A study on the effects of various inhibitors on the ATPase activity in, and fluid secretion by Malpighian tubules of Locusta Migratoria L.

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A STUDY ON THE EFFECTS OF VARIOUS INHIBITORS ON THE ATPase ACTIVITY IN, AND FLUID SECRETION BY MALPIGHIAN TUBULES OF LOCUSTA MIGRATORIA L.

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Being a Thesis presented in candidature for the Degree of Doctor of Philosophy of the University of Durham

June 1985
University of Durham

DECLARATION

I hereby declare that the work presented in this document is based on research carried out by me, and that this document has not been presented anywhere else for a degree and has not been published before.

Kalule-Sabiti, Margaret Jannat

TO MY LATE BELOVED FATHER

YAFESI MUSOKE

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CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF PLATES

ABSTRACT

A study of the effects of some inhibitors on both the activities of ATPases isolated from Locusta Malpighian tubules microsomal preparations and in vitro Malpighian tubule fluid production has been carried out. Inhibitors investigated included ouabain, vanadate, furosemide, acetazolamide, SITS and SCN^-.

It was important to establish the effects of temperature and $[K^+]$ on the inhibitory action of both ouabain and vanadate. Ouabain did not significantly inhibit either Na^+, K^+-ATPase or in vitro Malpighian fluid production at temperatures below 30°C. In contrast vanadate was negatively influenced by temperature. Vanadate was most effective at temperaturesbetween 20 and 30°C. High $[K^+]$ enhanced vanadate but antagonised ouabain inhibition of Na^+, K^+-ATPase. Under optimal conditions ouabain inhibited both Na^+, K^+-ATPase (pI_{50} = 6.8) and in vitro Malpighian tubule fluid production (pI_{50} = 4.3). Similarly vanadate inhibited Na^+, K^+-ATPase (pI_{50} = 5.8) and in vitro Malpighian tubule fluid secretion (pI_{50} = 4.8). Vanadate was found to be nonspecific as it also inhibited both Mg^{2+},-ATPase and Mg^{2+}, HCO_3^- -ATPase activities. With the exception of SITS, all other inhibitors studied inhibited in vitro Malpighian tubule fluid section. However, SITS was found to be a strong inhibitor of Mg^{2+}, HCO_3^- -ATPase. All results are discussed with reference to the role of ATPases in the process of ion and water transport across the Malpighian tubules.

Cytochemical and biochemical studies based on ERNST (1972a,b) technique were carried out as the first attempt to localise Na^+,K^+ - ATPase in Locusta Malpighian tubule cells. K^+-NPPase was localised mainly along the cytoplasmic side of the basal cell membrane infoldings but was inconsistently inhibited by ouabain.

Biochemical studies showed that paraformaldehyde fixation of the Malpighian tubules and inclusion of 20mM $SrCl_2$ in the standard incubation medium reduced the total NPPase activity by 73%. Use of β-glycerophosphate indicated that the reaction product observed in the present study was not due to nonspecific alkaline phosphatase activity. However, the results were inconclusive and led to further questioning of the validity of ERNST (1972b) procedure in localisation of Na^+,K^+-ATPase in insect epithelia.

GLOSSARY

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
B.S.A.	bovine serum albumen
cAMP	cyclic adenosine 3', 5'-monophosphate
D.D.S.A	dodecenyl succinic anhydride
D.M.P.30	2-4-6 tri (dimethylaminomethyl) phenol
E.D.T.A.	ethylene diamine tetra-acetic acid
g	gram
HEPES	N-2 hydroxylpiperazine-N'-2 ethanesulfonic acid
Isc	short-circuit current
K^+-pNPPase	potassium activated p-nitrophenolphosphatase
Mg^{2+}-ATPase	magnesium activated adenosine triphosphatase
Mg^{2+}, HCO_3^- - ATPase	magnesium dependent, bicarbonate-stimulated ATPase
Na^+, K^+ - ATPase	magnesium dependent, sodium and potassium stimulated adenosine triphosphatase
NP	p-nitrophenol
NPP	p-nitrophenyl phosphate
pI_{50}	Negative Log_{10} of concentration of any inhibitor which abolishes 50% of activity
PD	transepithelial potential difference
SDH	succinate dehydrogenase
S.E.M.	standard error of the mean
SITS	4-acetamido-4'-isothiocyanatostilbene 2'-disulphonic acid
Tris	tris (hydroxymethy) aminomethane
T.S.	transverse section
Vanadate	sodium orthovanadate
SCN^-	sodium thiocyanate

CHAPTER I

GENERAL INTRODUCTION

The mechanism of ion and water transport across isolated Malpighian tubules of insects was first studied by RAMSAY (1954, 1955, 1956, 1958) using *in vitro* preparations of *Carausius* tubules. This technique, with a few modifications, has been employed by several workers in subsequent studies of fluid secretion in different insect species (e.g. *Calliphora*, BERRIDGE, 1969; *Rhodnius*, MADDRELL, 1969, *Locusta*, ANSTEE and BELL, 1975; ANSTEE *et al.*, 1979; DONKIN, 1981; present study). Extensive reviews on fluid secretion studies are given by BERRIDGE (1967), MADDRELL (1971, 1977), and PHILLIPS (1981).

From these studies, a number of suggestions have been made : (1) that ion movements give rise to water movements; (2) in a majority of insects K^+ ions are the primary movers in urine production. It is the active pumping of K^+ from the haemolymph to the lumen which creates the osmotic imbalance necessary for water to flow passively (RAMSAY, 1958; BERRIDGE, 1968; MADDRELL and KLUNSUWAN, 1973). However, there are exceptions, such as *Rhodnius* which can produce urine in the presence of either Na^+ or K^+ (MADDRELL, 1969) and *Glossina* which uses Na^+ as the prime mover (GEE, 1975, 1976a,b); (3) in a majority of insects, fluid secreted by Malpighian tubules, is said to be marginally but consistently hyperosmotic to the bathing fluid over a wide range of osmotic concentrations of the bathing solution (MADDRELL, 1977). The rates of fluid production by Malpighian tubules are in close inverse relationship to the osmotic concentration of the bathing solution. In *Locusta*, for example, Malpighian tubule secrete fluid which is iso-osmotic to the bathing solution and at a rate which depends on the K^+ concentration and inversely on the osmotic

concentration of the bathing medium (HIGHNAM et al., 1965; MORDUE, 1969; MADDRELL, 1969); (4) the rate of solute movement is fairly constant but water movements change so that the fluid produced is slightly hypertonic to the bathing medium (MADDRELL, 1977). Despite the extensive studies, the whole mechanism of solute coupled water movement is not yet fully understood. A number of theories have been proposed:

(1) Co-diffusion theory (KEDEM, 1965 and DIAMOND, 1965).

This model was originally proposed by KEDEM (1965) and later applied to gall bladder by DIAMOND (1965). According to the co-diffusion theory, the molecules of solutes diffuse across the membrane through the same channels as water molecules. As a result of friction, the solute molecules drag water molecules along with them. This theory is strongly opposed and thought to be vague by HILL (1977). It was also opposed by BELL (1977) in her study of fluid secretion by Malpighian tubules of Locusta.

(2) Standing-gradient osmotic flow (DIAMOND and BOSSERT 1967, 1968).

The most widely supported model is that of standing-gradient osmotic flow designed by DIAMOND and BOSSERT (1967, 1968). This theory is given a functional geometric explanation. The transport channels are both functionally and structurally closed at one end. Solutes are pumped into the closed ends of the spaces from the adjacent cytoplasm making the region hyperosmotic to the cell. Water in turn moves into the space from the adjacent cytoplasm so that towards the open end of the space, the fluid is isomotic to the cytoplasm. A diagrammatic description of the standing-gradient along the length of the channel is seen in Figure 1.1.

FIGURE 1.1

Application of the standing-gradient osmotic flow hypothesis of solute-linked water transport as a model for 'urine' production by insect Malpighian tubules (DIAMOND and BOSSERT, 1968). The density of the black dots indicate the solute concentration. Solid lines indicate the active transport of solutes and the broken lines show passive water movements.

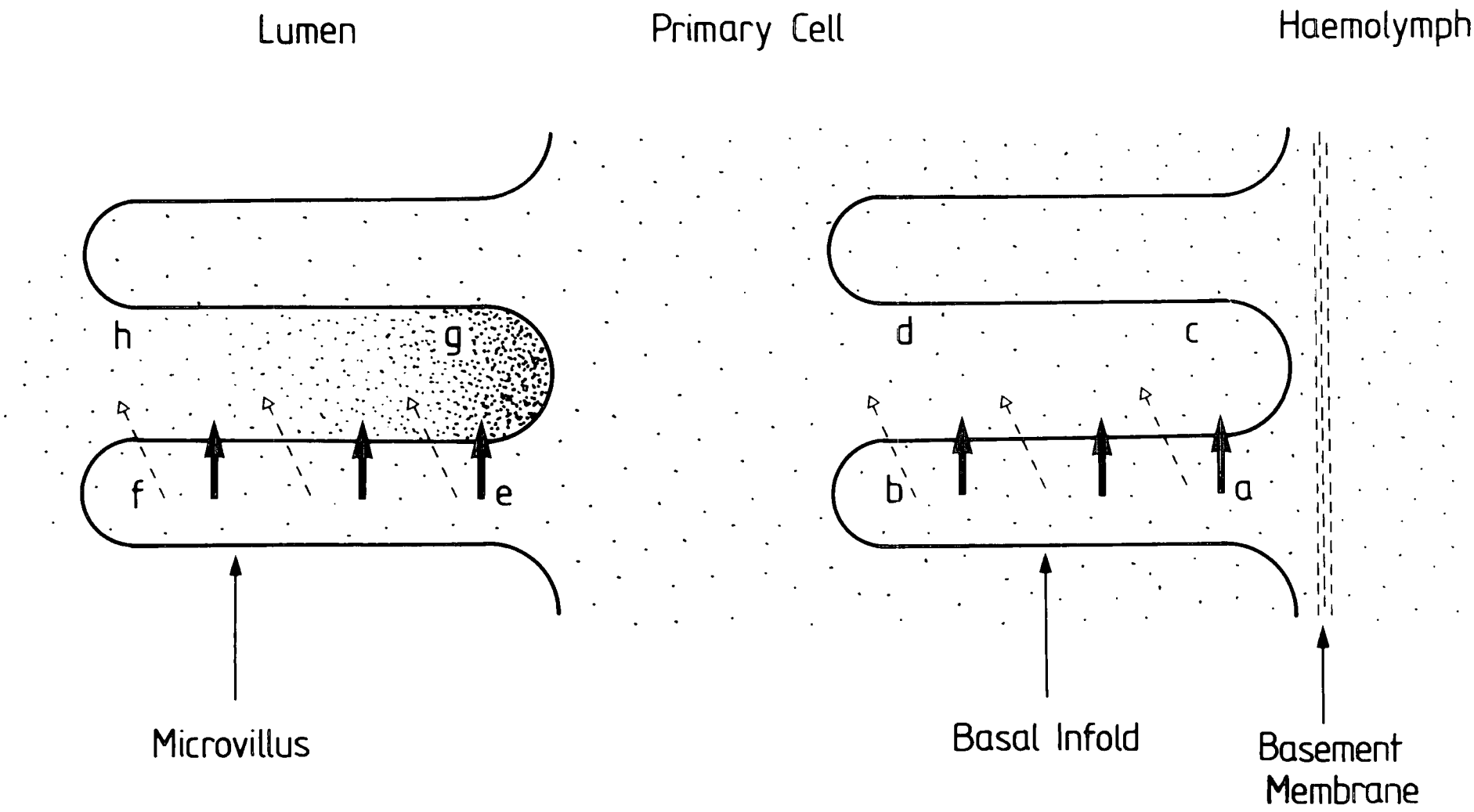
Basal surface

Solute is actively transported out of the channels (a-b) across its walls, making the channel fluid hypotonic. As the solute diffuses down its concentration gradient towards the closed end, more and more water leaves the channel across its walls into the cytoplasm (c-d) owing to the osmotic gradient. In a steady state a standing osmotic gradient will be maintained in the channel (a-b) by active solute transport, with the osmolarity decreasing progressively from the open end to the closed end; and a fluid of fixed osmolarity will constantly enter the channel mouth and be secreted across its walls.

Apical surface

Solute is actively transported from the microvilli (e-f) into the channel (g-h) across its walls making the channel fluid hypertonic and causing the water to move into the channel from adjacent cytoplasm. As solute moves down the channel due to diffusion along its concentration gradient, more and more water will enter the channel reducing the osmolarity until the fluid emerging at the open end would virtually be isotonic. In a steady state, a standing osmotic gradient would be maintained in the channel (g-h) by active solute transport, the osmolarity decreasing continuously from the open end, and fluid of fixed osmolarity would constantly flow from the open end.

Fig. 1.1


Lumen
Primary Cell
Haemolymph
h
g
f
e
d
c
b
a
Microvillus
Basal Infold
Basement Membrane

Several workers have applied this theory to insect Malpighian tubules fluid secretion (e.g. DIAMOND and BOSSERT, 1968; BERRIDGE and OSCHMAN, 1969; MADDRELL, 1971, 1980; OSCHMAN and BERRIDGE, 1971). However, TAYLOR (1971a) and MADDRELL (1977) found it difficult to apply this model to insect Malpighian tubules whose infoldings and microvilli are too short compared to fluid transporting tissues such as the gall bladder and the rectal proximal tubules of vertebrate kidney for which the model was originally designed. HILL (1975 a and b) also had a more fundamental criticism of the model. He pointed out that biological membranes do not have a sufficiently high osmotic permeability to allow isotonic flow in epithelia to occur in the process of local osmosis, and osmotic equilibration must play little part in the process under normal physiological conditions. In Rhodnius GUPTA et al., (1976) and Calliphora, GUPTA et al., (1977) found that Na^+ and K^+ and Cl^- were more concentrated towards the lumen than at the closed end of the channels; which is different from what is expected of the standing-gradient osmotic flow theory.

(3) Electro-osmotic theory (Hill 1975b, 1977)

In electro-osmosis, the transmembrane potential can move water because there is a specific frictional interaction between water and solutes as it moves out of the cell down an electrochemical gradient carrying water with it. MADDRELL (1977) has explained how this theory could be applied to Malpighian tubules. The electrogenic cation pumps situated on the apical microvilli of tubules produces an electrical potential difference across the membrane; and the resulting electrochemical gradient would draw Cl^- out from the cell through the membrane; and on crossing the membrane the Cl^- would frictionally interact with water molecules which would also move out of the cell.

Because this model depends on the maintenance of a potential gradient across the cell membrane, it would be important that the apical wall should be so arranged that it is not bathed by fluids other than its own secretion. MADDRELL (1977) found that the apical membrane infoldings of the Malpighian tubule cells would serve such a purpose and would allow a greater density of 'pump' sites because of the increased surface area.

Finally it is possible that urine of Locusta Malpighian tubules is always slightly hypertonic to the bathing medium (BELL, 1977; ANSTEE et al., 1979) in which case a simple local osmosis (DIAMOND, 1964) may be responsible for water movement across the tubules. This theory suggests that the cytoplasm is marginally hypertonic to the haemolymph as a result of solute actively 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 osmotic permeability of the membrane. This theory is also supported by MADDRELL (1972) and TAYLOR (1971a) for other insect Malpighian tubules. They suggest that the folding of basal and apical cell membranes is primarily to increase the effective passive permeability of the cells to solute and that the overall osmotic pressure difference between lumen and haemolymph gives rise to water movement.

In general, all models proposed agree to one factor that fluid production by insect Malpighian tubules is a consequence of solute transport.

Although active cation transport has been established in insect Malpighian tubules, (RAMSAY, 1953, 1955; BERRIDGE, 1967, 1968;

PILCHER, 1970a,b; MADDRELL, 1971, 1977; BELL, 1977; ANSTEE and BELL, 1975; ANSTEE et al., 1979; FATHPOUR, 1980; DONKIN, 1981) the nature of the ion pumps remain obscure. Considering the different models proposed, it is generally suggested that an electrogenic cation pump is located on the apical cell membrane (BERRIDGE, 1967; BERRIDGE and OSCHMAN, 1969; MADDRELL, 1977). Controversy is centred on how solute is transported across the basal cell membrane.BERRIDGE (1967) and BERRIDGE and OSCHMAN, (1969) suggested that movement across the basal cell membrane occurs as a result of a coupled Na^+- K^+ exchange pump. This pump requires energy and ATP and simultaneous presence of Na^+ and K^+ are essential for its activity. In the majority of tissues studied the enzyme responsible for the Na^+- K^+ exchange 'pump' is a Mg^{2+}-dependent Na^+ K^+-stimulated ATPase which is, for example, found in crab nerve (SKOU, 1957) mammalian kidney, (WHITTAM and WHEELER, 1961; SKOU, 1962) dog pancreas (RIDDERSTAP and BONTING, 1969) frog and rat brain (BOWLER and DUNCAN, 1968 b) Locusta Malpighian tubules (ANSTEE and BELL, 1975; ANSTEE et al., 1979; FATHPOUR, 1980; DONKIN, 1981; present study).

However, despite this vast literature there is controversy concerning the involvement of Na^+K^+-ATPase in secretion of fluid by Malpighian tubules of insects. The conflicting reports are mainly attributed to failure by some investigators to demonstrate that urine production by isolated Malpighian tubules is sensitive to ouabain, a specific inhibitor of Na^+,K^+-ATPase (SKOU 1965, 1969). A review by ANSTEE and BOWLER (1979) explained some of the causes of these conflicting reports.

MADDRELL (1971) reports ouabain-insensitivity in Malpighian tubules of Calliphora and Carausius and therefore proposes an

alternative hypothetical model to explain the mechanism of Malpighian tubule function in these insects. The main features of this model are that active ion transport occurs both at the basal and apical cell membrane. It is suggested 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 cell surface, Na^+ and K^+ are transported into the lumen by electrogenic 'pumps', whilst the transport of Cl^- is again considered to be passive (See Figure 1.2).

MADDRELL (1971, 1972) proposed a different model for Malpighian tubules of Rhodnius where the electrogenic Na^+ and K^+ pumps are found on the basal cell membrane. Here each pump is stimulated by a different cation, i.e. Na^+ pump is stimulated by K^+ and vice versa; and Cl^- enters the cell passively. He suggested that in this way the three ions, K^+, Na^+ and Cl^- are made available to three electrogenic 'pumps' for Na^+, K^+ and Cl^- situated on the apical cell membrane and these are responsible for transporting these ions into the lumen.

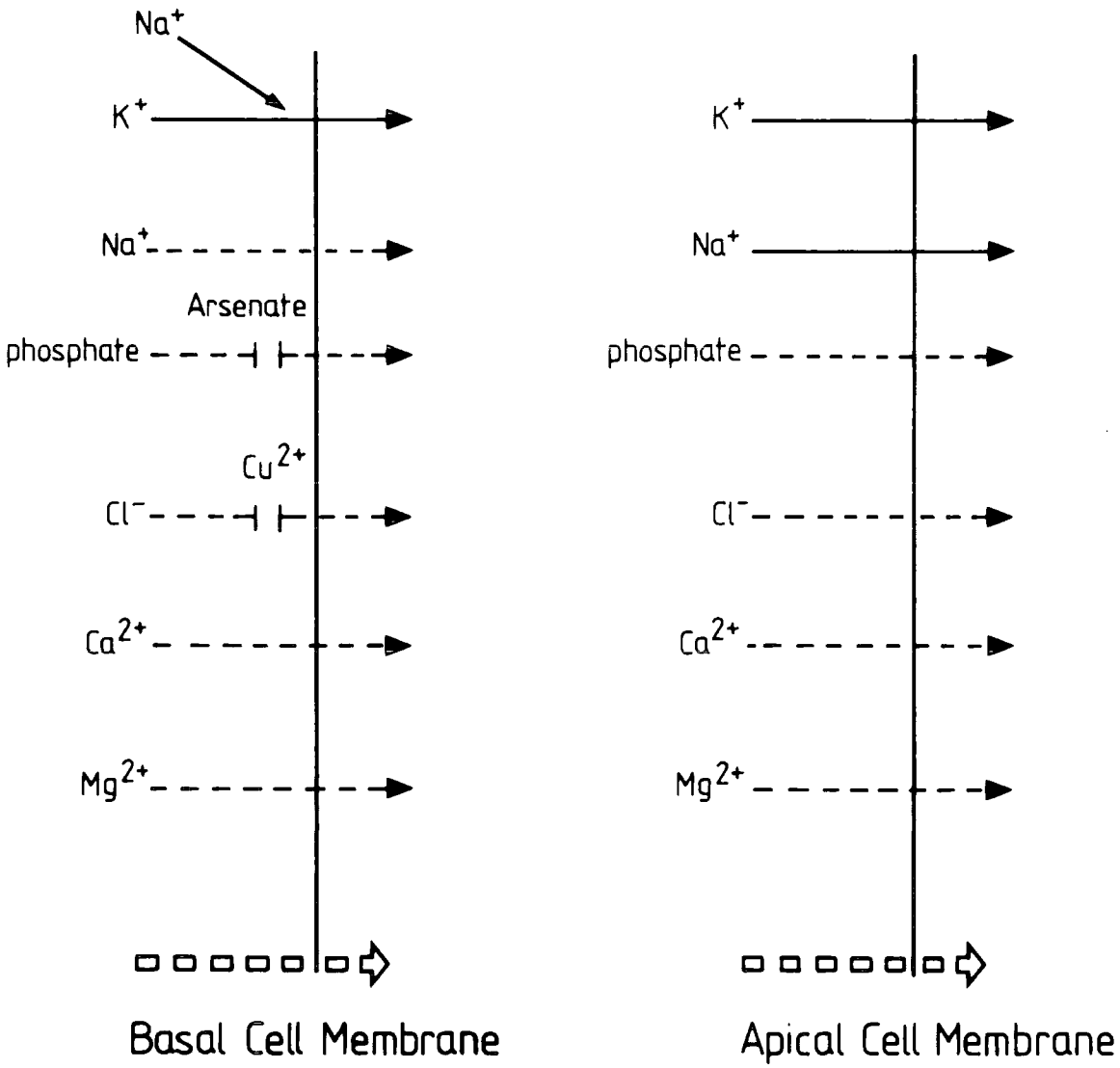
More recently MADDRELL (1977) designed a model which is supposed to suit transport through Malpighian tubules of any insect regardless of which ion is the prime mover (K^+ or Na^+). This model attempts to explain how one mechanism can account for the secretion of Na^+ rather than K^+ in different species of insects. In this model, it suggested that an electrogenic cation pump is situated on the membrane facing the tubule lumen and that it has a higher affinity for Na^+ than K^+. This pump acts to maintain the intracellular level of Na^+ ions lower than that of K^+ and the actual rate at which cations are pumped across the tubules from the bathing medium into the lumen is dependent on both the affinity of the pump for the two ions and on how fast these ions enter the cell. It is proposed that K^+, Na^+

FIGURE 1.2

A hypothetical scheme for operation of Malpighian tubules of *Carausius* and *Calliphora*.

Active movements are indicated by unbroken lines and passive movement by broken lines.
(Drawn from MADDRELL, 1971).

