studies.

Overall, results from the present and numerous other studies show without doubt that a Na^++K^+-ATPase is present in insect Malpighian tubules and various other tissues. Indeed, Berridge (1980) has proposed that under resting conditions there is a custodial Na^++K^+-ATPase performing its normal function in the salivary glands of Calliphora, but during fluid secretion other processes take place which are ouabain insensitive. Similarly, Maddrell (personal communication) has stated that cellular gradients are maintained by a Na^++K^+-ATPase in the Malpighian tubules of Rhodnius.

Vanadate

In several studies on the Na^++K^+-ATPase from vertebrate tissues (Phillips et al., 1983; Nechay et al., 1986), vanadate has been shown to be a potent inhibitor of the enzyme (Cantley et al., 1977), but it is not a specific inhibitor as other classes of ATPase, including Ca^{2+}+Mg^{2+}-ATPase and K^++H^+-ATPase (O'Neal et al., 1979) are also inhibited by this ion. Vanadate has also been shown to activate

adenylate cyclase (Cuthbert et al., 1980). In physiological conditions, vanadium is expected to occur principally in two oxidation states, vanadyl (+4) and vanadate (+5). It is vanadium in the latter oxidation state which exerts a potent inhibitory effect on the Na^++K^+-ATPase, whereas the vanadyl ion has only a minimal impact on the activity of the enzyme (Cantley and Aisen, 1979).

Very few studies on the effect of vanadate on either the activity of the Na^++K^+-ATPase from insect tissues or on transport of ions across insect epithelial tissues have been published. Anstee and Bowler (1984) showed this inhibitor has about the same potency as ouabain on Na^++K^+-ATPase activity in Locusta Malpighian tubule microsomal preparations. Further, 1 mM vanadate has been shown in both the present and previous studies (Kalule-Sabiti, 1985) to inhibit fluid secretion even more than ouabain. However, orthovanadate (2.3×10^{-6} M) effected substantial stimulation of fluid secretion by the Malpighian tubules of Rhodnius (Evans and Mills, 1980). Evans and Mills (1980) explain this effect on the basis of vanadate activation of adenyl cyclase increasing intracellular c AMP levels (see later). In mammalian kidney, vanadate has been shown to inhibit fluid reabsorption (Edwards and Grantham, 1983). English and Cantley (1984) found that 2×10^{-4} M vanadate caused an inhibition of ^{86}Rb-K^+ uptake and increased the Na^+ level in cells from a Manduca embryonic cell line CHE, indicating an effect on the Na^++K^+-ATPase. These workers, however, found the vanadate effects were not induced by ouabain.

The addition of 1 mM vanadate in Normal saline caused a biphasic depolarization of both the apical and basal membrane potentials with a quicker 5 min depolarization followed by a slower potential reduction over the remaining exposure time. T.E.P. became less positive over

the first 5 min and was maintained at the new level for the remaining time in vanadate. Vanadate at concentrations of 4×10^{-4} M and 4×10^{-3} M depolarized the resting membrane potential of mouse skeletal muscle fibre (Dlouhà et al., 1981). In contrast to the present study, 10^{-4} M vanadate caused a rapid decrease in the lumen-negative T.E.P. of rabbit cortical collecting tubule (Edwards and Grantham, 1983).

As with ouabain treatment, the overall action of vanadate may be explained by an inhibition of the Na^++K^+-ATPase (leading to an increase in $[Na^+]_i$ and a decrease in $[K^+]_i$) preventing the maintenance of the membrane potentials. A lot of variation occurred in the vanadate-induced membrane depolarization and this may be related to the speed of conversion of vanadate to the less active vanadyl within the cells (Cantley and Aisen, 1979). This observation may also be related to the fact that K^+ enhances the inhibitory effect of vanadate on the Na^++K^+-ATPase (Higashino et al., 1983).

If an apical membrane K^+ pump occurs in the tubules of Locusta, it is unlikely to be affected by vanadate. Wieczorek et al. (1986) have shown that the K^+-ATPase activity (which may be a possible key component of the lepidopteran K^+pump) of goblet cell apical membranes in Manduca midgut is insensitive even to high concentrations of vanadate.

Amiloride

The diuretic amiloride has been extensively used to investigate sodium transport in epithelia, being a well known inhibitor of conductive Na^+ entry and Na^+-H^+ antiport activity (Benos, 1982). It has been shown that in a variety of epithelia low concentrations (10^{-5} to 10^{-4} M) of amiloride block Na entry (Bentley, 1968; Frizzell and Turnheim, 1978), while high concentrations (> 1 mM) inhibit

Na^+-H^+ exchange (Aronson, 1981). Aronson (1981) points out that at concentrations $\geq$ 1 mM, amiloride may have additional effects, such as to inhibit paracellular cation permeability and to inhibit Na^++K^+-ATPase. However, Soltoff and Mandel (1983) found that it took very high concentrations of amiloride (> 10 mM) to inhibit this enzyme.

In insects, amiloride has been shown to inhibit fluid secretion by the Malpighian tubules of Locusta (present study; Fathpour et al., 1979) and Glossina (Gee, 1976) and by the salivary glands of Calliphora (Berridge et al., 1976). This shows a similarity with vertebrate tissues, in which the drug inhibits fluid transport in Necturus gallbladder (Reuss, 1984), frog skin (Nagel, 1980) and rabbit cortical collecting duct (O'Neil and Sansom, 1984). In the present study, 1 mM amiloride did not affect V_B but slowly reduced the apical potential. As a consequence, T.E.P. slowly reduced in positivity towards zero. This is consistent with the findings of Fathpour et al. (1983) who found that the inclusion of amiloride in the bathing medium caused a fall in T.E.P. across Locusta tubules. In contrast, amiloride had no effect on T.E.P. across the tubules of Aedes (Williams and Beyenbach, 1984) and in shark rectal gland tubules (Greger and Schlatter, 1984). In the latter tissue, however, amiloride up to 1 mM was devoid of effect on V_B when present on either epithelial side (Greger and Schlatter, 1984). Application of luminal amiloride at concentrations of 10^{-5} M and 5×10^{-5} M has been shown to hyperpolarize V_A in frog skin (Nagel, 1980) and rabbit cortical collecting duct (O'Neil and Sansom, 1984) but had little effect on V_B in the latter case. In Necturus gallbladder, 10^{-5} M luminal amiloride had no effect on membrane potential, whereas 1 mM depolarized V_A (Reuss, 1984). Luminal amiloride did not alter T.E.P. in rabbit nephron (Greger, 1985) but caused a sudden decrease in both cell

membrane potentials in toad and frog urinary bladder (Davis and Finn, 1982).

An apical amiloride-sensitive Na^+ conductance has been proposed to explain the hyperpolarization of V_A in the Na^+ absorbing frog skin (Nagel, 1980) and rabbit cortical collecting duct (O'Neil and Sansom, 1984). If such a conductance occurs in the tubules of Locusta, Na^+ would probably enter the cell across the apical membrane down its concentration and with its electrical gradient (see Fig. 27). However, amiloride inhibition of such a conductance would result in a hyperpolarization of V_A and not the observed depolarization. Thus, it is unlikely that an apical Na^+ conductance occurs in the tubules of Locusta. Furthermore, the lack of effect of amiloride on V_B (and the relative impermeability of the basal membrane to Na^+, as shown by Na^+ free saline) also suggests that a significant conductance pathway for Na^+ on the basal membrane can probably be excluded from this tissue. The latter suggestion agrees with the results of Greger and Schlatter (1984) in shark rectal gland tubules.

The fact that amiloride greatly reduced fluid secretion and gradually decreased V_A suggests that perhaps amiloride affects a Na^+-H^+ exchanger in the tubules of Locusta. Indeed, this electroneutral transporter has been found in a variety of renal tissue (Aronson, 1981; 1983). In the salivary glands of Calliphora, Berridge et al. (1976) have concluded that amiloride may inhibit fluid secretion by interfering with the movement of cations across the basal membrane. Fathpour et al. (1983) have explained the action of amiloride in the tubules of Locusta on the basis of an inhibition of Na^+ entry into the cell across the basal membrane. These authors suggest that the reduction in $[Na^+]_i$ would affect the normal functioning of the Na^++K^+-ATPase thereby reducing the transport of K^+ across the tubule wall. Such

a reducing affect on the Na^++K^+-ATPase activity, however, would probably result in a depolarization of V_B which was not observed in the present study. It may be that although a Na^+-H^+ exchanger was inhibited by amiloride, Na entered the cell by other means to maintain the activity of the Na^++K^+-ATPase and thus the basal potential. Thus, although V_B was not affected, an increase in the activity of a basal membrane NaCl or NaKCl cotransporter to supply Na^+ to the Na^++K^+ pump may occur following inhibition of Na^+-H^+ exchange. This may lead to sufficient changes in $[Cl^-]_i$ to produce a depolarization of V_A as Cl^- exit increases. It may be that the latter mechanism and the high $[K^+]_o$-induced Cl^- exit discussed earlier are related. To support this speculation, there is no evidence for an effect of amiloride on NaCl or NaKCl cotransport systems (Davis and Finn, 1985). Finally, amiloride inhibition of a Na^+-H^+ exchange could also alter the pH of the Locusta Malpighian tubule cell, perhaps affecting an apical K^+ pump, reducing K^+ exit and depolarizing V_A. It is now known that the Na^+-H^+ antiporter plays an important role in the maintenance of cytosolic $[H^+]$ in a variety of cells and can thus control the pH of the cell cytoplasm (Scoble et al., 1986).

Barium

Since the early demonstration by Pacifico et al. (1969) that basal application of the alkali earth metal Ba^{2+} inhibits basolateral K^+ conductance in gastric mucosa, evidence for Ba^{2+}-sensitive permeability of both basolateral and apical membranes has been obtained in at least nine epithelia and the list continues to grow (Lewis et al., 1984). Indeed, Ba^{2+} is reported to be a specific blocker of K^+ permeability (in the concentration range 0.5-5 mM) in such transporting epithelia as frog skin (Nagel, 1980), tracheal mucosa

(Welsh, 1983), salivary gland acini (Petersen and Maruyama, 1984), cortical collecting tubules (Hunter et al., 1984) and locust rectum (Hanrahan et al., 1986). The action of Ba^{2+} is to inhibit K^+ transport through various plasma membrane K^+ channels (Latorre and Miller, 1983).

In the present study, 1 mM Ba^{2+} in Normal saline caused a reversible maintained hyperpolarization of both membrane potentials and a reduction in the positivity of T.E.P. to a slightly negative value. Associated with this action, 1 mM Ba^{2+} greatly decreased fluid secretion across Locusta tubules. In contrast to these results, Greger and Schlatter (1984) found that 3 mM Ba^{2+} depolarized V_B (but reduced T.E.P.) in shark rectal gland tubules. A large depolarization of V_B has also been found in various other vertebrate tissues upon application of Ba^{2+} (Cox and Helman, 1986; O'Neil and Sansom, 1984; Candia and Cook, 1986; Schlatter and Greger, 1985) and has been explained as an inhibition of K^+ transport through a basolateral K^+ conductance. In view of the established K^+ permeability of the basal membrane of the tubules of Locusta (see earlier) one must conclude that Ba^{2+} is acting somewhat differently in the present situation, i.e. Ba^{2+} may not be acting directly on the K^+ conductance. Support for this observation comes from the studies of Hanrahan et al. (1986), who found that Ba^{2+} treatment resulted in hyperpolarization of the basolateral membrane in locust rectum preparations. These workers state that although simple changes in basolateral conductance could account for the observed hyperpolarization in locust rectum, they could not exclude the contribution of other factors such as the Na^++K^+-ATPase. Moffett and Koch (1985) suggested that Ba^{2+} may block K^+ permeability in Manduca midgut by acting on the K^+ uptake step rather than on the apical pump. They add, however, that the effect of Ba^{2+} cannot easily be accounted for by simple competition with K^+ for basal membrane uptake sites.

The possibility that the Ba^{2+}-induced hyperpolarization of both membrane potentials was due to an unmasking or activation of the $Na^{+}+K^{+}$-ATPase was investigated by combining Ba^{2+} treatment with exposure to ouabain. Assuming $3\,Na^{+}$ moved out of the cell in exchange for $2\,K^{+}$ moved in across the basal membrane (Cantley, 1981) such an activation would hyperpolarize V_B. The excess K^{+} derived from the Ba^{2+} activation of the pump may exit the cell across the apical membrane, without Cl^{-} following as a counterion, increasing V_A. The results of such experiments in which ouabain was included in a Ba^{2+} saline are inconsistent with the proposal that the hyperpolarization of the membrane potentials was due to an electrogenic action of the $Na^{+}+K^{+}$-ATPase, although ouabain reduced the extent to which the increase in potential developed during Ba^{2+} treatment. The presence of Ba^{2+} in ouabain saline increased the decline of both membrane potentials suggesting that Ba^{2+} may be increasing the exit of K^{+} from the cell across the basal membrane. This, however, does not fit with the role of Ba^{2+} as a K^{+} channel blocker and provides evidence for an inexplicable action for this cation.

The fact that results show the Ba^{2+}-induced hyperpolarization of V_B and V_A to be abolished by Cl^{-} free saline (even in the presence of Ba^{2+}), suggests that Ba^{2+} may be affecting a change in anion transport across both membranes. This change is unlikely to involve a basal NaKCl transporter as this mechanism is thought to be electroneutral (Palfrey and Rao, 1983). Furthermore, Greger and Schlatter (1984) have concluded that Ba^{2+} does not impede the NaKCl carrier of shark rectal gland tubules. A Ba^{2+}-induced increase in membrane potential, however, may occur as a result of Ba^{2+} substituting for Ca^{2+}, as has been shown in other tissues (Mandel and Murphy, 1984), leading to an increase in an independent Cl^{-} entry across the basal membrane and perhaps an increase in the activity of a K^{+} pump across the apical membrane (see later).

Monensin

Monensin is a carboxylic acid ionophore that forms an uncharged complex with Na^+ and protons and catalyzes the electroneutral exchange of the two ions across membranes (Christensen, 1975). Thus, monensin produces an initial large H^+ efflux in exchange for Na^+ influx, greatly increasing $[Na^+]_i$ across a favourable Na gradient (Tartakoff, 1983). On this basis, monensin was used to increase Na^+ entry into the tubule cells of Locusta.

Very little information is available on the effects of monensin on insect tissue. The change from Normal saline to Normal saline containing 10^{-4} M monensin in the present study produced an initial depolarization (10 mV and 8 mV for V_B and V_A respectively) over 1-4 mins followed by a secondary hyperpolarization (23 mV and 20 mV for V_B and V_A respectively) over 2 to 7 mins in both membrane potentials. The hyperpolarized potentials were greater than the original potentials but were not maintained and decreased slowly; T.E.P. was not affected by monensin. Monensin at 10^{-5} M has no effect on either membrane potential and this may suggest a threshold concentration occurs for monensin in this system. Similarly, in studies on toad urinary baldder, Hardy and Ware (1985) found that monensin at concentrations $< 10^{-5}$ M had little or no effect. The ionophore has been shown to induce an increase in membrane potential in mouse neuroblastoma - rat gliomia hybrid and HeLa cells (Lichtshtein et al., 1979; Alonso and Carrasco, 1982). Indeed, in the former tissue, Lichtshtein et al., (1979) found that local application of monensin induced a 20-30 mV hyperpolarization.

The initial depolarization followed by a secondary hyperpolarization of both V_B and V_A, in the tubules of Locusta, during monensin treatment are difficult to explain in terms of ion fluxes. The initial decrease of V_B may be due to an inward movement of Na^+ increasing $[Na^+]_i$ as a

result of monensin increasing the permeability of the basal membrane to this ion. That an increase in $[Na^+]_i$ should follow monensin treatment has been found in various tissues including neuroblastoma-gliomia cells (Lichtshtein et al., 1979), mouse fibroblasts (Smith and Rozengurt, 1978) and midgut cells of Calliphora (Taylor 1984). Furthermore, it has been shown in mouse fibroblasts and neuroblastoma-gliomia hybrid cells that monensin greatly activates the Na^++K^+-ATPase by increasing internal Na^+ (Smith and Rozengurt, 1978; Lichtshtein et al., 1979). Hence, the secondary hyperpolarization of V_B in monensin may be the result of an electrogenic component of the Na^++K^+-ATPase developing as this enzyme pumps the excess Na^+ out of the cell in exchange for extracellular K^+. (The increased $[K^+]_i$ due to this activity may exit the cell across the apical membrane without Cl^- as a counterion, hyperpolarizing V_A.) However, although results showed that the introduction of Normal saline containing monensin and the Na^++K^+-ATPase inhibitor ouabain (after a period of ouabain treatment) caused a disappearance of the initial depolarization of both membrane potentials, an immediate small hyperpolarization of V_B and V_A occurred instead. Similarly, an immediate hyperpolarization (although enhanced) of both potentials occurred with the introduction of Na^+ free saline + monensin. Thus, the presence of a hyperpolarization in ouabain and Na^+ free saline containing monensin suggests that it was probably not due to the action of the Na^++K^+-ATPase. In contrast, Lichtshtein et al. (1979) found that monensin had no effect on the resting potential in the presence of ouabain and the monensin-induced increase in membrane potential required the presence of Na^+ in neuroblastoma-gliomia cells.

The monensin effect, observed with tubules of Locusta may be a consequence of changes in other ions such as Cl^- and Ca^{2+}. This is supported by the observations that: a) with Cl^- free saline

+ 10^{-4} M monensin the initial depolarization of both membrane potentials still occurred but the new potentials were maintained and not followed by the secondary hyperpolarization and b) with Ca^{2+} free saline + 10^{-4} M monensin no change occurred in either membrane potential. Thus, the monensin-induced decrease in both V_B and V_A may be due to the initial entry of Na^+, in a process controlled by Ca^{2+}. The secondary hyperpolarization may be due to a stimulation of a basal membrane Cl^- entry and an apical membrane K^+ pump, produced by a monensin-induced change in $[Ca^{2+}]_i$ (see later).

Finally, caution is needed regarding the application of an ionophore such as monensin (Reed, 1979). Primary cation movement by ionophores may result in secondary cation or anion fluxes (Pfeiffer et al., 1976) making it all but impossible to attribute an ionophore effect directly to transport of a specific cation.

Type A and Type B Response

The introduction of high K^+ saline immediately after treatment in Normal saline + 1 mM ouabain exposed Type B behaviour for both membranes in cells which originally exhibited the Type A response to high K^+ saline. In contrast, the introduction of high K^+ saline containing 1 mM vanadate or amiloride after pretreatment in the respective inhibitor did not reveal the Type B response in cells which originally exhibited Type A behaviour to high K^+ saline. Furthermore, vanadate pretreatment abolished the naturally occurring Type B response in high K^+ saline + vanadate. The fact that a Type B response was induced after a period of ouabain treatment showed that this unusual behaviour was not due to an electrogenic component of the Na^++K^+-ATPase "switching on". Indeed, ouabain would be expected to inhibit the response if it were the result of Na^++K^+-ATPase activity.

As was mentioned earlier the Type B response may be related to an increase in $[Na^+]_i$. Both K^+ free and ouabain treatments, which probably increase the cellular concentration of Na^+, induce this response to 128 mM $[K^+]_o$. However, high K^+ saline + vanadate, after vanadate treatment, which would also be expected to increase $[Na^+]_i$ did not result in a subsequent Type B response to 128 mM $[K^+]_o$. This may be related to the negativity of V_B and V_A immediately before the addition of high K^+ saline. Thus, vanadate treatment by producing a much greater depolarization of the membrane potentials than K^+ free or ouabain salines may result in cellular conditions different from those which are necessary for the Type B response to occur. This was supported by the fact that vanadate pretreatment abolished the Type B response in cells which originally demonstrated this behaviour. However, the observation that a novel introduction of vanadate in high K^+ saline resulted in: a) some Type B behaviour for both membranes in cells which exhibited the Type A response in high K^+ saline and b) did not alter the Type B response in cells which exhibited this naturally, suggests that overall an increase in $[Na^+]_i$ may be related to the production of the Type B response. Support for this comes from the observation that treatment in amiloride saline, which unlike K^+ free, ouabain and vanadate treatments, may lead to a reduction in $[Na^+]_i$ (as discussed earlier) did not induce the Type B response, which was also the case after Na^+ free treatment.

Barium induced a Type B response in high K^+ saline + Ba^{2+} for cells which originally exhibited the Type A response. Pretreatment with monensin also resulted in the inducement of Type B behaviour in cells which originally exhibited the Type A response. As for K^+ free and ouabain treatments, the postulated increase in $[Na^+]_i$ during exposure to monensin may be related to the production of the Type B

response. The Ba^{2+}-induced Type B behaviour is more difficult to explain. However, the latter response was probably not related to an inhibition of the K^+ conductance as Ba^{2+} does not seem to act on the K^+ permeability (see earlier). Thus, the complete disappearance of the marked depolarization of V_B induced by a K^+ concentration downward step in rectal gland tubules of shark due to the presence of Ba^{2+} interacting with the K^+ conductance of the basolateral membrane (Greger and Schlatter, 1984) does not seem to be applicable to the tubules of Locusta.

It was suggested earlier that the actions of both monensin and Ba^{2+} may be related to changes in $[Ca^{2+}]_i$. Hence, it may be that not only an increase in $[Na^+]_i$, but also an increase in $[Ca^{2+}]_i$ which leads to the inducement of the Type B response.

Section 4: Electrophysiological Studies on V_B, V_A and T.E.P. Using Furosemide-, Bumetanide-, Thiocyanate-, SITS- and HCO_3^--free + Acetazolamide-Containing Salines

Effect of 1 mM Furosemide

The effect of Normal saline containing 1 mM furosemide on V_B, V_A and T.E.P. can be seen in Table 28a and Fig. 33. It was found that V_B and V_A hyperpolarized 9.9 ± 0.6 mV and 8.1 ± 0.9 mV respectively, over the first min of furosemide treatment, to values which slightly increased over the next 19 mins. Thus, V_B and V_A increased to 13.5 ± 3.5 mV and 11.0 ± 1.0 mV respectively more negative than the original resting values after 20 mins exposure to furosemide saline. T.E.P. exhibited a small decrease in positivity, due to V_B hyperpolarizing more than V_A, during the first min of Normal saline + furosemide but, thereafter, exhibited little change in value.

Table 28a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM furosemide.

Table 28b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM furosemide to 128 mM K^+ saline + 1 mM furosemide in cells which exhibited the Type A response.

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

Table 28a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10	15	20
V_B	Normal	-72.3 ± 1.1	Normal	-82.2 ± 1.5	-83.0 ± 1.3	-83.8 ± 2.7	-83.5 ± 2.5	-85.0 ± 4.0
V_A	Saline	-84.8 ± 5.9	Saline + 1 mM	-92.9 ± 5.8	-93.5 ± 5.6	-92.0 ± 7.3	-92.7 ± 2.3	-92.5 ± 5.5
T.E.P.		+ 8.3 ± 4.7	Furosemide	+ 5.5 ± 4.2	+ 4.8 ± 4.1	+ 2.4 ± 5.3	+ 2.3 ± 5.8	+ 3.5 ± 0.5
n	-	9	-	9	9	5	4	3

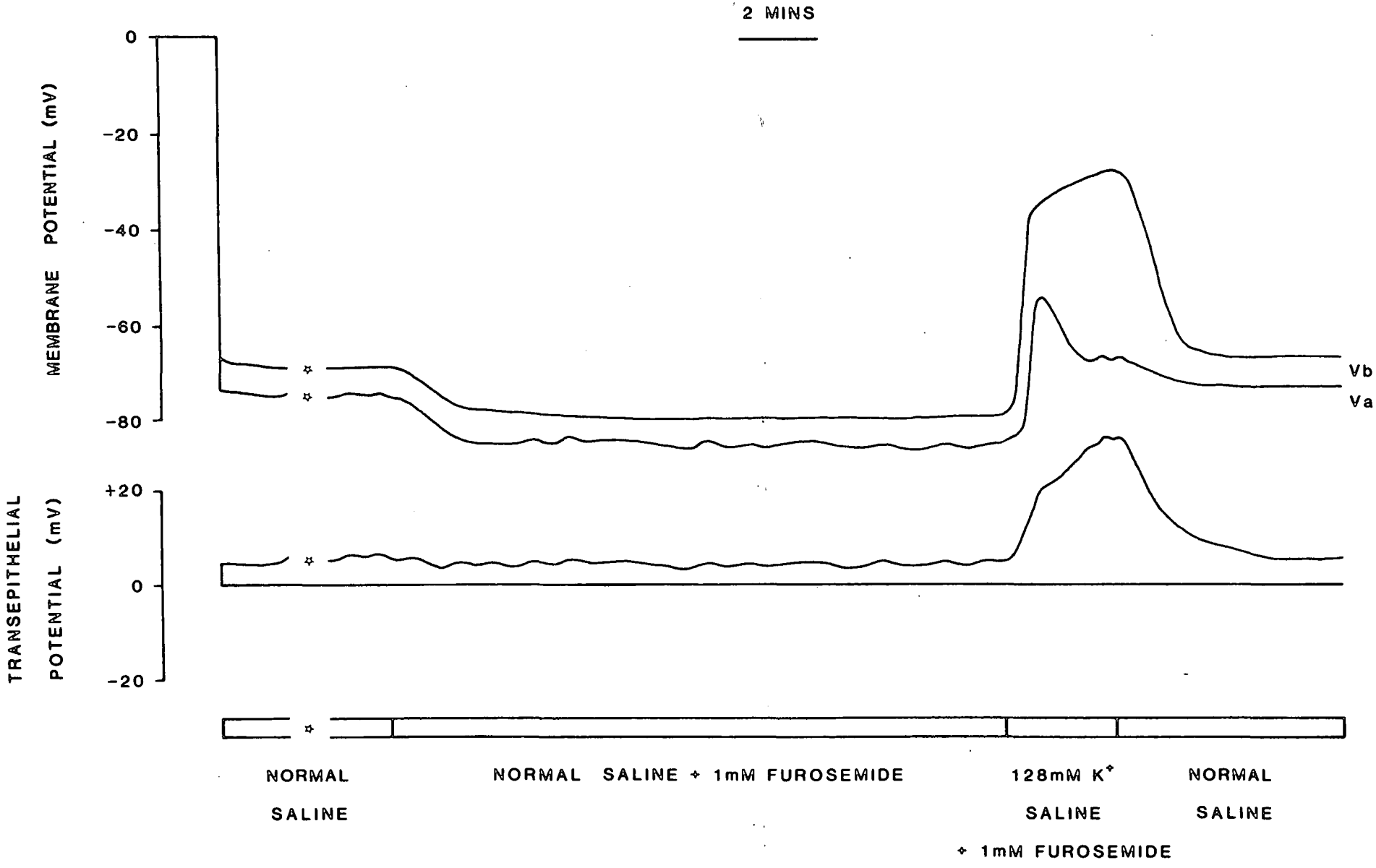
Table 28b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-72.9 ± 0.6	Normal	-83.5 ± 2.7	128mM K^+	-38.1 ± 5.2	-	Normal	-	-71.0 ± 2.6	8
V_A	Saline	-87.4 ± 5.9	Saline + 1 mM	-93.1 ± 5.1	Saline + 1 mM	-57.6 ± 5.6	-70.5 ± 6.0	Saline	-	-87.1 ± 6.1	8
T.E.P.		+ 7.6 ± 3.0	Furosemide	+ 5.3 ± 2.7	Furosemide	+19.5 ± 2.7	-		-	+ 8.0 ± 3.4	8

Fig. 33

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM furosemide before the subsequent addition of 128 mM K^+ saline + 1 mM furosemide, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -8 mV, V_A = -45 mV, T.E.P. = +36 mV).


2 MINS
0
-20
-40
-60
-80
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
NORMAL SALINE + 1mM FUROSEMIDE
128mM K+ SALINE + 1mM FUROSEMIDE
NORMAL SALINE

The amplitude of the oscillations of V_A (and T.E.P.) decreased significantly ($p < 0.001$) from 6.0 ± 0.3 mV in Normal saline to 3.8 ± 0.4 mV in Normal saline + 1 mM furosemide, with oscillations occurring in 67% of cells.

It was found that Normal saline containing 1 mM furosemide significantly reduced ($p < 0.001$) fluid secretion by the Malpighian tubules of Locusta by 77% (n=24) (see Appendix, Table 1). This result compares favourably with the significant 62% reduction in fluid secretion (n=30) found by Kalule-Sabiti (1985) in the same tissue.

Effect of 128 mM K^+ Saline + 1 mM Furosemide After Treatment in Normal Saline + 1 mM Furosemide

Table 28b and Fig. 32 show the effect of 128 mM K^+ saline + 1 mM furosemide on V_B, V_A and T.E.P. directly after a period of 5 to 20 mins treatment in Normal saline + 1 mM furosemide, in cells which exhibited the Type A response. It can be seen that pretreatment in furosemide saline induced Type B behaviour for both membrane potentials. Thus, V_B and V_A initially depolarized in high K^+ saline + furosemide to potentials (component c, Fig. 20) similar ($p = 0.1-0.2$ and $p = 0.5-0.6$ respectively) to those values found in the naturally occurring Type B response; T.E.P. increased in positivity to a value similar ($p = 0.8-0.9$) to that found in the Type B response. However, after depolarizing, V_B and V_A behaved differently. Hence, V_B slowly depolarized by a further 4.6 ± 0.7 mV over 2 to 3 mins, in 128 mM K^+ saline + furosemide, whilst V_A exhibited a repolarizing change (component d, Fig. 20) of 12.9 ± 1.7 mV over 1 to 2 mins. The resulting maintained V_A (component e, Fig. 20) was not significantly different ($p = 0.4-0.5$) from the equivalent potential found in the naturally occurring Type B response. This differing behaviour of V_B and V_A,

in high K^+ saline containing furosemide, resulted in T.E.P. increasing to a value significantly more positive ($p = 0.001-0.01$) than in the Type B response but similar ($p > 0.9$) to the potential found in the Type A response.