Fig. 1.2


Na+
K+
Na+
Arsenate
phosphate
Cu2+
Cl-
Ca2+
Mg2+
Basal Cell Membrane
K+
Na+
phosphate
Cl-
Ca2+
Mg2+
Apical Cell Membrane

and Cl^- enter the cells passively at a rate depending on the permeability of the membrane to these irons and on the electrochemical gradients across the cell membrane. Earlier MADDRELL (1971) thought it was not feasible for K^+ to move passively from an external concentration of, for example, 5 mM K^+ when it is known that most cells have an intracellular concentration of between 50 and 200 mM K^+.

Although K^+ and Na^+ have been considered to be the prime movers, anions too play an important role in urine production. BERRIDGE (1969) and MADDRELL (1969, 1971) have demonstrated that K^+ alone will not induce water transport unless accompanied by an appropriate anion. Supposing Cl^- bathing isolated tubules of Calliphora is replaced with a non-transporting anion such as sulphate, fluid transport ceases (BERRIDGE 1969). As previously described, it is generally thought that Cl^- transport across the basal cell membrane is passive and that Cl^- follows K^+ out of the cell on the luminal side (MADDRELL, 1977). However, active transport of Cl^- has also been reported in Malpighian tubules of Rhodnius (MADDRELL 1971, 1977). Chloride transport is widely observed in several insect epithelia including locust rectum (HANRAHAN and PHILLIPS, 1983), insect Malpighian tubules (MADDRELL, 1971, 1978; PHILLIPS,1981), salivary glands of Dermacentor andersoni (KAUFMAN and PHILLIPS, 1973), hindgut of Aedes taeniorhynchus, (BRADLEY and PHILLIPS, 1977). Recently, two mechanisms have been proposed to explain Cl^- transport through a variety of epithelia (see reviews by FRIZZELL et al., 1979, PHILLIPS, 1981; HANRAHAN and PHILLIPS, 1983), 1. Na^+-coupled electrogenic Cl^- secretion, 2. Cl^-/HCO_3^- exchange pump at the entry step.

1. Na^+-coupled electrogenic Cl^- secretion : All NaCl cotransport models, whether absorptive or secretory postulate an uphill transport of Cl^- into epithelial cells (energy-requiring entry) driven by the inward flow of Na^+ down a favourable electrochemical gradient. Exit of Na^+ from the cell at the serosal membrane occurs via the ubiquitous ouabain-sensitive Na^+K^+ ATPase which explains why ouabain inhibits Cl^- transport (HANRAHAN and PHILLIPS, 1983). Na^+ is actively recycled while Cl^- moves passively from the cell into mucosal sides through a cAMP-stimulated conductance. This Na^+-coupled chloride secretion has been described in several epithelia (CANDIA, 1972; ZADUNAISKY, 1966, 1979; FRIZZEL et al., 1979; HANRAHAN and PHILLIPS 1983). It is also thought to occur in Rhodnius Malpighian tubules (PHILLIPS, 1981). In Rhodnius the Na^+ driven component of fluid secretion specifically requires Cl^- whereas various other anions, e.g. NO_3 will sustain K^+-driven secretion in the absence of external NaCl. Furosemide which inhibits the coupled entry of NaCl into vertebrate epithelial cells, completely stops fluid secretion by Rhodnius Malpighian tubules (review by PHILLIPS, 1981). The latter suggested that there is a separate Cl^- (basal) and a K^+ (Apical) transport process in Rhodnius. Conceivably Na^+ (as previously described) drives Cl^- entry into the cell by a cotransport and is then pumped out in exchange for K^+. In this way active local recycling of Na^+ would help drive both Cl^- and K^+ influx into the cell without net secretion of Na^+ stimulation of both necessarily occurring. This would explain Na^+ stimulation of both K^+ and Cl^- secretion by insect Malpighian tubules (see Figure 1.3B).

With the use of furosemide, more recently, O'DONELL and MADDRELL (1984) propose that in Rhodnius, Cl^- does not enter the cells through passive channels in the basal membrane as in salivary gland and Malpighian tubules of Calliphora (BERRIDGE, 1969) nor is it actively

pumped as in locust rectum (HANRAHAN and PHILLIPS, 1983) rather Cl^- appear to enter the Malpighian tubule basal cell membrane through cotransport with Na^+ and K^+. Cl^- probably crosses the apical membrane into the lumen passively in response to a favourable electrochemical gradient. The apical cation pump maintains Na^+ at low intracellular concentration by making a favourable gradient for entry of Na^+ through the proposed basal cotransport. The suggested stoichiometry is Na^+: K^+: $2Cl^-$ (O'DONELL and MADDRELL, 1984).

2. Cl^-/HCO_3^- exchange pump : A second widely accepted mechanism of epithelial Cl^- transport involves Cl^-/HCO_3^- exchange at the entry. This has been proposed for frog skin, GARCIA-ROMEU et al., (1969); fish gills, De RENZIS and MAETZ (1973); rabbit colon, POWELL, (1979); mosquito larvae anal papillae, STOBBART, (1967). The energy source for this process is unknown, but it has been suggested that Cl^- influx is energized in part by an ATPase or possibly by a passive outflow of HCO_3 from the cell (FRIZZEL et al., 1979) (see Figure 1.3C).

There is at present no evidence for a primary transport mechanism for Cl^- in animals, but there are some recent reports on the existence of anion-dependent ATPases sensitive to Cl^- in insect rectum (HERRERA et al., 1978, KOMNICK et al., 1980), fish gills (BORNANCIN et al., 1980), rat small intestine (HUMPHREYS and CHOU, 1979) and plant cells (HILLS and HANKE, 1979).

The nature of the transport of HCO_3^- across different epithelia is also not yet fully understood. In the water boatman, Cenocorixa bifida, for example, it is thought that HCO_3^- might be excreted by the Malpighian tubules in order to regulate the haemolymph pH. These animals live in saline lakes with high HCO_3^- concentrations (SZIBBO and SCUDDER, 1979). These workers suggest that the transport

FIGURE 1.3 A-D : Diagrammatic description of various models proposed for sodium-coupled Cl^- absorption (A) and secretion (B) and for bicarbonate-coupled Cl^- absorption (C,D). Proposed sites of action of inhibitors and stimulant, cAMP, in some systems are indicated. Mucosal side on left in all diagrams

The models for various epithelia differ in the proposed mechanism for Cl^- exit from the cell, e.g. K^+ and HCO_3^- -coupled steps have been suggested where serosal membrane conductance is not sufficient to permit diffusion of Cl^- from the cell (Figure 1.3A) (e.g. gall bladder epithelium REUSS and GRADY, 1979). To explain electrogenicity of transepithelial Cl^- absorption in flounder intestine, in the presence of electroneutral NaCl coentry, it is postulated that some Na^+ leaks back to the mucosal side from the lateral intercellular spaces via tight junctions (Figure 1.3Ai). Na^+-coupled electrogenic Cl^- secretion is found in a wide variety of epithelia including cornea (CANDIA, 1972) rabbit ileum (NELLANS et al., 1973) dogfish rectal gland and Killifish operculum (review by FRIZZELL et al., 1979). In these systems (Figure 1.3B) Na^+ is believed to be actively recycled at the serosal border while Cl^- moves passively from the cell to the mucosal side through a cAMP-enhanced Cl^- conductance, (KLYCE and WONG, 1977).

Fig. 1.3C shows the proposed Cl^-/HCO_3^- exchange 'pump' as previously discussed in the text.

Fig. 1.3D - finally WHITE (1980) has provided evidence for an electrogenic Cl^- transport at the apical membrane of Amphiuma intestine without specifying the energy source. This Cl^- mechanism is not strictly Na^+-coupled but does require both intracellular Na^+ for Na^+/H^+ exchange (which reduces backflux of Cl^- from cell to lumen) and also serosal HCO_3^- for Cl^-/HCO_3^- exchange at the serosal membrane.

Source: HANRAHAN and PHILLIPS, (1983) (see also text).

Fig. 1.3

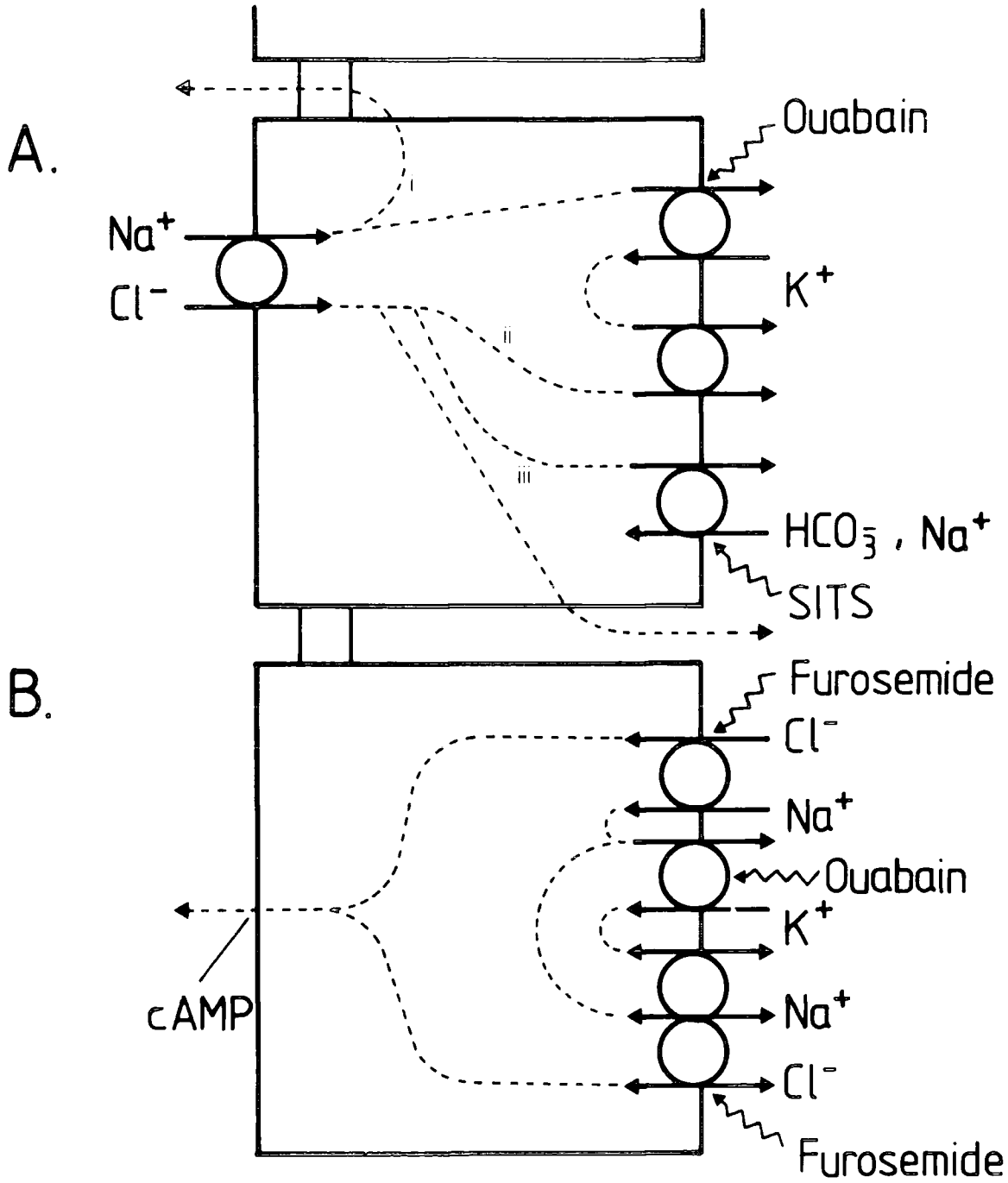
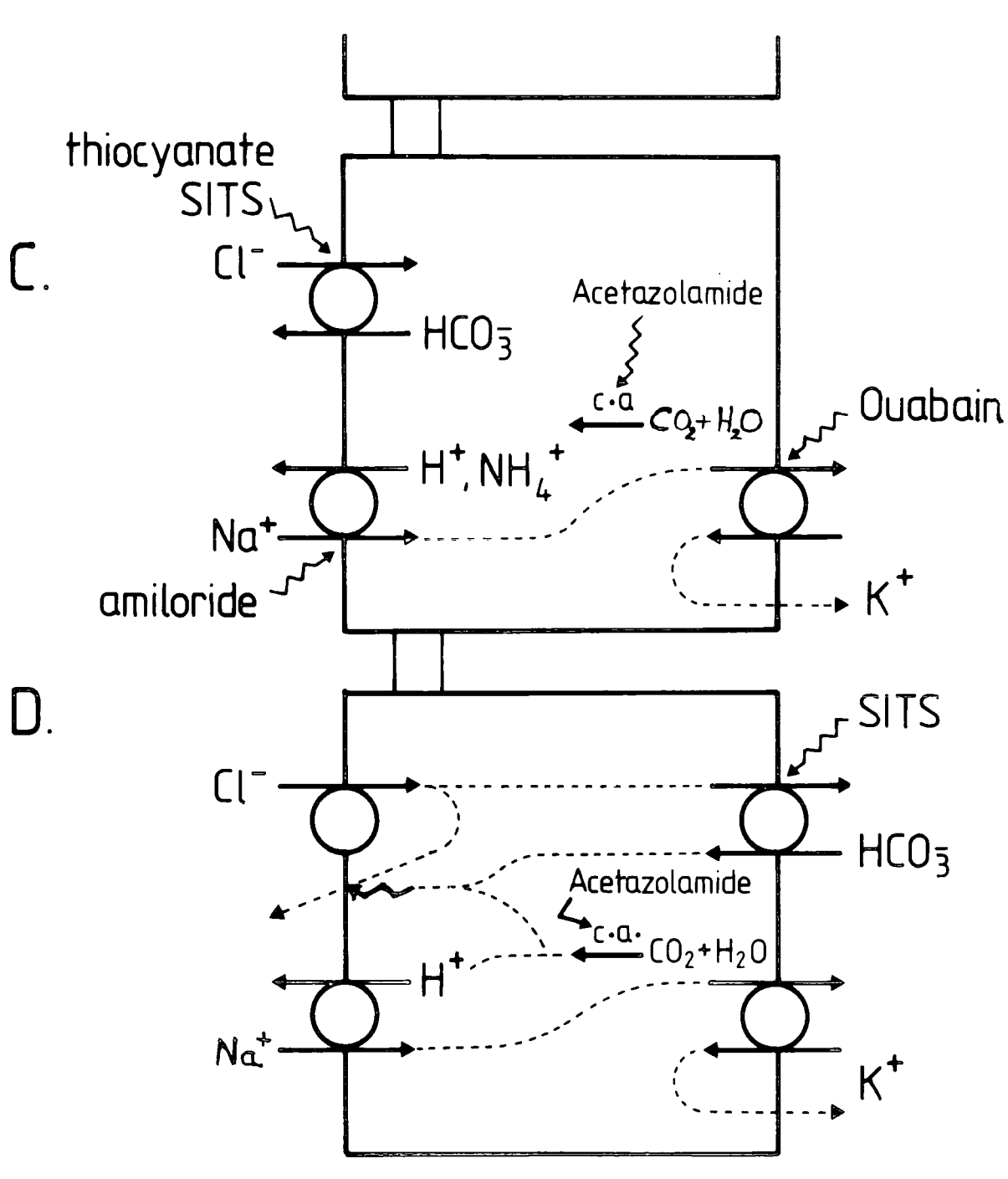

A.
Ouabain
Na+
Cl-
K+
HCO3-, Na+
SITS
B.
Furosemide
Cl-
Na+
Ouabain
K+
cAMP
Na+
Cl-
Furosemide
MUCOSAL SIDE
CELL
SEROSAL SIDE

Fig. 1.3


thiocyanate
SITS
C.
Cl⁻
HCO3⁻
Acetazolamide
c.a
CO2+H2O
Ouabain
H+, NH4+
Na+
amiloride
K+
D.
SITS
Cl⁻
HCO3⁻
Acetazolamide
c.a.
CO2+H2O
H+
Na+
K+
MUCOSAL SIDE
CELL
SEROSAL SIDE

of HCO_3 may either be passive or active via a lumen-directed HCO_3^- -'pump'. A HCO_3^- -stimulated ATPase has been demonstrated in a variety of tissues from different species where it has been implicated in a number of ion transport processes. For example, acid secretion and HCO_3^- transport in gastric mucosa (BLUM et al., 1971; SACHS et al., 1972B), H^+/HCO_3^- in submandibular gland (IZUTSU and SIEGEL, 1972); HCO_3^- transport and Na^+/H^+ exchange in renal proximal tubules (KINNE-SAFFAREN and KINNE, 1974; LIANG and SACKTOR, 1976); HCO_3^-/Cl^- exchange in locust rectum HERRERA et al., 1977; 1978), and HCO_3^- transport in mammalian pancreas (SIMON and THOMAS, 1972; VAN AMELSVOORT et al., 1978B). Unlike NaK^+ ATPase, HCO_3-stimulated ATPase is insensitive to ouabain; and although it is shown to be inhibited by SCN^- in several epithelia, there is lack of a specific inhibitor for this enzyme (ANSTEE and FATHPOUR, 1979; FATHPOUR, 1980; present study). The former workers have identified the Mg^{2+}, HCO_3^- -ATPase in Locusta Malpighian tubule preparations; and also implicated this enzyme in the transport of water and anions across the Malpighian tubules. However in view of the scanty information concerning the nature of ion coupled water transport across insect Malpighian tubules, the present study also investigates further into the presence of Mg^{2+},HCO_3^- -ATPase in Locusta Malpighian tubules. With the use of various inhibitors, an attempt has been made to establish Mg^{2+},HCO_3^- -ATPase involvement the transport of ion and water across the Malpighian tubules. Some of the proposed schemes for the transport of anions across different epithelia are shown in Fig. 1.3 A-D.

As the study of water and ion transport across insect epithelia is still underway, and the precise location of the Na^+,K^+-ATPase 'pump' is uncertain, an attempt has also been made to localise

Na^+, K^+-ATPase in Locusta Malpighian tubule cells by employing a modified WACHSTEIN and MEISEL (1957) technique designed by ERNST (1972a,b). The controversy surrounding the validity of this procedure in localising Na^+,K^+-ATPase in insect tissues is disscussed in Chapter 6.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Maintenance of Insects

A stock population of Locusta migratoria L. was maintained in an insectary at 28 ± 0.5°C and a relative humidity of 60 ± 5% with a constant photoperiod of 12 hours light and 12 hours dark. Continuous air exchange with the outside was provided by means of a fan-driven ventilator (Xpelair) and circulation within the room was effected by three wall-mounted electric fans. Humidity was controlled by 3 humidifiers, (Lumatic Humidifier Group, Bromley, Kent, England). The locusts were reared in perspex-fronted cages 41 cm x 41 cm x 60 cm, (supplied by Phillip Harris Biological Supplies Ltd., Oldmixon, Weston-super-Mare, Avon). Each cage was illuminated by a 40 watt bulb and consequently the temperature within the cage varied from 30 - 40°C depending on the distance from the bulb and with the photoperiod. Humidity within each cage also varied due to the addition of fresh grass, water and Bemax on which the insects were fed daily. Throughout their development the animals were reared at sufficiently high population density to prevent reversion to the solitaria phase (JOLY and JOLY, 1953).

2.2 Chemicals

All chemicals used were the purest available and were supplied either by Sigma Co., Kingston-uponThames, Surrey, U.K., or B.D.H., Poole, Dorset, U.K.

2.3 Glassware

Pyrex glassware was used throughout. Prior to use it was cleaned by soaking overnight in 2% Quadralene laboratory detergent followed by several rinses in hot tap water and a final thorough rinse in distilled

water. Except for the glass/teflon homogenisers which were allowed to drain at room temperature, all other glassware was dried in ovens.

2,4 Statistical Analysis

Statistical comparisons of data were performed by using a computer program based on methods described by SNEDECOR and COCHRAN (1967). Means and S.E.M. were calculated as 't' tests were applied. Statistical tables of FISHER and YATES (1963) were used to determine probability values. Probability values of or less than 0.05 were taken as significant.

2.5 Preparation of Tris ATP

(Tris Salt of adenosine triphosphate).

The disodium salt of ATP was converted into Tris ATP by using an iron exchange Dowex 50 resin (H^+ form) according to SCHWARTZ et al., (1962). The Dowex resin was first well rinsed with distilled water in a Buchner funnel and the wet weight noted. It was then washed in 3N HCl (Anala R), using 30 mls of acid per 5g wet weight of Dowex resin. The resin was washed again in distilled water until the effluent had a pH between 3-4. At this stage all the residual acid was removed from the resin and it was in its charged (H^+) form. It was resuspended in its own volume of distilled water and stored at 0-4°C until required.

A known quantity of disodium ATP was dissolved in a known small volume of distilled water (approximately 10 mls) and thoroughly mixed using a magnetic stirrer. A layer of the charged Dowex was placed in a Buchner funnel lined with 2-3 layers of moistened filter paper. The Dowex was evenly spread in the funnel. Using a Pasteur pipette drops of the disodium ATP solution were gently passed through the Dowex resin. The effluent was collected in a conical flask as the ATP in

its (H^+) form. This was then converted to Tris salt by the addition of drops of 2M Tris until the pH was 7.2. It was then made up to the required volume and stored at 20°C.

2.6 Analysis of Inorganic Phosphate

Inorganic phosphate (Pi) liberated in the presence of the enzyme was determined by reference to a standard calibration curve prepared by assay of standard phosphate solutions. The standard phosphate solutions were prepared from a stock solution containing 20 µg phosphorus (as KH_2PO_4/ml). Serial dilution of this stock solutions gave samples of 20, 15, 10, 5, 2, 1 and 0 µgPi/ml. To 2 mls of each sample, 4 mls of Cirrasol solution were added (see Chapter 3 Section 3.1 for composition of Cirrasol solution). The tubes were allowed to stand at room temperature for 10 minutes before measuring the absorbance at 390 nm. A standard calibration curve was drawn by plotting the amount of inorganic phosphate (Pi) in nmoles against absorbance. A typical example of such a curve is shown in Figure 2.1.

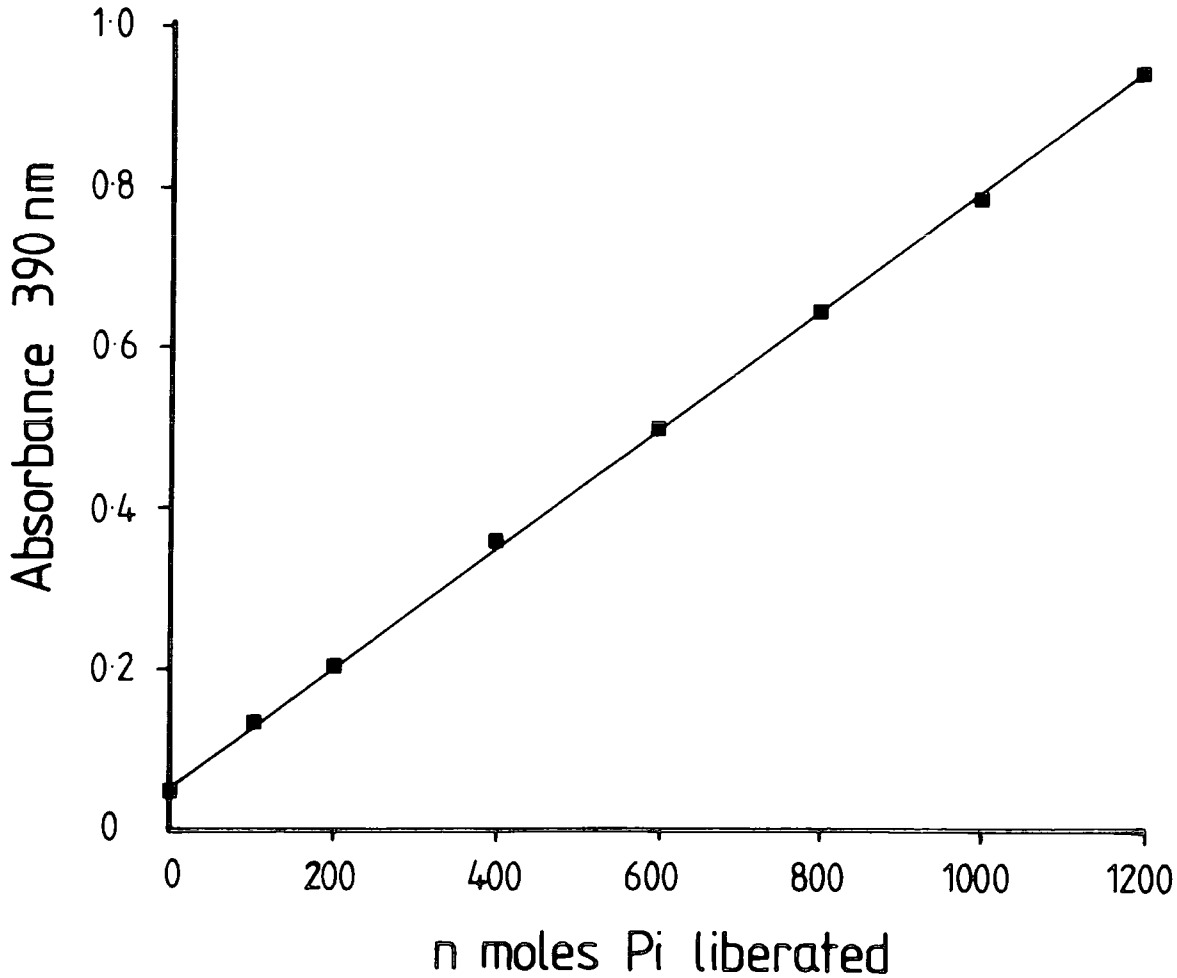
FIGURE 2.1

A standard calibration curve for determination of inorganic phosphate.

Ordinate : Absorbance 390 nm

Abscissa : n moles Pi liberated

Fig. 2.1


1·0
0·8
0·6
0·4
0·2
0
Absorbance 390 nm
0
200
400
600
800
1000
1200
n moles Pi liberated

2. 7 Estimation of Protein

The method employed is essentially the same as that described by LOWRY et al., (1951) using bovine serum albumin (B.S.A.) Fraction V as Standard Reagents:

(i) 2% Na_2CO_3

(ii) 0.5% $CuSO_4$

(iii) 1% KNa Tartrate

Folin Solution A :

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

Folin Solution B :

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

Method

Bovine serum albumin (B.S.A.), Fraction V was serially diluted to give concentrations of 400, 300, 200, 150, 100, 50 and 0 µg/ml protein. 3 mls of Folin Solution A were added to 0.2 mls of protein solution and this allowed to stand for 30 minutes at room temperature. 0.3 ml of Folin Solution B was then added to each tube and the resulting solutions allowed to stand for a further 60 minutes at room temperature. The absorbance was measured at 500 nm. A standard calibration graph of protein concentration against absorbance was constructed. From this curve the unknowns could be determined. A new calibration curve was prepared each time an assay was carried out. A typical standard protein calibration curve is shown in Figure 2.2.

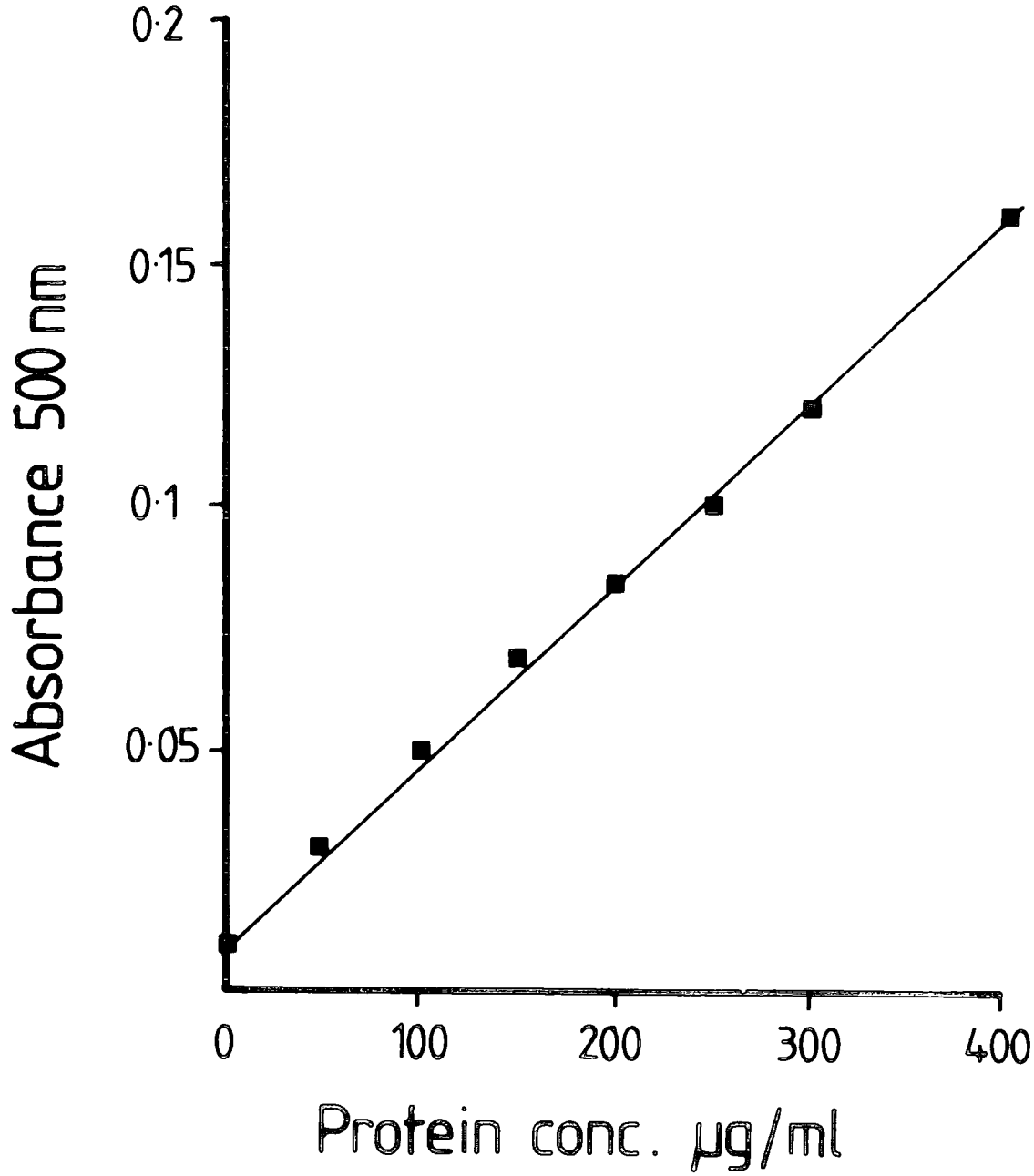
FIGURE 2.2

A standard calibration curve for determination of protein.

Ordinate : Absorbance at 500 nm

Abscissa : Concentration of Protein in µg/ml

Fig. 2.2


0·2
0·15
0·1
0·05
Absorbance 500 nm
0
100
200
300
400
Protein conc. µg/ml

2.8 Calculation of energies from Arrhenius Plots

Activation energies (Ea) were calculated from the Arrhenius equation:

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

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

CHAPTER 3

BIOCHEMICAL STUDIES OF Na^+, K^+-ATPase ISOLATED FROM MALPIGHIAN TUBULES OF LOCUSTA MIGRATORIA L.

Introduction

There is increasing evidence in the literature pointing to the fact that the enzyme responsible for movement of Na^+ and K^+ and fluid through different transporting epithelia is a magnesium-dependent, sodium and potassium stimulated adenosine triphosphatase (Na^+, K^+-ATPase) (E.C.3.6.1.3 SKOU, 1965). This enzyme was first recognised by SKOU (1957) in a crab nerve preparation. Subsequently, it has been isolated from numerous animal tissues (e.g. erythrocyte membranes, DUNHAM and GLYNN, 1961; POST et al., 1960; mammalian kidney, WHITTAM and WHEELER, 1961; SKOU, 1962, dog pancreas, RIDDERSTAP and BONTING, 1969; frog and rat brain, BOWLER and DUNCAN 1968a,b; several insect tissues, see Table 3.1).

To date there are relatively reasonable numbers of demonstrations of ATPases from insect tissues. Early biochemical studies using sucrose extraction techniques failed to demonstrate any Na^+, K^+-ATPase activity in cockroach muscle (WAREHAM et al., 1968), and BERRIDGE and GUPTA (1968) also failed to demonstrate Na^+, K^+-ATPase activity in preparations from Calliphora rectum despite their histochemical demonstration of Mg^{2+}-ATPase activity.

One of the first successful demonstrations of Na^+, K^+-ATPase activity in insect tissue was carried out by GRASSO (1967) using cockroach nerve cord. Later, Na^+, K^+-ATPase activity in the brain (CHENG and CUTKOMP, 1975) and in Malpighian tubules of Schistocerca gregaria and Jamaicana flava (PEACOCK et al., 1972), Locusta (ANSTEE and BELL, 1975, 1978); (PEACOCK, 1975, 1976); (BELL, 1977);

(FATHPOUR, 1980); DONKIN, 1981). Similarly the present study has demonstrated the presence of Na^+,K^+-ATPase in Malpighian tubule preparations of Locusta. Other recent studies on Na^+,K^+-ATPase in insect tissue include that of PEACOCK et al., (1976) who report its presence in Malpighian tubules of Homorocoryphus nitidulus vicinus and rectal epithelium of Periplaneta americana. Thus Na^+,K^+-ATPase has been identified in a number of tissues from some 20 insect species listed in Table 3.1 which is largely taken from ANSTEE and BOWLER (1984). Examples of some vertebrate animals species from which Na^+,K^+-ATPase has been isolated are shown in Table 3.2.

From these studies on a wide variety of tissues from different animal species, it is evident that Na^+,K^+-ATPase is widely distributed in animal cell plasma membranes (BONTING, 1970). The distribution of Na^+,K^+-ATPase activity in 39 tissues of man and cat was reported by BONTING (1970) and he demonstrated that the enzyme was present in all but 6 of these tissues. The ubiquity of the enzyme, and its high activity in ion transporting epithelia suggests that it has a functional role in ion and probably fluid secretion.

Considerable effort has been made to elucidate the reaction mechanism of the Na^+,K^+-ATPase. SKOU (1965) describes the basic characteristics of this enzyme as being:

(1) Located in cell membranes.

(2) Capable of hydrolysing ATP and thus converting the energy from ATP into the cation movement.

(3) Requires Mg^{2+}.

(4) Stimulated by the simultaneous presence of Na^+ and K^+.

TABLE 3.1 : Presence and Properties of Na^+, K^+-ATPase from various insect and one tick species

Species	Tissue	Preparation	Specific activity	Assay temp °C	pI ouabain	Na^+ (mM) app. Km	Na^+ (mM) (max)	K^+ (mM)	K^+ (mM) (max)	pH	ATP (mM) app. Km	Reference
Locusta migratoria	Recta	Lyophilate	120	30	-	-	-	-	-	-	-	Peacock (1979)
Locusta migratoria	Malpighian tubules	Microsomes	229	30	6.1	-	-	-	-	-	-	Anstee and Bell(1975)
Locusta migratoria	Malpighian tubules	Microsomes	292	30	-	-	-	-	-	-	-	Donkin and Anstee(1980)
Locusta migratoria	Malpighian tubules	Microsomes	213	30	-	28	100	1	20	7.5	0.18	Anstee and Bell(1978)
Locusta migratoria	Recta	Microsomes	105	30	-	-	-	-	-	-	-	Peacock(1976a)
Locusta migratoria	Recta	Microsomes	80	30	-	-	-	-	-	-	-	Peacock(1978)
Locusta migratoria	Recta	Lyophilate	140	30	6.0	-	-	-	-	-	-	Peacock(1981a)
Periplaneta americana	Nerve cord	Microsomes	*	37	6.4	-	100	5	20	7.6	-	Grasso(1967)
Periplaneta americana	"Brain"	Crude supernatant	2	24	-	-	-	-	-	7.4	0.07	Piccione and Baust(1977)
Periplaneta americana	Nerve cord	Mitochondrial pellet	438	27	-	-	-	-	-	-	-	Cheng and Cutcomp(1975)
Periplaneta americana	Rectum	Lyophilate	500	-	5.3	19.6	120	2.7	10	7.05	-	Tolman and Steel(1976)
Periplaneta americana	Antenna	Microsomes	250	30	6.2	6.3	90	3.7	20	9.0	0.25	Norris and Cary(1982)
Manduca sexta	Nerve cord	Microsomes	73	-	6.0	-	54	3.7	54	7.5	-	Rubin et al.(1980)
Manduca sexta	Head	Crude homogenate	**	-	5.0	-	-	2.2	-	-	-	Vaughan and Jungreis(1977)
Hyalophora cecropia	Head	Crude homogenate	**	-	5.3	-	-	-	-	-	-	Vaughan and Jungreis(1977)
Danaus plexippus	Head	Crude homogenate	**	-	<3.0	6.0	-	2.2	-	-	-	Vaughan and Jungreis(1977)
Calliphora erythrocephala	Eye	Crude homogenate	320	-	5.75	30	100	2	15	7.6	-	Rivera(1975)
Calliphora erythrocephala	Brain	Crude homogenate	330	-	-	-	-	-	-	-	-	Rivera(1975)
Musca domestica	Brain	Microsomes	1000	25	5.6	20	-	1	-	-	0.5	Jenner and Donnellan(1976)
Aedes aegypti	Larvae	Mitochondrial pellet	80	37	-	-	-	-	-	7.2	-	Yap and Cutcomp(1970)
Drosophila melanogaster	Imaginal discs	Homogenate	23	-	-	-	-	-	-	-	-	Fristrom and Kelly(1976)
Paragnetina media	Gill	Microsomes	630	37	-	-	-	-	-	7.4	-	Kapoor(1980)
Paragnetina media	Malpighian tubules	Microsomes	1087	37	-	-	-	-	-	-	-	Kapoor(1980)
Paragnetina media	Rectum	Microsomes	700	37	-	-	-	-	-	-	-	Kapoor(1980)
Glossina morsitans	Midgut	Lyophilate	120	30	6.0	-	-	-	-	-	-	Peacock(1982)
Glossina morsitans	Midgut	Lyophilate	157	30	-	-	-	-	-	-	-	Peacock(1981b)
Glossina morsitans	Hindgut	Lyophilate	97	30	-	-	-	-	-	-	-	Peacock(1981b)
Sarcophaga nodosa	Midgut	Lyophilate	13	30	-	-	-	-	-	-	-	Peacock(1981b)
Sarcophaga nodosa	Recta	Lyophilate	79	30	-	-	-	-	-	-	-	Peacock(1981b)
Bombyx mori	Ileum	Lyophilate	0	30	-	-	-	-	-	-	-	Peacock(1981b)
Bombyx mori	Rectum	Lyophilate	0	30	-	-	-	-	-	-	-	Peacock(1981b)
Homorocoryphus nitidulus	Malpighian tubules and rectum	Microsomes	240	30	6.5	-	-	3	20-25	7.4	0.14	Peacock et al.(1976)
Blaberus craniifer	Rectum	Microsomes	86	30	-	-	-	-	-	-	-	Peacock(1977)
Schistocerca gregaria	Rectum	Microsomes	75	30	-	-	-	-	-	-	-	Peacock(1977)
Schistocerca gregaria	Rectum	Microsomes	67	30	-	-	-	-	-	-	-	Peacock et al(1972)
Jamaicana flava	Rectum	Microsomes	18	30	-	-	-	-	-	-	-	Peacock et al(1972)
Rhodnius prolixus	Follicle cells	Microsomes	40	37	-	-	-	-	-	-	-	Abu-Hakima and Davey(1979)
Rhodnius prolixus	Follicle cells	Microsomes	90	37	6.0	37	100	2.2	10	7.4	0.79	Ilenchunk and Davey(1982)
Chironomus thummi	Salivary gland	Crude homogenate	*	37	6.92	3.5	10	1.5	5	7.6	0.5	Schin and Kroeger(1980)
Amblyomma hebraeum (tick)	Salivary gland	Microsomes	516	37	6.52	21	-	1.2	-	7.0	-	Rutti et al(1980)

Specific activity in nanomoles Pi liberated per milligram protein per minute. * Quoted activity not convertible to specific activity
** Quoted activity not convertible to an equivalent specific activity. app.Km = the apparent Michaelis constant (Km) of the enzyme for the ligand.
[max] = concentration of ligand giving maximal enzyme activity.

TABLE 3.2 : Ouabain-sensitivity of Na^+, K^+-ATPase from some vertebrate animal tissues

Source of Enzyme	pI_{50} (Ouabain)	Reference
Mammalian Kidney	6.8	SKOU, (1962)
Guinea Pig Kidney	6.7	KINSOLVING et al., (1963)
Rabbit Kidney	5.7	WHITTAM and WHEELER (1961)
Dog Pancreas	6.8	RIDDERSTAP (1969)
Rat Liver	3.9	BAKKEREN and BONTING (1968)
Shark rectum	6.8	BONTING (1966)
Dogfish rectum	6.8	BONTING (1966)

(5) Inhibited by the cardiac glycoside, ouabain.

(6) The rate of ATP hydrolysis is dependent on the intracellular concentration of Na^+ and requires K^+ outside the cell. There is a sodium site(s) at the internal face and a potassium site(s) at the external face of the membrane. The cations are translocated across the membranes by the enzyme by a mechanism which is not yet fully understood. In red blood cells it has been shown that $3Na^+$ ions are extruded and $2K^+$ ions are accumulated per molecule of ATP hydrolysed by Na^+,K^+-ATPase.

(7) Characteristically, a second ATPase enzyme is found in membrane preparations containing Na^+,K^+-ATPase. This is a Mg^{2+} ATPase which is not inhibited by ouabain. The function of Mg^{2+} ATPase is not yet understood. It is not directly involved in monovalent cation transport and is thought to be a separate enzyme (NAKAO et al., 1963). It has been suggested that it may be important in controlling passive permeability (BOWLER and DUNCAN, 1967) or in calcium transport (WINS and SCHOFFENIELS, 1966 and SCHATZMAN and VINCENZI, 1969).

Due to its firm association with the plasma membrane it has not been easy to obtain pure samples of Na^+,K^+-ATPase. In SKOU's (1962) work isolation of Na+, K^+-ATPase was carried out by homogenisation of the tissue in a sucrose medium containing a chelating agent such as ethylenediaminetetra-acetic acid (E.D.T.A). This was followed by differential centrifugation to yield a microsomal preparation. This method was later modified by chemically breaking up the membrane into vesicles which contain the enzyme. NAKAO et al., (1963) introduced the use of sodium iodide in extracting the enzyme; AHMED and JUDAH (1964)

used mannitol instead of sucrose in the homogenising medium, and JØRGENSEN and SKOU (1969) added sodium deoxycholate in the homogenisation medium. ROBINSON, (1967) combined the use of mannitol, sodium deoxycholate and sodium iodide and this significantly improved the Na^+, K^+-ATPase ; Mg^{2+} ATPase activity ratio.

Subsequently a variety of techniques have been employed in the separation of Na^+, K^+-ATPase (UESEGI et al., 1971; KYTE, 1971; HOKIN et al., 1973; HOKIN, 1974; JØRGENSEN, 1974a,b). According to their studies Na^+, K^+-ATPase consists of two polypeptide chains, a large polypeptide of MW 85,000 - 95,000 and a sialoglycoprotein of MW 50,000 - 60,000. HART and TITUS (1973) suggest that it is the large protein chain that possesses the binding sites for Na^+, K^+, ATP and ouabain and which becomes phosphorylated when ATP is split (UESUGI et al., 1971).

In an attempt to explain the involvement of Na^+, K^+-ATPase in the ion and water transport system through the epithelia, several models have been proposed to describe the stepwise mechanism of the enzymatically catalyzed hydrolysis of ATP (see reviews by SIEGEL and ALBERS, 1967; ALBERS, 1967; SKOU, 1975; WHITTAM and CHIPPERFIELD, 1975; STEKHOVEN and BONTING, 1981). The most recent model has been proposed by JØRGENSEN (1982) (see Figure 3.1). The common feature in these models is that transport occurs in a series of partial reactions related to cyclic phosphorylation and dephosphorylation. Following the binding of ATP to the enzyme (E_1 + ATP), the first step is the formation of an intermediary phosphorylated compound. This reaction depends on Na^+ and Mg^{2+} and is accompanied by a breakdown of ATP to ADP as illustrated below:

$$\text{Step 1} \qquad E_1 + ATP \underset{Na}{\overset{Mg^{2+}}{\rightleftharpoons}} E_1 \sim P + ADP.$$

FIGURE 3.1 : Minimal Scheme illustrating the relationship of ion movements and principal conformational changes of Na^+, K^+-ATPase operating in a normal Na^+/K^+ exchange mode as identified in kinetic studies.

(SKOU, 1965; ALBERS, 1967)

Inside the cell magnesium ATP binds to the enzyme with high affinity to form an enzyme-ATP complex (E_1). While in this cytoplasmic phase E_1 preferentially binds to Na^+. i.e. E_1 exchanges K^+_{cyt}. for Na^+cyt. E_1 is phosphorylated by MgATP but not by Pi. Then the enzyme bound Na^+ is translocated to the outside of the cell where it is exchanged for K^+. K^+ binding to the phophoenzyme induces the second conformational change (E_2). E_2 exchanges Na^+_{ext} for K^+_{ext} in the extracellular medium. This transition from E_1Na — E_2K is very fast whereas the reversal is slow (JORGENSEN, 1982) E_2 then translocates K^+ from outside the cell to the inside of the cell. E_2 is phosphorylated by Pi but not ATP. In the E_2K form, potassium ions are occluded and binding of ATP at a low affinity site accelerates release of K^+_{cyt} inside the cell and the enzyme enters another cycle. With a reversal of the pump, each of the steps shown in Fig.3.1, is reversed.