Overall, it seems that furosemide treatment induced a 'typical' Type B response for V_A but a 'reduced' variation of this response for V_B.

Effect of 10^{-5} M or 10^{-4} M Bumetanide

Tables 29a and 29b and Fig. 34 show the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline containing 10^{-5} M or 10^{-4} M bumetanide. Results shown that whilst V_B did not change even after 45 mins and 20 mins of 10^{-5} M and 10^{-4} M bumetanide treatment respectively, a small and gradual hyperpolarization of V_A occurred over the same exposure time. Thus, V_A hyperpolarized to values 4.6 ± ±.1 mV and 3.1 ± 1.7 mV more negative than the original resting potential after 20 mins of 10^{-5} M and 10^{-4} M bumetanide treatment respectively; this value had increased to 8.3 ± 3.2 mV more negative after 45 mins exposure to 10^{-5} M bumetanide. T.E.P. followed the hyperpolarization of V_A by exhibiting a slow and gradual increase in positivity during exposure to both concentrations of bumetanide.

Oscillations of V_A (and T.E.P.) occurred in 50% of cells exposed to Normal saline + 10^{-5} M bumetanide and had a mean amplitude of 4.0 ± 1.7 mV. Similarly, oscillations of V_A (and T.E.P.) occurred in 57% of cells treated with Normal saline + 10^{-4} M bumetanide and had a mean amplitude of 7.5 ± 1.3 mV. The frequency of amplitude of these oscillations, at both concentrations of bumetanide, were not significantly different ($p = 0.2-0.3$ for both treatments) from the values found in Normal saline.

Table 29a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10^{-5} M bumetanide.

Table 29b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10^{-4} M bumetanide.

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

Table 29a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 5	10	15	20	30	45
V_B	Normal Saline	-72.5 ± 2.3	Normal Saline + 10^{-5} M Bumetanide	-	-	-	-	-	-
V_A		-90.1 ± 4.5		-91.6 ± 4.5	-92.6 ± 4.8	-94.7 ± 5.5	-95.6 ± 5.5	-98.2 ± 6.7	-100.7 ± 13.1
T.E.P.		+11.5 ± 2.0		+13.1 ± 2.4	+14.0 ± 2.4	+14.9 ± 2.8	+15.3 ± 2.9	+17.5 ± 3.9	+ 17.7 ± 8.6
n	-	8	-	8	8	7	7	6	3

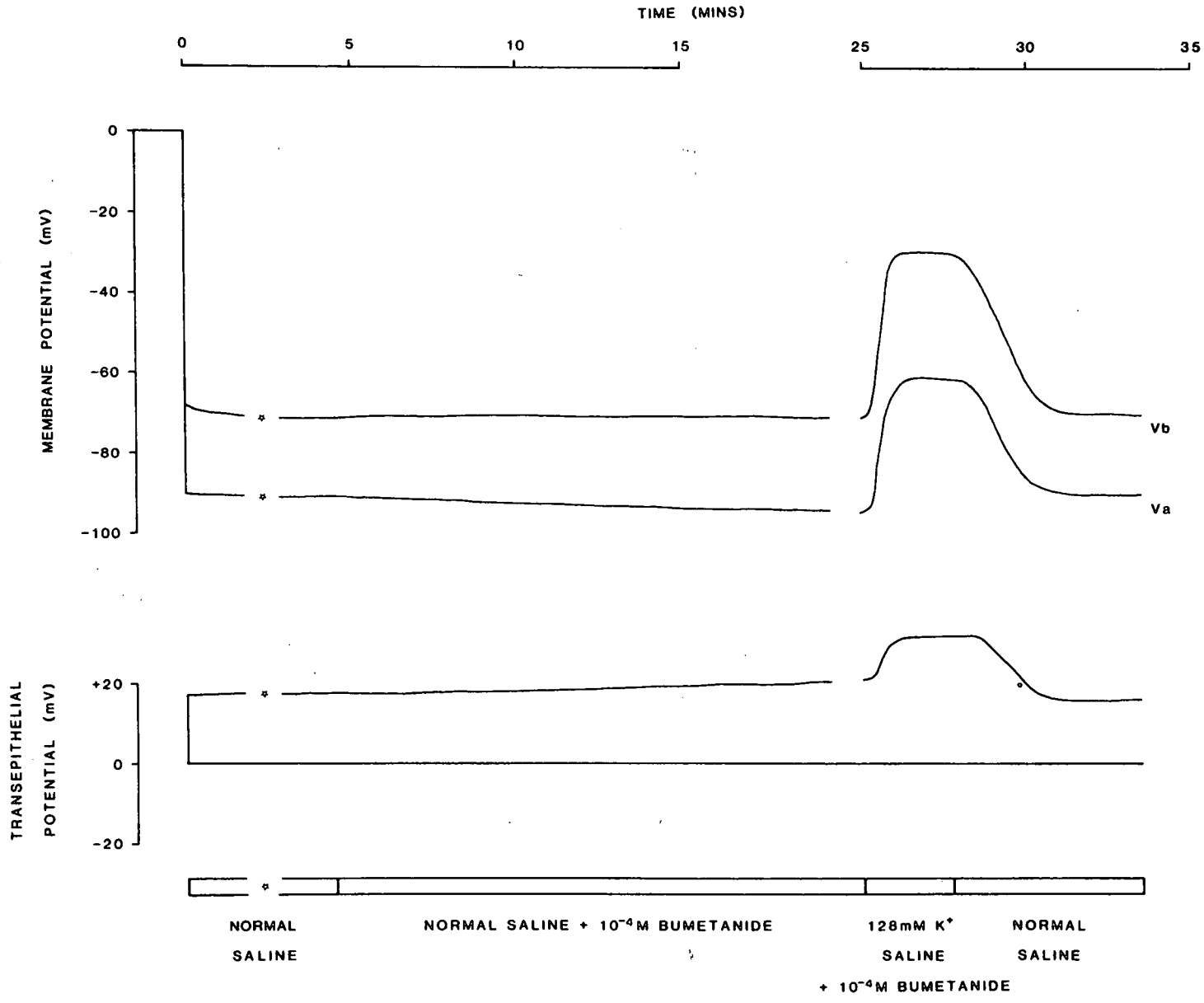
Table 29b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 5	10	15	20
V_B	Normal Saline	-76.9 ± 2.6	Normal Saline + 10^{-4} M Bumetanide	-	-	-	-
V_A		-91.4 ± 5.6		-92.0 ± 5.6	-92.9 ± 5.7	-93.7 ± 5.8	-94.6 ± 6.0
T.E.P.		+ 8.7 ± 2.2		+ 9.3 ± 1.9	+ 9.6 ± 1.7	+10.0 ± 1.5	+10.3 ± 1.4
n	-	7	-	7	7	7	7

Fig. 34

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10^{-4} M bumetanide before the subsequent addition of 128 mM K^+ saline + 10^{-4} M bumetanide, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -9 mV, V_A = -47 mV, T.E.P. = +33 mV).


TIME (MINS)
0
5
10
15
25
30
35
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
-100
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
NORMAL SALINE + 10^{-4}M BUMETANIDE
128mM K^+ SALINE + 10^{-4}M BUMETANIDE
NORMAL SALINE

It was found that the 16% inhibition of fluid secretion by the Malpighian tubules of Locusta in Normal saline containing 10^{-5} M bumetanide was not significant ($p = 0.7-0.8$; $n = 15$) (see Appendix, Table 1). In contrast, Normal saline containing 10^{-4} M bumetanide significantly reduced ($p < 0.001$) fluid secretion in this tissue by 59% ($n = 12$) (see Appendix, Table 1).

Effect of 128 mM K^+ Saline + 10^{-5} or 10^{-4} M Bumetanide After Treatment in Normal Saline + 10^{-5} M or 10^{-4} M Bumetanide

It was found that introducing 128 mM K^+ saline + 10^{-5} M bumetanide directly after a period of 20 to 45 mins treatment in Normal saline + 10^{-5} M bumetanide, in cells which exhibited the Type A response, did not alter this behaviour ($n = 8$). Thus, the depolarized values (component c, Fig. 20) of V_B and V_A were not significantly different ($p = 0.7-0.8$ and $p = 0.6-0.7$ respectively) from the equivalent values found during the Type A response.

In contrast, changing the perfusate to 128 mM K^+ saline + 10^{-4} M bumetanide after a period of 6 to 20 mins treatment in Normal saline + 10^{-4} M bumetanide resulted in the production of a 'reduced' Type B response for V_B and V_A, in cells which originally exhibited the Type A response. Results are shown in Table 30 and Fig. 34. It can be seen that the introduction of high K^+ saline + 10^{-4} M bumetanide produced depolarized potentials (component c, Fig. 20) that were not significantly different ($p = 0.1-0.2$ for V_B and $p = 0.3-0.4$ for V_A) from the equivalent values found in the naturally occurring Type B response. Although these depolarized potentials were maintained in most cases, about one third of cells studied exhibited a repolarizing change (component d, Fig. 20) of 12.0 ± 5.4 mV for V_B and 11.5 ± 4.1 mV for V_A to maintained repolarized potentials (component e, Fig. 20). The

Table 30

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 10^{-4} bumetanide to 128 mM K^+ saline + 10^{-4} M bumetanide in cells which exhibited the Type A response.

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

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-75.9 ± 1.0	Normal	-75.9 ± 1.0	128mM K^+	-27.0 ± 1.9	-	Normal	-	-75.1 ± 1.2	6
V_A	Saline	-92.6 ± 3.0	Saline + 10^{-4} M	-95.1 ± 2.8	Saline + 10^{-4} M	-58.0 ± 3.8	-	Saline	-	-91.0 ± 3.2	6
T.E.P.		+ 9.9 ± 0.5	Bumetanide	+13.0 ± 0.6	Bumetanide	+30.2 ± 2.1	-		-	+ 9.7 ± 2.0	6

initial depolarization (component b, Fig. 20) of V_A in 128 mM K^+ saline + 10^{-4} M bumetanide was 76% of that found for V_B, which compares favourably with the value (71%) found during the Type B response. However, T.E.P. in high K^+ saline + bumetanide was significantly more positive ($p < 0.001$) than the value found in naturally occurring Type B behaviour and not significantly different ($p = 0.1$-0.2) from that found in the Type A response. The latter observation may be related to the initially high value of T.E.P. which resulted from the hyperpolarization of V_A during Normal saline + bumetanide pretreatment.

Effect of 10 mM Sodium Thiocyanate

Table 31a and Fig. 35 show the effect of Normal saline + 10 mM sodium thiocyanate (NaSCN) on V_B, V_A and T.E.P. It was found that V_B and V_A hyperpolarized 5.9 ± 0.7 mV and 5.2 ± 1.5 mV respectively over the first min of SCN^- treatment to values which were maintained or slightly hyperpolarized over the next 19 mins. Thus V_B and V_A increased to 6.1 ± 0.8 mV and 9.8 ± 2.4 mV respectively more negative than the original resting values after 20 mins exposure to SCN^- saline. Due to both membrane potentials changing by similar amounts, T.E.P. changed little over 20 mins in Normal saline + SCN^-. Just under one third of cells exposed to SCN^- saline were returned to Normal saline; V_B and V_A in these cells depolarized, over 1 min, to the original resting potentials.

Oscillations of V_A (and T.E.P.) occurred in only 9% of cells in Normal saline + SCN^- and were 4.0 ± 1.0 mV in amplitude. Although the frequency of these oscillations was greatly reduced during SCN^- treatment, the mean amplitude was not significantly different ($p = 0.05$-0.1) from the value found in Normal saline.

It was found that Normal saline containing 10 mM SCN^- significantly

Table 31a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10 mM SCN^-.

Table 31b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 10 mM SCN^- to 128 mM K^+ saline + 10 mM SCN^- in cells which exhibited the Type A response.

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

Table 31a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10	15	20
V_B	Normal	-69.5 ± 2.3	Normal	-75.4 ± 2.3	-75.4 ± 2.3	-75.4 ± 2.3	-76.7 ± 2.6	-76.7 ± 2.6
V_A	Saline	-75.6 ± 4.4	Saline + 10 mM	-81.3 ± 4.4	-81.3 ± 4.4	-82.4 ± 4.4	-85.1 ± 3.9	-87.4 ± 4.0
T.E.P.		+ 2.0 ± 3.3	SCN^-	- 0.3 ± 3.4	- 0.3 ± 3.4	+ 0.6 ± 3.4	+ 2.6 ± 2.4	+ 3.8 ± 2.3
n	-	11	-	11	11	11	9	9

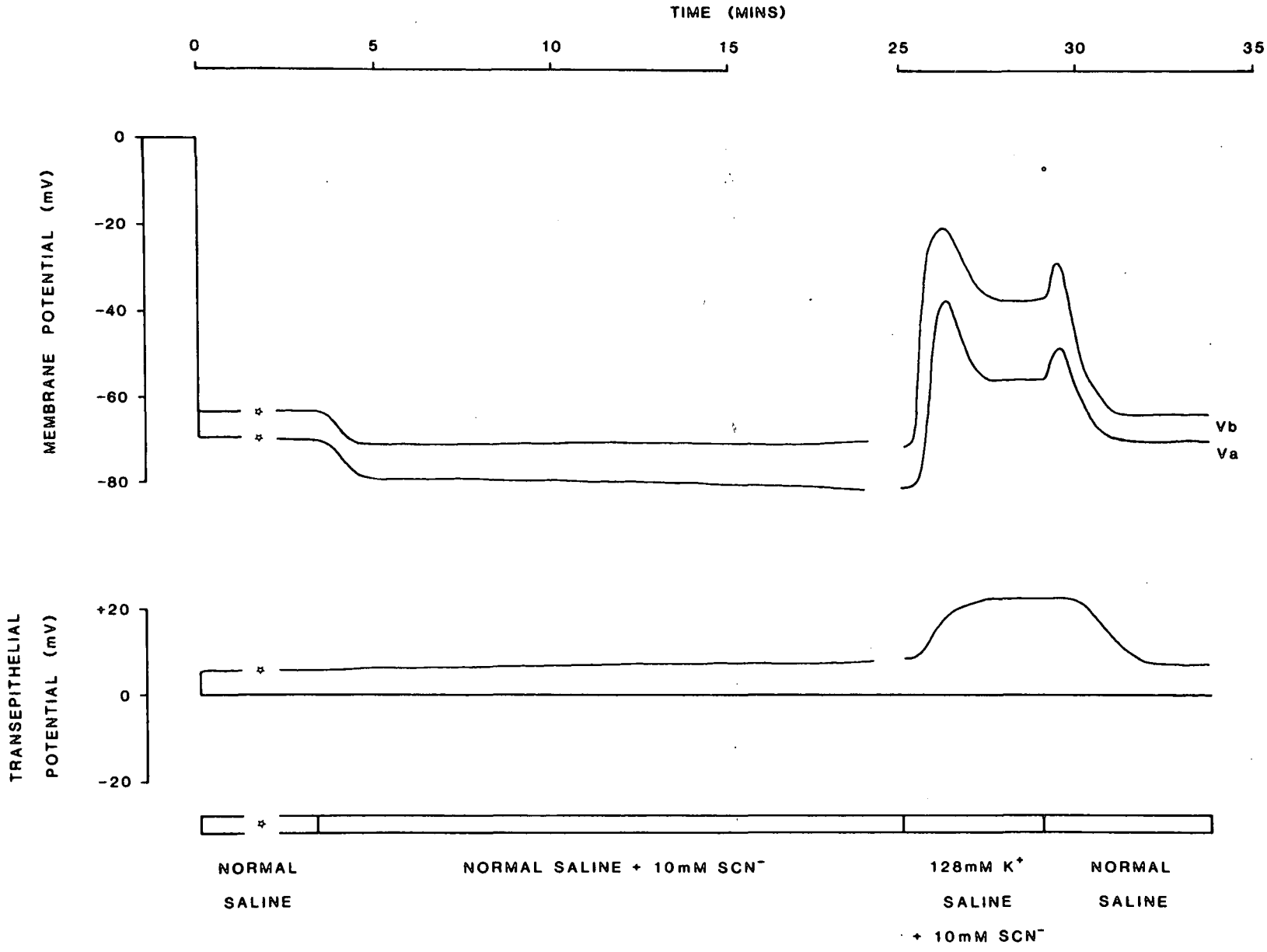
Table 31b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-69.8 ± 2.4	Normal	-74.5 ± 2.1	128mM K^+	-21.6 ± 2.6	-40.6 ± 6.8	Normal	+9.3 ± 3.2	-68.5 ± 3.1	10
V_A	Saline	-76.3 ± 3.8	Saline + 10 mM	-82.8 ± 2.4	Saline + 10 mM	-41.7 ± 4.2	-61.7 ± 5.2	Saline	+8.0 ± 2.9	-76.7 ± 3.4	10
T.E.P.		+ 1.4 ± 2.2	SCN^-	+ 2.9 ± 2.5	SCN^-	+24.3 ± 1.6	-		-	+ 2.0 ± 2.7	10

Fig. 35

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 10 mM SCN^- before the subsequent addition of 128 mM K^+ saline + 10 mM SCN^-, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -7 mV, V_A = -43 mV, T.E.P. = +34 mV).


TIME (MINS)
0
5
10
15
25
30
35
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
NORMAL SALINE + 10mM SCN⁻
128mM K⁺ SALINE + 10mM SCN⁻
NORMAL SALINE

reduced ($p < 0.001$) fluid secretion by the Malpighian tubules of Locusta by 57% (n=15) (see Appendix, Table 1). This result agrees favourably with the significant 62% reduction in fluid secretion (n=32) found by Kalule-Sabiti (1985) in the same tissue.

Effect of 128 mM K^+ Saline + 10 mM SCN^- After Treatment in Normal Saline + 10^{-4} M SCN–

Table 31b and Fig. 35 show the effect of introducing 128 mM K^+ saline + 10 mM SCN^- on V_B, V_A and T.E.P. after a period of 5 to 20 mins in Normal saline + 10^{-4} M SCN^-, in cells which exhibited the Type A response. It can be seen that SCN^- pretreatment induced a 'reduced' Type B response for V_B and V_A. Thus, the initial depolarization (component b, Fig. 20) of V_B and V_A in 128 mM K^+ saline + SCN^- produced depolarized potentials (component c, Fig. 20) that were significantly less negative ($p = 0.02-0.03$ and $p = 0.01-0.02$ respectively) than the equivalent values found in the naturally occurring Type B response. However, V_B and V_A repolarized (component d, Fig. 20) after depolarizing. Although the resulting potentials (component e, Fig. 20) were significantly less negative ($p = 0.02-0.03$ and $p = 0.01-0.02$ respectively) than the equivalent values found in the naturally occurring Type B response, these maintained V_B and V_A values were similar ($p = 0.1-0.2$ for both potentials) to the depolarized potentials (component c, Fig. 20) found during Type B behaviour. T.E.P. increased in positivity in 128 mM K^+ saline + SCN^- to a value which was similar ($p = 0.05-0.1$) to that found in the Type B response.

Effect of 1 mM SITS

The effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM SITS is shown in Table 32a

and Fig. 36. It can be seen that a gradual depolarization of both membrane potentials occurred in this new saline which increased in rate after about 10 mins. Thus, V_B and V_A declined by only 3.6 ± 1.2 mV and 3.7 ± 1.2 mV respectively after 10 mins, but by 17.1 ± 5.0 mV and 14.8 ± 4.5 mV respectively after 30 mins. As a consequence of the similar changes of V_B and V_A, little change occurred in T.E.P. throughout exposure to SITS saline. However, although the overall picture in many cells was a depolarization of both membrane potentials, SITS saline had no effect on either V_B or V_A, over 30 mins, in one third of cells studied.

Oscillations of V_A (and T.E.P.) occurred in 39% of cells exposed to Normal saline + 1 mM SITS and had a mean amplitude of 9.1 ± 2.0 mV. Although the frequency of these oscillations was reduced during SITS treatment, the amplitude was not significantly different ($p = 0.1-0.2$) from the value found in Normal saline.

Effect of 128 mM K^+ Saline + 1 mM SITS After Treatment in Normal Saline + 1 mM SITS

Table 32b and Fig. 36 show the effect of introducing 128 mM K^+ saline + 1 mM SITS on V_B, V_A and T.E.P. directly after a period of 10 to 30 mins in Normal saline + 1 mM SITS, in cells which exhibited the Type A response. It can be seen that SITS pretreatment resulted in the inducement of a 'reduced' Type B response for both V_B and V_A. Thus, the introduction of high K^+ saline + SITS produced depolarized potentials (component c, Fig. 20) that were not significantly different ($p = 0.3-0.4$ for V_B and $p > 0.9$ for V_A) from the equivalent values found in the naturally occurring Type B response. Although the majority of cells were maintained at these depolarized potentials, V_B and V_A in a small number of cases (ca. 30%) exhibited a repolarizing

Table 32a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM SITS.

Table 32b

Effect on V_B, V_A and T.E.P. on changing the perfusate from Normal saline + 1 mM SITS to 128 mM K^+ saline + 1 mM SITS, in cells which exhibited the Type A response.

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

Table 32a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 5	10	15	20	30
V_B	Normal Saline	-71.6 ± 1.2	Normal Saline + 1 mM SITS	-70.7 ± 1.3	-68.3 ± 1.7	-64.8 ± 2.8	-58.9 ± 3.6	-53.6 ± 4.7
V_A		-80.5 ± 3.6		-78.5 ± 3.6	-76.5 ± 4.3	-71.8 ± 5.8	-66.2 ± 7.0	-64.1 ± 9.2
T.E.P.		+ 5.7 ± 3.4		+ 5.1 ± 3.3	+ 5.9 ± 3.7	+ 5.7 ± 4.8	+ 5.9 ± 5.9	+ 5.8 ± 7.9
n	-	23	-	23	22	15	12	8

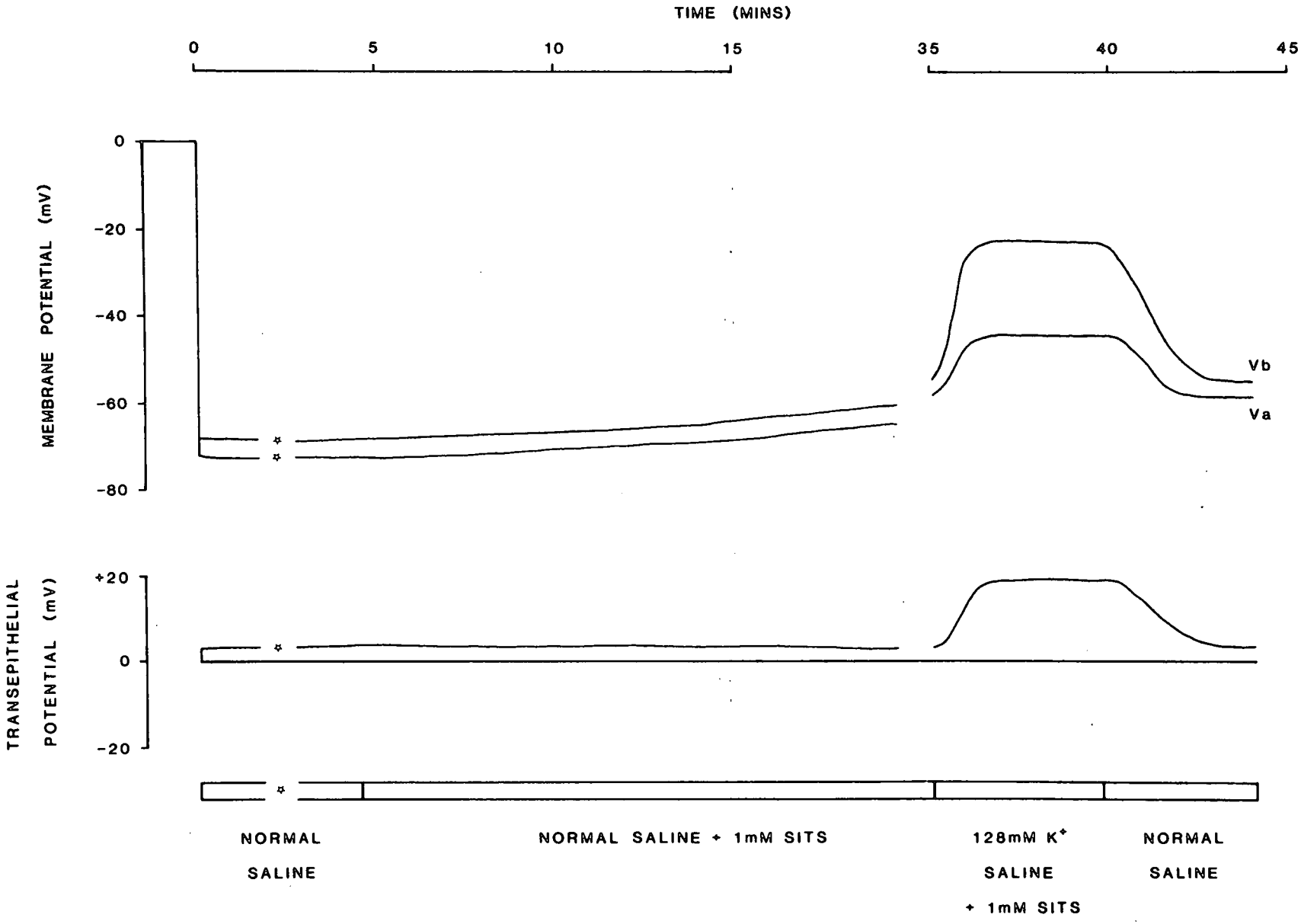
Table 32b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal Saline	-74.3 ± 1.1	Normal Saline + 1 mM SITS	-65.4 ± 4.0	Normal Saline + 1 mM SITS	-27.8 ± 3.0	-	Normal Saline	-	-65.4 ± 3.9	14
V_A		-81.7 ± 3.2		-77.3 ± 6.1		-53.6 ± 6.3	-		-	-78.4 ± 5.7	14
T.E.P.		+ 5.2 ± 3.4		+ 8.4 ± 4.5		+21.9 ± 6.9	-		-	+ 9.2 ± 5.3	14

Fig. 36

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM SITS before the subsequent addition of 128 mM K^+ saline + 1 mM SITS, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -10 mV, V_A = -45 mV, T.E.P. = +30 mV).


TIME (MINS)
0
5
10
15
35
40
45
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
NORMAL SALINE + 1mM SITS
128mM K+ SALINE + 1mM SITS
NORMAL SALINE

change (component d, Fig. 20) of 14.3 ± 5.1 mV and 11.8 ± 1.7 mV respectively to maintained repolarized potentials (component e, Fig. 20). T.E.P. increased in positivity in 128 mM K^+ saline + SITS to produce a potential similar ($p = 0.7-0.8$) to that found in the Type B response. On re-introduction of Normal saline, V_B and V_A only reached values, after 2-3 mins, similar to those found before the addition of high K^+ saline + SITS and did not recover further. This indicates some irreversibility in the effect of SITS.

The effect of SITS on V_B, V_A and T.E.P. in high K^+ saline was examined further, in cells which exhibited the naturally occurring Type B response, by changing the perfusate to 128 mM K^+ saline + SITS after a period of 5 to 20 mins in Normal saline + SITS. Results are shown in Table 33a. It was found that SITS pretreatment altered the naturally occurring Type B behaviour of both membrane potentials in these cells. Thus, on introduction of high K^+ saline containing SITS, both V_B and V_A depolarized to maintained potentials (component c, Fig. 20) that were not significantly different ($p = 0.7-0.8$) for V_B, but were significantly less negative ($p = 0.02-0.03$) for V_A, compared with the repolarized potentials (component e, Fig. 20) found during the Type B response. Indeed, the depolarized V_A (component c, Fig. 20) was not significantly different from the equivalent value found during this behaviour. These results show that SITS pretreatment produced Type B values for V_B and V_A without a repolarizing change (component d, Fig. 20). Furthermore, this SITS-induced alteration of the Type B response affected V_A more than V_B. Indeed, the initial depolarization of V_A (component b, Fig. 20) in high K^+ saline + SITS was 106% of that for V_B and this was much higher than the value (71%) found in the Type B response. This produced little change in T.E.P., which was only -1.2 ± 7.7 mV and significantly different ($p < 0.001$) from

Table 33a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM SITS to 128 mM K^+ saline + 1 mM SITS in cells which exhibited the Type B response.

Table 33b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to 128 mM K^+ saline + 1 mM SITS in cells which exhibited the Type A response (i.e. a novel addition of SITS in high K^+ saline).