Note: For simplicity, a single cation binding is shown here, although the pump normally exchanges 3 internal Na^+ for 2 external K^+ ions.
(E_1 and E_2) define different conformational states of the enzyme.
(E_1P and E_2P) are the phosphorylated forms of E_1 and E_2.
(cyt) cytoplasmic (ext) extracellular.

Note also that Mg^{2+} is required for phosphorylation, conversion between E_1P and E_2P, and for dephosphorylation. More details are given by JØRGENSEN (1982) from whom this model has been largely drawn.

With reference to the present study, it appears that ouabain binds outside the cell membrane when the enzyme assumes the Na^+-induced form (TAI, 1977) whereas vanadate binds on the cytoplasmic side when the enzyme is K^+-bound (also see Chapter 3).

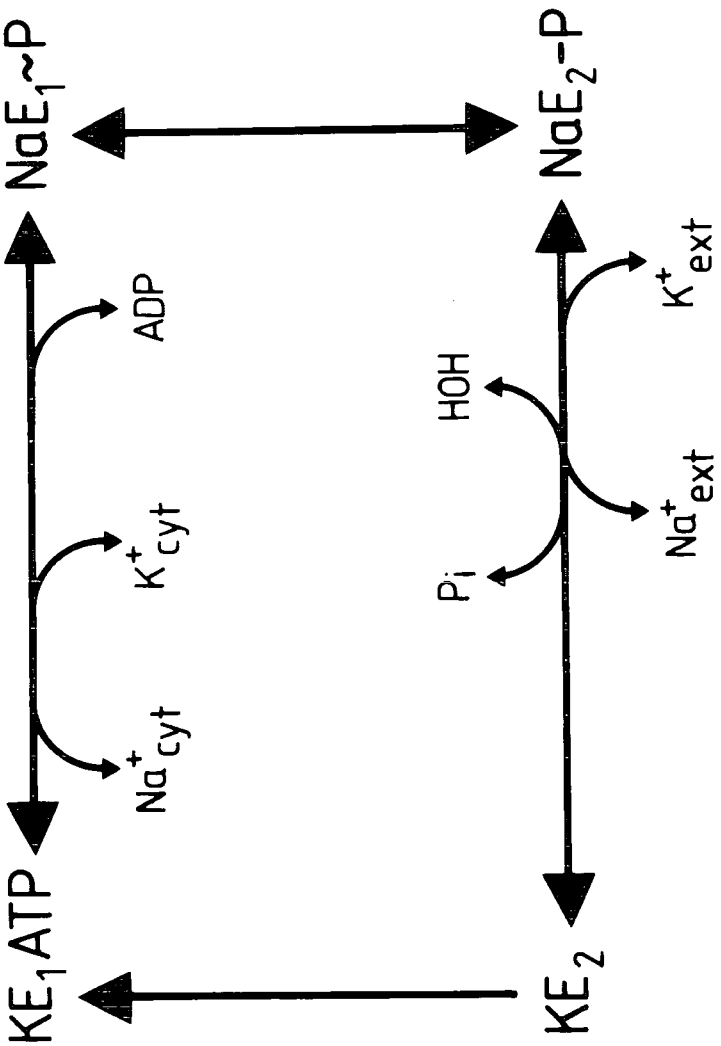


Fig. 3.1

Adapted from Jørgensen (1982)

[The resulting phosphorylated intermediate ($E_1 \sim P$) must be a high-energy compound since it can be made to react with ADP to form ATP (i.e. reversal of Step I)].

Step 2 $E_1 \sim P \rightleftharpoons E_2 - P$

This is a probable step at which high-affinity Na^+ sites on the inside of the cell membrane become outward oriented and change to K^+ affinity sites. As a result Na^+ unloads and K^+ loads. The resulting state of the phosphorylated enzyme is apparently that of low energy compound indicated by the symbol (E_2 - P). It does not react with ADP but will decompose in the presence of K^+ (STEKHOVEN and BONTING,1981). Although it has been questioned by KLODOS and SKOU (1975, 1977) this indicates that ($E_1 \sim P$) and (E_2 - P) are two different states of the enzyme.

Step 3 K^+-stimulated dephosphorylation.

This involves the K^+-stimulated dephosphorylation during which the K^+-binding sites (on the outside) turn inward and the enzyme returns to its original conformation

$$E_2 - P + H_2O \underset{\text{Ouabain inhibits}}{\overset{K+}{\rightleftharpoons}} E_2 + P_i$$

Step 4. This is the reverse of the cationic sites on the inside and outside of the membrane and results in restoration of the initial orientation and affinities.

$$E_2 \rightleftharpoons E_1 \quad .$$

It is important to remember that it is the K^+-stimulated phosphatase activity which represents the final reaction step and is inhibited by ouabain. This component of Na^+, K^+ -ATPase activity is often assayed in the presence of p-nitrophenylphosphate. (NPP) as a substrate (see Chapter 6). The hydrolysis of NPP leads to the direct

formation of E_2 - P which is then dephosphorylated (STEKHOVEN and BONTING, 1981).

The ouabain-binding site is on the outside of the cell membrane. Binding of cardiac glycosides is modulated by ligands and its rate parallels the inhibition of the microsomal Na^+, K^+-ATPase (ALBERS et al., 1968; ALLEN and SCHWARTZ, 1970). Results reported by these workers have indicated that ouabain binds to the (E_2 - P) form of the enzyme which means that ouabain and K^+ compete for the same conformational state of the enzyme. Adding K^+, discharges this form of the enzyme (E_2 - P $\xrightarrow{K^+}$ E_2 + Pi) and so K^+ produces a form of the enzyme which does not bind to ouabain. Mg^{2+} appear to be a pre-requisite for ouabain-enzyme interaction but JØRGENSEN and KARLISH (1980) pointed out that ouabain can inhibit Na^+, K^+-ATPase activity regardless of the ligand composition. The kinetics of ouabain-binding are such that the dissociation of the enzyme-ouabain complex is very slow, thus preventing further K^+-stimulated dephosphorylation or ion translocation (WHITTAM and CHIPPERFIELD, 1975).

The first report of ouabain inhibition was given by SCHATZMAN (1953) and later confirmed by SKOU (1957) and DUNHAM and GLYNN (1961) when they demonstrated a specific inhibition of Na^+, K^+-ATPase activity by ouabain. Several cardiac glycosides have been used in elucidating aspects of the reaction mechanism catalysed by Na^+,K^+-ATPase. Ouabain (strophanthin-G) has been the most extensively used glycoside, mainly because of its high solubility in aqueous solution. However, several workers failed to recognise conditions optimal for ouabain action. It has been established that ouabain inhibition of Na^+, K^+-ATPase activity is extremely temperature-sensitive both in mammalian

(CHARNOCK et al., 1975) and in insect preparations (PEACOCK et al., 1976; DONKIN and ANSTEE,1980; and the present study). The inhibitory effect of ouabain decreases as temperature is lowered below 30°C.

The effect of K^+ concentration on ouabain inhibitory action is also another factor which has been overlooked by some workers. BONTING (1970), GLYNN and KARLISH, (1975) have demonstrated K^+ antagonism of oubain inhibition. AHMED and JUDAH (1965) pointed out that the effect of temperature on ouabain action results from an enhanced K^+ affinity at low temperatures. As clearly explained by ANSTEE and BOWLER (1979) and later in Chapter 5, failure to realise the effect of temperature and K^+ on ouabain inhibition may be the cause of the conflicting reports in literature on ouabain-sensitivity.

The present study also investigates the possible use of vanadate as an inhibitor of Na^+, K^+-ATPase. Vanadate is also highly soluble in water. Interest in the use of vanadate has been raised by the report that the contamitant found in Sigma 'Grade' ATP derived from muscle has been identified as Pentavalent vanadate (+ 5 oxidation state of vanadium) (CANTLEY et al., 1977, 1978). Vanadate occurs naturally in tissues and has been estimated to be present at sufficient concentration in some mammalian tissues to act as a specific regulator of Na^+, K^+-ATPase activity (JOSEPHSON and CANTLEY, 1977; CANTLEY et al., 1977). The latter point out that this could be the cause of the anomalous kinetics of Na^+, K^+-ATPase activity reported by some workers using vanadate containing ATP (e.g. CANTLEY and JOSEPHSON, 1976; FAGAN and RACKER, 1977). This conclusion was in agreement with previous report by CHARNEY et al., (1975) and a subsequent report by BEAUGÉ and GLYNN (1977). In view of these reports, during the present study, in one set of experiments, vanadate-contaminated Sigma 'Grade' ATP was used and in another set, vanadate-free ATP was used.

Little is known about vanadate inhibition of Na^+,K^+-ATPase from insect tissues but its inhibition of this enzyme has been reported in several mammalian tissues (e.g. rat renal cortex, BALFOUR et al., 1978; mouse neuroblastoma cells, MONTERO et al., 1981; cat capillary muscle, HACBARTH et al., 1978; human kidney and red cell, NEIDER et al., 1979; human red cell, CANTLEY et al., 1978; dog kidney and human red cell, BOND and HUDGINS, 1981). However, these studies have been complicated by the observation that in the human red cell, for example, much of the cytoplasmic vanadium was not in a form capable of inhibiting Na^+,K^+-ATPase (CANTLEY et al., 1978). There is need to understand fully the oxidation-reduction reactions which occur in the cytoplasm which may alter the concentration and valency of vanadate ions. It was realised that vanadate is a poor inhibitor when applied extracellularly (CANTLEY et al., 1978) but it a potent inhibitor of Na^+,K^+-ATPase in purified membrane preparations.

Unlike ouabain which binds from the outside (ALBERS et al.,1968) vanadate's inhibitory site for cation transport is on the cytoplasmic side of the cell membrane (CANTLEY et al., 1978). When applied extracellularly, vanadate crosses the cell membrane and the reducing agents in the cytoplasm change vanadate to the +4 oxidation state which has a low inhibitory power (CANTLEY et al., 1978; AKERA et al., 1979; BEAUGÉ and GLYNN, 1977, 1978; CANTLEY and AISEN, 1979).

It is known that vanadate binds to the free form (E_2) of the enzyme and inhibits its activity by blocking the $E_2P \rightarrow E_1P$ conformational change (see Figure 3.1) which is thought to be a hydrolytic limiting step and not a subsequent conformational change (BEAUGÉ and GLYNN, 1979). This means that vanadate inhibits the enzyme activity by binding to the site where phosphate (Pi) is released during the overall turnover

(CANTLEY et al., 1978) and that vanadate is not a competitive inhibitor with ATP (NEIDER et al., 1979).

As indicated previously ouabain and K^+ compete for the same conformational state of the enzyme (ERDMANN and SCHONER, 1973) and ouabain binding to the enzyme is antagonised by high concentrations of K^+. On the contrary, vanadate binding to the Na^+,K^+-ATPase is facilitated by potassium (CANTLEY et al., 1978) and is antagonised by ATP. Vanadate requires the presence of Mg^{2+} and K^+ for the formation of the $Mg^{2+}.E_2KVO_3$ form (CANTLEY et al., 1978; JØRGENSEN and KARLISH, 1980). When Na^+, K^+-ATPase preparations catalyze the hydrolysis of ATP in the absence of K^+, the rate limiting step is thought to be the hydrolytic step and not the subsequent conformational change. In the absence of K^+, therefore, it is expected that the hydrolytic activity would be very much less sensitive to vanadate.

Although a number of workers have demonstrated vanadate inhibition of Na^+,K^+-ATPase, DLOUHA et al., (1981) reported that in their experiments even high vanadate concentrations (e.g. 4×10^{-4} and 4×10^{-5} M) had no appreciable inhibitory effect on the electrogenic $Na^+ + K^+$ pump. They suggested that the cation potentials were reduced by mechanisms which did not involve inhibition of Na^+, K^+-ATPase activity; and that vanadate did not have any specific blocking effect on any particular ion channel but affected the membrane passive permeability for Na^+ ions which led to changes in membrane resistance; or that vanadate inhibited some energy-producing pathway (BYCZKOWSKI et al., 1979). In support of this interpretation, MONTERO et al., (1981) reported that in mouse neuroblastoma cells, vanadate increased the cell membrane permeability in a dose-dependent manner. But it was important when he pointed out that he made parallel measurements which demonstrated equal inhibition of Na^+, K^+-ATPase by vanadate.

JØRGENSEN (1982) pointed out that the inhibition by ouabain and vanadate suggests that the passive fluxes are coupled to conformational transitions in the protein of Na^+, K^+-ATPase. The present study investigates the effect of the cardiac glycoside , ouabain, vanadate and other inhibitors on the activity of Na^+, K^+-ATPase and other ATPases in microsomal preparations of Locusta Malpighian tubules. The purpose being to compare their effect on the enzyme with their effect on the in vitro fluid secretion by the Malpighian tubules so that the role of Na^+, K^+-ATPase and other enzymes in fluid secretion can be assessed.

Materials and Methods

3.1 Preparation of a microsomal fraction with Na^+, K^+-ATPase (E.C.3.6.1.3., SKOU, 1965) activity from the Malpighian tubules of Locusta migratoria L.

Reagents (Final concentrations)

Homogenisation 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 M
EDTA	10 mM

Washing Medium (pH 7.2)

NaCl	5 mM
EDTA	5 mM

Ionic reaction medium for total ATPase (pH 7.2) (final concentrations)

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

Ionic reaction medium for Mg^{2+}, -ATPase (pH 7.2) (final concentrations)

$MgCl_2$	4 mM
Histidine	50 mM

Reaction Stopping solution (Cirrasol mixture) (ATKINSON et al., 1973)

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

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

Substrate used : (final concentration)

Tris. ATP 3 mM

See method of preparation in Chapter 2.

3.2 Preparation of the membrane microsomal fraction

The method of extraction of Na^+, K^+-ATPase used in the present study is essentially the same as that described by PEACOCK et al.,(1972). Equal numbers of mature adult female and male locusts were used and the number killed was dependent on the size of the experiment planned. Sufficient numbers of animals were used to give a minimum of 80 µg/ml protein content in the final microsomal preparation.

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 and the whole gut bearing the Malpighian tubules was then carefully

drawn out through the thorax with the head still attached. The heads were removed and discarded. The Malpighian tubules together with a small 'collar' of the gut to which they are attached were quickly dissected free from the rest of the alimentary tract under ice-cold homogenising medium. The tubules were then placed in 10 mls of fresh ice-cold homogenisation medium in a glass Potter-Elvehjem homogenising tube. The homogenising medium in the dissecting dish was changed after every third dissection to avoid contamination by gut contents.

Homogenisation was carried out in a Potter-Elvehjem homogeniser with a Teflon pestle (clearance 0.1 to 0.15 mm) giving 20 passes of the plunger at 2,000 r.p.m. The resulting homogenate was extracted with an equal volume of ice-cold NaI (10 mls) for 30 minutes at 0°C (NAKAO et al., 1965). The extract was then diluted to 50 mls with deionised ice-cold water and centrifuged in thin-walled polypropylene centrifuging tubes at 50,000 g at 0°C for 30 minutes. The resulting pellet was discarded and the supernatant centrifuged at 100,000 g for 60 minutes. 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 minutes. This washing procedure was repeated twice, centrifuging for 20 minutes each time. The final microsomal pellet was suspended in an appropriate volume of ice-cold deionised water and was gently homogenised to ensure an even suspension.

3.3 Assay of ATPase activity

Unless otherwise stated in the text, all incubations were carried out at 30°C for 30 minutes. Pairs of boiling tubes containing 1.0 ml of the appropriate ionic medium and 0.5 ml 12 mM Tris ATP were set up and equilibriated at 30°C for 10 minutes. The reaction was

started by the addition of 0.5 ml of the microsomal fraction and stopped by the addition of 4 mls of freshly prepared cirrasol solution (ATKINSON et al., 1973). A reagent blank (i.e. Control) measuring non-enzymatic hydrolysis of ATP was set up for each experiment by addition of the cirrasol solution before the enzyme was introduced. The tubes were then left to stand at room temperature for 10 minutes for the yellow colour to develop. Any protein which precipitated was removed by centrifugation at 1000 g for 15 minutes at 4°C. The tubes were immediately stored on ice.

3.4 Analysis of inorganic phosphate

Following centrifugation to remove any protein, the supernatant in each tube was transferred to a cuvette and the absorbancy, which was proportional to the amount of inorganic phosphate (Pi) present was measured at 390 nm in a Pye Unicam 1800 Dual Beam spectrophotometer. The amount of (Pi) released was determined by reference to a standard calibration curve relating absorbancy to (Pi) concentration (ATKINSON et al., 1973). The standard curve was prepared as previously described (see Fig. 2.2, Chapter 2).

3.5 Estimation of ATPase activity

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

3.6 To determine the effect of Ouabain, Vanadate and Furosemide on the activity of Na^+,K^+-ATPase from Malpighian tubules of Locusta

A membrane microsomal fraction with Na^+, K^+-ATPase activity was prepared from the Malpighian tubules of Locusta as described previously. The ATPase activity was assayed in the reaction ionic media (also described previously) in the absence or presence of a named inhibitor. The inhibitors investigated included ouabain, vanadate and furosemide at concentrations ranging from 0-1 mM. Levels of inhibition were assessed by reference to the tubes containing no inhibitor.

3.7 To determine the effect of different concentrations of potassium and the inhibitory action of Ouabain and Vanadate

The Na^+, K^+-ATPase assay conditions with respect to temperature, pH and concentration of all essential ligands were as previously described except for K^+ concentration. All experimental reaction media contained 10^{-6}M vanadate or ouabain, and the potassium concentration ranged from 0-100 mM. A control reaction medium containing no inhibitor was set up for each potassium concentration.

3.8 To determine the effect of temperature on Ouabain-sensitivity and Vanadate-sensitivity of Na^+, K^+-ATPase

Water baths at different temperatures namely 10°C, 20°C, 30° and 40°C were set up. The temperature of the individual baths was controlled by a 1000 watt immersion heater (TE-7 Tempette, Techne (Cambridge) Limited, Duxford, Cambridge, England). A thermometer was placed in each bath. Both the control and experimental reaction media were thermoequilibrated for 15 minutes before the ATPase activity was assayed at the various temperatures both in the absence and presence of 10^{-6}M ouabain or vanadate. The effect of each inhibitor was assessed by reference to the ATPase activity observed in the absence of the inhibitor. Two sets of experiments were set up, one

using vanadium-free ATP and another using vanadium-contaminated ATP. As previously mentioned this was done to determine whether the vanadium in Sigma 'Grade' ATP would influence the enzyme kinetics as has been reported (CANTLEY et al., 1977, 1978).

RESULTS

3.9 Na^+, K^+-ATPase activity in membrane microsomal preparation of Malpighian tubules of Locusta

The results presented in Table 3.3 show that there were two types of ATPase present in the microsomal preparations of the Malpighian tubules: (1) a magnesium-dependent ATPase (Mg^{2+} - ATPase) assayed in the presence of Mg^{2+} +ATP and (2) a magnesium-dependent, sodium and potassium-stimulated ATPase (Na^+, K^+-ATPase) that was evident only by the inclusion of Na^+ and K^+ in the media. The Na^+, K^+-ATPase showed relatively high activity and the results show that Mg^{2+} ATPase activity constituted only 18.5% of the total ATPase activity and the remaining 81.5% was due to the Na^+, K^+-ATPase activity. In another set of determinations, however, Mg^{2+} ATPase activity constituted a higher percentage (37.8%) of the total ATPase activity (see Table 3.4). Results in Table 3.3 show that (1 mM) ouabain totally inhibited the Na^+, K^+-ATPase component but did not significantly affect the Mg^{2+} ATPase component. This is illustrated in Table 3.3 by the amount of ATPase activity in condition C (with ouabain) being not significantly different from that in condition A (in presence of Mg^{2+} alone). The results presented in Table 3.4 also clearly show that over a wide range of concentrations (10^{-9} - 10^{-3}M) ouabain did not significantly inhibit the Mg^{2+} ATPase activity. Whilst Figure 3.2a shows a sigmoid curve for the effect of different concentrations of ouabain on Na^+,K^+ -ATPase activity in microsomal preparations of Locusta Malpighian tubules, it

TABLE 3.3 : ATPase activity in Locusta Malpighian tubule microsomal preparations

ATPase activity in presence of :				
Mg^{2+} (A)	Mg^{2+}, Na^{+}, K^{+} (B)	Mg^{2+}, Na^{+}, K^{+} +(1 mM) ouabain (C)	B - A	B - C
27.7 ± 2.3	149.8 ± 7.8	24.8 ± 2.7	122.1 ± 7.0	124.9 ± 7.9

Conditions : (A) 4 mM Mg^{2+}; (B) 4 mM Mg^{2+}, 100 mM Na^{+}, 20 mM K^{+}; (C) 4 mM Mg^{2+}, 100 mM Na^{+}, 20 mM K^{+} + 1 mM ouabain

Each medium was made in 50 mM Histidine/HCl buffer, pH 7.2 and contained 3 mM ATP.

Incubation temperature : 30°C.

Activity expressed in nmoles Pi liberated/mg Protein/min ± SEM and is the mean of 17 separate preparations.

TABLE 3.4 : The effect of different concentrations of ouabain on Mg^{+}-ATPase activity in Locusta Malpighian tubule microsomal preparations. (n = 5)

Ouabain (M)	Mg^{+}-ATPase activity nmoles Pi/mg Protein/min	% activity remaining ± SEM
10^{-3}	57.2 ± 7.9	94.3 ± 4.0
10^{-4}	57.8 ± 7.4	95.6 ± 3.5
3×10^{-5}	56.4 ± 9.0	90.6 ± 6.0
10^{-5}	60.7 ± 9.5	98.8 ± 3.5
3×10^{-6}	60.2 ±10.8	96.0 ± 6.0
10^{-6}	60.5 ± 9.2	98.9 ± 3.5
3×10^{-7}	59.3 ± 9.6	95.7 ± 4.2
10^{-7}	61.3 ± 8.4	101.1 ± 3.7
3×10^{-8}	62.8 ± 8.4	103.4 ± 1.9
10^{-8}	57.7 ± 8.3	94.9 ± 3.5
10^{-}	61.5 ± 7.9	101.6 ± 3.6
0	61.1 ± 9.1	100.0 ± 0.0

Conditions as in Table 3.5

shows almost a straight line for the effect of the same concentrations of ouabain on the Mg^{2+} ATPase activity in the same preparation.

3.10 Effect of Ouabain on Na^+, K^+-ATPase activity

Results in Table 3.5 show the effect of different concentrations of ouabain on Na^+, K^+-ATPase activity in Malpighian tubule microsomal preparations. It is clear that the level of ouabain inhibition of the Na^+, K^+-ATPase activity increased with concentration from 10^{-9}M to 10^{-3}M. At the high concentrations of 10^{-4}M and above, inhibition was almost complete. This dose-dependent nature of inhibition is illustrated by the typical sigmoid shape curves in Fig.3.2 a,b. This curve is transformed into a straight line using Probits in Figure 3.3 which indicates that the negative logarithm of the ouabain concentration causing 50% inhibition (pI_{50})was 6.6.

3.11 The effect of vanadate on Na^+, K^+-ATPase and Mg^{2+}ATPase from microsomal preparations of Locusta Malpighian tubules

The effect of different concentrations of vanadate on Na^+,K^+-ATPase activity is shown in Table 3.6. This table shows only the data obtained when vanadate-free ATP was used as substrate. It is seen that vanadate inhibition increased with concentration between 10^{-9} to 10^{-4}M. At concentrations of 10^{-4}M and higher, inhibition was complete. Figures 3.4a and 3.5 show the dose-response curves for vanadate inhibition of Na^+, K^+-ATPase the former when vanadium-free ATP was used and the latter when vanadium-contaminated ATP was used. Both Figure 3.4a and Figure 3.5 are transformed into straight lines in Figure 3.6 and Figure 3.7 respectively. The values for the negative logarithm of vanadate concentration causing 50% inhibition (pI_{50}) were not significantly different despite the different types of ATP used; being 5.9 and 5.8 with vanadate-free and vanadate-contaminated ATP respectively.

TABLE 3.5 : The effect of different ouabain concentrations on

Ouabain concentration (M)	Na^+, K^+-ATPase activity nmoles Pi/mg Protein/min (± SEM)
10^{-3}	0.3 ± 0.3
10^{-4}	3.0 ± 3.0
3×10^{-5}	8.6 ± 6.0
10^{-5}	10.0 ± 4.0
3×10^{-6}	23.0 ± 5.0
10^{-6}	32.7 ± 6.0
3×10^{-7}	57.2 ±14.0
10^{-7}	75.5 ±13.0
3×10^{-8}	78.0 ±11.0
10^{-8}	93.1 ±14.0
10^{-9}	96.6 ±16.0
0	100.7 ±16.0

Reaction media used: (- Ouabain for controls)
(+ Ouabain for experiments)

(1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}. 100 mM Na^+, 20 mM K^+ in Histidine/HCl buffer pH 7.2 at 30°C. Each medium contains 3 mM Tris ATP (final concentration). The values of the specific Na^+, K^+-ATPase activity shown were calculated by subtracting the values of activity in the presence of Mg^{2+} alone from the values of total activity in the presence of Mg^{2+}, Na^+ and K^+. Mean values of 5 separate experiments ±SEM are shown.

FIGURE 3.2a

The effect of ouabain on the activity of Na^+, K^+-ATPase and Mg^{2+} ATPase from Locusta Malpighian tubule microsomal preparations.

The results shown are the mean value ± SEM of five separate preparations.

Ordinate : Activity remaining expressed as % of activity in the absence of ouabain.

Abscissa : Negative Log_{10} of ouabain concentration (M).
Vertical lines represent ± 2 SEM.

Conditions:

(1) 4 mM Mg^{2+}, 3 mM Tris ATP;

(2) 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 3 mM Tris ATP in 50 mM Histidine/HCl buffer pH 7.2.

Incubation temperature : 30°C

Data for Na^+, K^+ -ATPase as in Fig.3.2b

Mean value for 100% enzyme activity was 27.7 ± 2.3 (Mg^{2+}-ATPase) and 148.9 ± 7.8 (Na^+,K^+-ATPase).
in nmoles Pi/mg Protein/min.

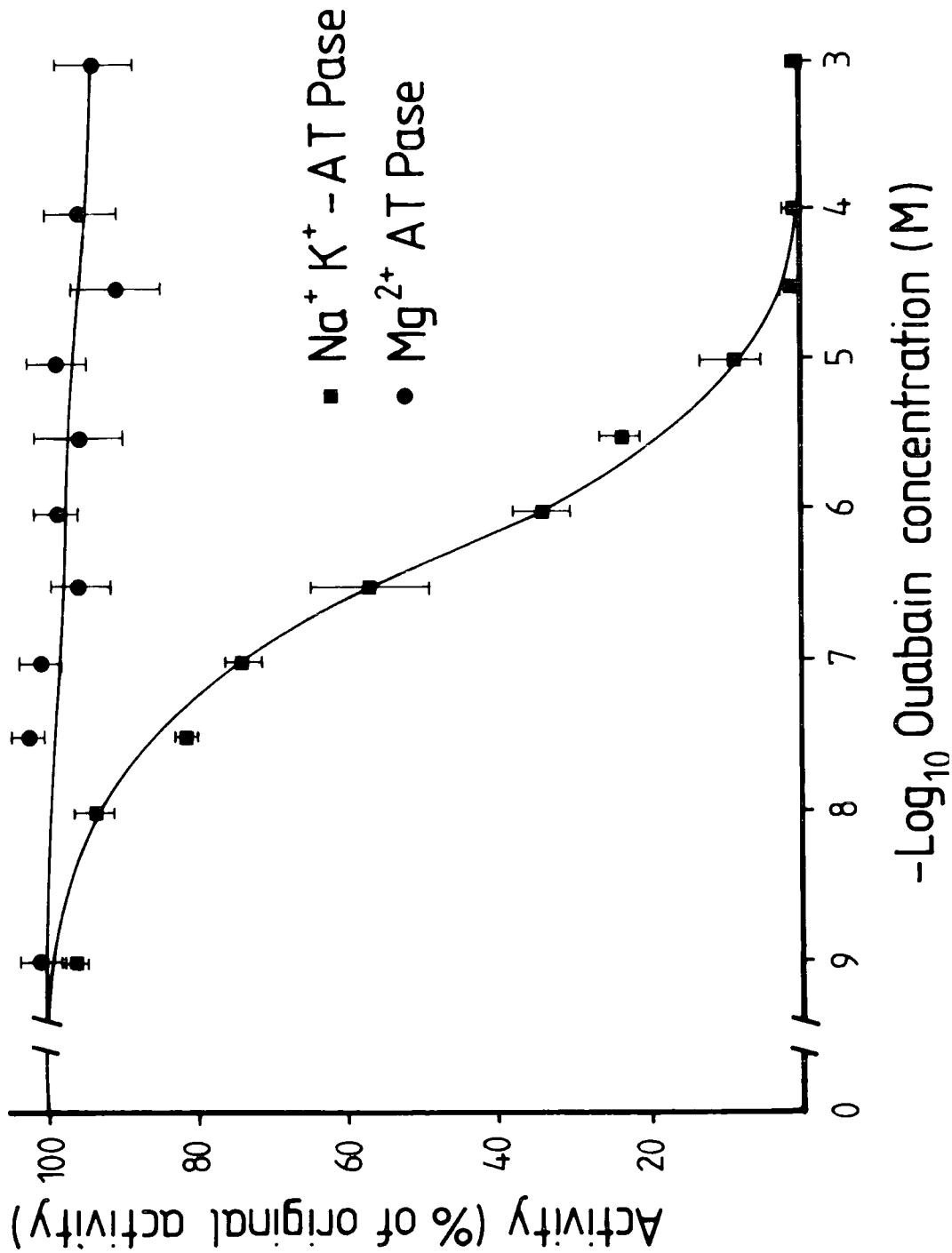

Activity (% of original activity)
100
80
60
40
20
0
9
8
7
6
5
4
3
-Log10 Ouabain concentration (M)
■ Na+ K+ - AT Pase
● Mg2+ AT Pase

Fig. 3.2a

FIGURE 3.2b

The effect of ouabain on activity of Na^+, K^+-ATPase from Locusta Malpighian tubule preparations.
The results shown are the mean values ± SEM of five separate preparations.

Ordinate : Activity remaining expressed in nmoles Pi liberated/mg Protein/min.

Abscissa : Negative Log_{10} of ouabain concentration (M).
Vertical lines represent ± 2 SEM

Conditions : as described in Figure 3.2a.

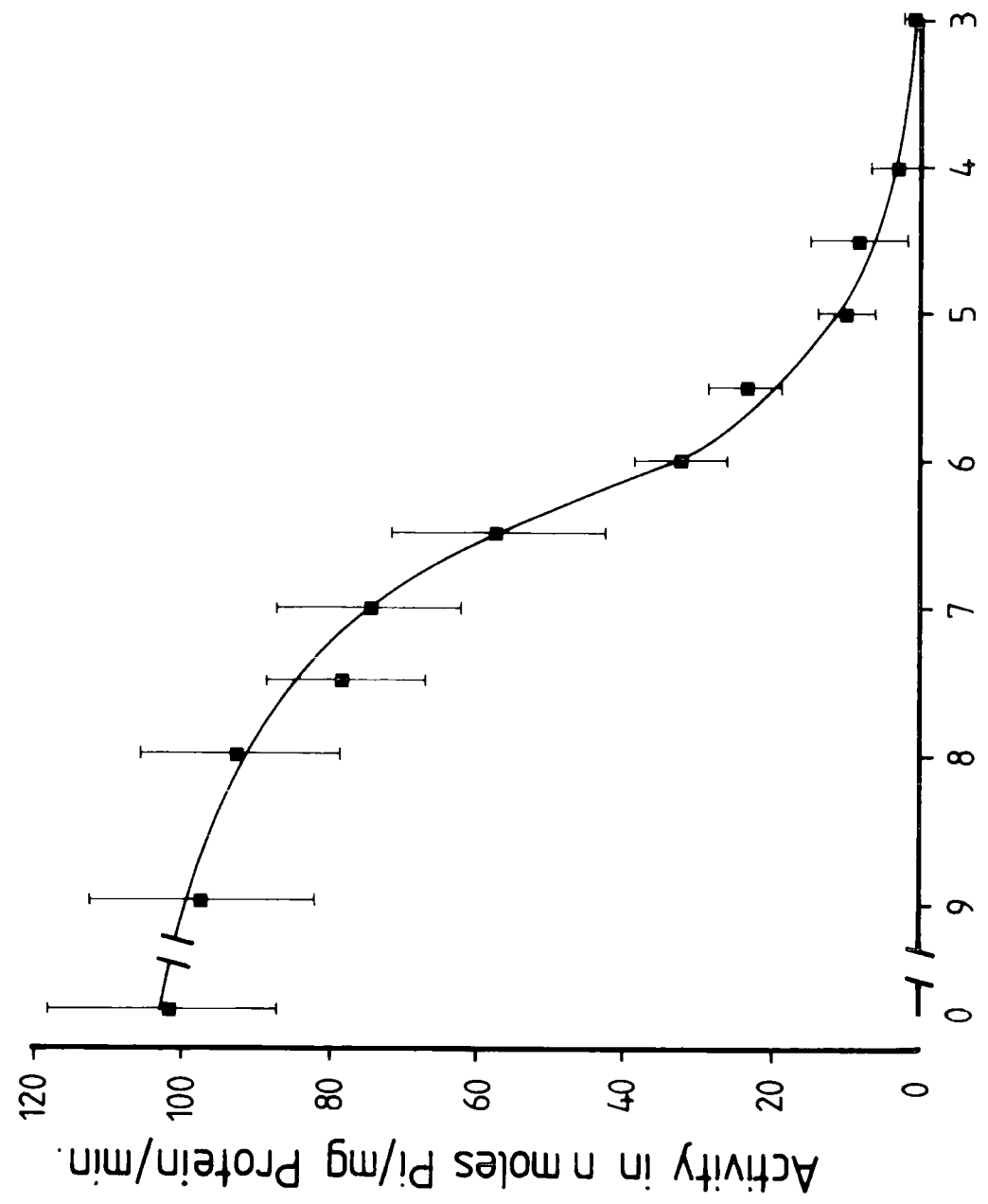

Activity in n moles Pi/mg Protein/min.
120
100
80
60
40
20
0
0
9
8
7
6
5
4
3
– Log10 (Ouabian) [M]

Fig. 3.2b

FIGURE 3.3

Effect of varying concentrations of ouabain on Na^+, K^+ - ATPase in Locusta Malpighian tubule preparations.

Ordinate: Fraction of activity remaining transformed to Probits

Abscissa: Negative Log_{10} of ouabain concentration (M)

Experimental conditions: As described in Fig.3.2a

Different symbols represent data from separate preparations.

Line fitted by least squares regression analysis.

Fig. 3.3

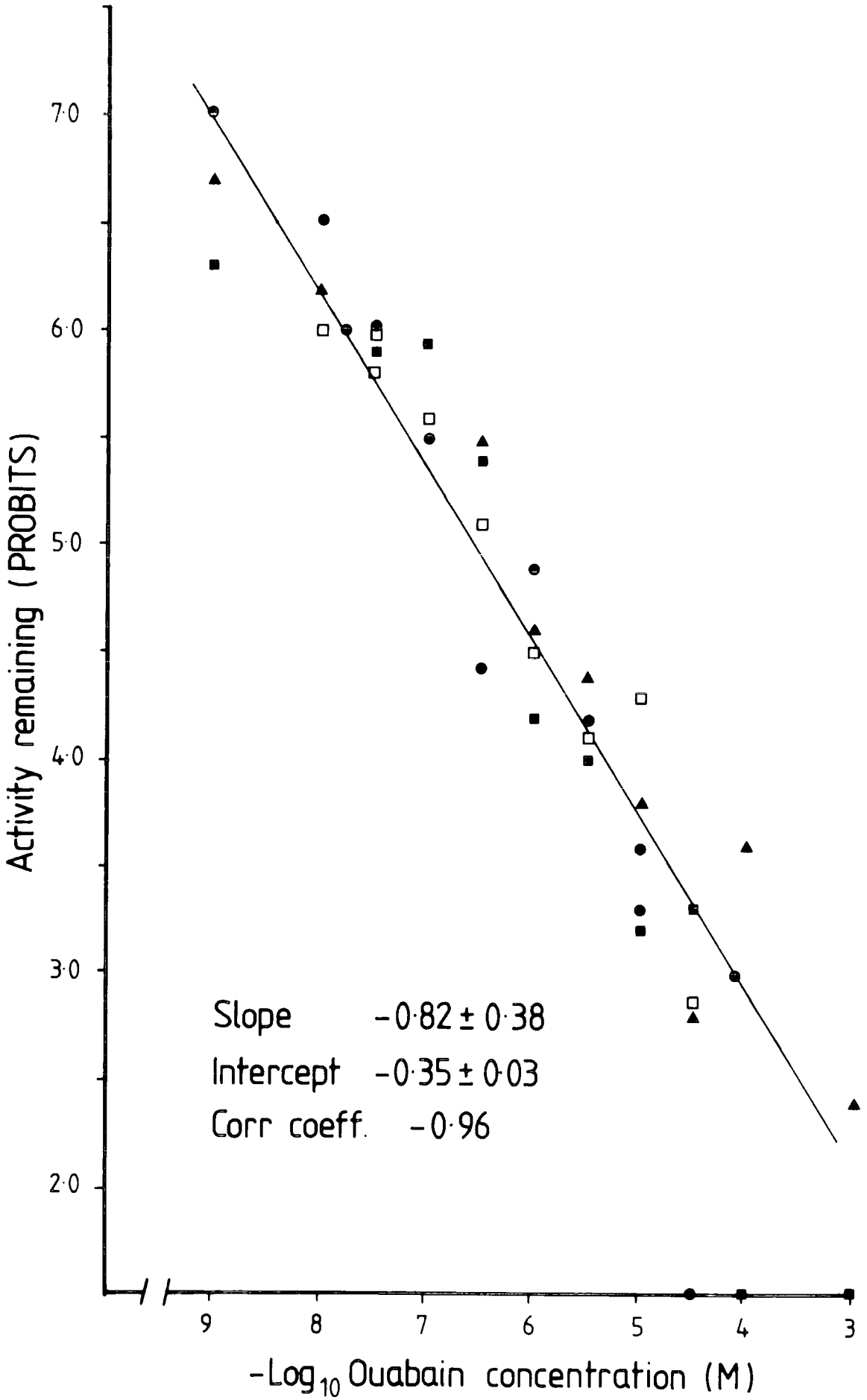

7·0
6·0
5·0
4·0
3·0
2·0
Activity remaining (PROBITS)
Slope -0·82 ± 0·38
Intercept -0·35 ± 0·03
Corr coeff. -0·96
9
8
7
6
5
4
3
-Log10 Ouabain concentration (M)

TABLE 3.6

Effect of different concentrations of vanadate on Na^+, K^+-ATPase activity in preparations of Malpighian tubules of Locusta.

Reaction media used:

(1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, Each medium was made in 50 mM Histidine/HCl buffer, pH 7.2, and each medium contained 3 mM Tris ATP (-vanadate) in control and (+ vanadate) in experimental media.

Temperature : 30°C

The values of activity shown were calculated by subtracting the values of activity in the presence of Mg^{2+} + ATP alone from the values for total ATPase activity in the presence of Mg^{2+}, Na^+, K^+ + ATP. Mean values of 4 separate experiments (± SEM) are shown.

Note Vanadate-free ATP was used.

TABLE 3.6 : Effect of different concentrations of vanadate on Na^+, K^+-ATPase activity in Locusta Malpighian tubule microsomal preparations

Vanadate Concentration (M)	Na^+, K^+-ATPase activity nmoles Pi/mg Protein/min ±SEM (n = 4)
10^{-3}	4.8 ± 0.7
10^{-4}	12.8 ± 1.0
3×10^{-5}	11.2 ± 3.6
10^{-5}	45.0 ± 1.8
3×10^{-6}	134.0 ± 7.4
10^{-6}	161.0 ± 8.6
3×10^{-7}	184.5 ± 9.3
10^{-7}	183.2 ± 8.8
3×10^{-8}	194.4 ± 9.9
10^{-8}	190.1 ± 9.4
10^{-9}	188.6 ± 9.4
0	205.5 ±10.5

FIGURE 3.4a and b

Effect of varying concentrations of vanadate on Na^+, K^+-ATPase activity (a) and Mg^+-ATPase activity (b) in Locusta migratoria L. Malpighian tubule microsomal preparations.

Ordinate : Activity remaining expressed as % of original activity in the absence of vanadate.

Abscissa: Log_{10} of vanadate concentration in (M).

Experimental conditions:

(1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+ + 3 mM Tris ATP (vanadium-free) in 50 mM Histidine/HCl buffer pH 7.2

Temperature : 30°C.

Each point on the graph represents a mean value of 4 separate preparations. Fig.3.4a data as for Fig.3.7

Mean value of 100% activity in n moles Pi/mg Protein/min was 116.4 ± 7.2 (Na^+, K^+-ATPase) and 36.8 ± 3.7 Mg^+-ATPase

Fig. 3.4a

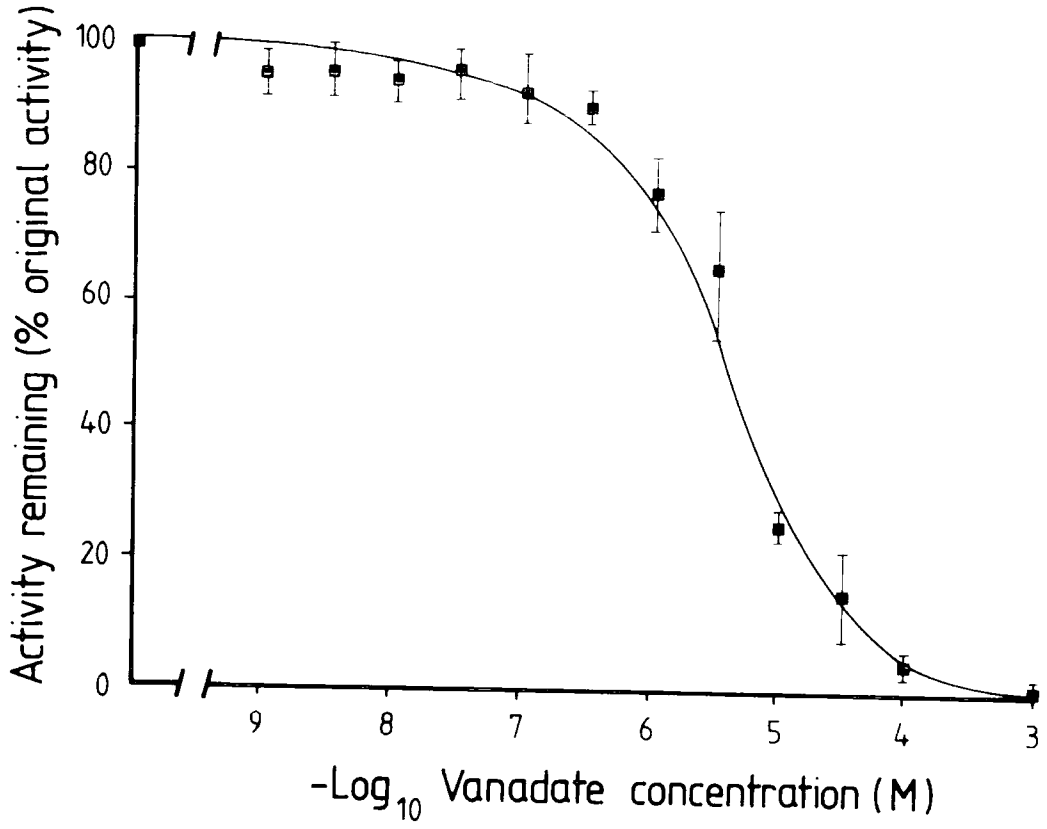


Fig. 3.4b

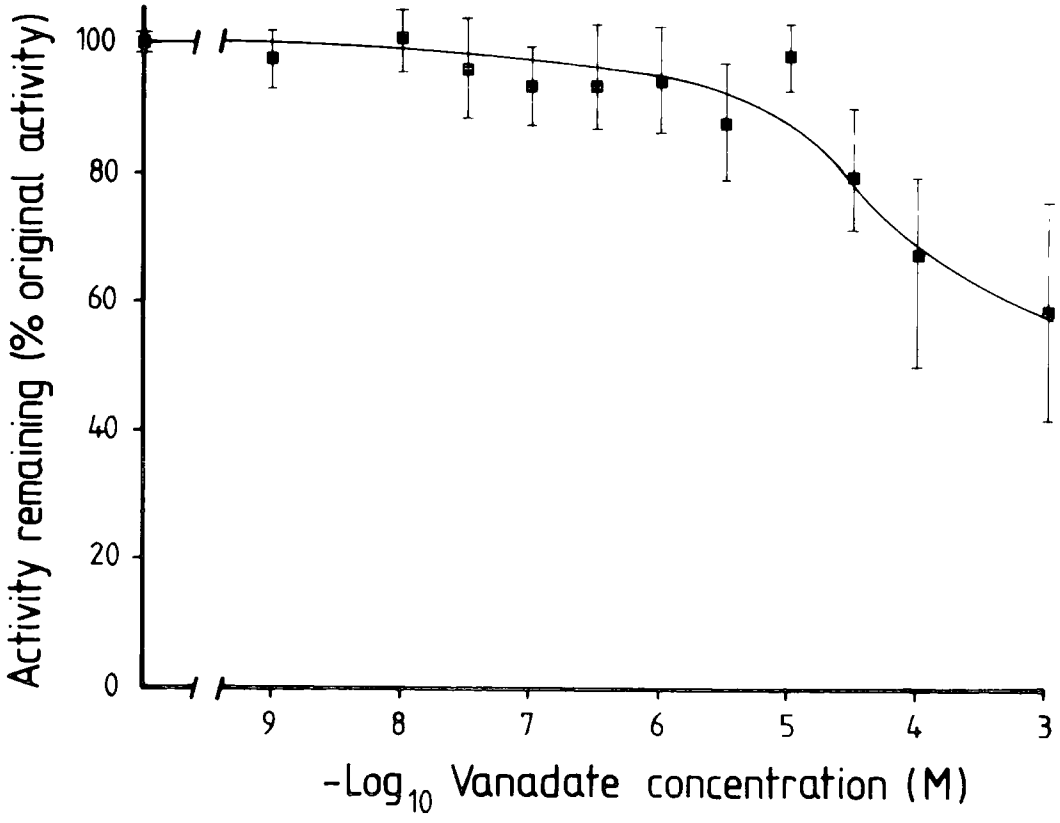


FIGURE 3.5

Effect of varying concentration of vanadate on Na^+, K^+-ATPase activity in Locusta Malpighian tubule microsomal preparations.