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

Table 33a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-69.0 ± 2.6	Normal	-48.8 ± 2.6	128 mM K^+	-32.2 ± 4.7	-	Normal	-	-42.4 ± 2.7	5
V_A	Saline	-79.2 ± 3.8	Saline + 1 mM	-50.4 ± 7.1	Saline + 1 mM	-32.8 ± 6.5	-	Saline	-	-42.4 ± 7.5	5
T.E.P.		+ 5.5 ± 3.6	SITS	- 2.0 ± 6.5	SITS	- 1.2 ± 7.7	-		-	- 4.0 ± 6.6	5

Table 33b

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-67.8 1.5	128 mM K^+	- 8.2 ± 2.2	-23.4 ± 2.3	Normal	-	-66.8 ± 1.2	5
V_A	Saline	-74.6 ± 4.1	Saline + 1 mM	-44.8 ± 4.5	-51.2 ± 4.7	Saline	-	-69.6 ± 2.7	5
T.E.P.		+ 2.8 ± 3.9	SITS	+35.6 ± 3.3	+24.8 ± 4.0		-	+ 0.6 ± 2.9	5

the +20.0 ± 1.7 mV value found in the original Type B response.

Results in Table 33b and Fig. 37 show that the introduction of high K^+ saline + SITS to cells which exhibited the Type A response and had no previous exposure to this inhibitor, induced Type B behaviour for V_B and V_A. It can be seen, however, that this induced Type B response was slow to develop. Thus, the introduction of SITS in 128 mM K^+ saline produced depolarized potentials (component c, Fig. 20) that were not significantly different ($p = 0.4-0.5$ for both V_B and V_A) from those found during the Type A response. After depolarizing, V_B and V_A exhibited repolarizing changes (component d, Fig. 20) of 15.2 ± 3.6 mV and 6.4 ± 1.9 mV respectively over 5 to 10 mins (cf. 1 to 2 mins in the Type B response). The extent to which this gradual hyperpolarization developed was not followed. Following the changes of V_B and V_A, T.E.P. increased in positivity, in high K^+ saline + SITS, to a value similar ($p = 0.5-0.6$) to that found in the Type A response, then decreased 10.8 ± 3.1 mV over the next 5 to 10 mins, as V_B hyperpolarized more than V_A, to a value similar ($p = 0.4-0.5$) to that found during Type B behaviour.

Finally, it was found that the presence of SITS in 128 mM K^+ saline did not qualitatively or quantitatively alter the Type B behaviour of either membrane potential when compared with results in the absence of SITS (n=4). Thus, the novel introduction of 1 mM SITS in high K^+ saline, in cells which originally exhibited Type B behaviour, had no significant effect ($p = 0.8-0.9$) on the Type B response values of V_B, V_A and T.E.P.

Effect of HCO_3^--free Saline + 1 mM Acetazolamide

Table 34 shows the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to HCO_3^- free saline + 1 mM acetazolamide

Fig. 37

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to 128 mM K^+ saline + 1 mM SITS, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -5 mV, V_A = -42 mV, T.E.P. = +31 mV).

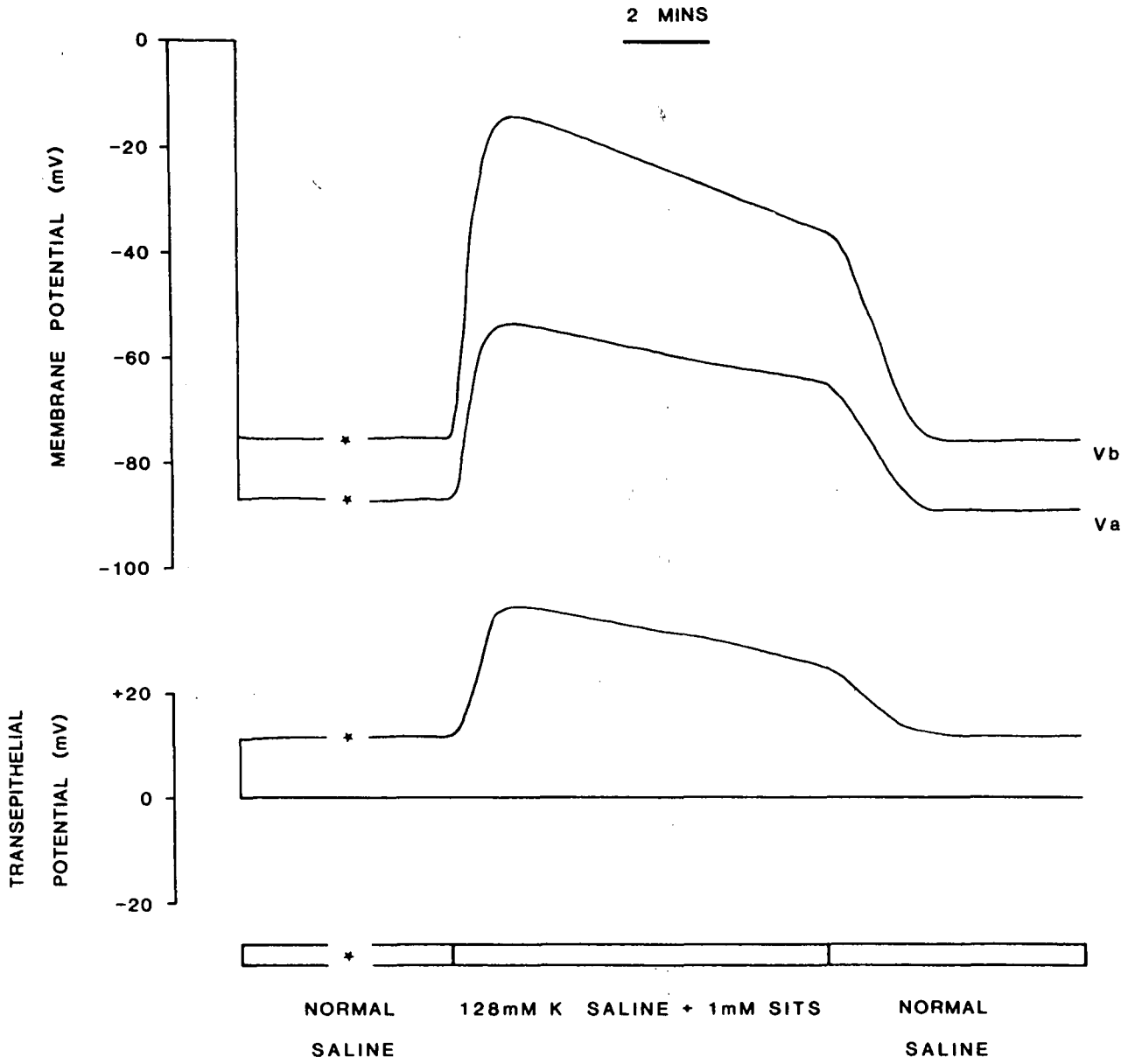

2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
128mM K SALINE + 1mM SITS
NORMAL SALINE

Table 34

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to HCO_3^- free saline + 1 mM acetazolamide.

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

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 5	10	15	20	30
V_B	Normal	-71.0 ± 0.8	HCO_3^- free	-	-	-	-	-
V_A	Saline	-86.1 ± 2	Saline + 1 mM	-87.3 ± 2.7	-88.6 ± 2.5	-89.8 ± 2.9	-88.5 ± 4.5	-88.5 ± 4.5
T.E.P.		+ 6.5 ± 3.2	Acetazolamide	+ 7.1 ± 3.1	+ 7.8 ± 2.9	+ 8.8 ± 2.7	+ 8.5 ± 2.5	+ 8.5 ± 2.5
n	-	8	-	8	8	6	2	2

(Diamox). ($NaHCO_3$ was omitted from Normal saline and the pH was corrected to pH 7.2 with NaOH.) It can be seen that whilst V_B did not change even after 30 mins exposure to HCO_3^- free saline containing acetazolamide, V_A hyperpolarized gradually over the first 10-15 mins in this modified solution before levelling after about 15 mins. The new maintained V_A was 3.0 ± 1.3 mV more negative than the original resting value. T.E.P. followed the change of V_A, becoming more positive in HCO_3^- free saline + acetazolamide.

The amplitude of the oscillations of V_A (and T.E.P.) decreased significantly ($p < 0.001$) from 6.0 ± 0.3 mV in Normal saline to 2.7 ± 0.7 mV in HCO_3^- free saline containing acetazolamide, with oscillations occurring in 38% of cells.

Effect of 128 mM K^+,HCO_3^- free Saline + 1 mM Acetazolamide After Treatment in HCO_3^- free Saline + 1 mM Acetazolamide

It was found that introducing 128 mM K^+,HCO_3^- free saline + 1 mM acetazolamide directly after a period of 10 to 30 mins in HCO_3^- free saline + 1 mM acetazolamide, in cells which exhibited the Type A response, did not alter this behaviour for either V_B or V_A (n=7). Thus, the depolarized values (component c, Fig. 20) of V_B and V_A (-10.9 ± 2.7 mV and -39.4 ± 2.1 mV respectively) in high K^+,HCO_3^- free saline + acetazolamide were not significantly different ($p = 0.5$-0.6 for both potentials) from the equivalent values found during the Type A response. However, although not significantly different, these depolarized values arose as a result of V_A initially depolarizing 85% of that for V_B (cf. 65% found during the Type A response) and resulted in a T.E.P. value (+18.3 ± 4.6 mV), in 128 mM K^+,HCO_3^- free saline + acetazolamide, that was significantly less positive ($p < 0.001$) than the T.E.P. value found in the Type A response.

Discussion

Furosemide and Bumetanide

It was mentioned earlier that Na^+ and Cl^- entry across the basal membrane of the Malpighian tubules of Locusta may be linked in the form of a NaKCl cotransporter as described for the tubules of Rhodnius (O'Donnell and Maddrell, 1984). This was examined using furosemide and bumetanide. These agents are examples of so-called "high-ceiling" diuretics or loop diuretics and are thought to block sodium chloride co-transport by inhibition of NaCl and NaKCl cotransport in various epithelia (Frizzell et al., 1979; Hanrahan and Phillips, 1983; 1984; Palfrey and Rao, 1983).

Results showed that 1 mM furosemide and 10^{-4} M bumetanide caused a significant reduction in fluid secretion in the tubules of Locusta, whilst 10^{-5} M bumetanide did not have a significant effect. Similarly, O'Donnell and Maddrell (1984) found that fluid secretion was severely reduced in the tubules of Rhodnius by 10^{-4} M furosemide and 4×10^{-6} - 10^{-5} M bumetanide. The introduction of 1 mM furosemide in the present study caused a hyperpolarization of both V_B and V_A (and a reduction in the positivity of T.E.P.) and this was followed by a gradual but very slow continual increase in both potentials (but maintained T.E.P.). The introduction of Normal saline containing 10^{-5} or 10^{-4} M bumetanide resulted in no change in V_B but V_A hyperpolarized in a slow gradual manner (with a slow increase in T.E.P.). In the tubules of Rhodnius, 10^{-4} M furosemide treatment (in saline containing the secretagogue 5-HT) resulted in a large decrease in T.E.P. which was largely due to a hyperpolarization of the apical membrane, although a small depolarization of V_B was also observed (O'Donnell and Maddrell, 1984). In contrast, 1 mM furosemide had no effect on T.E.P. in tubules from Aedes (Williams and Beyenbach, 1984).

Among vertebrate tissue, furosemide leads to a hyperpolarization of V_B in mammalian nephron (Greger and Schlatter, 1983) and shark rectal gland tubules (Greger and Schlatter, 1984) and an inhibition of fluid secretion and a hyperpolarization of V_B in shark renal proximal tubules (Beyenbach and Frömter, 1985). Furosemide (1 mM) also inhibited fluid secretion in the canine tracheal epithelium (Welsh, 1983). These results have all been related to an inhibition of NaCl cotransport by furosemide. Patarca et al. (1983) found that furosemide and bumetanide both resulted in a hyperpolarization of V_B and V_A (especially the latter) in frog cornea with a gradual recovery of V_B back towards the original value.

Results from the present study have shown the basal membrane of the tubules of Locusta to be largely K^+ selective with a low permeability to Na^+ and Cl^-. If Na^+ and Cl^- enter the cells by electroneutral NaCl of NaKCl cotransport, it is difficult to explain why V_B should hyperpolarize during furosemide treatment but not change during bumetanide treatment, if such systems are inhibited by both these loop diuretics. Indeed, if NaKCl cotransport was the sole mechanism for Cl^- entry, treatment with furosemide and bumetanide would, in theory, depolarize V_B due to a reduction in anion entry. However, such an inhibition of Cl^- entry may be balanced by a reduction in Na^+ and/or K^+ entry. Overall, it may be that these loop diuretics are not affecting Na•KCl cotransport in the tubules of Locusta. Williams and Beyenbach (1984) have reported that the coupled cation-Cl secretion found during stimulation with head extract in the tubules of Aedes was not a furosemide-sensitive system. Furthermore, Greger (1985) has pointed out that an effect on ion transport by furosemide and bumetanide does not permit the conclusion that the NaKCl carrier is present in the preparation under study. Loop diuretics have been

shown to inhibit carbonic anhydrase, the Na^+K^+ATPase, OH^-/Cl^- exchange, basolateral anion carrier systems and electroneutral KCl exit at concentrations $\geq 10^{-4}$ M (Greger, 1985). Thus, the membrane changes and the inhibition of fluid secretion observed during furosemide and bumetanide treatments in the present study may not be related to a NaKCl cotransporter but to other non-specific effects.

An inhibition of Cl entry into the cell by furosemide and bumetanide may lead to the observed hyperpolarization of V_A as Cl was not available to follow K^+ extruded across the apical surface by a K^+ pump. O'Donnell and Maddrell (1984) have suggested that the V_A hyperpolarization observed in furosemide saline in the tubules of Rhodnius was evidence for co-transport of Na^+ and Cl^- as this agent inhibited Cl^- flux. However, Patarca et al. (1983) have postulated that the loop diuretics induced hyperpolarization of V_A in the frog cornea may be due to these agents decreasing the Cl^- permeability of the apical membrane and not by affecting Na^+-coupled Cl^- transport. Such a decrease in permeability in the tubules of Locusta would also lead to an increase in V_A due to the inability of Cl^- to follow the actively extruded K^+.

Sodium Thiocyanate

Sodium thiocyanate has been shown to inhibit anion transport across a variety of epithelial systems. As well as inhibiting Na-dependent active Cl^- transport (Epstein et al., 1973; Zadunaisky et al., 1971), an inhibitory effect on anion-stimulated ATPase activity by SCN^- has been found in various tissues (Gerenscer and Lee, 1983). In the present study, 10 mM NaSCN resulted in a reversible hyperpolarization of both membrane potentials. The new potentials were maintained or slightly increased during SCN^- exposure and T.E.P. did not alter. In addition to these effects, fluid secretion was greatly reduced

in 10 mM SCN^- (present study; Fathpour, 1979; Kalule-Sabiti, 1985). In contrast, Dalton and Windmill (1980) found that 10 mM SCN^- stimulated tubule fluid secretion across the tubules of Musca.

The maintained hyperpolarization of both membrane potentials found with SCN^- was similar to that found with furosemide. Thus, the hyperpolarization of V_A may be due to a reduced availability of Cl^- (due to a SCN^- inhibited Cl^- entry) to follow the active extrusion of K^+ across the apical membrane. However, it is difficult to explain how the inhibition of Cl^- entry across the basal membrane by SCN^- could be lead to the observed hyperpolarization of V_B on evidence currently available.

SITS

The stilbene derivation, SITS, blocks epithelial anion transport in a variety of tissue (White, 1980; Ehrenspeck and Brodsky, 1976; Rothstein et al., 1976). The introduction of 1 mM SITS in Normal saline resulted in a gradual depolarization of both V_B and V_A and little change in T.E.P. It must be noted, however, that SITS had no effect on either V_B or V_A in one third of tubules examined. In contrast to the tubules of Locusta, a hyperpolarization of V_A in Amphiuma intestinal cells occurred in 1 mM SITS (White, 1986). Furthermore, Greger and Schlatter (1984) found that 1 mM SITS had an inhibitory effect on T.E.P. in shark rectal gland tubules.

As SITS affects anion transport but does not inhibit NaCl or Na K Cl cotransport (Warnock et al., 1983), the gradual depolarization of both V_B and V_A in the present study during SITS treatment may be due to this stilbene derivation directly preventing Cl^- entry

into the cell (decreasing V_B). However, it is difficult to speculate why such a SITS-induced reduction in $[Cl^-]_i$ should decrease V_A. Although stilbene derivatives have been used as specific inhibitors of anion exchange, the use of these inhibitors in epithelial tissues is complicated by the possibility of effects on alternative mechanisms (Smith et al., 1986). Indeed, SITS has been shown to inhibit the Na^++K^+-ATPase in turtle bladder (Ehrenspeck and Brodsky, 1976). This inhibitory affect may be responsible for the decrease of both V_B and V_A through a decline of the cellular gradient as described earlier. Such an inhibition of the Na^++K^+-ATPase may also decrease Cl^- entry if Na^+ (and K^+) Cl cotransport occurs in the tubules of Locusta as the Na^+ gradient across the cell, which operates this carrier, is established by the Na^++K^+-ATPase (Phillips and Lewis, 1983).

Overall it is difficult to interpret whether SITS was having an effect on anion transport in Locusta tubules. It is of interest that Greger and Schlatter (1984) had no ready explanation for the inhibitory effect of SITS on T.E.P. in shark rectal gland tubules which possess a basolateral NaKCl cotransporter. However, SITS had no effect on Cl secretion by the canine tracheal epithelium which is thought to utilize NaCl cotranport (Welsh, 1983). Furthermore, if Cl^- enters the tubule cells of Locusta by a means other than NaKCl cotransport, such as Cl^--HCO_3^- exchange (see later), then SITS need not affect this either. Indeed, Strange and Phillips (1984) have shown that 0.5 mM SITS did not have an affect on CO_2 (and thus HCO_3^-) flux in the rectal glands of Aedes in which a HCO_3^--Cl^- exchange has been demonstrated.

HCO_3^- Substitution + Acetazolamide

It is possible that Cl^- transport into the tubule cells of

Locusta is associated with HCO_3^-. As mentioned in the introduction, electrically silent Cl^--HCO_3^- exchange has been shown in a variety of epithelial tissues (Gerencser and Lee, 1983). The possibility of such an exchange was examined in the present study using HCO_3^--free saline in the presence of acetazolamide (Diamox). Acetazolamide acts by inhibiting the enzyme carbonic anhydrase (Rector et al., 1965) which catalyses the reversible dehydration of carbonic acid (Gay, 1982). The latter, in solution, dissociates into H^+ and HCO_3^-. Acetazolamide will thus prevent or reduce the production of intracellular HCO_3^-. The introduction of HCO_3^--free saline + 1 mM acetazolamide produced no change in V_B but a hyperpolarization of V_A (and increase in the positivity of T.E.P.) over 10-15 mins to a new maintained potential. Similarly, 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 (Strange and Phillips, 1984).

The fact that HCO_3^--free saline containing acetazolamide produced a similar result to Cl^- free saline in the present study, suggests that Cl^- and HCO_3^- transport may be related. If the former treatment produced a reduction in $[Cl^-]$ through an inhibition of a Cl^--HCO_3^- exchange, then V_A may hyperpolarize as in Cl^- free saline, due to the reduced availability of Cl^- to act as a counterion for an apical K^+ pump. In contrast to the hyperpolarization of V_B following treatment with furosemide and SCN^- saline acetazolamide, which has been shown to be a good Cl^- transport inhibitor (White, 1980), did not effect any change in V_B. This observation, however, would be expected if HCO_3^--free saline containing acetazolamide inhibited electroneutral Cl^--HCO_3^- exchange in the tubules of Locusta.

Although controversial, various recent studies have provided evidence which suggests a possible involvement of an anion Cl^- + HCO_3^--stimulated ATPase in various tissues which is involved in the net movement of Cl up its electrochemical gradient across plasma membranes (see Introduction; Gerencser and Lee, 1983). Indeed, Anstee and Fathpour (1979; 1981) have reported the presence of a Mg^{2+}-dependent HCO_3^--stimulated ATPase in microsomal preparations from Malpighian tubules of Locusta. Furosemide has been shown to be an inhibitor of Cl^-+HCO_3^--ATPase activity in various tissues, including insect rectum (Gassner and Komnick, 1982) and SCN^- inhibited anion-stimulated ATPase activity in microsomal preparations of Locusta tubules (Anstee and Fathpour, 1979). If a Cl^-+HCO_3^--ATPase occurs in the basal membrane of the latter tissue, and is inhibited by furosemide and SCN^-, the hyperolarization of V_A found during treatment with these modified salines may be explained once again, by the reduced availability of intracellular Cl^- to follow the active extrusion of K^+.

Recently, Peacock (1986) has suggested that a HCO_3^--stimulated ATPase may be involved in diuresis in Glossina upon finding an inhibition of this process in 4 mM acetazolamide (and 1-5 mM SCN^-). Gerencser (1983) and Gerencser and Lee (1985) have postulated a Cl^-+HCO_3^--stimulated ATPase which is electrogenic and independent of Na^+, in the basolateral membrane of Aplysia intestinal epithelium. These authors showed that the mechanism of Cl^- movement was independent of the presence or movement of Na^+ (no effect on Cl^- flux with furosemide, amiloride or ouabain), and independent of the simultaneous counter transport of another anion (no effect with SITS) but required the movement of HCO_3^- (a block of Cl^- flux with acetazolamide and SCN^-). The unusual electrogenic Cl^- transport system proposed by Hanrahan and Phillips (1983; 1984) in the rectum of Schistocerca

is activated and stimulated directly by K^+ and is independent of Na^+ and HCO_3^-. This mechanism was proposed as experimental evidence excluded the obvious ion gradients (Na^+,K^+,H^+,OH^- and HCO_3^-) that might drive Cl^- transport (Hanrahan and Phillips, 1983; 1984). Thus ouabain, furosemide and SITS treatments had no effect on electrogenic Cl^- flux in locust rectum (Hanrahan and Phillips, 1983; 1984) although an inhibitory effect on Cl^- flux was shown in this tissue upon application of acetazolamide (Herrera et al., 1978).

Overall, results from the present study, do not preclude the presence of either Cl^--HCO_3^- exchange or a Cl^-+HCO_3^--ATPase in the basal membrane of Locusta. However, in the latter case, Anstee and Fathpour (1979; 1981) could not resolve the question as to whether the anion-stimulated ATPase activity in microsomal preparations from the tubules of Locusta was located in the plasma membrane or was due to mitochondrial contamination.

Type A and Type B Response

The treatment of tubules in Normal saline with 1 mM furosemide, 10^{-4} M bumetanide, 10 mM SCN^- and 1 mM SITS all resulted in an inducement of Type B behaviour with the introduction of high K^+ saline including the appropriate inhibitor, in cells with originally exhibited the Type A response. These results suggest than an induced Type B response still occurs even though treatment with these Cl^- transport inhibitors, as discussed above, may inhibit Cl^- entry. However, if the Type B response is related to an increase, and not a decrease, in basal Cl^- entry (see Chapter 4) then the presence of a Type B response after treatment with furosemide-, bumetanide-, SCN^-- and SITS-containing salines suggests that the Cl^- entry mechanism is not reduced by the action of these inhibitors. Thus,

the means of the furosemide-, bumetanide-, SCN^-- and SITS-induced Type B behaviour remains, as yet, unclear. It is also unclear a) why the depolarization of V_B and V_A found during SITS pretreatment, in cells which exhibited the naturally occurring Type B response should, upon introduction of high K^+ saline + SITS, result in a change to Type A-like behaviour and b) why a novel introduction of high K^+ saline containing SITS resulted in a very slow development of the Type B response.

Finally, it was noted that the introduction of high K^+,HCO_3^- free saline + 1 mM acetazolamide after HCO_3^- free + 1 mM acetazolamide treatment did not alter the Type A response. This result would be expected if the Type B response is related to a basal Cl^- entry and HCO_3^- free saline + acetazolamide reduced Cl^- entry, as noted earlier, by inhibiting the functioning of a Cl^--HCO_3^- exchanger.

Section 5: Electrophysiological Studies on V_B,V_A and T.E.P. Using Ca^{2+} free, Ca^{2+} free + A23187 and Verapamil-Containing Salines

Effect of Ca^{2+} free Saline + E.G.T.A.

Table 35a and Fig. 38 show the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Ca^{2+} free saline + E.G.T.A. ($CaCl_2$ was replaced with the calcium chelator E.G.T.A.).

Table 35a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Ca^{2+} free saline + E.G.T.A.

Table 35b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Ca^{2+} free saline + E.G.T.A. to 128 mM K^+, Ca^{2+} free saline + E.G.T.A. in cells which exhibited the Type A response.

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

Table 35a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10	15
V_B	Normal	-70.0 ± 1.9	Ca^{2+} free	-77.3 ± 1.7	-77.5 ± 1.6	-76.3 ± 1.1	-76.0 ± 2.0
V_A	Saline	-84.2 ± 5.7	Saline +	-91.2 ± 4.8	-92.3 ± 4.9	-90.8 ± 4.1	-90.0 ± 7.0
T.E.P.		+10.1 ± 4.4	E.G.T.A.	+ 9.0 ± 3.4	+ 9.2 ± 3.4	+ 8.7 ± 1.3	+ 9.0 ± 3.0
n	-	7	-	7	6	4	2

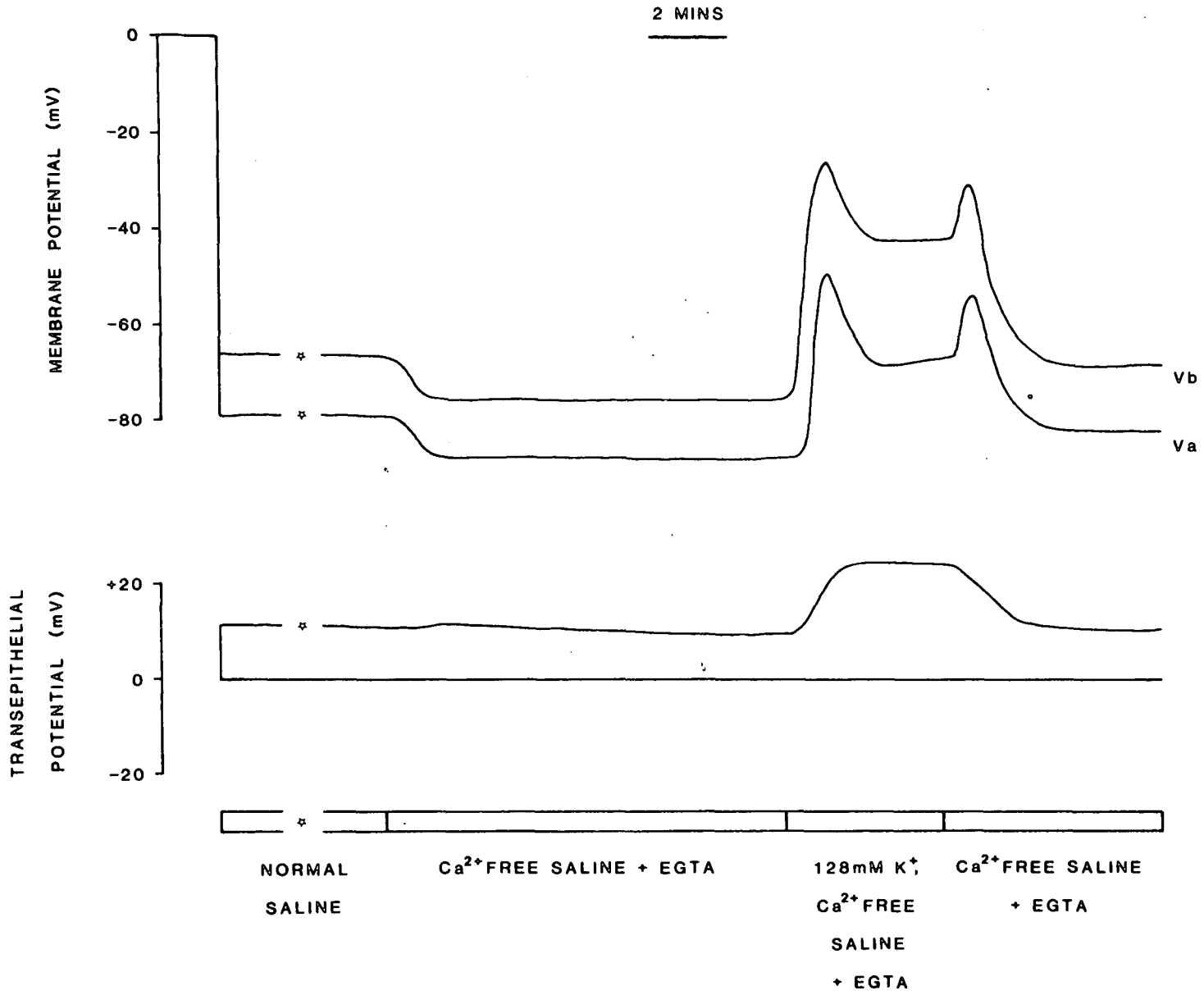
Table 35b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-70.5 ± 2.1	Ca^{2+} free	-75.4 ± 0.9	128 mM K^+	-17.6 ± 1.2	-32.1 ± 5.3	Ca^{2+} free	-	-75.7 ± 1.1	6
V_A	Saline	-82.9 ± 6.1	Saline +	-87.4 ± 3.3	Ca^{2+} free	-46.3 ± 3.6	-62.0 ± 1.5	Saline +	-	-88.7 ± 4.3	6
T.E.P.		+ 9.4 ± 4.8	E.G.T.A.	+ 6.9 ± 2.5	Saline + E.G.T.A.	+28.6 ± 3.9	-	E.G.T.A.	-	+ 8.0 ± 3.9	6

Fig. 38

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Ca^{2+} free saline + E.G.T.A. before the subsequent addition of 128 mM K^+,Ca^{2+} free saline + E.G.T.A. in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -11 mV, V_A = -46 mV, T.E.P. = +29 mV).