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

Abscissa: Negative Log_{10} of vanadate in (M)

Experimental conditions : were essentially the same as described for Fig. 3.4a and b, except that vanadium-contaminated ATP was used.

(n = 3)

Mean value of 100% activity in nmoles Pi/mg Protein/min was 151.4 ± 8.2.

Fig. 3.5

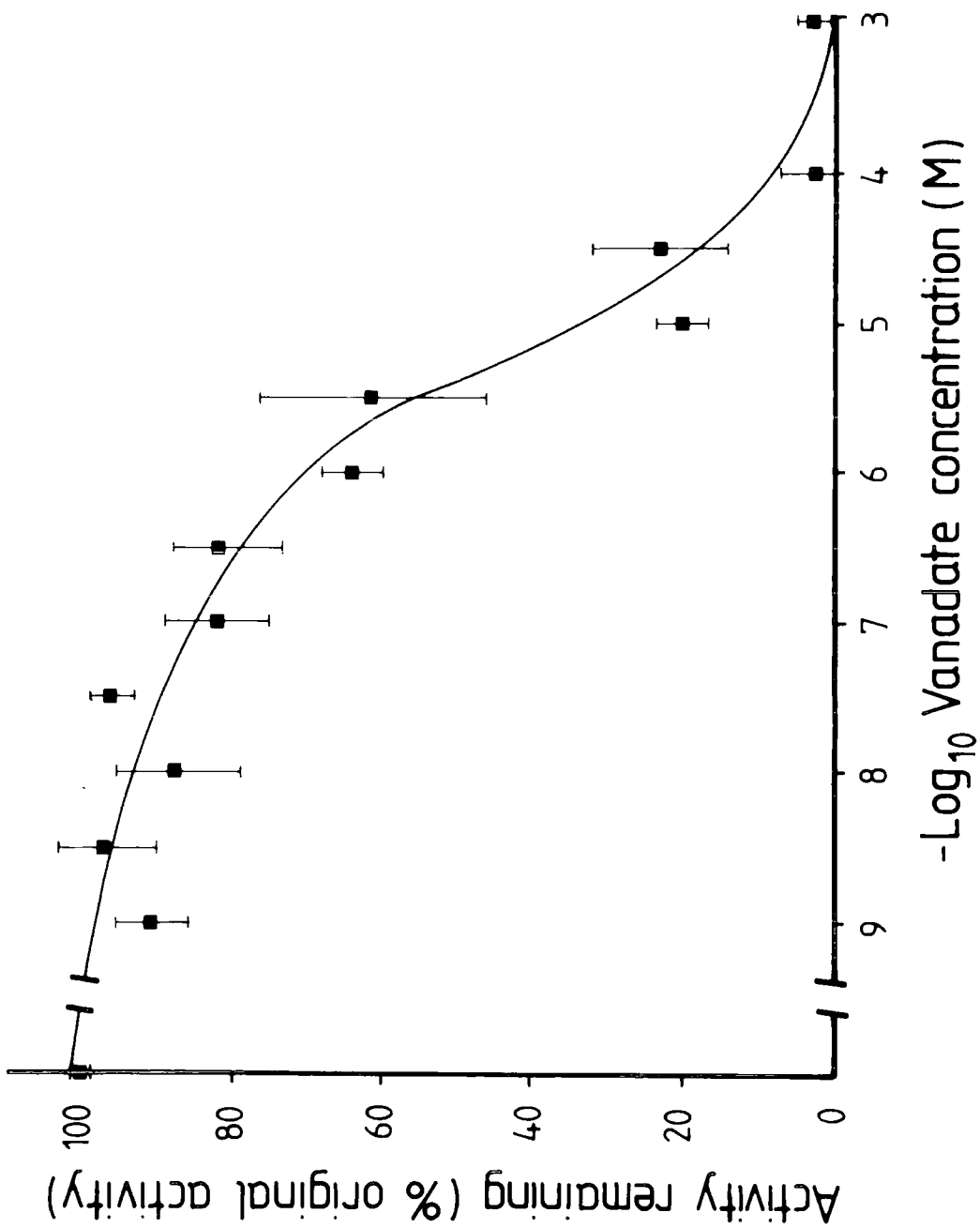

Activity remaining (% original activity)
100
80
60
40
20
0
9
8
7
6
5
4
3
-Log10 Vanadate concentration (M)

FIGURE 3.6

Effect of varying concentrations of vanadate on Na^+, K^+-ATPase activity in Locusta Malpighian tubule microsomal preparations.

Ordinate : Fraction of activity remaining transformed into probits.

Abscissa : Negative Log_{10} of vanadate concentration in (M).

Conditions : (1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}, 100 mM Na^+, 2) mM K^+ + 3 mM Tris ATP (Vanadium-free) in 50 mM Histidine| HCl buffer pH 7.2.

Temperature : 30°C.

Different symbols represent data from separate preparations

Line fitted by least squares regression analysis.

Fig. 3.6

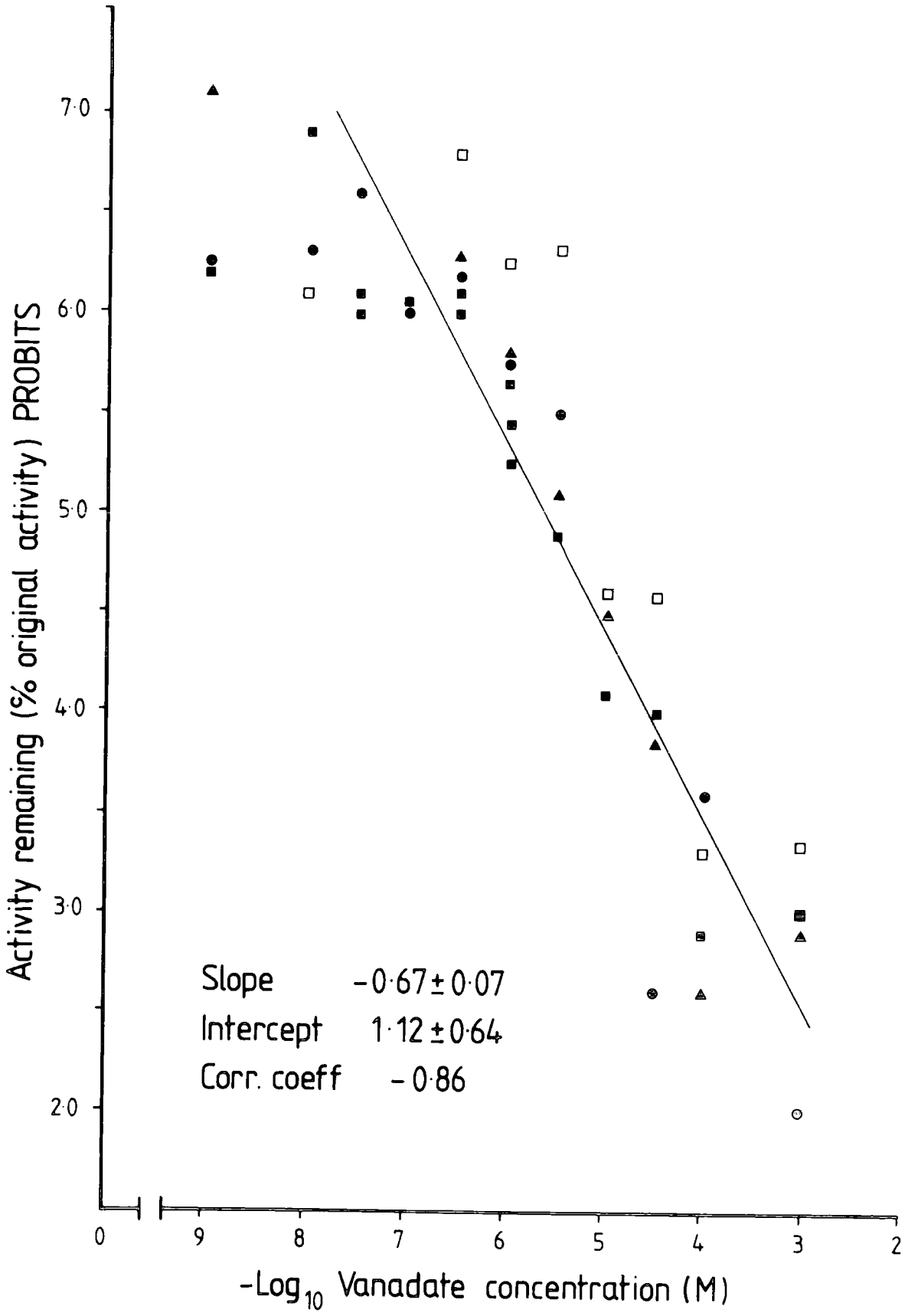

Activity remaining (% original activity) PROBITS
7·0
6·0
5·0
4·0
3·0
2·0
Slope -0·67 ± 0·07
Intercept 1·12 ± 0·64
Corr. coeff -0·86
0
9
8
7
6
5
4
3
2
-Log10 Vanadate concentration (M)

FIGURE 3.7

Effect of different concentrations of vanadate on Na^+, K^+-ATPase activity in Locusta Malpighian tubule preparations.

Ordinate : Probit transformation of fraction of enzyme activity remaining.

Abscissa : Negative Log_{10} vanadate concentration (M).

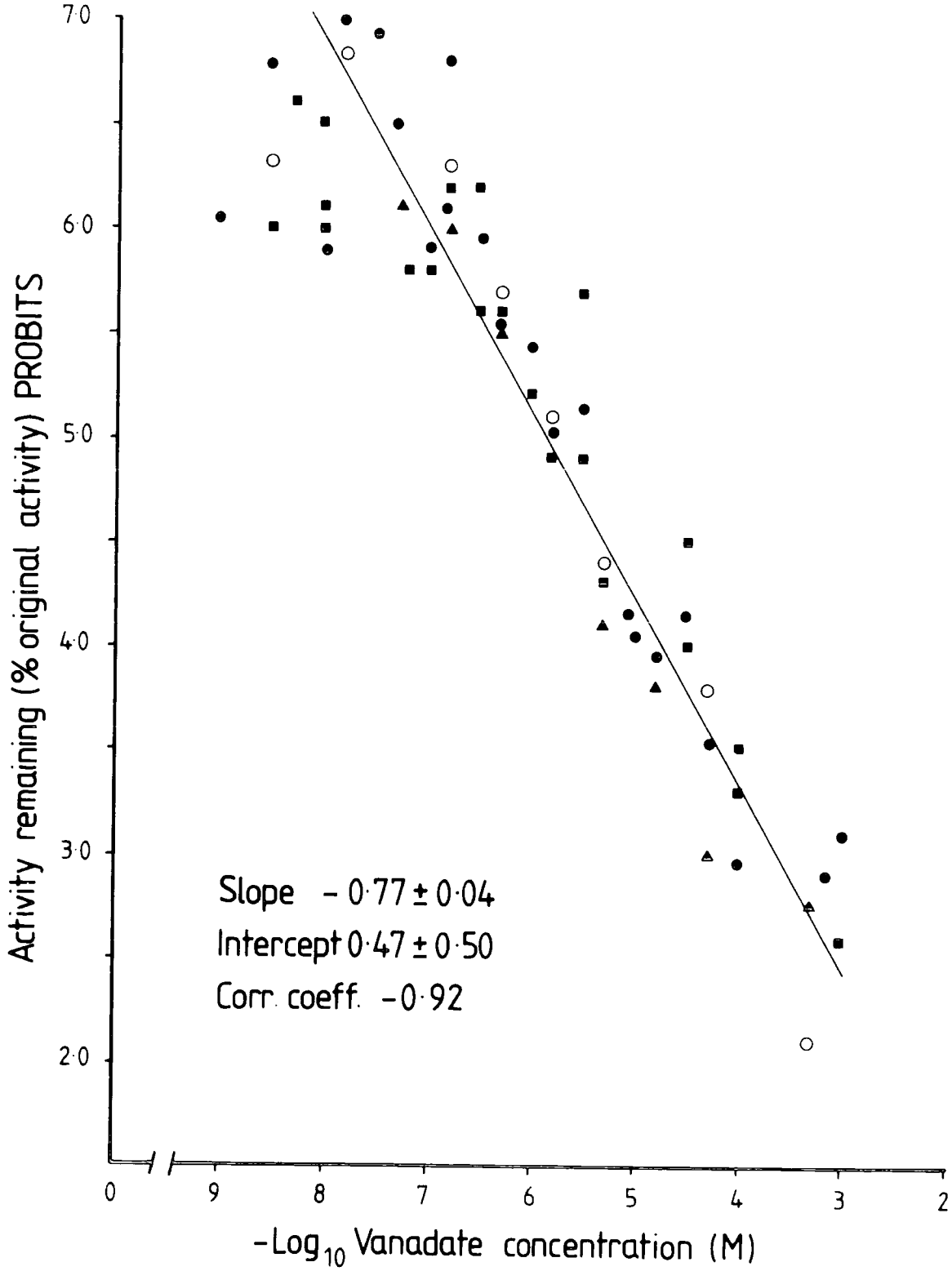
Conditions : (1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, each median contained 3 mM Tris ATP (vanadium-contaminated) and all media were made in 50 mM Histidine | HCl buffer pH 7.2.

Temperature : 30°C.

Line was fitted by least squares regression analysis. (SNEDECOR and COCHRAN, 1967).

Different symbols represent data from separate experiments.

Fig. 3.7


7·0
6·0
5·0
4·0
3·0
2·0
Activity remaining (% original activity) PROBITS
Slope - 0·77 ± 0·04
Intercept 0·47 ± 0·50
Corr. coeff. -0·92
0
9
8
7
6
5
4
3
2
-Log10 Vanadate concentration (M)

The results shown in Table 3.7 show that high concentrations of vanadate (10^{-5} - 10^{-3}M) inhibited the Mg^{2+} ATPase activity. Table 3.8 shows results from a similar experiment when vanadate-contaminated ATP was used. In Table 3.8 results from two separate determinations are shown. These show that in the case of Mg^{2+} ATPase, vanadate was effective over only 2 degrees of magnitude 10^{-5} to 10^{-3} and lower concentrations had no significant effect on Mg^{2+} ATPase activity. This is clearly illustrated in Figure 3.4b.

3.12 The effect of different concentrations of potassium on the inhibitory action of Ouabain and Vanadate

(a) Ouabain

Results presented in Figure 3.8 show Na^+, K^+-ATPase activity in the presence and absence of ouabain (10^{-6}M) at different concentrations of potassium. In the absence of ouabain, the activity of Na^+, K^+-ATPase rises with the increase in K^+ concentration between 2 mM - 30 mM K^+ (Figure 3.8). At concentrations of K^+ in excess of 30 mM there is a slight drop in enzyme activity to a level not further affected by K^+ concentration up to 100 mM K^+. This indicates that at these high concentrations, K^+ is competitive with Na^+ (ANSTEE and BELL, 1975).

Examination of Figure 3.8 also shows that ouabain inhibits Na^+, K^+-ATPase activity at all potassium concentrations studied. However, its inhibitory effect is reduced as K^+ concentration increases. This effect of K^+ concentration on ouabain-sensitivity is illustrated more clearly in Figure 3.9 where the activity at each concentration of K^+ both in the absence and presence of ouabain was expressed as a percentage of the activity observed in the presence of 20 mM K^+. At concentrations above 20 mM K^+, the inhibitory effect of ouabain on

TABLE 3.7 : The effect of different concentrations of vanadate on Mg^{2+}-ATPase activity in Locusta Malpighian tubule microsomal preparations

Vanadate	Mg^{2+}-ATPase activity nmoles Pi/mg Protein/min (n = 4)	% activity remaining
10^{-3}	21.8 ± 7.4	55.4 ±13.8
10^{-4}	24.9 ± 7.8	64.2 ±15.7
3×10^{-5}	29.4 ± 5.5	80.7 ± 9.2
10^{-5}	36.2 ± 4.8	98.0 ± 5.4
3×10^{-6}	31.9 ± 6.8	84.4 ± 7.8
10^{-6}	34.5 ± 5.5	92.6 ± 4.9
3×10^{-7}	34.3 ± 6.2	91.4 ± 5.6
10^{-7}	34.3 ± 6.2	91.6 ± 6.2
3×10^{-8}	35.1 ± 6.1	93.6 ± 5.5
10^{-8}	36.9 ± 6.0	99.0 ± 4.8
10^{-9}	35.6 ± 6.1	95.3 ± 5.8
0	36.8 ± 4.1	100.0 ± 0.0

Conditions as in Table 3.6

Note: Vanadate-free ATP was used.

TABLE 3.8 : The effect of varying the concentrations of vanadate on Mg^{+}-ATPase activity in Locusta migratoria L. Malpighian tubule microsomal preparations

Vanadate (M)		Mg^{+}-ATPase activity in nmoles Pi/mg Protein/min	Activity expressed as % of activity in medium without the inhibitor
10^{-3}	a	6.45	23.1
	b	8.83	22.5
10^{-4}	a	16.29	58.6
	b	25.78	65.7
$3x10^{-5}$	a	7.13	25.6
	b	11.66	29.7
10^{-5}	a	30.55	107.8
	b	41.32	105.3
$3x10^{-6}$	a	16.97	60.9
	b	30.73	78.3
10^{-6}	a	22.07	79.3
	b	40.62	103.5
$3x10^{-7}$	a	23.08	82.9
	b	38.50	98.1
10^{-7}	a	20.07	72.1
	b	40.62	103.5
$3x10^{-8}$	a	25.80	92.7
	b	35.32	90.0
10^{-8}	a	26.14	93.9
	b	40.97	104.4
10^{-9}	a	27.16	97.6
	b	41.32	105.3
0	a	27.82	100
	b	39.21	100

Conditions: Experimental conditions are essentially the same as described for Table 3.6 except that vanadate-containing ATP was used. a and b represent 2 separate preparations.

Na^+,K^+-ATPase decreases such that in the presence of 100 mM K^+ and ouabain the level of activity was 135% of that observed in the presence of 20 mM K^+ plus ouabain (10^{-6}M).

(b) Vanadate

The data showing the effect of vanadate (10^{-6}M) in the presence of different concentrations of K^+ was analysed in the same way as that described for ouabain. Figure 3.10 shows activity (in n moles Pi/mg Protein/min) in the presence and absence of vanadate plotted against different concentrations of K^+. The results show that vanadate does not inhibit Na^+, K^+-ATPase activity at K^+ concentrations less than 10 mM, but as K^+ increases vanadate inhibition increases from 9% at 30 mM K^+ to 40% at 100 mM K^+. Figures 3.10 and 3.11 show that in the absence of vanadate maximal activity is reached at 20 mM K^+ after which the curve dips slightly and then reaches a plateau. The maximum activity in the presence of vanadate is at 10 mM K^+.

Figure 3.12 compares the effect of vanadate inhibition with ouabain inhibition of the Na^+, K^+-ATPase at various concentrations of K^+. The activity remaining at each K^+ in the presence of the inhibitor (vanadate/ouabain) expressed as a percentage of the activity in the absence of the inhibitor, at each particular K^+ concentration, are plotted as a function of $[K^+]$. The curve for vanadate inhibition, shows an initial stimulation at K^+ concentrations at 5 and 10 mM after which inhibition increases as K^+ concentration increases.

The curve for ouabain inhibition on the other hand shows that inhibition decreased linearly with increasing K^+ concentration. The straight line showing ouabain inhibition was fitted by regression analysis ($r = 0.98$). The level of ouabain inhibition dropped from ca. 62% at 2 mM K^+ to ca 40% at 100 mM K^+.

FIGURE 3.8

Effect of K^+ concentration on the inhibition of the Na^+, K^+-ATPase from Locusta. Malpighian tubule preparations by 10^{-6}M ouabain.

Ordinate : Na^+, K^+-ATPase activity in nmoles Pi liberated/mg Protein/min.

Abscissa : K^+ concentration (mM).

Conditions : (1) 4 mM Mg^{2+} + 3 mM Tris ATP. (2) 4 mM Mg^{2+}, 100 mM Na^+, 3 mM Tris ATP + varying K^+ concentrations (2-100 mM). All media made in 50 mM Histidine/HCl buffer pH 7.2.

Temperature 30°C.

Na^+, K^+-ATPase activity was estimated by subtracting amounts of Pi liberated in condition (1) from that liberated in condition (2).

Fig. 3.8

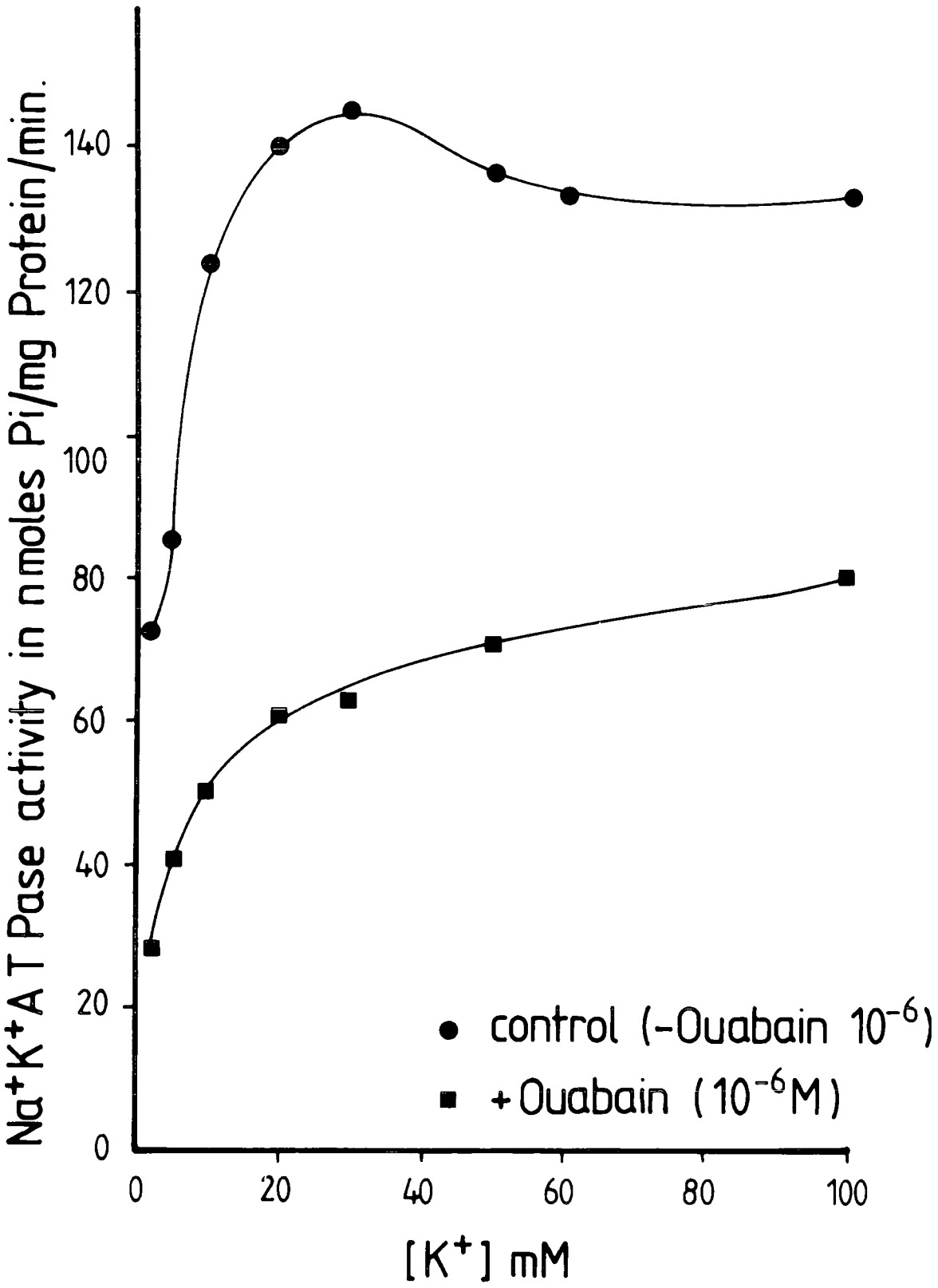

Na+K+A T Pase activity in n moles Pi/mg Protein/min.
0
20
40
60
80
100
120
140
0
20
40
60
80
100
[K+] mM
control (-Ouabain 10-6)
+Ouabain (10-6M)

FIGURE 3.9

Effect of varying concentrations of K^+ on the inhibitory effect of ouabain on the Na^+, K^+-ATPase from Locusta Malpighian tubule preparations. Activity remaining expressed as a percentage of the activity in the presence of 20 mM K^+.