2 MINS
MEMBRANE POTENTIAL (mV)
0
-20
-40
-60
-80
Vb
Va
TRANSEPITHELIAL POTENTIAL (mV)
+20
0
-20
NORMAL SALINE
Ca2+ FREE SALINE + EGTA
128mM K+, Ca2+ FREE SALINE + EGTA
Ca2+ FREE SALINE + EGTA

It was found that V_B and V_A hyperpolarized 7.3 ± 1.4 mV and 7.0 ± 1.6 mV respectively over the first min of Ca^{2+} free treatment to values which were maintained over the next 14 mins. Due to both membrane potentials altering by similar amounts, T.E.P. changed little over 15 mins exposure to Ca^{2+} free saline.

Oscillations of V_A (and T.E.P.) occurred in 83% of cells in Ca^{2+} free saline and were 3.8 ± 1.1 mV in amplitude. Although the frequency of these oscillations was increased, the amplitude was not significantly different ($p = 0.05-0.1$) from the value found in Normal saline.

Effect of 128 mM K^+, Ca^{2+} free Saline + E.G.T.A. After Treatment in Ca^{2+} free Saline + E.G.T.A.

Table 35b and Fig. 38 show the effect of 128 mM K^+,Ca^{2+} free saline + E.G.T.A. on V_B, V_A and T.E.P. directly after a period of 5 to 15 mins treatment in Ca^{2+} free saline + E.G.T.A., in cells which exhibited the Type A response. It was found that pretreatment of cells with Ca^{2+} free saline resulted in the inducement of a 'reduced' Type B response for both V_B and V_A. Thus, the introduction of high K^+,Ca^{2+} free saline produced a depolarized V_B (component c, Fig. 20) that was half-way between, and significantly different ($p < 0.001$) from, the depolarized V_B in either the Type A or Type B response. In contrast, V_A depolarized to a value (component c, Fig. 20) that was not significantly different ($p = 0.1-0.2$) from the depolarized V_A in either the Type A or Type B response. Although these depolarized potentials (component c, Fig. 20) were maintained in many cases, V_B and V_A in about 50% of cells exhibited a repolarizing change (component d, Fig. 20). The resulting repolarized potentials

(component e, Fig. 20) were not significantly different ($p = 0.8-0.9$ for V_B and $p > 0.9$ for V_A) from the depolarized potentials (component c, Fig. 20) found in the naturally occurring Type B response. The 'reduced' nature of the Ca^{2+} free saline-induced Type B response was reflected in T.E.P. which increased in positivity to a value similar ($p = 0.2-0.3$) to that found in the Type A response but significantly more positive ($p = 0.02-0.03$) to that found in the Type B response.

Effect of Ca^{2+} free Saline + E.G.T.A. + 5 x 10^{-6} M A23187

The effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Ca^{2+} free saline + E.G.T.A. + 5 x 10^{-6} M A23187 is shown in Table 36a. It can be seen that this modified saline produced changes in V_B and V_A similar to those found in Ca^{2+} free saline lacking A23187. Thus, V_B and V_A hyperpolarized 9.6 ± 0.9 mV and 9.2 ± 1.5 mV respectively over the first min in Ca^{2+} free saline + A23187 treatment to values which were maintained over the next 9 mins. Due to both membrane potentials altering by similar values, T.E.P. changed little over 10 mins exposure to Ca^{2+} free saline + A23187.

Oscillations of V_A (and T.E.P.) occurred in 27% of cells in Ca^{2+} free saline containing A23187 and were 4.0 ± 1.2 mV in amplitude. Although the frequency was reduced, the amplitude of these oscillations was not significantly different ($p = 0.05-0.1$) from the value found in Normal saline.

Effect of 128 mM K^+,Ca^{2+} free Saline + E.G.T.A. + 5 x 10^{-6} M A23187 After Treatment in Ca^{2+} free Saline + E.G.T.A. + 5 x 10^{-6} M A23187

The effect of introducing 128 mM K^+,Ca^{2+} free saline + E.G.T.A. + 5 x 10^{-6} M A23187 on V_B, V_A and T.E.P. after a period of 5 to

Table 36a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Ca^{2+} free saline + E.G.T.A. + 5 x 10^{-6} M A23187.

Table 36b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Ca^{2+} free saline + E.G.T.A. + 5. x 10^{-6} M A23187 to 128 mM K^+,Ca^{2+} free saline + E.G.T.A. + 5 x 10^{-6} M A23187 in cells which exhibited the Type A response.

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

Table 36a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10
V_B	Normal	-67.3 ± 1.3	Ca^{2+} free Saline +	-76.9 ± 1.5	-76.9 ± 1.5	-76.7 ± 1.8
V_A	Saline	-80.5 ± 3.8	E.G.T.A. +	-89.7 ± 4.1	-92.1 ± 3.8	-92.3 ± 4.7
T.E.P.		+ 7.4 ± 2.9	5×10^{-6} M A23187	+ 7.7 ± 3.2	+10.0 ± 2.9	+ 9.6 ± 2.7
n	-	15	-	15	15	11

Table 36b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-67.3 ± 1.9	Ca^{2+} free Saline +	-75.6 ± 1.4	128mM K^+, Ca^{2+} free	-28.8 ± 2.8	-37.4 ± 4.7	Normal	-	-69.0 ± 1.5	12
V_A	Saline	-80.0 ± 3.3	E.G.T.A.	-90.3 ± 4.0	Saline +	-52.0 ± 3.5	-60.5 ± 4.4	Saline	-	-79.2 ± 3.9	12
T.E.P.		+ 7.8 ± 3.8	5×10^{-6} M A23187	+ 7.8 ± 2.7	E.G.T.A. + 5×10^{-6} M A23187	+25.2 ± 2.5	-		-	+ 8.1 ± 4.4	12

10 mins in Ca^{2+} free saline + E.G.T.A. + 5 x 10^{-6} M A23187, in cells which exhibited the Type A response, is shown in Table 36b. It can be seen that pretreatment of cells with Ca^{2+} free saline + A23187 induced a 'reduced' Type B response for both V_B and V_A. Thus, the initial depolarization (component b, Fig. 20) of V_B and V_A in 128 mM K^+,Ca^{2+} free saline + A23187 produced depolarized values (component c, Fig. 20) that were not significantly different (p = 0.4-0.5 and p = 0.6-0.7 respectively) from the equivalent potentials found in the naturally occurring Type B response. After depolarizing, approximately 50% of cells were maintained at this potential. However, V_B and V_A in the remaining cells repolarized (component d, Fig. 20) to maintained values (component e, Fig. 20) that were significantly less negative (p = 0.001-0.01 for both potentials) than the equivalent potentials found in the naturally occurring Type B behaviour. Following the changes of V_B and V_A, T.E.P. increased in positivity in high K^+,Ca^{2+} free saline + A23187, to a value similar (p = 0.05-0.1) to that found in the Type B response.

Finally, it is of interest to note that the pattern of membrane changes and resulting potentials in high K^+ saline produced by Ca^{2+} free + A23187 pretreatment were similar to those found after Ca^{2+} free pretreatment. Indeed, the depolarized potentials (component c, Fig. 20) and repolarized potentials (component e, Fig. 20) for V_B and V_A in high K^+,Ca^{2+} free saline + A23187 were not significantly different (p = 0.4-0.5 and p = 0.7-0.8 respectively) from the values in high K^+,Ca^{2+} free saline lacking A23187. Thus, the addition of 5 x 10^{-6} M A23187 to Ca^{2+} free saline and to the ensuing 128 mM K^+,Ca^{2+} free saline did not have a significant effect on the induced Type B behaviour when compared to results in which A23187 was absent.

Effect of 1 mM Verapamil

The effect of Normal saline + 1 mM verapamil on V_B, V_A and T.E.P. is shown in Table 37a and Fig. 39. It was found that V_B and V_A hyperpolarized 13.7 ± 3.0 mV and 7.9 ± 3.9 mV respectively over the first min of verapamil treatment. Thereafter, V_B and V_A depolarized to values 4.0 ± 3.0 mV and 7.3 ± 1.7 mV respectively less negative than the original resting potentials over the next 9 mins. T.E.P. decreased in positivity due to V_B hyperpolarizing more than V_A during the first min of Normal saline + verapamil treatment but, thereafter, exhibited little change in value.

None of the cells examined exhibited oscillations of V_A (or T.E.P.) in Normal saline containing 1 mM verapamil.

Effect of 128 mM K^+ Saline + 1 mM Verapamil After Treatment in Normal Saline + 1 mM Verapamil

Table 37b and Fig. 39 show the effect on V_B, V_A and T.E.P. of changing the perfusate to 128 mM K^+ saline + 1 mM verapamil after a period of 5 to 10 mins in Normal saline + 1 mM verapamil in cells which exhibited the Type A response. It was found that pretreatment in Normal saline + verapamil induced the Type B response for both V_B and V_A. Thus, on introduction of 128 mM K^+ saline + verapamil V_B and V_A depolarized (component b, Fig. 20) to new potentials (component c, Fig. 20) that were similar ($p = 0.6$-0.7) for V_B and significantly more negative ($p = 0.02$-0.05) for V_A than the equivalent values found in the naturally occurring Type B response. After depolarizing, however, V_B and V_A hyperpolarized to repolarized potentials (component e, Fig. 20) that were not significantly different ($p = 0.1$-0.2 and $p = 0.5$-0.6 respectively) from the repolarized values found in the Type B response. Following the changes of V_B and V_A,

Table 37a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM verapamil.

Table 37b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM verapamil to 128 mM K^+ saline + 1 mM verapamil in cells which exhibited the Type A response.

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

Table 37a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10
V_B	Normal	-67.6 ± 1.0	Normal	-81.3 ± 2.5	-72.1 ± 3.2	-64.0 ± 3.0
V_A	Saline	-86.4 ± 3.5	Saline + 1 mM	-94.3 ± 3.3	-86.3 ± 4.1	-78.0 ± 6.5
T.E.P.		+10.9 ± 2.4	Verapamil	+ 4.4 ± 1.8	+ 5.4 ± 3.0	+ 5.3 ± 7.4
n	-	7	-	7	5	3

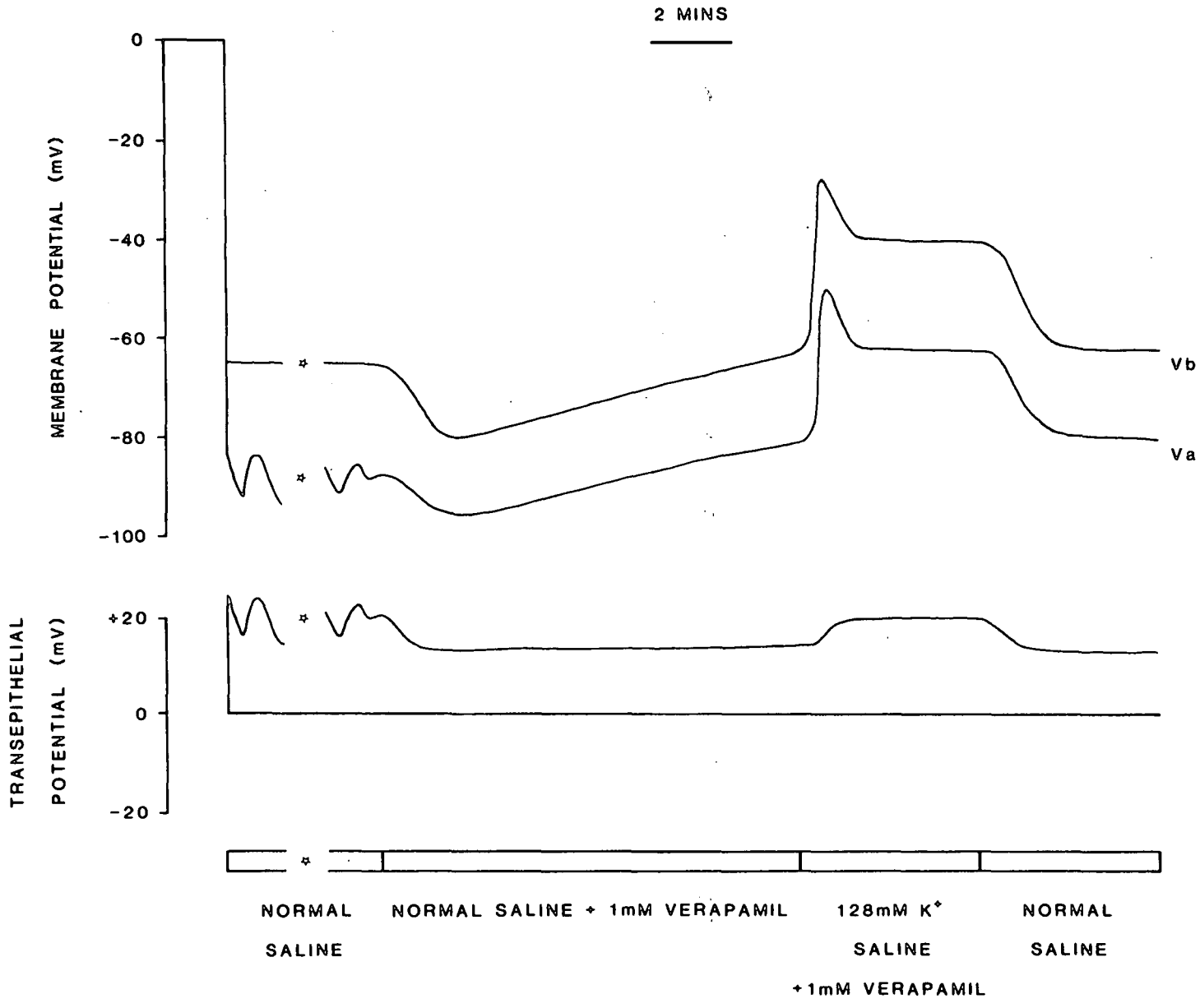
Table 37b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-68.3 ± 2.4	Normal	-64.8 ± 2.6	128 mM K^+	-33.6 ± 5.5	-43.3 ± 8.0	Normal	-	-70.2 ± 1.8	5
V_A	Saline	-83.0 ± 2.5	Saline + 1 mM	-82.0 ± 2.2	Saline + 1 mM	-63.8 ± 3.8	-72.1 ± 5.2	Saline	-	-81.9 ± 3.1	5
T.E.P.		+12.3 ± 1.8	Verapamil	+11.6 ± 0.9	Verapamil	+26.8 ± 6.0	-		-	+10.9 ± 2.4	5

Fig. 39

Typical example of the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM verapamil before the subsequent addition of 128 mM K^+ saline + 1 mM verapamil, in cells which exhibited the Type A response.

* indicates a period of 10 mins during which a Type A response to 128 mM K^+ saline was established (V_B = -8 mV, V_A = -46 mV, T.E.P. = +34 mV).


2 MINS
0
-20
-40
-60
-80
-100
MEMBRANE POTENTIAL (mV)
Vb
Va
+20
0
-20
TRANSEPITHELIAL POTENTIAL (mV)
NORMAL SALINE
NORMAL SALINE + 1mM VERAPAMIL
128mM K+ SALINE +1mM VERAPAMIL
NORMAL SALINE

T.E.P. increased in positivity, in high K^+ saline + verapamil, to a value similar ($p = 0.2-0.3$) to that found in the Type B response.

Results in Table 38 show that the introduction of high K^+ saline + verapamil, to cells which exhibited the Type A response and had no previous exposure to this inhibitor, induced Type B behaviour for V_B and V_A. Indeed, this induced Type B response developed strongly. Thus, the novel introduction of verapamil in high K^+ saline produced depolarized potentials (component c, Fig. 20) and repolarized potentials (component e, Fig. 20) that were not significantly different ($p = 0.8-0.9$ and $p = 0.1-0.2$ respectively for both potentials) from the equivalent values found in the naturally occurring Type B response. In addition, although the initial depolarization of V_A (component b, Fig. 20) was 88% of that for V_B (cf. 71% in the Type B response), the resulting T.E.P. value in 128 mM K^+ saline + verapamil was similar ($p = 0.2-0.3$) to the value found in the naturally occurring Type B response.

Discussion

Ca^{2+} Substitution

It has been postulated that calcium probably enters the cells of the Malpighian tubules of various insects by moving down its electrochemical gradient (Maddrell, 1971). If this is the case in Locusta, then calcium transport across the tubules is probably largely passive. The introduction of Ca^{2+} free saline in the presence of the calcium chelator, E.G.T.A., effected a hyperpolarization of both V_B and V_A over 1 min to a maintained value with no change in T.E.P. A different response was found in the salivary glands of Calliphora, where removal of Ca^{2+} from the bathing medium (in the

Table 38

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to 128 mM K^+ saline + 1 mM verapamil in cells which exhibited the Type A response (i.e. a novel addition of verapamil in high K^+ saline).

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

Parameter	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-73.0 ± 3.4	128 mM K^+	-31.9 ± 5.0	-45.7 ± 5.8	Normal	-	-72.8 ± 3.5	8
V_A	Saline	-88.9 ± 3.6	Saline + 1 mM	-52.8 ± 4.8	-64.8 ± 6.0	Saline	-	-88.3 ± 2.9	8
T.E.P.		+13.6 ± 1.8	Verapamil	+17.6 ± 1.5	-		-	+13.8 ± 1.6	8

presence of the agonist 5-HT) increased T.E.P. due to a marked hyperpolarization of V_A (Prince and Berridge, 1973).

It may be expected that Ca^{2+} free saline affects the functioning of Ca^{2+}-transporting mechanisms in the form of a Ca^{2+}-ATPase or a Na^+-Ca^{2+} exchanger. Evidence from the present study does not preclude the presence of a Ca^{2+}-ATPase in the tubules of Locusta, but it is difficult to explain the observed hyperpolarization in membrane potentials in Ca^{2+} free saline on the basis of this transporter. If a basal membrane Na^+-Ca^{2+} exchanger, which moved Ca^{2+} out of the cell in exchange for an inward movement of Na^+ (Scoble et al., 1985; Taylor and Windhager, 1979), occurred in the tubule cells of Locusta, the absence of Ca^{2+} in the perfusate may reduce its functioning. As this exchange is thought to be electrogenic with 3-5 Na^+ transported for every Ca^{2+} (Mullins, 1979; Chase, 1984), a reduction in its function may lead to a hyperpolarization of V_B as less Na^+ was moved into the cell. However, whether the Na^+-Ca^{2+} exchanger is electrogenic enough to produce the observed hyperpolarization of V_B, or indeed, extrude Ca^{2+} from the tubules of Locusta, remains to be seen. Support for this comes from Chase (1984) who 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 < 10 mM. When $[Na^+]_i$ is higher, the exchanger reverses, sending Ca^{2+} into the cell. This threshold value is similar to the estimated value of 13 mM $[Na^+]_i$ for Malpighian tubules (based on Rhodnius, Gupta et al., 1976).

An explanation for the Ca^{2+} free saline result in the present study is made difficult due to the fact that Ca^{2+} has been shown to be involved in the regulation of many cellular processes (Rasmussen and Goodman, 1977). The hyperpolarization of V_B (which

may be the result of a Ca^{2+} free-induced exposure of an electrogenic Na^{+}-Ca^{2+} exchanger) and V_A may be related to the observation that the removal of Ca^{2+} has been shown to have potentially damaging effects on cellular histology (Donowitz, 1983). In addition, reported values for intracellular free Ca^{2+} are low (10^{-5}-10^{-8} M, Schatzmann, 1975). Thus, the presence of Ca^{2+} free saline may not effect significant changes in intracellular Ca^{2+} in the short term.

Finally, a calcium-induced hyperpolarization has been attributed to the activity of Ca^{2+}-dependent K^{+} channels as a result of an increase in $[Ca^{2+}]_i$ in various tissues (Gárdos et al., 1976; Hoffmann et al., 1986). It is unlikely that the V_B hyperpolarization in the present study was due to the activation of such channels due to the absence of Ca^{2+} in the perfusate.

A23187

The cation ionophore A23187 has been widely used to assess the role of divalent cations in physiological processes (Pressman, 1976). This divalent ionophore has a well-documented ability to transport Ca^{2+} in biological systems in exchange for intracellular H^{+}, or Mg^{2+}, and is predominantly selective for divalent ions over monovalent ions (Reed, 1979). Thus, it is capable of stimulating various Ca^{2+}-dependent biological reactions without directly disturbing preexisting balances of Na^{+} and K^{+} (Pressman, 1976; Reed, 1979).

In the present study, A23187 was supplied in the perfusate in calcium-free saline (+ E.G.T.A.), to allow its incorporation into the basal membrane. Chandler and Williams (1977) have shown

that various tissues may need to be preincubated with A23187 before addition of calcium in order for an effect to be seen. The addition of 5 x 10^{-6} M A23187 in Ca^{2+} free saline lead to a maintained and reversible hyperpolarization of both V_B and V_A with no change in T.E.P. The changes in both membrane potentials in Ca^{2+} free saline + ionophore were almost identical to those found with Ca^{2+} free saline lacking A23187. Thus, the increase in membrane potentials in the former saline may simply be a Ca^{2+} free effect. The fact that A23187 did not alter the Ca^{2+} free saline effect, plus the observation that the membrane potentials return to their original values in Normal saline suggests that a) the ionophore may not be inserted into the basal membrane or b) treatment with A23187 does not greatly alter the Ca^{2+} gradient in the tubules of Locusta. The presence of Ca^{2+}-activated K^+ channels has been indicated by a membrane hyperpolarization in the presence of A23187 in a Ca^{2+} containing medium in Necturus gallbladder (Bello-Reuss et al., 1981) and Ehrlich Ascites tumour cells (Hoffmann et al., 1986). However, a similar system in the tubules of Locusta is difficult to envisage due to the absence of Ca^{2+} in the A23187 containing medium.

Verapamil

The Ca^{2+} antagonist verapamil has been shown to be a relatively specific blocker of voltage-activated Ca^{2+} channels (Janis and Scriabine, 1983). As a result of the action of this drug an inhibition of calcium transport has been reported in many tissues (Triggle, 1981; Pento and Johnston, 1983). Furthermore, 1 mM verapamil blocks calcium influx action potentials in Rhodnius oocytes (O'Donnell, 1985). In the present study, the introduction of Normal saline containing 1 mM verapamil resulted in an initial hyperpolarization

(and decrease in T.E.P. positivity) followed by a depolarization (but maintained T.E.P.) of both V_B and V_A. An explanation of these changes found during verapamil treatment is not obvious but may be related to the nonspecific action of this drug. Indeed, a variety of nonspecific effects have been documented for verapamil and other calcium channel inhibitors (at concentrations > 10^{-5} M) including inhibition of K^+, Na^+ and other ionic channels, interaction with hormone receptors and alterations of adenylate cyclase and Na^++K^+-ATPase activities (Triggle, 1981; Huff and Reinach, 1985; Janis and Scriabine, 1983). It may be that the decline of both membrane potentials after the initial hyperpolarization in verapamil-containing saline is related to a nonspecific effect, such as an inhibition of the Na^++K^+-ATPase, leading to an inability to maintain the cellular gradients.

General

It was suggested from Ca^{2+} replacement experiments that a Na^+-Ca^{2+} exchanger may occur in the basal membrane of Locusta tubule cells. It is of interest that an alteration in the functioning of such an exchanger during treatment with K^+ free, Na^+ free, ouabain, vanadate, amiloride and monensin salines may all increase $[Ca^{2+}]_i$ through inhibition of Na^+-Ca^{2+} exchange. An alteration of the Na^+ gradient has been shown to increase $[Ca^{2+}]_i$ during Na^+ free treatment (Taylor and Windhager, 1979; Mandel and Murphy, 1984) and ouabain treatment (Cruz-Soto et al., 1984; Lorenzen et al., 1984) due to a diminished calcium efflux from an inhibited Na^+-Ca^{2+} exchanger. Although vanadate does not directly inhibit Na^+-Ca^{2+} exchange (Ueda, 1983), the increase of $[Na^+]_i$ during treatment with this agent may prevent entry of Na^+ in exchange

for the outward movement of Ca^{2+}, increasing $[Ca^{2+}]_i$. An increase in cytosolic Ca^{2+} concentration as a result of vanadate has been shown in frog skin (Aboulafia and Lacaz-Viera, 1984). An increase in $[Na^+]_i$ as found with K^+ free, ouabain and vanadate treatments may also increase $[Ca^{2+}]_i$ through a reversal in the action of a Na^+-Ca^{2+} exchanger. As mentioned earlier, Chase (1984) has proposed that when $[Na^+]_i$ exceeds a value of approximately 10 mM, as after inhibition of the Na^++K^+ pump, the direction of the Na^+-Ca^{2+} exchanger reverses (in Na^+ transporting epithelia), sending Ca^{2+} into the cell. Amiloride has been shown to inhibit Na^+-Ca^{2+} exchange in various preparations (Schellenberg et al., 1983) and there are indications that this diuretic may inhibit the influx of Ca^{2+} in the salivary glands of Calliphora (Berridge et al., 1976). Davis and Finn (1985) have suggested that amiloride may affect intracellular calcium levels in frog urinary bladder by influencing basolateral membrane calcium pathways. As a result of an increased $[Na^+]_i$, monensin treatment may result in an increase in $[Ca^{2+}]_i$ due to an inhibition or reversal of a Na^+-Ca^{2+} exchange. Pressman and Painter (1983) have found that in different systems, monensin elicits the activation of mechanisms, the triggering of which is attributed to increases in $[Ca^{2+}]_i$. In addition, this ionophore produced a fast, large and sustained increase in cytosolic Ca^{2+} in toad urinary bladder (Hardy and Ware, 1985).

Overall, as will be discussed later, an increase in $[Ca^{2+}]_i$ brought about by an inhibition of Na^+-Ca^{2+} exchange, as described above, may explain many of the modified saline-induced membrane changes seen in the present study.

Type A and Type B Response

The introduction of high K^+,Ca^{2+} free saline, with or without the ionophore A23187, after Ca^{2+} free treatment (with or without A23187) resulted in the exposure of Type B behaviour for both V_B and V_A, in cells which originally exhibited the Type A response. Similarly, the introduction of high K^+ saline + 1 mM verapamil after Normal saline + 1 mM verapamil treatment resulted in an exposure of the Type B response for both membrane potentials. Results show that 1 mM verapamil pretreatment was not needed to induce the Type B behaviour as it occurred with a novel introduction of high K^+ saline + verapamil.

The application of Ca^{2+} free (with or without A23187) or verapamil saline would not be expected to increase $[Ca^{2+}]_i$. Thus, the Ca^{2+} free- and verapamil-induced Type B response in high K^+ saline seems to rule out the possibility that such behaviour is dependent on an increase in $[Ca^{2+}]_i$. However, this disagrees with an earlier suggestion (see Section 3) that the Type B response may be induced by an increase in $[Ca^{2+}]_i$. Furthermore, as will be discussed later (see Chapter 4), it is proposed that the Type B response is the result of an increase in $[Ca^{2+}]_i$ stimulating a basal Cl^- entry and the activity of an apical K^+ pump. It may be that the Ca^{2+} free- or verapamil-inducement of the Type B response cannot be explained on the basis of evidence currently available and may be related, as noted earlier, to the potentially damaging effects of Ca^{2+} free conditions on cellular histology (Donowitz, 1983).

Section 6: Electrophysiological Studies on V_B, V_A and T.E.P. Using 5-Hydroxytryptamine- and c AMP-Containing Saline

Effect of 1 mM 5-Hydroxytryptamine

The effect of Normal saline containing 1 MM 5-Hydroxytryptamine (5-HT) on V_B, V_A and T.E.P. was examined by exposing the cells to this agent for 10 to 20 mins (n=6). No change was observed in either membrane potential even after 20 mins in Normal saline + 5-HT.

Oscillations of V_A (and T.E.P.) occurred in 67% of cells in Normal saline + 1 mM 5-HT and were 4.8 ± 1.8 mV in amplitude. These results were not significantly different (p = 0.4-0.5) from values found in Normal saline.

Although Normal saline containing 1 mM 5-HT apparently increased fluid secretion by the Malpighian tubules of Locusta by 7%, this increase was not significant (p > 0.9; n=13) (see Appendix, Table 1).

Effect of 128 mM K^+ Saline + 1 mM 5-HT After Treatment in Normal Saline + 1 mM 5-HT

It was found that introducing 128 mM K^+ saline + 1 mM 5-HT directly after a period of 10-15 mins in Normal saline + 5-HT, in cells which exhibited the Type A response, did not alter this behaviour (n=6). Thus, the depolarized values (component c, Fig. 20) of V_B and V_A and the new T.E.P., in high K^+ saline + 5-HT, were not significantly different (p = 0.5-0.6, p = 0.8-0.9 and p = 0.3-0.4 respectively) from the equivalent values found in the Type A response.

Similarly, the introduction of 128 mM K^+ saline + 1 mM 5-HT directly after a period of 10-20 mins in Normal saline + 1 mM 5-HT in cells which exhibited the Type B response did not alter this behaviour (n=3). Thus the depolarized potentials (component c, Fig. 20)

and repolarized potentials (component e, Fig. 20) of V_B and V_A and the new T.E.P., in high K^+ saline + 5-HT, were not significantly different (p = 0.7-0.8, p = 0.3-0.4 and p = 0.1-0.2 respectively) from the equivalent values found in the Type B response.