Ordinate : Activity remaining expressed as a % of original activity in presence of 20 mM K^+.

Abscissa K^+ concentration (mM)

Conditions : As described in Figure 3.8

Fig. 3.9

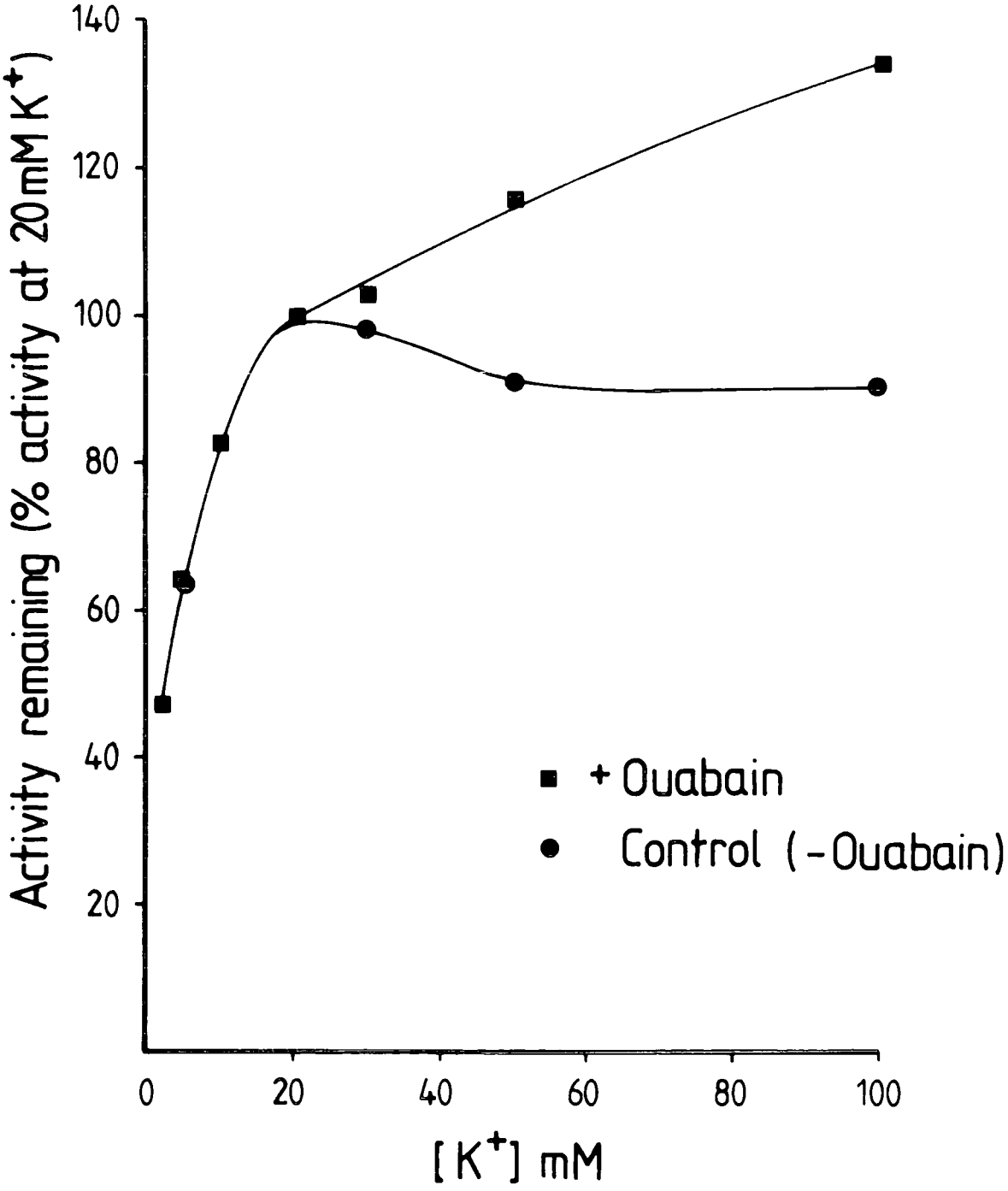

Activity remaining (% activity at 20mM K+)
140
120
100
80
60
40
20
0
20
40
60
80
100
[K+] mM
+ Ouabain
Control (-Ouabain)

FIGURE 3.10

Effect of varying concentrations of K^+ on Na^+, K^+-ATPase from Locusta Malpighian tubule preparations in the absence (●) or presence (■) of vanadate (10^{-6}M)

Ordinate : Activity in nmoles Pi liberated/mg Protein/min

Abscissa : K^+ concentration (mM)

Conditions : (1) 4 mM Mg^{2+}, 3 mM Tris ATP (2) 4 mM Mg^{2+}, 100 mM Na^+, 3 mM Tris ATP + varying concentrations of K^+. All media made in 50 mM Histidine/HCl buffer pH 7.2

Temperature : 30°C.

Na^+, K^+ -ATPase activity calculated by subtracting the amount of Pi liberated in condition (1) from that liberated in condition (2).

(●) Activity in absence of vanadate.

(■) Activity in presence of vanadate (10^{-6}M).

Fig. 3.10

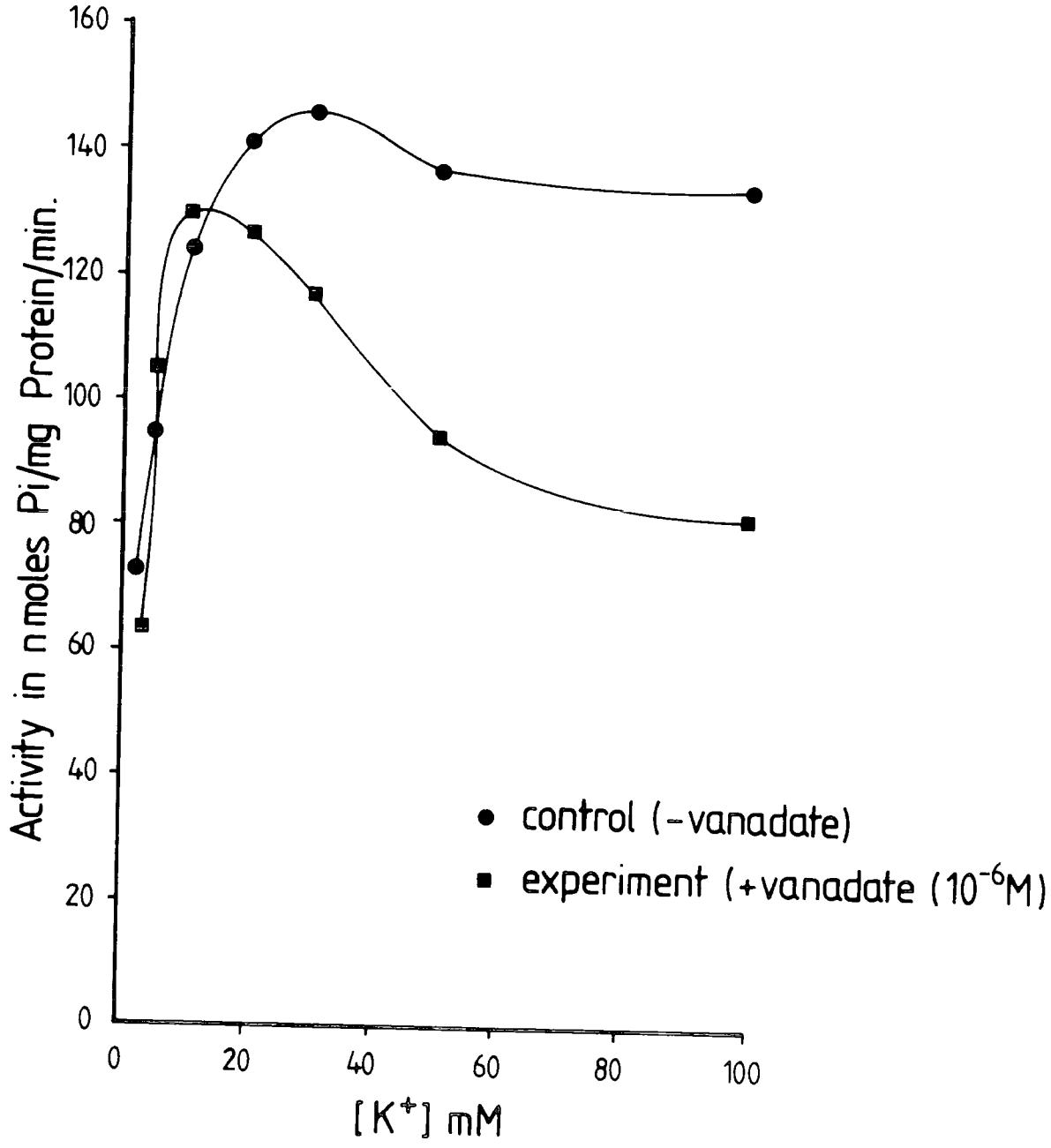

Activity in n moles Pi/mg Protein/min.
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
[K+] mM
control (-vanadate)
experiment (+vanadate (10-6M)

FIGURE 3.11

The effect of varying K^+ concentrations on Na^+, K^+-ATPase activity in absence or presence of vanadate (10^{-6}M).

Ordinate : activity remaining expressed as % of original activity at 20 mM K^+ in absence of vanadate.

Abscissa : K^+ concentration (mM)

Conditions : (1) 4 mM Mg^{2+} 3 mM Tris ATP. (2) 4 mM Mg^{2+}, 100 mM Na^+ + 3 mM Tris ATP (vanadium-free) + varying concentrations of K^+. All media made in 50 mM Histidine/HCl buffer pH 7.2

Temperature : 30°C .

Na^+, K^+ - ATPase activity estimated by subtracting the amount of Pi liberated under condition (1) from that liberated under condition (2).
(n = 4).

Fig. 3.11

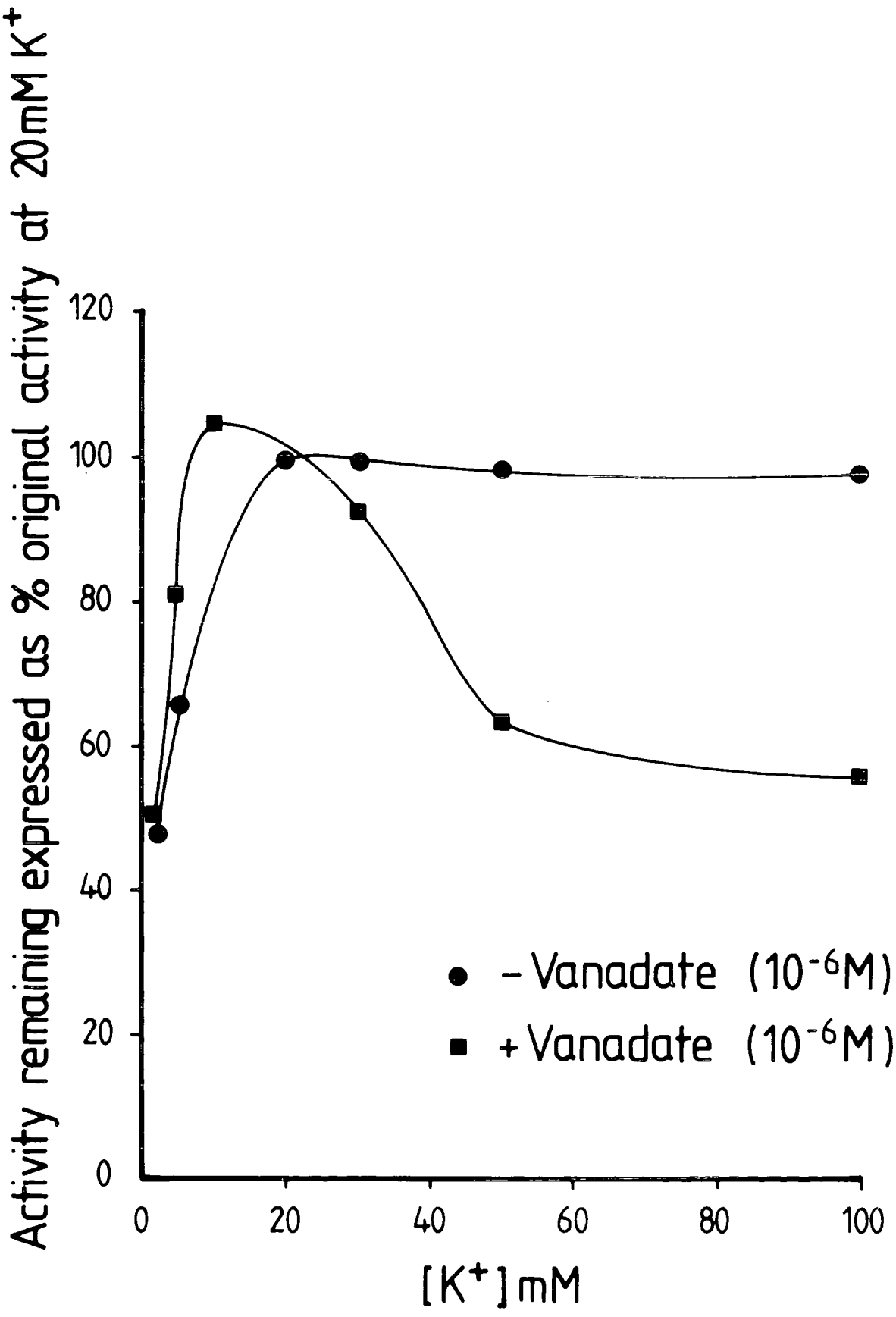

Activity remaining expressed as % original activity at 20mM K+
120
100
80
60
40
20
0
0
20
40
60
80
100
[K+] mM
- Vanadate (10-6M)
+ Vanadate (10-6M)

FIGURE 3.12

Effect of varying concentrations of K^+ on the activity of Na^+, K^+ -ATPase from Locusta Malpighian tubule preparations in the presence of ouabain (10^{-6}M) or vanadate (10^{-6}M).

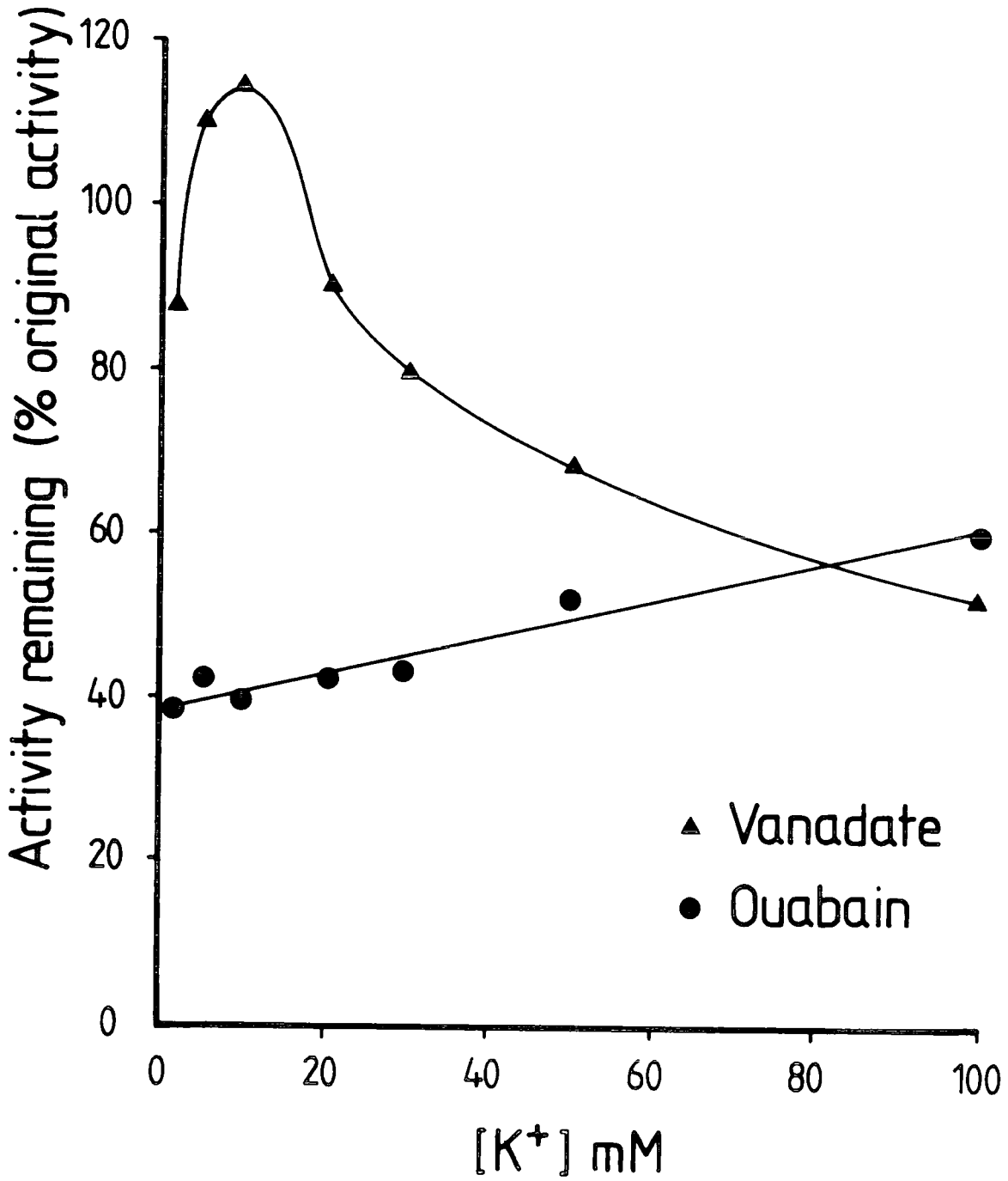
Ordinate : activity remaining expressed as % of original activity of each particular K^+ concentration in the absence of the inhibitor.

Abscissa : K^+ concentration (mM)

Assay conditions : (1) 4 mM Mg^{2+}, 3 mM Tris ATP. (2) 4 mM Mg^{2+}, 100 mM Na^+ 3 mM Tris ATP + different concentrations of K^+

Na^+, K^+-ATPase activity was calculated as previously described.
(n = 4)
Line for ouabain was fitted by a regression analysis.
Data as in Fig.3.8.

Fig. 3.12


Activity remaining (% original activity)
120
100
80
60
40
20
0
0
20
40
60
80
100
[K+] mM
Vanadate
Ouabain

3.13 The effect of temperature on the inhibitory function of Ouabain and Vanadate

(a) Ouabain

Figure 3.13 shows the effect of temperature on the activity of the uninhibited Na^+, K^+-ATPase. As can be seen the activity is markedly temperature-sensitive; Q_{10} values greater than 2 are found for the range 10° - 30° C and between 1.5 and 2 in the range 30° - 40°.

Figure 3.14a,c show the effect of temperature on ouabain inhibition of the Na^+, K^+-ATPase at 10°C ouabain (10^{-6}M) does not significantly lower activity ($p = >0.05$). Higher temperatures give a progressively greater degree of ouabain inhibition so that at 40°C some 85% of the enzyme activity is inhibited.

(b) Vanadate

Figure 3.14b,d shows the results of similar experiments using vanadate as an inhibitor. The temperature sensitivity of vanadate inhibition of the Na^+, K^+-ATPase appears to be complex with an apparent 'optimum' at about 30°C when vanadate (10^{-6}M) caused 70% inhibition. In contrast to ouabain, increasing temperature causes a reduction in the level of inhibition such that at 40°C no significant inhibition occurs.

3.14 Effect of Furosemide on Na^+, K^+-ATPase activity

Table 3.9 and Fig. 3.15 show the results obtain when Na^+,K^+-ATPase activity was assayed in the presence of different concentrations of furosemide ranging between 0 and 10^{-6}M. Results from two independent experiments clearly show that furosemide, at all concentrations, did not have any significant effect on Na^+, K^+-ATPase activity.

FIGURE 3.13

Arrhenius curve showing the effect of temperature on Na^+, K^+-ATPase activity in Locusta Malpighian tubule preparations.

Ordinate : Log_{10} of Na^+, K^+-ATPase activity in nmoles Pi/mg Protein/min.

Abscissa : $\frac{1}{T°A} \times (10^3)$

Each point on the curve represents a mean value of 4 separate determinations.