Effect of 1 mM c AMP

Table 39a shows the effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM c AMP. It was found that V_B and V_A hyperpolarized 4.1 ± 0.6 mV and 8.2 ± 1.3 mV respectively over the first min of c AMP treatment to values which levelled over the next 9 mins. Thus, V_B and V_A reached maintained values 6.9 ± 1.5 mV and 18.9 ± 3.2 mV respectively more negative than the original resting potentials after 10 mins exposure to c AMP saline. T.E.P. increased in positivity due to V_A hyperpolarizing more than V_B during the first min of Normal saline + c AMP treatment but, thereafter, exhibited little change in value. Just over 30% of cells exposed to c AMP saline were returned to Normal saline; V_B and V_A in these cells depolarized to the original resting values after a period of 5 to 8 mins.

The amplitude of the oscillations of V_A (and T.E.P.) increased significantly (p = 0.001-0.01) from 6.0 ± 0.3 mV in Normal saline to 11.5 ± 1.8 mV in Normal saline containing 1 mM c AMP, with oscillations occurring in 62% of cells.

Effect of 128 mM K^+ Saline + 1 mM c AMP After Treatment in Normal Saline + 1 mM c AMP

The effect of introducing 128 mM K^+ saline + 1 mM c AMP on V_B, V_A and T.E.P. after a period of 5 to 10 mins in Normal saline + 1 mM c AMP, in cells which exhibited the Type A response, is shown

Table 39a

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline to Normal saline + 1 mM c AMP.

Table 39b

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM c AMP to 128 mM K^+ saline + 1 mM c AMP in cells which exhibited the Type A response.

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

Table 39a

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	Time in New Saline (mins) 1	5	10
V_B	Normal	-74.1 ± 2.2	Normal	-78.2 ± 2.4	-80.2 ± 2.8	-81.6 ± 3.0
V_A	Saline	-88.1 ± 3.0	Saline + 1 mM	-96.2 ± 3.0	-104.5 ± 3.3	-107.4 ± 3.7
T.E.P.		+ 9.2 ± 2.3	c AMP	+12.4 ± 2.4	+18.4 ± 3.2	+18.8 ± 4.5
n	-	13	-	13	13	8

Table 39b

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-74.5 ± 1.5	Normal	-79.8 ± 2.8	128 mM K^+	-10.8 ± 2.4	-	Normal	-	-74.1 ± 2.1	4
V_A	Saline	-86.5 ± 2.5	Saline + 1 mM	-95.0 ± 5.8	Saline + 1 mM	-37.3 ± 7.1	-	Saline	-	-86.0 ± 2.9	4
T.E.P.		+ 6.3 ± 3.4	c AMP	+ 9.0 ± 6.7	c AMP	+18.5 ± 7.1	-		-	+ 7.0 ± 3.0	4

in Table 39b. It can be seen that c AMP pretreatment did not alter the original Type A response. Thus, the depolarized values (component c, Fig. 20) of V_B and V_A, in high K^+ saline + c AMP, were not significantly different ($p = 0.05-0.1$ and $p = 0.6-0.7$ respectively) from the equivalent values found during Type A behaviour. However, although not significantly different, depolarized values arose as a result of V_A initially depolarizing 84% of that for V_B (cf. 65% found during the Type A response) and resulted in a T.E.P. value, in 128 mM K^+ saline + c AMP, that was significantly less positive ($p < 0.001$) than T.E.P. found in the Type A response.

Table 40 shows the effect on V_B, V_A and T.E.P. of introducing 128 mM K^+ saline + 1 mM c AMP after a period of 10 to 15 mins in Normal saline + 1 mM c AMP, in cells which exhibited the Type B response. It was found that c AMP pretreatment altered the Type B behaviour. Thus, on introduction of high K^+ saline containing c AMP, both V_B and V_A depolarized to maintained potentials (component c, Fig. 20) that were not significantly different ($p > 0.9$ and $p = 0.05-0.1$ respectively) from the repolarized potentials (component e, Fig. 20) found during the Type B response. This shows that c AMP pretreatment produced the maintained repolarized potentials (component e, Fig. 20) of the Type B response, in high K^+ saline + c AMP, without V_B and V_A exhibiting a repolarizing change (component d, Fig. 20). However, although not significantly different, the depolarized V_A (component c, Fig. 20) in 128 mM K^+ saline + c AMP was 14 mV more negative than the Type B repolarized potential (component e, Fig. 20). This arose as a result of V_A initially depolarizing only 55% of that for V_B (cf. 71% found during the Type B response) and resulted in a T.E.P. value significantly more positive ($p > 0.9$) than the value found in the naturally occurring Type B response.

Table 40

Effect on V_B, V_A and T.E.P. of changing the perfusate from Normal saline + 1 mM c AMP to 128 mM K^+ saline + 1 mM c AMP in cells which exhibited the Type B response.

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

Parameter	Treatment	P.D. ± S.E.M. (mV)	Treatment	P.D. ± S.E.M. (component a, Fig.20) (mV)	Treatment	New P.D. ± S.E.M. (component c, Fig.20) (mV)	Repolarized P.D. ± S.E.M. (component e, Fig.20) (mV)	Treatment	Initial ΔP.D. ± S.E.M. (component f, Fig.20) (mV)	Re-established P.D. ± S.E.M. (component g, Fig.20) (mV)	n
V_B	Normal	-79.0 ± 3.0	Normal	-87.3 ± 2.2	128 mM K^+	-54.3 ± 6.2	-	Normal	-	-77.5 ± 1.9	3
V_A	Saline	-85.0 ± 7.0	Saline + 1 mM	-108.3 ± 3.3	Saline + 1 mM	-90.3 ± 7.2	-	Saline	-	-84.0 ± 5.3	3
T.E.P.		+ 8.0 ± 1.0	c AMP	+13.3 ± 2.0	c AMP	+33.7 ± 4.5	-		-	+ 7.1 ± 2.1	3

Finally, it was found that the introduction of high K^+ saline + c AMP, to cells which exhibited the Type A response and had no previous exposure to this agent, did not alter this behaviour (n=6). Thus, the depolarized potentials in cells so-treated were similar ($p = 0.2-0.3$) to those found in the Type A response.

Discussion

5-HT and c AMP

Fluid secretion by the Malpighian tubules and related ionic processes are believed to be under the control of diuretic hormone (D.H.) in many insects (Phillips, 1981; 1982). Although not examined in the present study, D.H. from the corpus cardiacum has been shown to greatly increase fluid secretion (Mordue, 1969; Donkin, 1981; Morgan and Mordue, 1984) but produced no significant change in membrane potentials (Morgan and Mordue, 1983a) in the Malpighian tubules of Locusta. Morgan and Mordue (1983a) have usggested that D.H. might act by increasing the permeability of the basal membrane to Na^+, achieving a greater influx of NaCl into the cell.

Recently, it has been shown that the biogenic amine 5-hydroxytryptamine (5-HT or serotonin) and the cyclic nucleotide (c AMP) mimic the action of D.H. on Malpighian tubule function in some insects but not others (Phillips, 1981; 1982). Various studies have demonstrated the stimulation of tubular fluid secretion (Maddrell et al., 1971; Nicolson and Miller, 1983), midgut fluid absorption (Farmer et al., 1981) and salivary gland activity (Berridge and Prince, 1972) by 5-HT. Other studies, however, have reported that Malpighian tubule fluid secretion is unaffected by 5-HT (Maddrell and Klunsuwan, 1973; Farquharson, 1974; Dalton and Windmill, 1980). Similarly, Anstee et al. (1980) and the present study found no significant stimulation

of fluid secretion in the presence of 5-HT in Normal saline in the tubules of Locusta, over the concentration range 10^{-8}-10^{-4} M, Rafaeli and Mordue (1982) have also reported the tubules of Locusta to be insensitive to 5-HT. In contrast, Morgan and Mordue (1984) have reported that 5-HT stimulates fluid secretion in this latter tissue with the threshold of stimulation lying between 10^{-8} and 10^{-7} M and maximum activation occurring at doses greater than 10^{-6} M.

In the present study, 1 mM 5-HT had no effect on V_B, V_A or T.E.P. In contrast, electrophysiological studies on the tubules of Rhodnius showed that T.E.P. changed in 3 distinct phases (a respective increase, decrease and then increase in negativity over about 10 min), largely due to changes in V_A, during exposure to 5-HT (Maddrell, 1971; O'Donnell and Maddrell, 1984). During prolonged 5-HT treatment, T.E.P. in the salivary glands of Calliphora decreased in positivity to a maintained value, while brief 5-HT exposure resulted in an initial T.E.P. decrease in positivity followed by a rapid increase, and then a slow return to the original value over about 2 min, with these changes being largely attributed to changes in V_A (Berridge and Prince, 1972).

The lack of effect of 5-HT on fluid secretion and membrane potentials points against any role for this biogenic amine in tubule function in Locusta. This differs from the tubules of Rhodnius in which 5-HT is a secretagogue and is thought to bring about changes in V_A through an exit of Cl^- from the cell into the lumen, then an activation of the apical cation pump followed by an increase in the apical membrane Cl^- conductance (Maddrell, 1971). No change in V_B occurred during the action of 5-HT in this tissue as the proposed $Na^+K^+2Cl^-$ mechanism is electrically silent (O'Donnell and Maddrell, 1984). Similarly, Berridge et al. (1976) and Berridge (1980) have suggested that exposure

to 5-HT in the salivary glands of Calliphora leads to changes in V_A through a Ca^{2+}-induced Cl^- permeability switching on and a c AMP activated apical K transport developing (see later). However, 5-HT has no effect on intracellular c AMP in the tubules of Locusta (Morgan and Mordue, 1984).

Rafaeli et al. (1984) have suggested that the increases in intracellular c AMP levels in response to D.H. (Rafaeli et al., 1984; Morgan and Mordue, 1984) in the tubules of Locusta, provide sufficient evidence for the involvement of c AMP in the coupling of hormonal stimulation to fluid secretion in this epithelium. This is supported by the fact that fluid secretion in the tubules of Locusta was greatly stimulated in c AMP (Donkin, 1981; Morgan and Mordue, 1981). Furthermore, with the exception of Musca (Dalton and Windmill, 1980), dibutryl c AMP acts as a secretagogue in all insect tubules studied (Donkin, 1981; Maddrell et al., 1971; Nicolson, 1976; Gee, 1976; Maddrell and Phillips, 1978; Szibbo and Scudder, 1979; Anstee et al., 1980; Morgan and Mordue, 1981; Rafaeli and Mordue, 1982; Williams and Beyenbach, 1983). This nucleotide stimulates fluid secretion in the salivary glands of Calliphora (Berridge, 1980) and fluid absorption by Rhodnius midgut (Farmer et al., 1981). In Locusta tubules, the threshold for stimulation lies between 10^{-4} and 3×10^{-4} M c AMP with maximum stimulation being observed in the presence of 10^{-3} M c AMP (Anstee et al., 1980).

In the present study, the addition of 1 mM dibutryl c AMP to Normal saline caused both membrane potentials to hyperpolarize (and T.E.P. to increase in positivity) to values which were maintained after about 10 mins. This effect of c AMP was found to be reversible. Similarly, Anstee et al. (1980) showed that 1 mM c AMP effected a reversible increase in T.E.P. positivity in Locusta tubules, with

a new stable potential being established after about 16 mins. In the salivary glands of Calliphora, c AMP induces a reversible increase in lumen positivity mainly by hyperpolarizing the apical membrane (Berridge, 1980). In contrast to the findings from the present study, Morgan and Mordue (1983a) have shown that no change in V_B was observed in response to c AMP in the tubules of Locusta. The suggestion by these authors that c AMP might stimulate coupled anion-cation transport across this membrane, therefore, may not be valid. In the Malpighian tubules of Aedes, c AMP caused a reversal and large depolarization of V_B and virtually no change in V_A (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985a). It has been suggested that this was due to an entry of Na^+ into the cell as a result of a selective increase in the basolateral Na^+ conductance (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985a).

In order to examine the role of c AMP in the tubules of Locusta, it can be noted that c AMP-mediated increases in the apical membrane Cl^- conductance have been reported for many tissues including shark rectal gland (Greger et al., 1984), cornea (Reuss et al., 1983) and canine trachea (Shorofsky et al., 1982; Welsh et al., 1983). Furthermore, it has been suggested that intracellular c AMP increases both Cl^- permeability and electrogenic transport of cations at the apical membrane in the tubules of Rhodnius (Phillips, 1982) and a c AMP activated apical K^+ transport has been proposed for the salivary glands of Calliphora (Berridge et al., 1976; Berridge, 1980). In the tubules of Locusta, an increase in the activity of an electrogenic apical K^+ pump, due to c AMP, may hyperpolarize V_A. In contrast, a c AMP-induced increase in apical Cl^- exit would probably lead to a depolarization of V_A and not the observed hyperpolarization. However, a c AMP-induced

increase in Cl^- entry across the basal membrane may explain the hyperpolarization of V_B in c AMP-containing saline. It is of interest that the action of c AMP, suggested above, may be mediated by Ca^{2+}. A mobilization of intracellular Ca^{2+} by c AMP has been proposed for a number of transporting epithelia (Berridge, 1980). Berridge (1980) has suggested that c AMP may be capable of releasing Ca^{2+} from intracellular reservoirs in the salivary glands of Calliphora. Furthermore, the ability of Ca^{2+} to regulate Cl^- permeability has been described in many transporting tissues (Berridge, 1980; Nauntofte and Poulsen, 1986). In the salivary glands of Calliphora, Berridge (1980) has postulated a Ca^{2+}-induced increase in Cl^- conductance (albeit in the apical membrane) whilst Berridge et al. (1975) have hinted at a role for Ca^{2+} in increasing the activity of the apical K^+ pump in the same tissue.

As mentioned in the Introduction, Morgan and Mordue (1984) have proposed a hypothetical model to describe hormone-stimulated fluid secretion by the Malpighian tubules of Locusta, similar to that found for Calliphora salivary glands (see Introduction; Berridge, 1980, Berridge and Heslop, 1982). In the model of Morgan and Mordue (1984), two distinct sites are thought to exist on the surface of the tubule cells; one which results in an increase in c AMP synthesis (R_1) and the other perhaps leading to an increase in intracellular Ca^{2+} concentration (R_2). Diuretic hormone is thought to stimulate both receptors (R_1 and R_2) activating maximum stimulation, with 5-HT stimulating fluid secretion by acting at R_2. The latter observation was made by Morgan and Mordue (1984) upon finding that 5-HT had a [illegible]tory effect on fluid secretion in the tubules of Locusta. However, as mentioned earlier, results from the present study, Anstee et al. (1980) and Rafaeli and Mordue (1982)

have shown the 5-HT had no effect on fluid secretion in this epithelium. Thus, as it stands, the viability of the model for hormonal control of fluid secretion by Morgan and Mordue (1984) may be invalid.

Type A and Type B Response

The introduction of high K^+ saline + 1 mM 5-HT after treatment in 1 mM 5-HT did not alter the Nernstian Type A response nor did the presence of 5-HT alter the naturally occurring Type B response. These results support the observation mentioned earlier that 5-HT does not appear to have a role in the functioning of the Malpighian tubules of Locusta.

The introduction of high K^+ saline + 1 mM c AMP after 1 mM c AMP pretreatment did not alter the Type A response nor the production of the naturally occurring Type B response. The former observation seems to rule out a role for c AMP in inducing Type B behaviour. This seems odd if, as mentioned in Section 5, the Type B response is induced by an increase in $[Ca^{2+}]_i$, and $c AMP_i$ leads to a mobilization of intracellular Ca^{2+}. However, as discussed for the final model (see Chapter 4), it may be that the postulated increase of $[c AMP]_i$ and $[Ca^{2+}]_i$ during treatment with c AMP-containing saline affects different Cl^- entry mechanisms, one leading to the Type A response, the other inducing the Type B response. Thus, whatever mechanism is dominant, at a given time, may determine whether a Type A or Type B response is exhibited in high K^+ saline.

Oscillations

Results from the Malpighian tubules of Locusta throughout the present study showed that cyclic oscillations of V_A and T.E.P.

occurred in Normal saline in just over 50% of cells. The amplitude of these oscillations was increased only in high K^+ saline and Normal saline containing ouabain and cAMP, but decreased in HCO_3^- free saline + acetazolamide and Normal saline with furosemide. The frequency of oscillation was increased only in Ca^{2+} free saline but decreased in K^+ free, vanadate, amiloride, Cl^- free, A23187 (Ca^{2+} free) and SCN^- salines.

Transient oscillations of T.E.P., similar to those found in the present study, have been found in the Malpighian tubules of Locusta (Morgan and Mordue, 1981), Carausius (Pilcher, 1970) and Aedes (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985a) and the salivary glands of Calliphora (Rapp and Berridge, 1981). The present findings show that the oscillations were solely a feature of the apical membrane, being copied exactly by T.E.P. This result differs from that of Aedes tubules in which the spontaneous transient oscillations are due to changes in the basolateral membrane (Sawyer and Beyenbach, 1985a). Sawyer and Beyenbach (1985a) suggest that these changes may be due to a transient increase of the K conductance at this membrane. It is not clear what role these oscillations play in the mechanism of fluid secretion in Malpighian tubules (Williams and Beyenbach, 1984). Morgan and Mordue (1981) have suggested that these changes are somehow related to control of the secretion rate by cyclical fluxing of anions and cations across the tubule wall. Similarly, Rapp and Berridge (1981) have suggested that the oscillations of T.E.P. in Calliphora salivary glands may be driven by an oscillation in the intracellular concentration of cAMP and calcium and that oscillatory control provides a reliable strategy for controlling secretion rate and regulating ion transport across epithelia.

However, despite the speculation above, results from the present study do not provide a clear picture to explain the cause and function of V_A oscillations in the tubules of Locusta.

CHAPTER 4

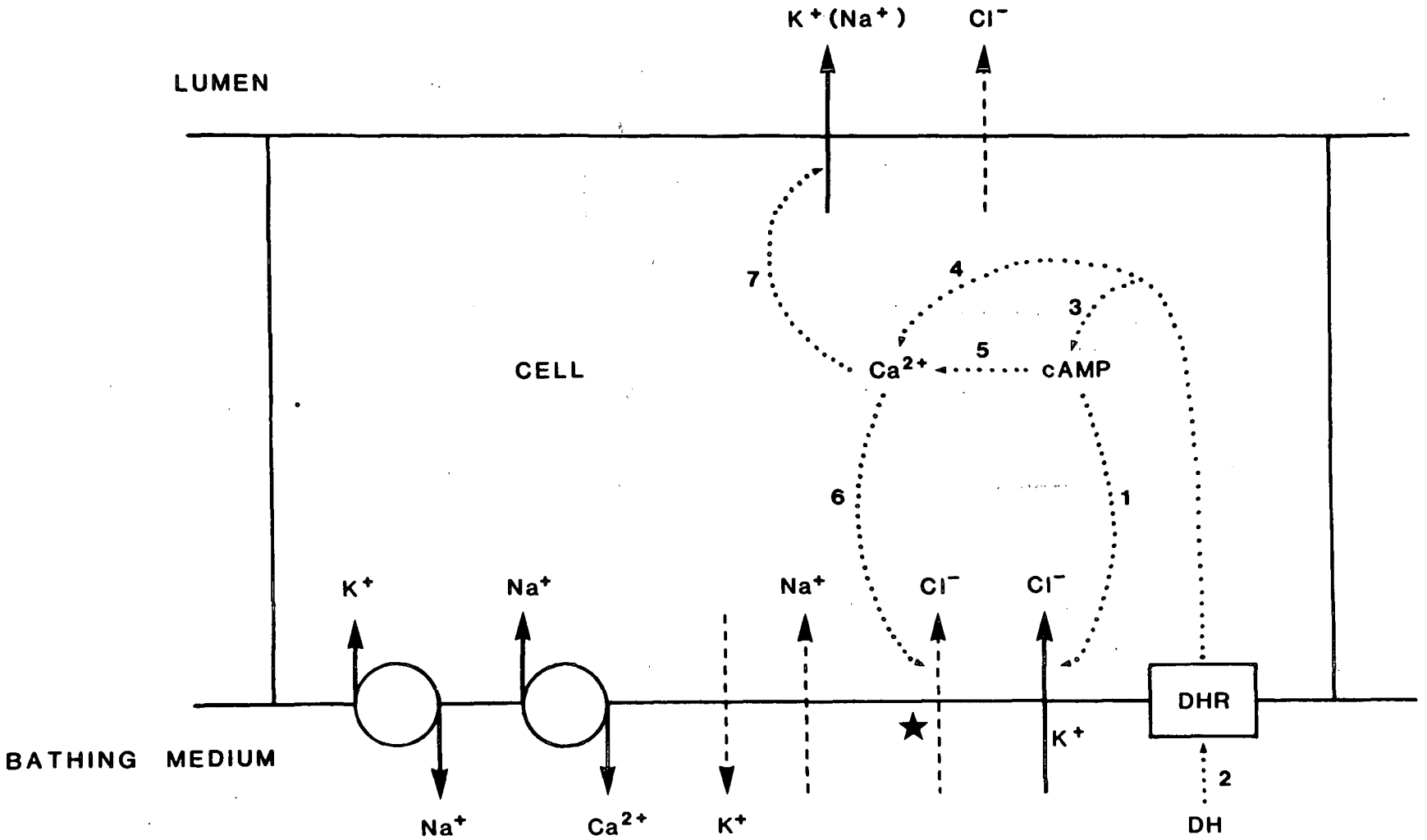
Conclusion

With the information obtained from ion substitution and the application of different pharmacological agents in Chapter 3, a hypothetical model can be postulated to describe the ionic fluxes which support fluid secretion by the Malpighian tubule of Locusta. This model can be seen in Fig. 40. As mentioned earlier, Na^++K^+-ATPase activity has been reported in this tissue (Anstee and Bell, 1975; 1972; Donkin and Anstee, 1980) and has been confirmed in the present study (also see Anstee et al., 1986). Ouabain-binding studies on microsomal preparations from Malpighian tubules of Locusta showed that the pump-site density and turnover activity of the Na^++K^+-ATPase is adequate to account for substantial K^+ transport across the tubules of Locusta. Thus, in Fig. 40, it is suggested that a Na^++K^+-ATPase occurs in Locusta tubule cells and since in the majority of tissues studied (Di Bona and Mills, 1979; Ernst et al;, 1980) this enzyme is associated with the basolateral membranes, it is proposed that it is responsible for the entry of K^+ into the cell in exchange for Na^+ across the basal membrane, thereby maintaining the Na gradient. This suggestion is supported by the observation, in the present study, that the Na^++K^+ pump inhibitors, ouabain and vanadate (Wallick et al., 1980, Kazazoglou et al., 1983; Phillips et al:, 1983; Nechay et al., 1986), both decreased the membrane potentials. Thus, inhibition of the enzyme probably results in an inability to maintain the K^+ and Na^+ gradient (decreasing V_B) with an ensuing reduction in K^+ exit across the apical membrane (decreasing V_A). In common with the present study, a Na^++K^+-ATPase has also been proposed to maintain the cellular gradients in the Malpighian tubules of

Fig. 40

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

In this model it is suggested that a basal membrane $Na^+ + K^+$-ATPase maintains the cellular gradients. Intracellular levels of 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 a passive entry of Na^+ with its concentration and electrical gradient occur across the basal membrane. It may be that a basal K^+ dependent Cl^- entry (which is stimulated [1] by $[c\,AMP]_i$) and an apical K^+ pump (which may also extrude Na^+) also occur in the tubules of Locusta. During fluid secretion, diuretic hormone (D.H.) may act (2) on a basal membrane receptor (D.H.R.) to elevate (3 and 4) intracellular Ca^{2+} and c AMP with c AMP, in turn, stimulating (5) a further increase in $[Ca^{2+}]_i$. The elevated $[c\,AMP]_i$ stimulates (1) K^+ dependent Cl^- entry whilst the elevated Ca^{2+} stimulates (6 and 7) an additional Cl^- permeability (★) and the activity of the apical K^+ pump. These steps ensure enough Cl^- crosses the cell to act as a counterion for K^+ during D.H.-stimulated fluid secretion. Finally, it is also proposed that Cl^- exit from the Malpighian tubule cells of Locusta may occur passively by following the active extrusion of K^+.


K+(Na+)
Cl−
LUMEN
7
4
3
5
CELL
Ca2+
cAMP
6
1
K+
Na+
Na+
Cl−
Cl−
DHR
K+
BATHING MEDIUM
2
Na+
Ca2+
K+
DH
----- PASSIVE MOVEMENTS
—— ACTIVE MOVEMENTS

Rhodnius (Maddrell, personal communication) and Glossina (Gee, 1975; 1976) and the salivary glands of Calliphora (Berridge, 1980).

The presence of a large negative apical membrane potential (ca. 80 mV) indicates an adverse electrical gradient for K^+ and Na^+ (but not Cl^-) exit (see Fig. 27, Chapter 3) across the apical membrane suggesting that some form of cation pump is required at this membrane in the tubules of Locusta. Hence, an apical electrogenic K^+ pump is proposed in Fig. 40 for similar reasons to that suggested elsewhere (Berridge, 1968; Maddrell, 1977; Morgan and Mordue, 1983a; O'Donnell and Maddrell, 1984). This pump is thought to be relatively unspecific, transporting Na^+ or K^+ out of the cells (Maddrell, 1977; O'Donnell and Maddrell, 1984). However, it is suggested that in K^+ secreting tubules which includes Locusta and the majority of other species of insects studied (Anstee and Bell, 1975; Maddrell, 1977), it is mainly K^+ which is extruded by the apical pump (Maddrell, 1977) with Cl^- following passively as proposed for Rhodnius (O'Donnell and Maddrell, 1984) and Locusta (Morgan and Mordue, 1983a).

In the tubules of Locusta, the basal membrane acts largely as a K^+ electrode with other ions contributing little to the resting potential. Indeed, results showed that the basal membrane is relatively impermeable to Na^+ and Cl^- with membrane permeability calculations (Goldman, 1943), indicating that this membrane was approximately 100 times more permeable to K^+ than Na^+. Similarly, O'Donnell and Maddrell (1984) have found the basal membrane of the tubules of Rhodnius to be largely impermeable to Na^+ and Cl^- and postulated these two ions may enter the tubule cell by means of a $Na^+K^+Cl^-$ cotransporter. Morgan and Mordue (1983a) have suggested that Cl^- entry may be Na^+ and/or K^+ dependent in the tubules of Locusta. In contrast, the Malpighian tubules of Aedes appear to

have basolateral conductances for K^+ and Na^+ of similar magnitude (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985a).

The hyperpolarization of V_A found in the present study with the NaCl and NaKCl cotransport inhibitors furosemide and bumetanide (Palfrey and Rao, 1985) and the NaCl transport inhibitor SCN^- (Epstein et al., 1973) may suggest that NaCl cotransport occurs in the tubules of Locusta, with inhibition of Cl^- entry reducing the availability of this anion to follow the extrusion of K^+ at the apical surface. However, if such a mechanism occurs in the tubules of Locusta, it is difficult to explain why Cl^- free saline had no effect on V_B and hyperpolarized V_A whilst application of Na^+ free saline resulted in a depolarization of both V_B and V_A. Thus, unlike the tubules of Rhodnius (O'Donnell and Maddrell, 1984) the lack of similarity of effect of Cl^- free and Na^+ free salines on V_B and V_A may suggest that Cl^- entry is not necessarily dependent on cotransport with Na^+ in Locusta. It may be, of course, that some Cl^- movements are possible across the basal membrane through a cotransport with K^+ under Na^+ free conditions. Indeed, O'Donnell and Maddrell (1984) have suggested that whilst Cl^- entry was normally through cotransport with both Na^+ and K^+ in the tubules of Rhodnius, with a suggested stoichimetry of $Na^+:K^+:2\,Cl^-$, in the absence of either cation other stoichimetries might be possible, such as $2\,Na^+:2\,Cl^-$ or $2\,K^+:2\,Cl^-$. The hyperpolarization of V_B observed in Locusta, in response to furosemide, is difficult to attribute to a direct action of this agent on a cotransport process, particularly as no such effect on V_B was observed with bumetanide.

The lack of firm evidence for the presence of a NaKCl cotransporter in the tubules of Locusta may suggest that Cl^- enters the cells by another mechanism. This mechanism may be in the form

of Cl^--HCO_3^- exchange, as proposed for Aedes rectal gland (Strange and Phillips, 1984), Cl^-+HCO_3^--ATPase as proposed for Aplysia intestinal epithelium (Gerenscer and Lee, 1983) or some form of KCl transport such as the KCl symporter of Necturus gallbladder and mammalian kidney diluting segment (Corcia and Armstrong, 1983; Greger, 1985) or the K^+-stimulated electrogenic Cl^- transport system of locust rectum (Hanrahan and Phillips, 1983). As mentioned in Chapter 3, the present or absence of a Cl^--HCO_3^- exchanger or a Cl^-+HCO_3^--ATPase in the basal membrane of Locusta tubule cells cannot be resolved on the basis of results obtained from the present study. Whilst anion-ATPase has been demonstrated in the Malpighian tubules of Locusta (Anstee and Fathpour, 1979; 1981), the controversy as to whether the latter enzyme is associated with plasma membrane fractions (Anstee and Fathpour, 1981; Du Pont and Bonting, 1981; Gerenscer and Lee, 1983) needs to be resolved before definite conclusions can be made about its role in ion transport in this tissue.

In Fig. 40 it is proposed that Cl^- entry occurs by means of a K^+ dependent mechanism. Evidence for this proposal comes from the observation that the depolarization of V_A in high K^+ saline was greatly reduced in the absence of Cl^- suggesting that when Cl^- was unavailable to enter the cell there was no short-circuiting of the extrusion of positive ions by the apical pump. Thus, under conditions in which Cl^- was available, a basal K^+ dependent Cl^- entry can be deduced from the fact that there is a greater depolarization of V_A in high $[K^+]_o$ saline due to increased short-circuiting of the electrogenic pump by Cl^- movement. The reduced level of depolarization of V_B in high K^+ saline (compared with the Type A response) observed in association with the Type B response, may be due to a short-circuiting of V_B by an increase in basal Cl^- permeability. However,

the latter is likely to be a process distinct from the K^+ dependent Cl^- entry referred to earlier. Thus, it is suggested that upon application of high K^+ saline to cells which exhibit the Type B response, V_B began to depolarize in a typical Nernstian fashion until a point when an increase in Cl^- permeability occurred, hyperpolarizing V_B. This is supported by the observation that the Type B hyperpolarization of V_B showed some Cl^- dependence. However, the fact that this hyperpolarization was not totally removed in Cl^- free conditions suggests that perhaps another anion may substitute, in part, for Cl^- when the latter is absent from high K^+ saline. The paradox is that if the Type A depolarization of V_A is due to Cl^- availability as a counterion, why, despite the apparent increase in Cl^- entry during the Type B response, V_A did not depolarize further than in a Type A response. Indeed, V_A hyperpolarized. This may be explained as follows. Initially, V_A depolarized in high K^+ saline presumably as a result of Cl^- 'shorting' the apical K^+ pump. The subsequent hyperpolarization of V_A (a Type B response) may be accounted for by an increase in the activity of the apical electrogenic cation pump. This is supported by the observation that the Type B hyperpolarization of V_A increased further when Cl^- was removed from the high K^+ bathing medium; indicating reduced counterion availability to follow K^+ out of the cell.

The question arises as to how the basal and apical membrane permeability changes might be coupled in the tubules of Locusta. Ca^{2+} and c AMP, in the form of secondary messengers, are well known as conveyors of information, linking both surfaces of the cell, in many tissues (Berridge, 1980). Furthermore, as mentioned in Chapter 3, Ca^{2+} has been reported to regulate Cl^- permeability in many transporting epithelia (Berridge, 1980; Nauntofte and Poulsen,

1986) whilst c AMP is thought to activate apical K^+ transport in the salivary glands of Calliphora (Berridge, 1980). In addition, intracellular c AMP has been reported to increase Cl^- permeability (albeit in the apical membrane) in the tubules of Rhodnius (Phillips, 1982) as well as leading to a mobilization of intracellular Ca^{2+} in a number of transporting epithelia (Berridge, 1980). In Fig. 40 it is suggested that the stimulation of basal Cl^- permeability and the apical K^+ pump which appear to be responsible for producing the Type B response may be due to changes in $[Ca^{2+}]_i$. Thus, it may be that in addition to possessing a K^+ dependent Cl^- entry step, increased intracellular Ca^{2+}, in cells which exhibit the Type B response, leads to a stimulation of a separate Cl^- permeability which results in a hyperpolarization of V_B. Increased $[Ca^{2+}]_i$ may also lead to an activation of the apical K^+ pump, hyperpolarizing V_A. Although the latter suggestion is speculative, a role for Ca^{2+} in controlling the K^+ pump in Calliphora salivary glands (but perhaps not directly) has been suggested by Berridge et al. (1975). It is further postulated in Fig. 40 that during the Type B response elevated $[c\,AMP]_i$ may increase basal Cl^- permeability and the activity of the apical pump, hyperpolarizing V_B and V_A, but this may be an indirect effect through an increase in $[Ca^{2+}]_i$. Evidence for the above speculation comes from the observation that, although the Type B response occurred naturally in only one fifth of cells, it could be artificially induced in all cells in high K^+ saline by pretreatment in a variety of modified normal K^+ salines, all of which may alter $[Ca^{2+}]_i$ (see later). In addition, the immediate hyperpolarization of V_B and V_A upon introduction of many of these modified normal K^+ salines may also be due to a Ca^{2+} stimulation of Cl^- entry and the activity of the apical K^+ pump.

A change in $[Ca^{2+}]_i$ may occur through an alteration in the functioning of a basal Na^+-Ca^{2+} exchanger (see Chapter 3). Such an exchanger is postulated in Fig. 40. Na^+-Ca^{2+} exchanger, which is thought to be electrogenic with 3-5 Na^+ being exchanged for 1 Ca^{2+} (Mullins, 1979; Chase, 1984), has been proposed to extrude excess intracellular calcium in various transporting epithelia (Taylor and Windhager, 1979; Scoble et al., 1986). The fact that Ca^{2+} free saline hyperpolarized V_B (perhaps by inhibiting Na^+-Ca^{2+} exchange through a reduction in the amount of Ca^{2+} extruded and exposing its electrogenicity) may provide some evidence for the presence of this mechanism in the tubules of Locusta. It may be that the hyperpolarization of V_B in various modified, normal K^+ salines in the present study was due to an inhibition of Na^+-Ca^{2+} exchange increasing $[Ca^{2+}]_i$ which stimulated Cl^- entry. Indeed, Chase (1984) has shown in transporting epithelia, that when Na^+ is raised, as after inhibition of the Na^++K^+ pump, the direction of the Na^+-Ca^{2+} exchanger reversed, sending Ca^{2+} into the cell. Because an increase in cell Na^+ has the additional effect of reducing the affinity of the exchanger for Ca^{2+}, the extrusion of calcium declines rapidly. Thus, the increase in $[Na^+]_i$ following treatment with monensin (Lichtshtein et al., 1979) and the non-specific inhibition of the Na^++K^+-ATPase (increasing $[Na^+]_i$) in furosemide- and verapamil-containing salines (Greger, 1985; Janis and Scriabine, 1983) may increase $[Ca^{2+}]_i$. This, in turn, may stimulate Cl^- entry, hyperpolarizing V_B. Similarly, the hyperpolarization of V_A in monensin-, furosemide- and verapamil-containing salines may be due to an increase in $[Ca^{2+}]_i$ resulting in a stimulation of the activity of the K^+ pump. In addition, the Type B response, for both V_B and V_A, found in high K^+ saline after pretreatment with

monensin-, furosemide- and verapamil-containing salines may be due to the increase in $[Ca^{2+}]_i$ stimulating Cl^- entry and the activity of the apical K^+ pump respectively. The Type B response, induced in cells following pretreatment with K^+ free-, ouabain- or SITS-containing saline may also be the result of a stimulation of a basal Cl^- entry and apical K^+ pump, by increased intracellular Ca^{2+}, due to a change in the Na^+ gradient. A reduction in the Na^+ gradient during K^+ free and ouabain treatment (Livengood and Kusano, 1972; Giraldez, 1984) has been shown to inhibit Na^+-Ca^{2+} exchange reducing Ca^{2+} efflux (Cruz-Soto et al., 1984; Lorenzen et al., 1984). The nonspecific inhibitory action of SITS on the Na^++K^+-ATPase (Ehrenspeck and Brodsky, 1976) may lead to an increase in $[Ca^{2+}]$, as mentioned earlier, due to a reduction in the functioning of the Na^+-Ca^{2+} exchange (Chase, 1984). Finally, as discussed in Chapter 3, it is difficult to explain the action of Ba^{2+}, in the present study, in terms of its reported inhibition of K^+ channels (Nagel, 1980; Welsh, 1983; Hanrahan et al., 1986). Thus, the hyperpolarization, in normal K^+,Ba^{2+}-containing and the ensuing Ba^{2+} induced Type B response in high K^+ saline, of both V_B and V_A, may be due to Ba^{2+} acting in a manner similar to Ca^{2+}, as has been shown elsewhere (Bylerly and Hagiwara, 1982, Mandel and Murphy, 1984) to stimulate a basal Cl^- entry and the activity of the apical K^+ pump.

If an elevation in $[Ca^{2+}]_i$ directly (or indirectly through the action of intracellular c AMP increasing $[Ca^{2+}]_i$) results in the stimulation of a basal Cl^- permeability and the apical K^+ pump, then it seems odd that normal K^+, c AMP-containing saline (which may increase $[Ca^{2+}]_i$) hyperpolarized V_B and V_A but did not result in a Type B response in high K^+ saline containing c AMP. In contrast, normal K^+,Ca^{2+} free saline (which would not be expected to increase $[Ca^{2+}]_i$) hyperpolarized V_B and V_A and resulted in a Type B response in high K^+,Ca^{2+} free saline. However, from the model in Fig. 40 (in which it is suggested that intracellular c AMP controls K^+ dependent Cl^- entry), it may be postulated that in normal K^+,c AMP-

containing saline a large elevation of $[c\,AMP]_i$ increases the action of the K^+ dependent Cl^- entry. In addition, increased intracellular c AMP stimulates a basal Cl^- permeability and the activity of the apical K^+ pump (hyperpolarizing V_A) by increasing $[Ca^{2+}]_i$ through a mobilization from intracellular stores. As the K^+ dependent Cl^- entry is limited by $[K^+]_o$, the Ca^{2+} activated Cl^- permeability increase is dominant, hyperpolarizing V_B. With the introduction of high K^+ saline however, $[K^+]_o$ is no longer limiting, resulting in K^+ dependent Cl^- entry increasing in significance. Thus, even though a Ca^{2+} stimulated Cl^- permeability and activity of the apical K^+ pump occurs in high K^+ saline, the increased K^+ dependent Cl^- entry in high levels of intracellular c AMP results in enough transcellular Cl^- movement to mask the predicted hyperpolarization of V_B and V_A (the Type B response) producing a Type A response. As mentioned in Chapter 3, the unexpected Type B response found in high K^+,Ca^{2+} free saline after Ca^{2+} free saline pretreatment cannot be explained on evidence currently available.

It may be asked how are increases in intracellular Ca^{2+} and c AMP, which may lead to the c AMP stimulated K^+ dependent Cl^- entry (basal membrane) and the Ca^{2+} stimulated Cl^- permeability (basal membrane) and K^+ pump (apical membrane) seen in the proposed model for the tubule cells of Locusta, physiologically controlled? The hormonal control mechanism of fluid secretion may be the answer. As mentioned in the Introduction, it is postulated in the salivary glands of Calliphora that hormonal interaction at a basal membrane receptor leads to an increase in intracellular Ca^{2+} and c AMP and these secondary messengers are then responsible for greatly accelerating the secretion of ions and water (Prince and Berridge, 1973; Berridge, 1980; Berridge and Heslop, 1982). In addition, it is thought that intracellular Ca^{2+} is further increased due

to mobilization by another secondary messenger, inositol 1,4,5-triphosphate, which is also generated by receptor activation (Berridge et al., 1984; Berridge, 1986). In the tubules of Locusta, Morgan and Mordue (1984) have suggested that diuretic hormone may stimulate two distinct receptors on the basal membrane, one leading to an increase in $[Ca^{2+}]_i$, the other $[c\,AMP]_i$.

Although beyond the scope of the present study, it can be suggested that the proposed systems for hormonal control in the salivary glands of Calliphora (Berridge and Heslop, 1982) and the tubules of Locusta (Morgan and Mordue, 1984) may be applied to the conclusions of the present study. Thus, from the model in Fig. 40 it can be postulated that under unstimulated conditions a c AMP-controlled K^+ dependent Cl^- entry step occurs in the basal membrane of Locusta tubules. Under these conditions, application of high K^+ saline leads to the production of the Type A response for both V_B and V_A as c AMP controlled K^+ dependent Cl^- entry is the dominant mechanism for anion entry. However, stimulation of fluid secretion by diuretic hormone may necessitate an increase in the amount of anion crossing the cell, to act as a counterion for the increased movement of K^+. Activation by diuretic hormone may increase $[c\,AMP]_i$ and $[Ca^{2+}]_i$, through stimulation of a basal receptor. In addition, $[Ca^{2+}]_i$ may be further increased through an intracellular mobilization by c AMP and/or inositol 1,4,5-triphosphate. These increases in intracellular c AMP and Ca^{2+} may then lead to an increase in K^+ dependent Cl^- entry (c AMP) and stimulation of a basal Cl^- permeability (Ca^{2+}) and the apical K^+ pump (Ca^{2+}). Under these conditions, application of high K^+ saline leads to the production of the Type B response for both V_B and V_A as the Ca^{2+} stimulated steps are dominant. Indeed, cells which exhibited the

naturally occurring Type B response in high K^+ saline in the present study may, in fact, have been activated with diuretic hormone before the tubules were dissected out. This would have resulted in the electroneutral c AMP stimulated K^+ dependent Cl^- entry being masked by the high $[Ca^{2+}]_i$ stimulated Cl^- permeability (hyperpolarizing V_B) and activity of the K^+ pump (hyperpolarizing V_A). In addition, many of the modified salines used in the present study may have induced the diuretic hormone activated state, artificially exposing the Ca^{2+} activation of the basal Cl^- permeability and the apical K^+ pump.

Finally, unlike the model to explain ion transport in the Malpighian tubules of Rhodnius (O'Donnell and Maddrell, 1984) the lack of strong evidence for a linked NaCl entry in Fig. 40 makes it necessary to explain how Na^+ enters the tubule cell of Locusta. Some Na^+ will enter through the functioning of a Na^+-Ca^{2+} exchange. In addition, although the basal membrane is relatively impermeable to Na^+, this cation may leak into the cell down its considerable electrical and concentration gradients (see Fig. 27, Chapter 3). Fig. 40 also shows how Na^+ may exit the cell across the apical membrane. Thus, it is speculated that, as proposed for Rhodnius tubules by O'Donnell and Maddrell (1984), although mainly K^+ extruding, the apical pump is unspecific enough to transport small amounts of Na^+ out of the Locusta tubule cell.

In conclusion, the results obtained in the present study, and incorporated in the hypothetical model (Fig. 40), indicate that both Ca^{2+} and c AMP play a central role in controlling ion movement across the basal and apical membranes of cells of the Malpighian tubules of Locusta.

REFERENCES

Aboulafia, J. and Lacaz-Vieira, F. (1984). Vanadate and ouabain: a comparative study in toad skin. Pflügers Arch. 401, 204-208.

Ahearn, G.A. (1980). Intestinal electrophysiology and transmural ion transport in freshwater prawns. Am. J. Physiol. 239, 1-10.

Albers, R.W., Koval, G.J. and Siegel, G.J. (1968). Studies on the interaction of ouabain and other cardio-active steroids with sodium-potassium-activated adenosine triphosphate. Mol. Pharmacol. 4, 324-336.

Alonso, M.A. and Carrasco, L. (1982). Molecular basis of the permeabilization of mammalian cells by ionophores. Eur. J. Biochem. 127, 567-569.

Andersen, E. and Harvey, W.R. (1966). Active transport by the cecropia midgut. II. Fine structure of the midgut epithelium. J. Cell Biol. 31, 107-34.

Anstee, J.H., Baldrick, P. and Bowler, K. (1986). Studies on ouabain-binding to $(Na^+ + K^+)$-ATPase from Malpighian tubules of the locust, Locusta migratoria L. Biochim. biophys. Acta 860, 15-24.

Anstee, J.H. and Bell, D.M. (1975). Relationship of $Na^+ + K^+$-activated ATPase to fluid production by Malpighian tubules of Locusta migratoria. J. Insect Physiol. 21, 1779-1784.

Anstee, J.H. and Bell, D.M. (1978). Properties of $Na^+ + K^+$-activated ATPase from the excretory system of Locusta. Insect Biochem. 8, 3-9.

Anstee, J.H., Bell, D.M. and Fathpour, H. (1979). Fluid and cation secretion by the Malpighian tubules of Locusta. J. Insect Physiol. 25, 373-380.

Anstee, J.H., Bell, D.M. and Hyde, D. (1980). Some factors affecting Malpighian tubule fluid secretion and transepithelial potential in Locusta migratoria L. Experientia 36, 198-199.

Anstee, J.H. and Bowler, K. (1979). Ouabain-sensitivity of insect epithelial tissues. Comp. Biochem. Physiol. 62A, 763-769.

Anstee, J.H. and Bowler, K. (1984). Techniques for studying Na^+, K^+-ATPase. In Measurement of Ion Transport and Metabolic Rate in Insects. Ed. Bradley, T.J. and Miller, T.A., Springer-Verlag, New York. pp. 187-220.

Anstee, J.H. and Fathpour, H. (1979). The presence and properties of a Mg^{2+}-dependent HCO_3^- stimulated ATPase in the Malpighian tubules of Locusta migratoria. Insect Biochem. 9, 383-388.

Anstee, J.H. and Fathpour, H. (1981). Studies on the anion-sensitivity, oligomycin-sensitivity and sub-cellular localization of adenosine triphosphatase activity in Malpighian tubules of Locusta. Insect Biochem. 11, 103-115.

Armstrong, W.McD., Suh, T.K. and Gerencser, G.A. (1972). Stimulation by anoxia of active chloride transfer in isolated bullfrog small intestine. Biochim. biophys. Acta 255, 647-662.

Aronson, P.S. (1981). Identifying secondary active solute transport in epithelia. Am. J. Physiol. 240, 1-11.

Aronson, P.S. (1983). Mechanisms of active H^+ secretion in proximal tubule. Am. J. Physiol. 245, 647-659.

Aston, R.J. (1979). Studies on the diuretic hormone of Rhodnius prolixus. Some observations on the purification and nature of the hormone and the dynamics of its release in vitro. Insect Biochem. 9, 163-176.

Aston, R.J. and Hughes, L. (1980). Diuretic hormone-extraction and chemical properties. In Neurohormonal Techniques in Insects. Ed. Miller, T.A., Springer, New York, Chap. 4. pp. 91-115.

Atkinson, A., Gatenby, A.D. and Lowe, A.G. (1973). The determination of inorganic orthophosphate in biological systems. Biochim. biophys. Acta 320, 195-204.

Atzbacher, U., Hevert, F., Weber-Von Grotthuss, E. and Wessing, A. (1974). The influence of ouabain on the elimination of injected and orally applied dyes in Drosophila hydei. J. Insect Physiol. 20, 1989-1997.

Baker, P.F. and Willis, J.S. (1972). Binding of the cardiac glycoside ouabain to intact cells. J. Physiol. Lond. 224, 441-462.

Barkai, A.I. and Williams, R.W. (1983). The exchange of calcium in larvae of the mosquito Aëdes aegypti. J. exp. Biol. 104, 139-148.

Barry, J. and Diamond, J.M. (1970). Junction potentials, electrode standard potentials and other problems in interpreting electrical properties of membranes. J. Membrane Biol. 3, 93-122.

Bello-Reuss, E., Grady, T.P. and Reuss, L. (1981). Mechanism of action of the effect of cyanide on cell membrane potentials in Necturus gall bladder epithelium. J. Physiol., Lond. 314, 343-357.

Benos, D.J. (1982). Amiloride: a molecular probe of sodium transport in tissues and cells. Am. J. Physiol. 242, 131-145.

Bentley, P.J. (1968). Amiloride: a potent inhibitor of sodium transport across the toad bladder. J. Physiol., Lond. 195, 317-330.

Berridge, M.J. (1965). The physiology of excretion in the cotton stainer Dysdercus fasciatus (Signoret). 1. Anatomy, water excretion and osmoregulation. J. exp. Biol. 43, 511-521.

Berridge, M.J. (1967). Ion and water transport across epithelia. In Insects and Physiology. Ed. Beamont, J.W.L. and Treherne, J.E., Oliver and Boyd, Edinburgh and London. pp. 329-347.

Berridge, M.J. (1968). Urine formation by the Malpighian tubules of Calliphora. I. Cations. J. exp. Biol. 48, 159-174.

Berridge, M.J. (1980). The role of cyclic nucleotides and calcium in the regulation of chloride transport. Ann. N.Y. Acad. Sci. 341, 157-171.

Berridge, M.J. (1986). Inositol triphosphate and diacylglycerol as intracellular second messengers. In Mechanisms of Receptor Regulation. Ed. Poste, G. and Crooke, S.T., Plenum Press, New York and London. pp. 111-130.

Berridge, M.J., Buchan, P.B. and Heslop, J.P. (1984). Relationship of polyphosphoinositide metabolism to the hormonal activation of the insect salivary gland to 5-hydroxytryptamine. Mol. Cell. Endocrinol. 36, 37-42.

Berridge, M.J. and Heslop, J.P. (1982). Receptor mechanisms mediating the action of 5-hydroxytryptamine. In Neuropharmacology of Insects Ciba Foundation Symposium 88. Ed. Evered, D., O'Connor, M. and Whelan, J., Pitman, London. pp. 260-274.

Berridge, M.J., Lindley, B.D. and Prince, W.T. (1975). Stimulus-secretion coupling in an insect salivary gland: cell activation by elevated potassium concentrations. J. exp. Biol. 62, 629-639.

Berridge, M.J., Lindley, B.D. and Prince, W.T. (1976). Studies on the mechanism of fluid secretion by isolated salivary glands of Calliphora. J. exp. Biol. 64, 311-322.

Berridge, M.J. and Oschman, J.L. (1969). A structural basis for fluid secretion by Malpighian tubules. Tissue and Cell 1, 247-272.

Berridge, M.J. and Patel, N.G. (1968). Insect salivary glands: stimulation of fluid secretion by 5-hydroxytryptamine and adenosine-3', 5'-monophosphate. Science, N.Y. 162, 462-463.

Berridge, M.J. and Prince, W.T. (1972). Transepithelial potential changes during stimulation of isolated salivary glands with 5-hydroxytryptamine and cyclic AMP. J. exp. Biol. 56, 139-153.

Berridge, M.J. and Schlue, W.R. (1978). Ion-selective electrode studies on the effects of 5-hydroxtryptamine on the intracellular level of potassium in an insect salivary gland. J. exp. Biol. 72, 203-216.

Beyenbach, K.W. and Frömter, E. (1985). Electrophysiological evidence for Cl secretion in shark renal proximal tubules. Am. J. Physiol. 248, 282-295.

Blaustein, M.P. (1974). The interrelationship between sodium and calcium fluxes across cell membranes. Rev. Physiol. Biochem. Pharmacol. 70, 33-82.

Bonting, S.L. (1970). Sodium-potassium activated adenosine-triphosphatase and cation transport. In Membranes and Ion Transport (Vol. 1). Ed. Bittar, E.E., John Wiley & Sons Ltd., Woking and London. pp. 257-350.

Boron, W.F. and Boulpaep, E.L. (1983). Intracellular pH regulation in the renal proximal tubule of the salamander. J. Gen. Physiol. 81, 29-52.

Byerly, L. and Hagiwara, S. (1982). Calcium currents in internally perfused nerve cell bodies of Limnea stagnalis. J. Physiol., Lond. 322, 503-528.

Caldwell, P.C., Hodgkin, A.L., Keynes, R.D. and Shaw, T.I. (1960). Partial inhibition of the active transport of cations in the giant axons of Loligo. J. Physiol., Lond. 152, 591-600.

Candia, O.A. and Cook, P. (1986). Na^++K^+ pump stoichiometry and basolateral membrane permeability of frog corneal epithelium. Am. J. Physiol. 250, 850-859.

Cantley, L.C. (1981). Structure and mechanism of the (Na,K)-ATPase. In Current Topics in Bioenergetics, Vol. II. Ed. Sanadi, D.R., Academic Press, New York. pp. 201-237.

Cantley, L.C., Jr. and Aisen, P. (1979). The fate of cytoplasmic vanadium. Implications on (Na,K)-ATPase inhibition. J. Biol. Chem. 254, 1781-1784.

Cantley, L.C., Josephson, L., Warner, R., Yamagisawa, M., Lechene, C. and Guidotti, G. (1977). Vanadate is a potent (Na-K)ATPase inhibitor in ATP derived from muscle. J. Biol. Chem. 252, 7421-7423.

Carafoli, E. and Zurini, M. (1982). The Ca^{2+}-pumping ATPase of plasma membranes: purification, reconstitution and properties. Biochim. biophys. Acta 683, 279-301.

Cardinal, J., Lapointe, J.-Y. and Laprade, R. (1984). Luminal and peritubular ionic substitutions and intracellular potential of rabbit proximal convoluted tubule. Am. J. Physiol. 247, 352-364.

Chandler, D.E. and Williams, J.A. (1977). Intracellular uptake and α-amylase and lactate dehydrogenase releasing actions of the divalent cation ionophore, A23187 in dissociated pancreatic acinar cells. J. Membrane Biol. 32, 201-230.

Chappell, J.B. (1964). The oxidation of citrate, isocitrate and cisaconitate by isolated mitochondria. Biohcem. J. 90, 225-237.

Charnley, A.K. (1982). The ultrastructure of the Type 2 cells in the Malpighian tubules of Locusta migratoria. Micron 13, 45-48.

Chase, H.S., Jr. (1984). Does calcium couple the apical and basolateral membrane permeabilities in epithelia? Am. J. Physiol. 247, 869-876.

Chase, H.S. and Al-Awqati, Q. (1981). Regulation of the sodium permeability of the luminal border of toad bladder by intracellular sodium and calcium. Role of sodium-calcium exchange in the basolateral membrane. J. Gen. Physiol. 77, 693-712.

Christensen, A.K. (1975). Leydig cells. In Handbook of Physiology. Ed. Berne, R.M. Vol. 5. American Physiological Society, Bethesda, M.D. pp. 57-94.

Clark, L.C., Jr. (1956). Monitor and control of blood and tissue oxygen tension. Trans. Ann. Soc. artif. internal organs 2, 41-65.

Coast, G.M. (1969). Formation of urinary fluid by Malpighian tubules of an insect. J. Physiol., Lond. 202, 102P-103P.

Cohen, I.S. (1983). Can blocking the Na/K exchange pump lead to a reduction in intracellular sodium? Experientia 39, 1280-1282.

Colburn, T.R. and Schwartz, E.A. (1972). Linear voltage control of current passed through a micropipette with variable resistance. Med. and Biol. Eng. 10, 504-509.

Cole, C.H. (1979). Bicarbonate-activated ATPase activity in renal cortex of chronically acidotic rats. Can. J. Physiol. Pharmac. 57, 271-276.

Corcia, A. and Armstrong, W.M. (1983). KCl cotransport: a mechanism for basolateral chloride exit in Necturus gallbladder. J. Membrane Biol. 76, 173-182.

Cox, T.C. and Helman, S.I. (1986). Na^+ and K^+ transport at basolateral membranes of epithelial cells. II. K^+ efflux and stoichiometry of the Na^+,K^+-ATPase. J. Gen. Physiol. 87, 485-502.

Cruz-Soto, M., Benabe, J.E., López-Novoa, J.M. and Martinez-Maldonado, M. (1984). Na^+-K^+-ATPase inhibitors and renin release: relationship to calcium. Am. J. Physiol. 247, 650-655.

Cuthbert, A.W., Herrera, F.C., Schuz, A.D. and Wilson, S.A. (1980). Increase in epithelial cyclic adenosine 3',5'-monophosphate following vanadate. Br. J. Pharmacol. 69, 8-10.

Dalton, T. and Windmill, D.M. (1980). Fluid secretion by isolated Malpighian tubules of the housefly Musca domestica. J. Insect Physiol. 26, 281-286.

Datta, S. (1971). Effects of inhibitors and tonicity on the hindgut potential across the isolated intestine of the cockroach Byrostria fumigata. Indian J. Exp. Biol. 9, 36-39.

Davis, C.W. and Finn, A.L. (1982). Sodium transport inhibition by amiloride reduces basolateral membrane potassium conductance in tight epithelia. Science 216, 525-527.

Davis, W.C. and Finn, A.L. (1985). Effects of mucosal sodium removal on cell volume in Necturus gallbladder epithelium. Am. J. Physiol. 249, 304-312.

Deaton, L.E. (1983). Tissue K^+-stimulated ATPase and HCO_3^--stimulated ATPase in the tobacco hornworm Manduca sexta. Insect Biochem. 14, 109-114.

De Pont, J.J.H.H.M. and Bonting, S.L. (1981). Anion-sensitive ATPase $(K^+ + H^+)$-ATPase. In Membrane Transport. Ed. Bonting, S.L. and De Pont, J.J.H.H.M., Elsevier/North Holland Biomedical Press. pp. 209-234.

Diamond, J.M. (1964). The mechanism of isotonic water transport. J. Gen. Physiol. 48, 15-42.

Diamond, J.M. and Bossert, W.H. (1967). Standing-gradient osmotic flow: a mechanism for coupling water and solute transport in epithelia. J. Gen. Physiol. 50, 2061-2083.

Diamond, J.M. and Bossert, W.H. (1968). Functional consequence of ultrastructural geometry in "backwards" fluid transporting epithelia. J. Cell Biol. 37, 694-702.

Di Bona, D.R. (1972). Passive intercellular pathway in amphibian epithelia. Nature, Lond. 238, 179-181.

Di Bona, D.R. (1985). Functional analysis of tight junction organisation. Pflügers Arch. 405 (Suppl. 1), 559-566.

Di Bona, D.R. and Mills, J.W. (1979). Distribution of Na^+-pump sites in transporting epithelia. Fedn. Proc. Fedn. Am. Socs. exp. Biol. 38, 134-143.

Diez de los Rios, A., De Rose, N.E. and Armstrong, W. McD. (1981). Cyclic AMP and intracellular ionic activities in Necturus gallbladder. J. Membrane Biol. 63, 25-30.

Dlouhá, H., Teisinger, J. and Vyskočil, F. (1981). The effect of vanadate on the electrogenic Na^+/K^+ pump, intracellular Na^+ concentration and electrophysiological characteristics of mouse skeletal muscle fibre. Physiol. bohemoslov. 30, 1-10.

Donkin, J.E. (1981). Some effects of insect hormones on Na^+,K^+-ATPase and fluid secretion by the Malpighian tubules of Locusta migratoria L. Ph.D. Thesis, University of Durham.

Donkin, J.E. and Anstee, J.H. (1980). The effect of temperature on the ouabain-sensitivity of Na^+-K^+-activated ATPase and fluid secretion by the Malpighian tubules of Locusta. Experientia 36, 986-987.

Donowitz, M. (1983). Ca^{2+} in the control of active intestinal Na and Cl transport: involvement in neurohumoral action. Am. J. Physiol. 245, 165-177.

Doucet, A. and Katz, A.I. (1982). High-affinity Ca-Mg-ATPase along the rabbit nephron. Am. J. Physiol. 242, 346-352.

Douglas, W.W. (1968). Calcium-dependent links in stimulus-secretion coupling in the adrenal medulla and neurohypophysis. Br. J. Pharmacol. 34, 451-474.

Dow, J.A.T. (1981). Localization and characterization of water uptake in the midgut of the locust, Schistocerca gregaria Forsk. J. exp. Biol. 93, 269-281.

Dow, J.A.T., Gupta, B.L., Hall, T.A. and Harvey, W.R. (1984). X-ray microanalysis of elements in frozen-hydrated sections of an electrogenic K^+ transport system: The posterior midgut of tobacco hornworm (Manduca sexta) in vivo and in vitro. J. Membrane Biol. 77, 223-241.

Edwards, R.M. and Grantham, J.J. (1983). Inhibition of vasopressin action by vanadate in the cortical collecting tubule. Am. J. Physiol. 245, 772-777.

Ehrenspeck, G. and Brodsky, W.A. (1976). Effects of 4'-acetamido 4'-isothiocyano-2, 2'-disulphonic stilbene on ion transport in turtle bladders. Biochim. biophys. Acta 419, 555-561.

El Mernissi, G. and Doucet, A. (1984). Quantitation of [^{3}H] ouabain binding and turnover of Na-K-ATPase along the rabbit nephron. Am. J. Physiol. 247, 158-167.

English, L.H. and Cantley, L.C. (1984). Characterization of monovalent ion transport systems in an insect cell line (Manduca sexta embryonic cell line CHE). J. Cell. Physiol. 121, 125-132.

English, L., White, B. and Cantley, L. (1986). Comparison of the Na^+ pump and the ouabain-resistant K^+ transport system with other metal ion transport ATPases. In New Insights into Cell and Membrane Transport Proceses. Ed. Poste, G. and Crooke, S.T., Plenum Press, New York and London. pp. 249-259.

Epstein, F.H., Maetz, J. and Renzis, G. (1973). Active transport of chloride by the teleost gill: inhibition by thiocyanate. Am. J. Physiol. 224, 1295-1299.

Erdmann, E. (1981). Consequences of specific tritium-labelled ouabain binding to guinea pig left atria and cardiac cell membranes. In Handbook of Experimental Pharmacology. Ed. Greeff, K., Springer, Berlin. pp. 337-380.

Erdmann, E. and Hasse, W. (1975). Quantitative aspects of ouabain binding to human erythrocyte and cardiac membranes. J. Physiol. Lond. 251, 671-682.

Erdmann, E., Phillip, G. and Scholz, H. (1980). Cardiac glycoside receptor, (Na+K)-ATPase activity and force contraction in rat heart. Biochem. Pharmac. 29, 3219-3229.

Erdmann, E. and Schoner, W. (1973). Ouabain-receptor interactions in (Na^++K^+)-ATPase preparations from different tissues and species. Biochim. biophys. Acta 307, 386-398.

Ericson, A.-C. and Spring, K.R. (1982). Coupled NaCl entry into Necturus gallbladder epithelial cells. Am. J. Physiol. 243, 140-145.

Ernst, S.A., Riddle, C.V. and Karnaky, K.J., Jr. (1980). Relationship between localization of Na^++K^+-ATPase, cellular fine structure and reabsorption and secretory electrolyte transport. In Current Topics in Membranes and Transport, Vol. 14. Ed. Bronner, F. and Kleinzeller, A., Academic Press, New York. pp. 355-385.

Evans, A.L. and Mills, I.H. (1980). Stimulation of secretion of Malpighian tubules of Rhodnius prolixus by noradrenaline and vanadate. J. Physiol. Lond. 307, 32P.

Farmer, J., Maddrell, S.H.P. and Spring, J.H. (1981). Absorption of fluid by midgut of Rhodnius. J. exp. Biol. 94, 301-316.

Farquharson, P.A. (1974). A study of the Malpighian tubules of the pill millipede, Glomeris marginata (Villers) - II. The effect of variations in osmotic pressure and sodium and potassium concentrations on fluid secretion. J. exp. Biol. 60, 29-39.

Fathpour, H. (1980). Studies on fluid and ion secretion by the Malpighian tubules of Locusta with particular reference to the role played by ATPase enzymes. Ph.D. Thesis, University of Durham.

Fathpour, H. and Anstee, J.H. (1981). Effects of various inhibitors and 2,4-dinitrophenol on adenosine triphosphatase from Malpighian tubules of Locusta migratoria L. Experientia 37, 117-118.

Fathpour, H., Anstee, J.H. and Hyde, D. (1983). The effect of Na^+,K^+, ouabain, amiloride and ethacrynic acid on the transepithelial potential across Malpighian tubules of Locusta. J. Insect Physiol. 29, 773-778.

Fischer, R.A. and Yates, F. (1963). Statistical tables for biological, agricultural and medical research. 6th Ed. Oliver and Boyd.

Flier, J.S., Edwards, M.W., Daly, J.W. and Myers, C.W. (1980). Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na^+,K^+-ATPase. Science 208, 503-505.

Fricke, U. (1985). Erythrosin B inhibits high affinity ouabain binding in guinea-pig heart Na^+-K^+-ATPase without influence on cardiac glycoside induced contractility. Br. J. Pharmac. 85, 327-334.

Fricke, U. and Klaus, W. (1977). Evidence for two different Na^+-dependent [^{3}H]-ouabain binding sites of a Na^+-K^+-ATPase of guinea-pig hearts. Br. J. Pharmac. 61, 423-428.

Fristrom, J.W. and Kelly, L. (1976). Effects of β-ecdysone and juvenile hormone on the Na^+/K^+-dependent ATPase in imaginal disks of Drosophila melanogaster. Insect Physiol. 22, 1697-1707.

Frizzell, R.A., Field, M. and Schultz, S.G. (1979). Sodium-coupled chloride transport by epithelial tissues. Am. J. Physiol. 236, 1-8.

Frizzell, R.A. and Turnheim, K. (1978). Ion transport by rabbit colon. II. Unidirectional sodium influx and the effects of amphtericin B and amiloride. J. Membrane Biol. 40, 193-211.

Fromter, E. (1972). The route of passive ion movement through the epithelium of Necturus gallbladder. J. Membrane Biol. 8, 259-301.

Fromter, E. and Diamond, J. (1972). Route of passive ion permeation in epithelia. Nature, Lond. 235, 9-13.

Gárdos, G., Lassen, U.V. and Pape, L. (1976). Effect of antihistamines and chlorpromazines on the calcium-induced hyperpolarization of the Amphiuma red cell membrane. Biochim. biophys. Acta 448, 599-606.

Gárdos, G. (1958). Effect of ethylendiamic-tetraacetate on the permeability of human erythrocytes. Acta Physiol. Academ. Scient. Hung. 14, 1-5.

Garner, A., Peters, T.J. and Wilkes, J. (1983). Demonstration of HCO_3^--activated Mg^{2+}-dependent ATPase activity in rat duodenal microvillus membranes. J. Physiol., Lond. 362, 13P-14P.

Gassner, D. and Komnick, H. (1981). The loop diuretic furosemide as a non-competative inhibitor of Cl^-/HCO_3^--ATPase of vertebrate kidneys and insect rectum. Comp. Biochem. Physiol. 71C, 43-48.

Gay, C.V. (1982). On the stability of sulphydryl-containing carbonic anhydrases. Comp. Biochem. Physiol. 72A, 587-589.

Geck, P. and Heinz, E. (1986). The Na-K-2 Cl cotransport system. J. Membrane Biol. 91, 97-105.

Gee, J.D. (1975). The control of diuresis in the tsetse fly Glossina austeni: A preliminary investigation of the diuretic hormone. J. exp. Biol. 63, 391-401.

Gee, J.D. (1976). Fluid secretion by the Malpighian tubules of the tsetse fly Glossina morsitans: The effects of ouabain, ethacrynic acid and amiloride. J. exp. Biol. 65, 323-332.

Gerencser, G.A. (1983). Electrophysiology of chloride transport in Aplysia (mollusc) intestine. Am. J. Physiol. 244, 143-149.

Gerencser, G.A. and Lee, S.-H. (1983). Cl^--stimulated adenosine triphosphatase: existence, location and function. J. exp. Biol. 106, 143-161.

Gerencser, G.A. and Lee, S.-H. (1985). Cl^--HCO_3^--stimulated ATPase in intestinal mucosa of Aplysia. Am. J. Physiol. 248, 241-248.

Giraldez, F. (1984). Active sodium transport and fluid secretion in the gallbladder epithelium of Necturus. J. Physiol., Lond. 348, 431-455.

Giunta, C., De Bortoli, M., Sanchini, M. and Stacchini, A. (1985). Activatory effect of two cardioglycosides on Cavia cobaya kidney Na^+/K^+-ATPase activity. Gen. Pharmacol. 16, 183-188.

Giunta, C., De Bortoli, M., Stacchini, A. and Sanchini, M. (1984). Na^+/K^+-ATPase from Xenopus laevis (Daudin) kidney and epidermis: high sensitivity towards regulatory compounds. Comp. Biochem. Physiol. 79B, 71-74.

Glynn, I.M. (1957). The action of cardiac glycosides on sodium and potassium movements in human red blood cells. J. Physiol., Lond. 136, 148-173.

Glynn, I.M. and Karlish, J.D. (1975). The sodium pump. A. Rev. Physiol. 37, 13-53.

Gmaj, P., Zurini, M., Murer, H. and Carafoli, E. (1983). A high-affinity, calmodulin-dependent Ca^{2+} pump in the basal-lateral plasma membranes of kidney cortex. Eur. J. Biochem. 136, 71-76.

Godfraind, T., Castaneda-Hernandez, G., Ghysel-Burton, J. and De Pover, A. (1983). Hypothesis for the mechanism of stimulation of the Na/K pump by cardiac glycosides - role of endogenous digitalis-like factor. Curr. Top. Membr. Transp. 19, 913-915.

Godfraind, T., De Pover, A., Castaneda-Hernandez, G. and Fagoo, M. (1982). Cardioginin: endogenous digitalis-like material from mammalian heart. Archs int. Pharmacodyn. Ther. 258, 165-167.

Godfraind, T., De Pover, A. and Lutete, D.-N.T. (1980). Identification with potassium and vanadate of two classes of specific ouabain binding sites in a $(Na^+ + K^+)$ATPase preparation from the guinea-pig heart. Biochem. Pharmacol. 29, 1195-1199.

Goh, S. and Phillips, J.E. (1978). Dependence of prolonged water absorption by in vitro locust rectum on ion transport. J. exp. Biol. 72, 25-41.

Goldman, D.E. (1943). Potential, impedance and rectification in membranes. J. Gen. Physiol. 27, 37-60.

Grantham, J.J. (1980). The renal sodium pump and vanadate. Am. J. Physiol. 239, 97-106.

Greger, R. (1985). Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. Physiol. Reviews 65, 760-797.

Greger, R. and Schlatter, E. (1983). Properties of the basolateral membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. A model for secondary active chloride transport. Pflügers Arch. 396, 325-334.

Greger, R. and Schlatter, E. (1984). Mechanism of NaCl secretion in rectal gland tubules of spiny dogfish (Squalus acanthias). II. Effects of inhibitors. Pflügers Arch. 402. 364-375.

Greger, R., Schlatter, E., Wang, F. and Forrest, J.N., Jr. (1984). Mechanism of NaCl secretion in rectal gland tubules of spiny dogfish (Squalus acanthias). III. Effects of stimulation of secretion by cyclic AMP. Pflügers Arch. 402, 376-384.

Grotmol, T., Buanes, T., Brørs, O. and Roeder, M.G. (1986). Lack of effect of amiloride, furosemide, bumetanide and triamterene on pancreatic $NaHCO_3^-$ secretion in pigs. Acta Physiol. Scand. 126, 593-600.

Guggino, W.B., Boulpaep, E.L. and Giebisch, G. (1982). Electrical properties of chloride transport across the Necturus proximal tubule. J. Membrane Biol. 65, 185-196.

Guggino, W.B., London, R., Boulpaep, E.L. and Giebisch, G. (1983). Chloride transport across the basolateral cell membrane of the Necturus proximal tubule: dependence on bicarbonate and sodium. J. Membrane Biol. 71, 227-240.

Gupta, B.L., Berridge, M.J., Hall, T.A. and Moreton, R.B. (1978). Electron microprobe studies of fluid secretion in the salivary glands of Calliphora. J. exp. Biol. 72, 261-284.

Gupta, B.L. and Hall, T.A. (1979). Quantitative electron probe X-ray microanalysis of electrolyte elements within epithelial tissue compartments. Fedn. Proc. Fedn. Am. Socs. exp. Biol. 38, 144-153.

Gupta, B.L. and Hall, T.A. (1983). Ionic distribution in dopamine-stimulated NaCl fluid-secreting cockroach salivary glands. Am. J. Physiol. 244, 176-186.

Gupta, B.L., Hall, T.A., Maddrell, S.H.P. and Moreton, R.B. (1976). Disruption of ions in a fluid-transporting epithelium determined by electron-probe X-ray microanalysis. Nature, Lond. 264, 284-287.

Gupta, B.L., Hall, T.A., Maddrell, S.H.P. and Moreton, R.B. (1977). Electron probe X-ray microanalysis. In Transport of Ions and Water in Animals. Ed. Gupta, B.L., Moreton, R.B., Oschman, J.L. and Wall, B.J., Academic Press, London, New York, San Fransisco. pp. 83-143.

Gupta, B.L., Wall, B.J., Oschman, J.L. and Hall, T.A. (1980). Direct microprobe evidence of local concentration gradients and recycling of electrolytes during fluid absorption in the rectal papillae of Calliphora. J. exp. Biol. 88, 21-47.

Hakin, R.S. and Kafatos, F.C. (1974). The structure and salivary function of the labial gland in adult Manduca sexta. Tissue and Cell 6, 729-750.

Hanrahan, J.W. (1984). Ionic permeability of insect epithelia. Amer. Zool. 24, 229-240.

Hanrahan, J.W. and Phillips, J.E. (1983). Cellular mechanisms and control of KCl absorption in insect hindgut. J. exp. Biol. 106, 71-89.

Hanrahan, J.W. and Phillips, J.E. (1984). KCl transport across an insect epithelium. II. Electrochemical potentials and electrophysiology. J. Membrane Biol. 80, 27-47.

Hanrahan, J.W., Willis, N.K. and Phillips, J.E. (1986). Basolateral K channels in an insect epithelium. Channel density, conductance and block by barium. J. Gen. Physiol. 87, 443-466.

Hansen, O. (1971). The relationship between g-strophanthin binding capacity and ATPase activity in plasma membrane fragments from ox brain. Biochim. biophys. Acta 233, 122-132,

Hansen, O. (1976). Non-uniform binding of g-strophanthin binding sites of Na^++K^+-activated ATPase. Apparent conversion to uniformity by K^+. Biochim. biophys. Acta 433, 383-392.

Hardy, M.A. and Ware, H.M. (1985). Roles of Ca^{2+} and Na^+ on the modulation of antidiuretic hormone action on area permeability in toad urinary bladder. J. Clin. Invest. 75, 921-931.

Harms, V. and Wright, M. (1980). Some characteristics of Na/K-ATPase from rat intestinal basal lateral membrane. J. Membrane Biol. 53, 119-128.

Harvey, W.R. and Nedergaard, S. (1964). Sodium-independent active transport of potassium in the isolated midgut of the Cecropia silkworm. Proc. natn. Acad. Sci. U.S.A. 51, 757-65.

Harvey, W.R. and Zerahn, K. (1972). Active transport of potassium and other alkali metal by the isolated midgut of the silkworm. In Current Topics in Membranes and Transport. Ed. Bronner, F. and Kleinzeller, A., Academic Press, New York. Vol. 3. pp. 367-410.

Harvey, W.R., Cioffi, M., Dow, J.A.T. and Wolfersberger, M.G. (1983). Potassium ion transport ATPase in insect epithelia. J. exp. Biol. 106, 91-117.

Henry, R.P. (1984). The role of carbonic anhydrase in blood ion and acid base regulation. Amer. Zool. 24, 241-251.

Herrera, L., Jordana, R. and Ponz, F. (1976). Effects of inhibitors on chloride dependent transmural potential in the rectal wall of Schistocerca gregaria. J. Insect Physiol. 23, 677-682.

Herrera, L., Lopez-Moratalla, N., Santiago, E., Ponz, F. and Jordana, R. (1978). Effect of bicarbonate on chloride-dependent transmural potential and ATPase activity in the rectal wall of Schistocerca gregaria. Revta. esp. Fisiol. 34, 219-224.

Higashino, H., Bogden, J.D., Lavenhar, M.A., Bauman, J.W., Jr., Hirotsu, T. and Aviv, A. (1983). Vanadium, Na-K-ATPase, and potassium adaptation in the rat. Am. J. Physiol. 244, 105-111.

Hill, A.E. (1975a). Solute-solvent coupling in epithelia: a critical examination of the standing-gradient osmotic flow theory. Proc. R. Soc. Lond. B 190, 99-114.

Hill, A.V. (1909). The mode of action of nicotine and curari, determined by the form of the contraction curve and the method of temperature coefficients. J. Physiol. Lond. 39, 361-373.

Hill, A.E. (1975b). Solute-solvent coupling in epithelia: an electro-osmotic theory of fluid transfer. Proc. R. Soc. Lond. B 190, 115-134.

Hill, A.E. (1977). General mechanism of salt-water coupling in epithelia. In Transport of Ions and Water in Animals. Ed. Gupta, B.L., Moreton, R.B., Oschman, J.L. and Wall, B.J., Academic Press, London, New York, San Fransisco. pp. 183-214.

Hodgkin, A.J. and Keynes, R.D. (1955). Active transport of cations in giant axons from Sepia and Loligo. J. Physiol., Lond. 128, 28-60.

Hoffmann, E.K., Lambert, I.H. and Simonsen, L.O. (1986). Separate, Ca^{2+}-activated K^+ and Cl^- transport pathways in Ehrlich Ascites tumor cells. J. Membrane Biol. 91, 227-244..

House, C.R. and Ginsborg, B.L. (1982). Properties of dopamine receptors at a neuroglandular synapse. In Neuropharmacology of Insects. Ciba Foundation Symposium 88. Ed. Evered, E., O'Connor, M. and Whelan, J. Pitman, London. pp. 32-47.

Huff, J.W. and Reinach, P.S. (1985). Mechanism of inhibition of net ion transport across frog corneal epithelium by calcium channel antagonists. J. Membrane Biol. 85, 215-223.

Hunter, M., Lopez, A.G., Boulpaep, E.L. and Giebisch, G.H. (1984). Single channel recordings of calcium-activated potassium channels in the apical membrane of rabbit cortical collecting tubules. Proc. Natl. Acad. Sci. U.S.A. 81, 4237-39.

Irvine, H.B. and Phillips, J.E. (1971). Effects of respiratory inhibitors and ouabain on water transport by isolated locust rectum. J. Insect Physiol. 17, 381-393.

Janis, R.A. and Scriabine, A. (1983). Sites of action of Ca^{2+} channel inhibitors. Biochem. Pharmacol. 32, 3499-3507.

Joly, P. and Joly, L. (1953). Resultats de graffe de corpora allata chez Locusta migratoria L. Ann. Sci. nat. zool. ser. 15, 331-345.

Joiner, C.H. and Lauf, P.K. (1978). The correlation between ouabain binding and potassium pump inhibition in human and sheep erythrocytes. J. Physiol. Lond. 283, 155-175.

Jørgensen, P.L. (1974). Purification and characterization of $(Na^+{+}K^+)$-ATPase. IV. Estimation of the purity and of the molecular weight and polypeptide content per enzyme unit in preparations from the outer medulla of rabbit kidney. Biochim. biophys. Acta 356, 53-67.

Jørgensen, P.L. and Petersen, J. (1977). Purification and characterization of $(Na^+{+}K^+)$-ATPase. VI. Differential tryptic modification of catalytic functions of the purified enzyme in presence of NaCl and KCl. Biochim. biophys. Acta 466, 97-108.

Jungreis, A.M. (1979). Physiology of moulting in insects. Adv. Insect Physiol. 14, 109-184.

Jungreis, A.M. and Vaughan, G.L. (1977). Insensitivity of Lepidopteran tissues to ouabain: absence of ouabain binding and Na^+-K^+ ATPases in larval and adult midgut. J. Insect Physiol. 23, 503-509.

Kafatos, F.C. (1968). The labial gland: a salt-secreting organ of Saturniid moths. J. exp. Biol. 48, 435-453.

Kalule-Sabiti, J.M. (1985). A study on the effects of various inhibitors on the ATPase activity in, and fluid secretion by Malpighian tubules of Locusta migratoria L. Ph.D. Thesis, University of Durham.

Karnaky, K.J., Kinter, L.B., Kinter, W.B. and Stirling, C.E. (1976). Osmoregulation in euryhaline teleosts. II. Autoradiographic localization of Na,K-ATPase in gills of kilifish adapted to low and high salinity environments. J. Cell Biol. 70, 157-177.

Karnovsky, M.J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol 27, 137A-138A.

Kasbekar, D.K. and Durbin, R.P. (1965). An adenosine triphosphatase from frog gastric mucosa. Biochim. biophys. Acta 105, 472-482.

Kazazoglou, T., Renaud, J.-F., Rossi, B. and Lazdunski, M. (1983). Two classes of ouabain receptors in chick ventricular cardiac cells and their relation to (Na^+,K^+)-ATPase inhibition, intracellular Na^+ accumulation, Ca^{2+} influx, and cardiotonic effect. J. Biol. Chem. 258, 12163-12170.

Komnick, H. (1978). Osmoregulatory role and transport ATPases of the rectum of dragonfly larvae Odonatol. 7, 247-262.

Komnick, H. and Achenbach, U. (1979). Comparative biochemical, histochemical and autoradiographic studies of Na^+/K^+-ATPase in the rectum of dragonfly larvae (Odonata, Aeshnidae). Eur. J. Cell. Biol. 20, 92-100.

Lane, N.J. (1979). Freeze-fracture and tracer studies on the intercellular junctions of insect rectal tissues. Tissue and Cell 11, 481-507.

Latorre, R. and Miller, C. (1983). Conduction and selectivity in K^+ channels. J. Membrane Biol. 71, 11-30.

Leech, C.A. (1986). Resting potential and potassium-selective electrode measurements in locust skeletal muscles. J. exp. Biol. 122, 439-442.

Lewis, S.A., Eaton, D.C. and Diamond, J.M. (1976). The mechanism of Na^+ transport by rabbit urinary bladder. J. Membrane Biol. 28, 41-70.

Lewis, S.A., Hanrahan, J.W. and Van Driessche, W. (1984). Channels across epithelial cell layers. Curr. Top. Memb. Transp. 21, 253-293.

Lichtshtein, D., Dunlop, K., Kaback, H.R. and Blume, A.J. (1979). Mechanism of monensin-induced hyperpolarization of neuroblastoma-glioma hybrid NG108-15. Proc. Natl. Acad. Sci. U.S.A. 76, 2580-2584.

Lichtstein, D. and Samuelov, S. (1980). Endogenous "ouabain like" activity in rat brain. Biochem. Biophys. Res. Commun. 96, 1518-1523.

Livengood, D.R. and Kusano, K. (1972). Evidence for an electrogenic sodium pump in follower cells of the lobster cardiac ganglion. J. Neurophysiol. 35, 170-186.

Lord, B.A.P. and Di Bona, D.R. (1976). Role of septate junctions in the regulation of paracellular transepithelial flow. J. Cell Biol. 71, 967-972.

Lorenzen, M., Lee, C.O. and Windhager, E.E. (1984). Cytosolic Ca^{2+} and Na^+ activities in perfused proximal tubules of Necturus kidney. Am. J. Physiol. 247, 93-102.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurements with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Maddrell, S.H.P. (1963). Excretion in the blood-sucking bug, Rhodnius prolixus Stal. I. The control of diuresis. J. exp. Biol. 40, 247-256.

Maddrell, S.H.P. (1969). Secretion by the Malpighian tubules of Rhodnius. The movements of ions and water. J. exp. Biol. 51, 71-97.

Maddrell, S.H.P. (1971). The mechanism of insect excretory systems. Adv. Insect Physiol. 8, 199-331.

Maddrell, S.H.P. (1977). Insect Malpighian tubules. In Transport of Ions and Water in Animals. Ed. Gupta, B.L., Moreton, R.B., Oschman, J.L. and Wall, B.J., Academic Press, London, New York, San Fransisco. pp. 541-569.

Maddrell, S.H.P. (1980). Characteristics of epithelial transport in insect Malpighian tubules. In Current Topics in Membranes and Transport, Vol. 14. Ed. Bronner, F. and Kleinzeller, A., Academic Press, New York. pp. 427-463.

Maddrell, S.H.P. and Klunsuwan,S. (1973). Fluid secretion by in vitro preparations of the Malpighian tubules of the desert locust Schistocerca gregaria. J. Insect Physiol. 19, 1369-1376.

Maddrell, S.H.P. and Phillips, J.E. (1978). Induction of sulphate transport and hormonal control of fluid secretion by Malpighian tubules of larvae of the mosquito Aedes taeniorhynchus. J. exp. Biol. 72, 181-202.

Maddrell, S.H.P., Pilcher, D.E.M. and Gardiner, B.O.C. (1971). Pharmacology of the Malpighian tubules of Rhodnius and Carausius. The structure activity relationship of tryptamine analogues and the role of cyclic AMP. J. exp. Biol. 54, 779-804.

Mandel, L.J. and Murphy, E. (1984). Regulation of cytosolic free calcium in rabbit proximal renal tubules. J. Biol. Chem. 259, 11188-11196.

Marunaka, Y. (1986). Effects of internal Na concentration on ouabain-sensitive Na efflux and membrane potential. IRCS Med. Sci. 14, 601-602.

Moffett, D.F. and Koch, A.R. (1985). Barium modifies the concentration dependence of active potassium transport by insect midgut. J. Membrane Biol. 86, 89-97.

Mordue, W. (1969). Hormonal control of Malpighian tube and rectal function in the desert locust, Schistocerca gregaria. J. Insect Physiol. 15, 273-285.

Mordue, W. (1972). Hydromineral regulation in animals - Part I. Hormones and excretion in locusts. Gen. Comp. Endocr. 3, 289-298.

Mordue, W. and Goldsworthy, G.J. (1969). The physiological effects of corpus cardiacum extracts in locusts. Gen. Comp. Endocr. 12, 360-369.

Mordue, W. and Rafaeli-Bernstein, A. (1978). Glucose transport in Malpighian tubules of Locusta. J. Physiol., Lond. 278, 36P.

Morgan, P.J. and Mordue, W. (1981). Stimulated fluid secretion is sodium dependent in the Malpighian tubules of Locusta migratoria. J. Insect Physiol. 27, 271-279.

Morgan, P.J. and Mordue, W. (1983a). Electrochemical gradients across Locusta Malpighian tubules. J. Comp. Physiol. 151, 175-183.

Morgan, P.J. and Mordue, W. (1983b). Separation and characteristics of diuretic hormone from the corpus cardiacum of Locusta. Comp. Biochem. Physiol. 75B, 75-80.

Morgan, P.J. and Mordue, W. (1984). 5-hydroxytryptamine stimulates fluid secretion in locust Malpighian tubules independently of c AMP. Comp. Biochem. Physiol. 79C, 305-310.

Mullins, L.J. (1979). The generation of electric currents in cardiac fibres by a Na-Ca exchange. Am. J. Physiol. 236, 103-110.

Murer, H., Hopfer, J., Kinne, R. (1976). Sodium-proton antiport in brush-border membrane vesicles isolated from rat small intestine and kidney. Biochem. J. 154, 597-604.

Nagel, W. (1980). Rheogenic sodium transport in a tight epithelium, the amphibian skin. J. Physiol. Lond. 302, 281-295.

Nagel, W., Garcia-Diaz, J.F. and Armstrong, W. McD. (1981). Intracellular ionic activities in frog skin. J. Membrane Biol. 61, 127-134.

Nakao, T., Tashima, Y., Nagano, K. and Nakao, M. (1965). Highly specific sodium-potassium-activated adenosine triphosphatase from various tissues of rabbit. Biochem. Biophys. Res. Commun. 19, 755-758.

Nauntofte, B. and Poulsen, J.H. (1986). Effects of Ca^{2+} and furosemide on Cl^- transport and O_2 uptake in rat parotid acini. Am. J. Physiol. 251, 175-185.

Nechay, B.R. (1984). Mechanism of action of vanadium. Ann. Rev. Pharmacol. Toxicol. 24, 501-24.

Nechay, B.R., Nanninga, L.B., Nechay, P.S.E., Post, R.L., Grantham, J.J., Macara, I.G., Kubena, L.F., Phillips, J.D. and Nielsen, F.H. (1986). Role of vanadium in biology. Fed. Proc. 45, 123-132.

Nicolson, S.W. (1976). Diuresis in the cabbage white butterfly, Pieris brassicae: fluid secretion by the Malpighian tubules. J. Insect Physiol. 22, 1347-1356.

Nicolson, S.W. and Millar, R.P. (1983). Effects of biogenic amines and hormones on butterfly Malpighian tubules: dopamine stimulates fluid secretion. J. Insect Physiol. 29, 611-615.

Nijhout, H.F. and Carrow, G.M. (1978). Diuresis after a bloodmeal in a female Anopheles freebormi. J. Insect. Physiol. 24, 293-298.

Noel, F. and Godfraind, T. (1984). Heterogeneity of ouabain specific binding sites and $(Na^+ + K^+)$-ATPase inhibition im microsomes from rat heart. Biochem. Pharmac. 33, 47-53.

Norris, D.M. and Cary, L.R. (1981). Properties and subcellular distribution of Na^+K^+-ATPase and Mg^{2+}-ATPase in the antennae of Periplaneta americana. Insect Biochem. 11, 743-750.

O'Donnell, M.J. (1985). Calcium action potentials in the developing oocytes of an insect, Rhodnius prolixus. J. exp. Biol. 119, 287-300.

O'Donnell, M.J. and Maddrell, S.H.P. (1983). Paracellular and transcellular routes for water and solute movements across insect epithelia. J. exp. Biol. 106, 231-253.

O'Donnell, M.J. and Maddrell, S.H.P. (1984). Secretion by the Malpighian tubules of Rhodnius prolixus Stal: Electrical events. J. exp. Biol. 110, 275-290.

O'Donnell, M.J., Maddrell, S.H.P. and Gardiner, B.O.C. (1984). Passage of solutes through walls of Malpighian tubules of Rhodnius by paracellular and transcellular routes. Am. J. Physiol. 246, 759-769.

O'Neal, S.G., Rhoads, S.B. and Racker, E. (1979). Vanadate inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase and other ATPases. Biochem. Biophys. Res. Commun. 89, 845-850.

O'Neil, R.G. and Sansom, S.C. (1984). Characterization of apical cell membrane Na^+ and K^+ conductances of cortical collecting duct using microelectrode techniques. Am. J. Physiol. 247, 14-24.

O'Riordan, A.M. (1969). Electrolyte movement in the isolated midgut of the cockroach (Periplaneta americana L.). J. exp. Biol. 51, 699-714.

Pacifico, A.D., Schwartz, M., MacKrell, T.N., Spangler, S.G., Sanders, S.S. and Rehm, W.S. (1969). Reversal by potassium of an effect of barium on the frog gastric mucosa. Am. J. Physiol. 216, 536-541.

Palfrey, H.C. and Rao, M.C. (1983). Na/K/Cl co-transport and its regulation. J. exp. Biol. 106, 43-54.

Patarca, R., Cardia, O.A. and Reinach, P.S. (1983). Mode of inhibition of active chloride transport in the frog cornea by furosemide. Am. J. Physiol. 245, 660-669.

Peacock, A.J. (1975). Studies on the excretory and neuroendocrine systems of some orthopteran insects, with particular reference to Jamaicana flava (Caudell). Ph.D. thesis, University of Durham.

Peacock, A.J. (1981). Further studies of the properties of locust rectal Na^+-K^+-ATPase, with particular reference to the ouabain sensitivity of the enzyme. Comp. Biochem. Physiol. C 68, 29-34.

Peacock, A.J. (1982). Effects of sodium transport inhibitors on diuresis and midgut (Na^++K^+)-ATPase in the tsetse fly Glossina morsitans. J. Insect Physiol. 28, 553-558.

Peacock, A.J. (1986). Effects of anions, acetazolamide and copper on diuresis in the tsetse fly Glossina morsitans morsitans Westwood. J. Insect Physiol. 32, 157-160.

Peacock, A.J., Bowler, K. and Anstee, J.H. (1976). Properties of Na^+-K^+-dependent ATPase from the Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus. Insect Biochem. 6, 281-288.

Pento, J.T. and Johnson, M.E. (1983). The influence of verapamil on calcium transport and uptake in segments of rat intestine. Pharmacology 27, 343-349.

Petersen, O.H. and Maruyama, Y. (1984). Calcium-activated potassium channels and their role in secretion. Nature, Lond. 307, 693-696.

Pfeiffer, D.R., Hutson, S.M., Kauffman, R.F. and Lardy, H.M. (1976). Some effects of ionophore A23187 on energy utilization and the distribution of cations and anions in mitochomdria. Biochemistry 15, 2690-2697.

Phillips, J.E. (1964). Rectal absorption in the desert locust, Schistocerca gregaria Forskal. II. Sodium, potassium and chloride. J. exp. Biol. 41, 39-67.

Phillips, J.E. (1981). Comparative physiology of insect renal function. Am. J. Physiol. 241, 241-257.

Phillips, J.E. (1982). Hormonal control of renal functions in insects. Fed. Proc. 41, 2348-2354.

Phillips, J.E. and Lewis, S. (1983). Introduction: trends in epithelial transport and control. J. exp. Biol. 106, 3-8.

Phillips, J.E., Mordue, W., Meredith, J. and Spring, J. (1980). Purification and characteristics of chloride transport stimulating factor from locust corpora cardiaca: a new peptide. Can. J. Zool. 58, 1851-1860.

Phillips, T.D., Nechay, B.R. and Heidelbaugh, N.D. (1983). Vanadium: chemistry and the kidney. Fed. Proc. 42, 2969-2973.

Pilcher, D.E.M. (1970b). The influence of the diuretic hormone on the process of urine secretion by the Malpighian tubules of Caransius morosus. J. exp. Biol. 53, 465-484.

Pitts, B.J.R., Wallick, E.T., Van Winkle, W.B., Allen, J.C. and Schwartz, A. (1977). On the lack of inotropy of cardiac glycosides on skeletal muscle: a comparison of Na^+,K^+-ATPase from skeletal and cardiac muscle. Arch. Biochem. Biophys. 184, 431-440.

Podevin, R.A. and Boumendil-Podevin, E.F. (1972). Effects of temperature, medium K^+, ouabain and ethacrynic acid on transport of electrolytes and water by separated renal tubules. Biochim. biophys. Acta 282, 234-249.

Polya, J.B. and Wirtz, A.J. (1965). Studies on carbonic anhydrase. I. A review of recent investigations. Enzymol. 28, 355-366.

Post, R.L. and Jolly, P.C. (1957). The linkage of sodium, potassium and ammonium active transport across the human erythrocyte membrane. Biochim. biophys. Acta 25, 118-128.

Pressman, B.C. (1976). Biological application of ionophores. Ann. Rev. Biochem. 45, 501-530.

Pressman, B.C. and Painter, G.R. (1983). Mechanism of biological effects of carboxylic ionophores: modulation of intracellular calcium activity by sodium. In The Biochemistry of Metabolic Processes. Ed. Stratman, F.W., Lennon, D.L.F. and Zahlten, R.N. Elsevier-North Holland, Amsterdam. pp. 41-54.

Prince, W.T. and Berridge, M.J. (1972). The effects of 5-hydroxytryptamine and cyclic AMP on the potential profile across isolated salivary glands. J. exp. Biol. 56, 323-333.

Prince, W.T. and Berridge, M.J. (1973). The role of calcium in the action of 5-hydroxytryptamine and cyclic AMP on salivary glands. J. exp. Biol. 58, 367-384.

Proux, J., Proux, B. and Phillips, J.E. (1984). Source and distribution of factors in locust nervous system which stimulate rectal Cl^- transport. Cn. J. Zool. 63, 37-41.

Proverbio, F., Robinson, J.W.L. and Whittembury, G. (1970). Sensitivity of $(Na^+ + K^+)$-ATPase and Na^+ extrusion mechanisms to ouabain and ethacrynic acid in the cortex of the guinea pig kidney. Biochim. Biophys. Acta 211, 327-336.

Prusch, R.D. (1978). Active Na^+ uptake in the isolated midgut of larval Sarcophaga bullata. J. Insect Physiol. 24, 81-85.

Rabon, E., Cuppoletti, J., Malinowska, D., Smolka, A., Helander, H.F., Mendlein, J. and Sachs, G. (1983). Proton secretion by the gastric parietal cell. J. exp. Biol. 106, 119-133.

Rafaeli, A. and Mordue, W. (1982). The responses of the Malpighian tubules of Locusta to hormones and other stimulants. Gen. Comp. Endocrinol. 46, 130-135.

Rafaeli, A., Pines, M., Stern, P.S. and Applebaum, S.W. (1984). Locust diuretic hormone-stimulated synthesis and excretion of cyclic AMP: a novel Malpighian tubule bioassay. Gen. Comp. Endocrin. 54, 35-42.

Rafaeli-Bernstein, A. and Mordue, W. (1978). The transport of the cardiac glycoside ouabain by Malpighian tubules of Zonocerus variegatus. Physiol. Ent. 3, 59-63.

Rafaeli-Bernstein, A. and Mordue, W. (1979). The effects of phlorizin, phloretin and ouabain on the reabsorption of glucose by the Malpighian tubules of Locusta migratoria migratorioides. J. Insect Physiol. 25, 241-247.

Ramsay, J.A. (1953). Active transport of potassium by the Malpighian tubules of insects. J. exp. Biol. 30, 358-369.

Ramsay, J.A. (1954). Active transport of water by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera, Plasmidae). J. exp. Biol. 31, 104-113.

Ramsay, J.A. (1956). Excretion by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera, Plasmidae): calcium, magnesium, chloride, phosphate and hydrogen ions. J. exp. Biol. 33, 697-708.

Ramsay, J.A. (1958). Excretion by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera, Plasmidae): amino acids, sugars and urea. J. exp. Biol. 35, 871-891.

Rapp, P.E. and Berridge, M.J. (1981). The control of transepithelial potential in the salivary gland of Calliphora erythrocephala. J. exp. Biol. 93, 119-132.

Rasmussen, H. and Goodman, D.B.P. (1977). Relationship between calcium and cyclic nucleotides in cell activation. Physiol. Rev. 57, 421-509.

Rector, F.C., Jr., Carter, N.W. and Seldin, D.W. (1965). The mechanism of bicarbonate reabsorption in the proximal and distal tubules of the kidney. J. Clin. Invest. 44, 278-290.

Reed, P.W. (1979). Ionophores. In Methods in Enzymology, Biomembranes, Vol. LV. Ed. Fleischer, S. and Packer, L., Academic Press, New York, London, San Fransisco. pp. 435-454.

Reinach, P. and Nagel, W. (1985). Implications of an anomalous intracellular electrical response in bullfrog corneal epithelium. J. Membrane Biol. 87, 201-209.

Reuss, L. (1983). Basolateral KCl cotransport in a NaCl-absorbing epithelium. Nature, Lond. 305, 723-726.

Reuss, L. (1984). Independence of apical membrane Na and Cl entry in Necturus gallbladder epithelium. J. Gen. Physiol. 84, 423-447.

Reuss, L. and Finn, A.L. (1974). Passive electrical properties of toad urinary bladder epithelium: intercellular electrical coupling and transepithelial cellular and shunt conductances. J. Gen. Physiol. 64, 1-25.

Reuss, L., Lewis, S.A., Wills, N.K., Helman, S.I., Cox, T.C., Boron, W.F., Siebens, A.W., Guggino, W.B., Giebisch, G. and Schultz, S.G. (1984). Ion transport processes in basolateral membranes of epithelia. Fed. Proc. 43, 2488-2502.

Reuss, L., Reinbach, P., Weinman, S.A. and Grady, T.P. (1983). Intracellular ion activities and Cl^- transport mechanisms in bullfrog corneal epithelium. Am. J. Physiol. 244, 336-347.

Reuter, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature, Lond. 301, 569-574.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain. J. Cell Biol. 17, 208-212.

Robinson, J.D. and Flashner, M.S. (1979). The $(Na^+ + K^+)$-activated ATPase. Enzymatic and transport properties. Biochim. biophys. Acta 549, 145-176.

Ross, A. and Boron, W.F. (1981). Intracellular pH. Physiol. Rev. 61, 296-434.

Rothstein, A., Cabantchik, Z.I. and Knauf, P. (1976). Mechanism of anion transport in red blood cells: Role of membrane proteins. Fedn. Proc. Fedn. Am. Soc. exp. Biol. 35, 3-10.

Rubin, A.L., Clark, A.F. and Stahl, W.L. (1981). The insect brain $(Na^+ + K^+)$-ATPase binding of ouabain in the hawk moth, Manduca sexta. Biochim. biophys. Acta 649, 202-210.

Rugg, E.L., Simmons, N.L. and Tivey, D.R. (1986). An investigation of [^{3}H] bumetanide uptake in a cultured renal cell line (MDCK). Quat. J. Exp. Physiol. 71, 165-182.

Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M. and Saccomani, G. (1976). A nonelectrogenic H^+ pump in plasma membranes of hog stomach. J. Biol. Chem. 251, 7690-7698.

Sackin, H. and Boulpaep, E.L. (1975). Models for coupling of salt and water transport. Proximal tubular reabsorption in Necturus kidney. J. gen. Physiol. 66, 671-733.

Salkoff, L.B. and Tanouye, M.A. (1986). Genetics of Ion Channels. Physiol. Rev. 66, 301-329.

Sansom, S.C., Weinman, E.J. and O'Neil, R.G. (1984). Microelectrode assessment of chloride-conductive properties of cortical collecting duct. Am. J. Physiol. 247, 291-302.

Sato, N., Austin, G., Yai, H. and Maruhashi, J. (1968). The ionic permeability changes during acetycholine-induced responses of Aplysia ganglion cells. J. Gen. Physiol. 51, 321-345.

Sawyer, D.B. and Beyenbach, K.W. (1985a). Dibutyryl-c AMP increases basolateral sodium conductance of mosquito Malpighian tubules. Am. J. Physiol. 248, 339-345.

Sawyer, D.B. and Beyenbach, K.W. (1985b). Mechanisms of fluid secretion in isolated shark renal proximal tubules. Am. J. Physiol. 249, 884-890.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-672.

Schatzmann, H.J. (1966). ATP-dependent Ca^{2+}-extrusion from human red cells. Experientia 22, 364-368.

Schatzmann, H.J. (1975). Active calcium transport and Ca^{2+}-activated ATPase in human red cells. Curr. Top. Membr. Transport 6, 125-168.

Schellenberg, G.D., Anderson, L. and Swanson, P.D. (1983). Inhibition of Na^{+}-Ca^{2+} exchange in rat brain by amiloride. Mol. Pharmacol. 24, 251-258.

Schneeberger, E.E. and Lynch, R.D. (1984). Tight junctions. Their structure, composition and function. Circ. Res. 55, 723-733.

Schlatter, E. and Greger, R. (1985). cAMP increases the basolateral Cl^{-} conductance in the isolated perfused medullary thick ascending limb of Henle's loop of the mouse. Pflügers Arch. 405, 367-376.

Schuurmans Stekhoven, F. and Bonting, S.L. (1981). Transport adenosine triphosphates: properties and functions. Physiol. Rev. 61, 1-76.

Schwartz, A. (1983). Positive inotropic action of digitalis and endogenous factors: Na,K-ATPase and positive inotropy; "endogenous glycosides". Curr. Top. Membr. Transp. 19, 825-841.

Scoble, J.E., Varghese, Z., Sweny, P. and Moorhead, J. (1986). Renal physiology revisited; amiloride. The Lancet 8502, 326-328.

Shaver, J.L.F. and Stirling, C. (1978). Ouabain binding to renal tubules of the rabbit. J. Cell Biol. 76, 278-292.

Shorofsky, S.R., Field, M. and Fozzard, H.A. (1982). The cellular mechanism of active chloride secretion in vertebrate epithelia: studies in intestine and trachea. Phil. Trans. R. Soc. Lond. B 299, 597-609.

Silva, P., Epstein, J.A., Stevens, A., Spokes, K. and Epstein, F.H. (1983). Ouabain binding in rectal gland of Squalus acanthias. J. Membrane Biol. 75, 105-114.

Simon, B. and Thomas, L. (1972). HCO_3^{-}-stimulated ATPase from mammalian pancreas - properties and its arrangement with other enzyme activities. Biochim. biophys. Acta 288, 434-442.

Skou, J.C. (1957). The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. biophys. Acta 23, 394-401.

Skou, J.C. (1965). Enzymatic basis for active transport of Na^{+} and K^{+} across cell membranes. Physiol. Rev. 45, 596-617.

Skou, J.C. (1969). The role of membrane ATPase in the active transport of ions. In Molecular Basis of Membrane Function. Ed. Tostein, D.C., Prentice-Hall, Inc., Englewood Cliffs, New Jersey. pp. 455-482.

Skou, J.C. (1975). The $(Na^{+}+K^{+})$-activated enzyme system and its relation to transport of sodium and potassium. Q. Rev. Biophys. 7, 401-434.

Sloley, B.D. and Downer, R.G.H. (1984). Distribution of 5-hydroxytryptamine and indolealkylamine metabolites in the American cockroach Periplaneta americana L. Comp. Biochem. Physiol. 79C, 281-286.

Smith, J.B. and Rozengurt, E. (1978). Serum stimulates the Na^+,K^+ pump in quiescent fibroblasts by increasing Na^+ entry. Proc. Natl. Acad. Sci U.S.A. 75, 5560-5564.

Smith, P.L., Sullivan, S.K. and McCabe, R.D. (1986). Concentration-dependent effects of disulphonic stilbenes on colonic chloride transport. Am. J. Physiol. 250, 44-49.

Snedecor, G.W. and Cochran, W.G. (1967). Statistical Methods, 6th Ed. Iowa State University Press, U.S.A.

Snowdowne, K.W. and Borle, A.B. (1985). Effects of low extracellular sodium on cytosolic ionized calcium. Na^+-Ca^{2+} exchange as a major influx pathway in kidney cells. J. Biol. Chem. 260, 14998-15007.

Soltoff, S.P. and Mandel, L.J. (1983). Amiloride directly inhibits the Na,K-ATPase activity of rabbit kidney proximal tubules. Science 220, 957-959.

Spedding, M. (1985). Activators and inactivators of Ca^{++} channels: new perspectives. J. Pharmacol. (Paris) 16, 319-343.

Spenny, J.G., Shoemaker, R.L. and Sachs, G. (1974). Microelectrode studies of fundic gastric mucosa: cellular coupling and shunt conductance. J. Membrane Biol. 19, 105-128.

Staehelin, L.A. (1974). Structure and function of intercellular junctions. Int. Rev. Cytol. 39, 191-283.

Stern, P.A. (1985). Interactions of calcemic hormones and divalent cation ionophores on fetal rat bone in vitro. In Calcium in Biological Systems. Ed. Rubin, R.P., Weiss, G.B. and Putney, J.W., Jr., Plenum Press, New York and London. pp. 541-547.

Stewart, W.W. (1978). Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. Cell 14, 741-759.

Stobbart, R.H. (1971). Evidence for Na^+/H^+ and Cl^-/HCO_3^- exchanges during independent sodium and chloride uptake by the larva of the mosquito Aedes aegypti (L.). J. exp. Biol. 54, 19-27.

Strange, K. and Phillips, J.E. (1984). Mechanisms of CO_2 transport in rectal salt gland of Aedes. I. Ionic requirements of CO_2 secretion. Am. J. Physiol. 246, 727-734.

Szibbo, C.M. and Scudder, G.G.E. (1979). Secretory activity of the segmented Malpighian tubules of Cenocorixa bifida (Hung.) (Hemiptera, Corixidae). J. Insect Physiol. 25, 931-937.

Tartakoff, A.M. (1983). Perturbation of the structure and function of the Golgi complex by monovalent carboxylic ionophores. In Methods in Enzymology. Biomembranes, Vol. 98. Ed. Fleischer, S. and Fleischer, B. Academic Press, New York, London, San Fransisco. pp. 47-59.

Taylor, A. and Windhager, E.E. (1979). Possible role of cytosolic calcium and Na-Ca exchange in regulation of transepithelial sodium transport. Am. J. Physiol. 236, 505-512.

Taylor, C.W. (1984). Transcellular calcium transport by the midgut of the blowfly, Calliphora vicina. Cell Calcium 5, 377-390.

Taylor, H.H. (1971). Water and solute transport by the Malpighian tubules of the stick insect, Carausius morosus. The normal ultrastructure of the type 1 cells. Z. Zellforsch. mikrosk. Anat. 118, 333-368.

Thomas, M.V. and May, T.E. (1984). Active potassium ion transport across the caterpillar midgut. II. Intracellular microelectrode studies. J. exp. Biol. 108, 293-304.

Thurm, V. and Küppers, J. (1980). Epithelial physiology of insect sensilla. In Insect Biology in the Future, 'VBW 80'. Ed. Locke, M. and Smith, D.S., Academic Press, London, New York. pp. 735-764.

Towle, D.W. (1984). Membrane-bound ATPases in arthropod ion-transporting tissues. Am. Zool. 24, 177-185.

Treherne, J.E. (1966). The effect of ouabain on the efflux of sodium ions in the nerve cords of two insect species (Periplaneta americana and Carausius morosus). J. exp. Biol. 44, 355-362.

Triggle, D.J. (1981). Calcium antagonists: basic chemical and pharmacological aspects. In New Perspectives on Calcium Antagonists. Ed. Weiss, G.B., Williams and Wilkins, Baltimore. pp. 1-18.

Trimmer, B.A. (1985). Serotonin and control of salivation in the blowfly Calliphora. J. exp. Biol. 114, 307-328.

Ueda, T. (1983). Na^+-Ca^{2+} exchange activity in rabbit lymphocyte plasma membranes. Biochim. biophys. Acta 734, 342-346.

Usherwood, P.N.R. (1978). Permeability of insect muscle fibres to potassium and chloride ions. J. Physiol., Lond. 191, 29P-30P.

Van Driessche, W. and Zeiske, W. (1985). Ion channels in epithelial cell membranes. Physiol. Rev. 65, 833-903.

Villalobo, A., Brown, L. and Roufogalis, B.O. (1986). Kinetic properties of the purified Ca^{2+}-translocating ATPase from human erythrocyte plasma membrane. Biochim. biophys. Acta 854, 9-20.

Völkl, H., Geibel, J., Greger, R. and Lang, F. (1986). Effects of ouabain and temperature on cell membrane potentials in isolated perfused straight proximal tubules of the mouse kidney. Pflügers Arch. 407, 252-257.

Walker, R.J. (1984). 5-hydroxytryptamine in invertebrates. Comp. Biochem. Physiol. 79C, 231-235.

Wall, B.J., Oschman, J.L. and Schmidt-Nielsen, B. (1970). Fluid transport: concentration of the intercellular compartment. Science, N.Y. 167, 1497-1498.

Wallick, E.T., Dowd, F., Allen, J.C. and Schwartz, A. (1974). The nature of the transport adenosine triphosphatase-digitalis complex. II. Characteristics of ouabagenic-Na^+,K^+-adenosine triphosphatase interaction. J. Pharmacol. Exp. Ther. 189, 434-444.

Wallick, E.T., Lane, K.L. and Schwartz, A. (1979). Biochemical mechanism of the sodium pump. Am. Rev. Physiol. 41, 387-411.

Wallick, E.T., Pitts, B.J.R., Lane, L.K. and Schwartz, A. (1980). A kinetic comparison of cardiac glycoside interactions with Na^+,K^+-ATPases from skeletal and cardiac muscle and from kidney. Arch. Biochem. Biophys. 202, 442-449.

Wang, W., Messner, G., Oberleithner, H., Lang, F. and Deetjen, P. (1984). The effect of ouabain on intracellular activities of K^+,Cl^-,H^+ and Ca^{2+} in proximal tubules of frog kidneys. Pflügers Atch. 401, 6-13.

Warnock, D.G., Greger, R., Dunham, P.B., Benjamin, M.A., Frizzell, R.A., Field, M., Spring, K.R., Ives, H.G., Aronson, P.S. and Seifter, J. (1984). Ion transport processes in apical membranes of epithelia. Fed. Proc. 43, 2473-2487.

Weber-von Grotthuss, E., Hevert, F., Atzbacher, U. and Wessing, A. (1974). Influence of ouabain on Na^+ and K^+ concentration in haemolymph of Drosophila hydei and appearance of Malpighian tubules. J. Insect Physiol. 20, 1411-1420.

Welsh, M.J. (1983). Inhibition of chloride secretion by furosemide in canine tracheal epithelium. J. Membrane Biol. 71, 219-226.

Welsh, M.J., Smith, P.L. and Frizzell, R.A. (1983). Chloride secretion by canine tracheal epithelium. III. Membrane resistances and electromotive forces. J. Membrane Biol. 71, 209-218.

White, J.F. (1980). Bicarbonate-dependent chloride absorption in small intestine: ion fluxes and intracellular chloride activities. J. Membrane Biol. 53, 95-107.

White, J.F. (1986). Modes of Cl^- transport across the mucosal and serosal membranes of Urodele intestinal cells. J. Membrane Biol. 92, 75-89.

White, J.F., Ellingsen, D. and Burnup, K. (1984). Electrogenic Cl^- absorption by Amphiuma small intestine: dependence on serosal Na^+ from tracer and Cl^- microelectrode studies. J. Membrane Biol. 78, 223-233.

Whittam, R. and Wheeler, K.P. (1970). Transport across cell membranes. A. Rev. Physiol. 32, 21-60.

Wieczorek, H. (1982). A biochemical approach to the electrogenic potassium pump of insect sensilla: potassium sensitive ATPases in the labellum of the fly. J. Comp. Physiol. 148, 303-311.

Wieczorek, H. and Gnatzy, W. (1985). The electrogenic potassium pump of insect cuticular sensilla. Further characterization of ouabain- and azide-insensitive K^+-stimulated ATPases in the labellum of the blowfly. Insect Biochem. 15, 225-232.

Wieczorek, H., Wolfersberger, M.G., Cioffi, M. and Harvey, W. (1986). Cation-stimulated ATPase activity in purified plasma membranes from tobacco hornworm midgut. Biochim. biophys. Acta 857, 271-281.

Williams, J.C., Jr. and Beyenbach, K.W. (1983). Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of Aedes aegypti (L.). J. Comp. Physiol. 149, 511-517.

Williams, J.C., Jr. and Beyenbach, K.W. (1984). Differential effects of secretagogues on the electrophysiology of the Malpighian tubules of the yellow fever mosquito. J. Comp. Physiol. 154, 301-309.

Windhager, E.E. and Taylor, A. (1983). Regulatory role of intracellular calcium ions in epithelial Na transport. Annu. Rev. Physiol. 45, 519-532.

Wolfersberger, M.G., Harvey, W.R. and Cioffi, M. (1982). Transepithelial potassium transport in insect midgut by an electrogenic alkali metal ion pump. Curr. Topics Membr. Trans. 16, 109-133.

Wollberg, Z. and Cocos, R. (1981). Steady state potential in the developing oocytes of Locusta migratoria: passive and active components. J. exp. Biol. 92, 347-351.

Wright, M.S. and Cook, B.J. (1985). Distribution of calmodulin in insects as determined by radioimmunoassay. Comp. Biochem. Physiol. 80C, 241-244.

Zadunaisky, J.A., Lande, M.A. and Hafner, J. (1971). Further studies in chloride transport in the frog cornea. Am. J. Physiol. 221, 1832-1836.

Zerahn, K. (1977). Potassium transport in insect midgut. In Transport of Ions and Water in Animals. Ed. Gupta, B.L., Moreton, R.B., Oschman, J.L. and Wall, B.J., Academic Press, New York. pp. 381-401.

APPENDIX

Table 1

Agent	Concentration	Mean Rate of Fluid Secretion ± S.E.M. Expressed in nl/min		Expressed as % Original Rate	p	n
		Rate 1 (-Agent)	Rate 2 (+Agent)			
Vanadate	1 mM	4.06 ± 0.79	1.44 ± 0.98	18.24 ± 6.06	< 0.001	15
Amiloride	1 mM	3.55 ± 0.81	0.34 ± 0.08	12.26 ± 3.00	< 0.001	14
$BaCl_2$	1 mM	4.42 ± 0.42	1.62 ± 0.22	37.41 ± 3.11	< 0.001	34
Furosemide	1 mM	2.26 ± 0.27	0.43 ± 0.06	22.63 ± 3.04	< 0.001	24
Bumetanide	10^{-4} M	1.97 ± 0.40	0.76 ± 0.17	41.22 ± 5.96	< 0.001	12
Bumetanide	10^{-5} M	1.95 ± 0.36	1.64 ± 0.32	83.62 ± 5.63	NOT SIGNIFICANT	15
NaSCN	10 mM	2.76 ± 0.63	0.91 ± 0.20	43.13 ± 6.31	< 0.001	15
5-HT	1 mM	3.53 ± 1.07	3.28 ± 0.83	106.65 ± 9.32	NOT SIGNIFICANT	13

The effect of various agents on fluid secretion by the Malpighian tubules of Locusta. p values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.