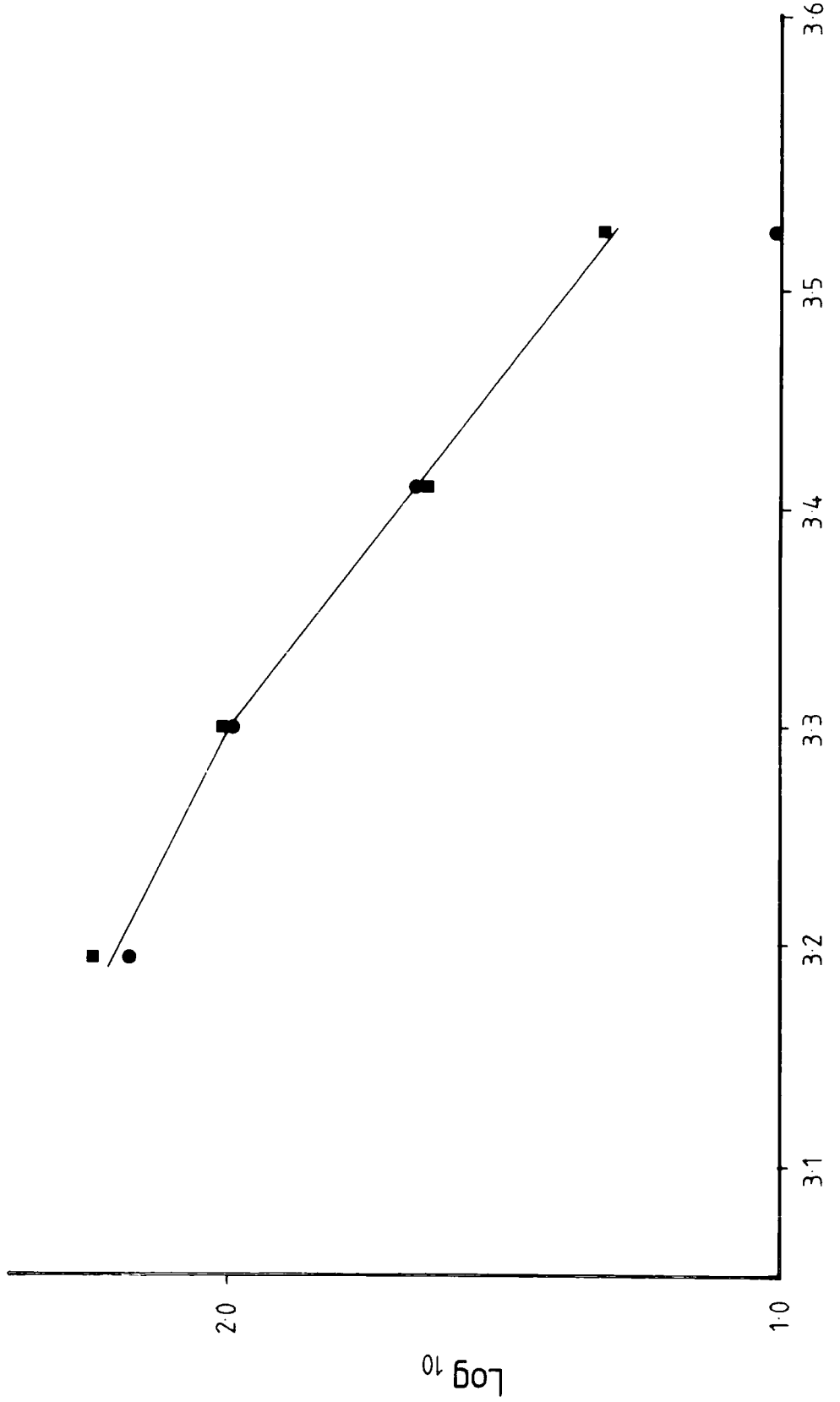


Fig. 3.13

FIGURE 3.14a,b

Effect of temperature on Na^+, K^+-ATPase from Locusta Malpighian tubule preparations (a) in presence of ouabain (10^{-6}M) and (b) vanadate (10^{-6}M).

Ordinate : % inhibition

Abscissa : Temperature °C

Conditions : (1) 4 mM Mg^{2+}, (2) 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+ + 3 mM Tris ATP in 50mM Histidine/HCl buffer pH 7.2

Temperature ranged between 0 - 40°C

Note Data as for Fig.3.14c,d.
Each point on the graph represents a mean of 4 separate preparations.

Fig. 3.14b

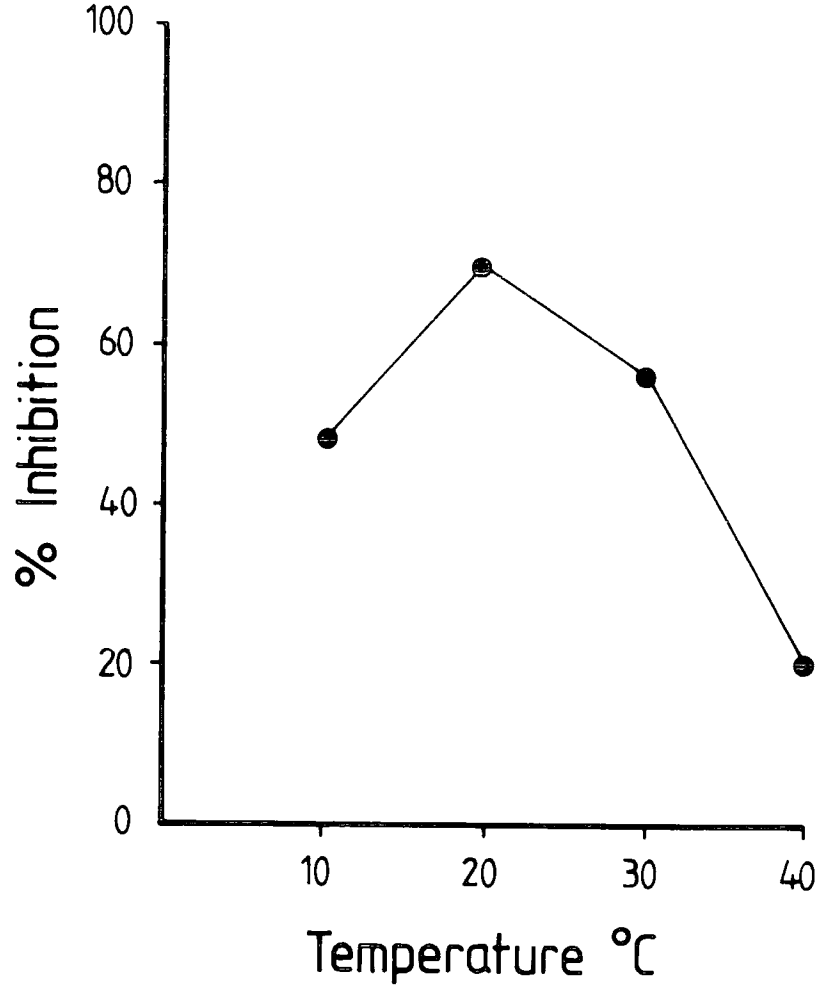


Fig. 3.14a

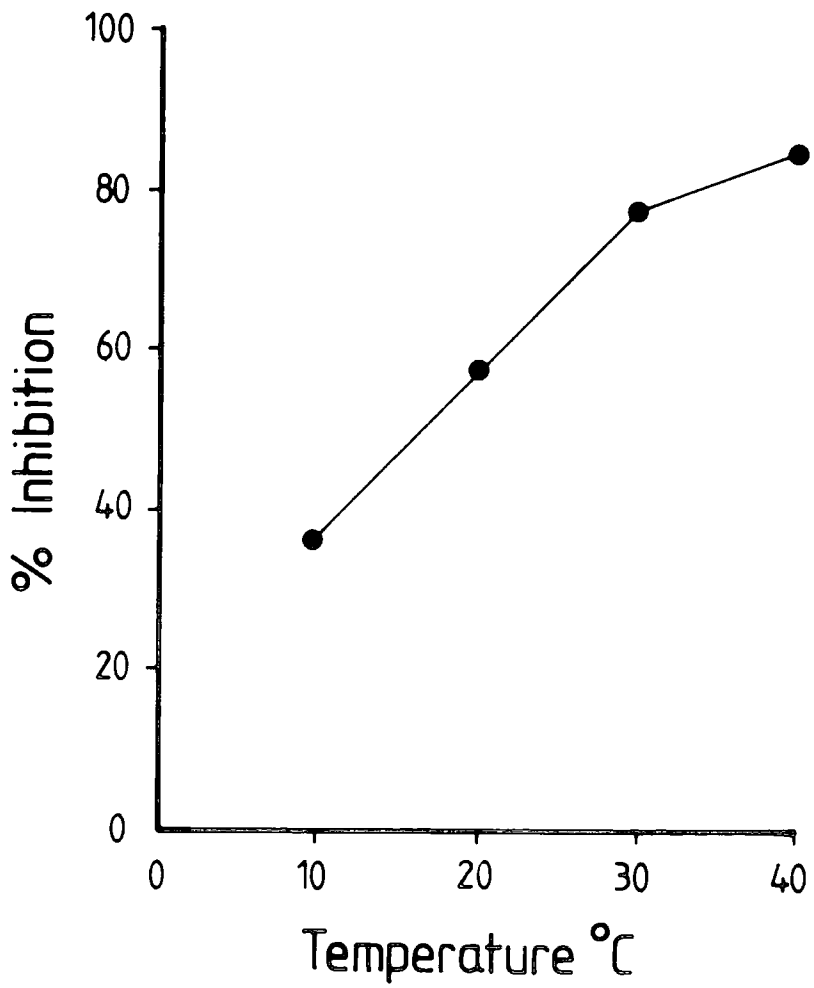


FIGURE 3.14c,d

Effect of temperature on Na^+, K^+ -ATPase activity in absence or presence of (10^{-6} M) ouabain (c) or vanadate (d)

Abscissa : Temperature (°C)

Conditions : (1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+. All media were made in 50 mM Histidine/HCl buffer, pH 7.2 and each medium contained 3 mM Tris ATP.

Control: (minus inhibitor)

Experiment 10^{-6} M ouabain or vanadate added to each medium.

Na^+, K^+-ATPase activity estimated by subtracting the amount of Pi liberated under condition (1) from that liberated under condition (2).

Vertical lines represent 2 SEM
Each point on the graph represents a mean of 4 separate determinations.

Fig. 3.14c

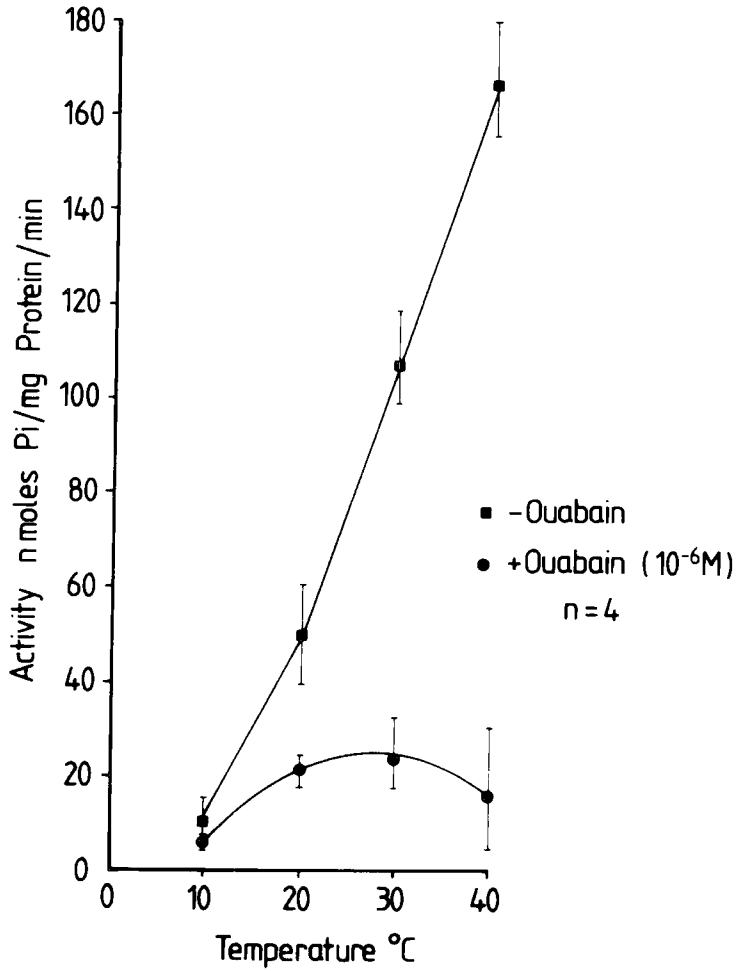


Fig. 3.14d

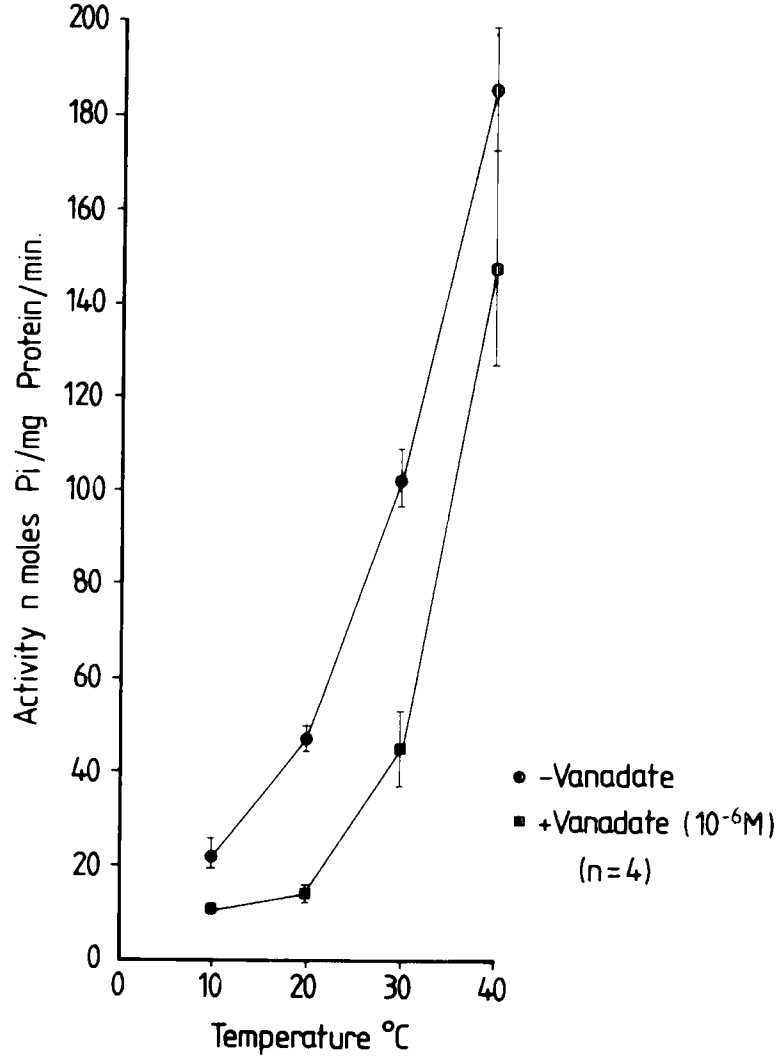


TABLE 3.9 : Effect of furosemide on Na^+, K^+ -ATPase activity from Locusta Malpighian tubules. Specific activity in nmoles Pi liberated/mg Protein/min

Furosemide (M)	Na^+, K^+-ATPase activity			
	EXPERIMENT 1		EXPERIMENT 2	
	Specific Activity	% Activity remaining	Specific Activity	% Activity remaining
0	208.0	100	110	100
10^{-9}	212.0	101.9	114	103.6
10^{-8}	196.0	94.2	116	105.4
3×10^{-8}	196.0	94.2	110	100
10^{-7}	186.8	89.7	98	90.1
3×10^{-7}	186.6	89.7	92	83.6
10^{-6}	206.0	99.0	112	101.8
3×10^{-6}	212.0	101.9	106	96.4
10^{-5}	190.0	91.3	110	100
3×10^{-5}	212.0	101.9	106	96.4
10^{-4}	192.0	92.3	100	90.9
10^{-3}	188.0	90.4	110	100

Conditions : 4 mM Mg^{2+}, 4 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+
Experiment (+ Furosemide), Control (- Furosemide).

All media made in 50 mM Histidine/HCl buffer 7.2

Temperature 30°C.

Each medium contained 3 mM Tris ATP.

Values shown for specific activity were calculated by subtracting activity in presence of Mg^{2+} alone from activity in presence of Mg^{2+}, Na^+ and K^+.

FIGURE 3.15

The effect of varying concentrations of furosemide on Na^+, K^+-ATPase activity in Locusta Malpighian tubule preparations.

Ordinate : Activity remaining expressed as % original activity in the absence of inhibitor.

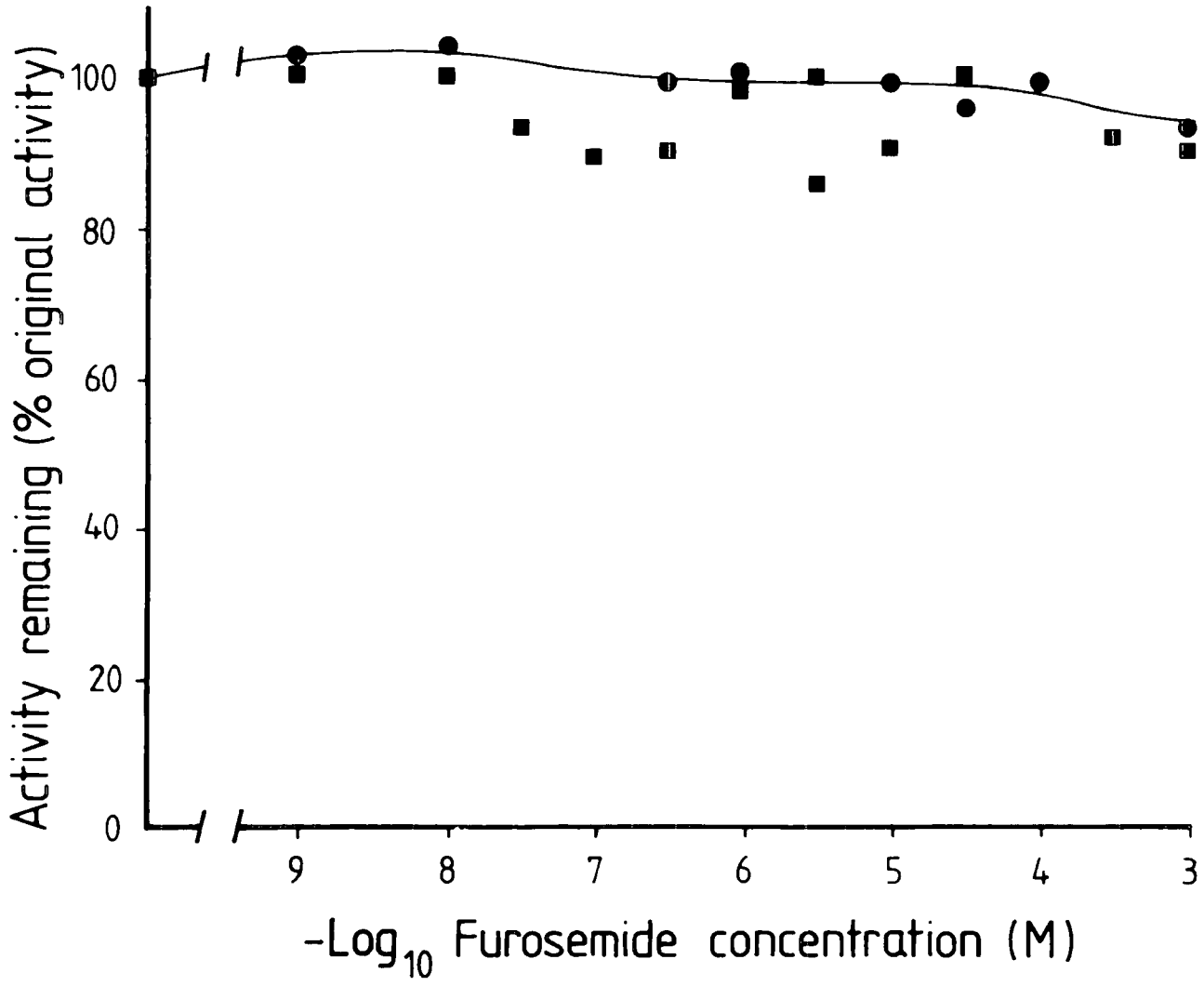
Abscissa : Negative Log_{10} furosemide concentration (M).

Conditions : (1) 4 mM Mg^{2+} (2) 4 mM Mg^{2+}, 100 mM Na^+ 20 mM K^+ + 3 mM Tris ATP in 50 mM Histidine/HCl buffer pH 7.2 (+ furosemide) Experiment and (-furosemide) Control.

Temperature : 30°C

Different symbols show data from separate experiments.

Fig. 3.15


Activity remaining (% original activity)
100
80
60
40
20
0
9
8
7
6
5
4
3
-Log10 Furosemide concentration (M)

DISCUSSION

The results presented here show that microsomal preparations of Malpighian tubules of Locusta migratoria are able to hydrolyse ATP and that they possess two types of ATPase namely, a magnesium-dependent ATPase (Mg^{2+}ATPase) which is active and is insensitive to ouabain; and a sodium-potassium activated, magnesium-dependent ATPase (Na^+,K^+-ATPase, E.C.3.6.1.3) (SKOU, 1965), which is inhibited by ouabain. Similar results have been reported for Locusta Malpighian tubules (ANSTEE and BELL, 1975, 1978) and Malpighian tubules of Homorocoryphus nitidulus vicinus (PEACOCK et al., 1976) microsomal preparations.

Mg^{2+}ATPase activity was routinely demonstrated during the present study because Na^+, K^+-ATPase is always accompanied by other ATPases (SKOU, 1965; ANSTEE and BOWLER, 1984). However, due to the deoxycholate.sodium iodide extraction procedure employed, the level of Mg^{2+}-ATPase activity was low (usually less than 20% of the total ATPase activity). The Na^+, K^+-ATPase : Mg^{2+}ATPase ratio value was 5.4. This ratio is slightly higher but compares favourably with the value of 3.47 reported for Locusta Malpighian tubules by BELL (1977) and 3.4 for cockroach nerve cord (GRASSO, 1967) and is somewhat lower than the value of 8.5 reported for Jamaicana Malpighian tubules (PEACOCK, 1975) preparations. However, the value of 59.7 for Locusta Malpighian tubules reported by PEACOCK (1975) is significantly higher than the present study value. This variation in the Na^+, K^+-ATPase : Mg^{2+}ATPase ratio is attributed to the differences in incubation temperature (BOWLER and DUNCAN, 1968a; and BELL, 1977) and composition of the ionic reaction media. Mg^{2+}ATPase is less sensitive to temperature than Na^+,K^+-ATPase (BOWLER and DUNCAN, 1968a) and it has been reported that

Mg^{2+}ATPase may be sensitive to monovalent cations such as Na^+ at concentrations used to activate tne Na^+, K^+-ATPase. Therefore, Mg^{2+}ATPase activity may not be accurately determined in a medium containing Mg^{2+} alone (BONTING et al., 1964; IZUTSU et al., 1974, SCHIN and KROEGER, 1980) which could be true for the present study as Mg^{2+}ATPase activity was assayed in the presence of Mg^{2+} alone. This could lead to a biased estimation of the Na^+, K^+-ATPase activity. However, the latter could be calculated in two ways according to ANSTEE and BOWLER (1984): (1) by subtracting the ouabain-insensitive ATPase component from the total ATPase, and (2) by subtracting the ATPase activity in the presence of Mg^{2+} alone from the total ATPase activity in the presence of Mg^{2+}, Na^+ and K^+. SCHIN and KEOEGER (1980); TOLMAN and STEELE (1976) point out that ouabain may not completely inhibit the Na^+, K^+-ATPase activity. However, the results in Table 3.3 show that this has not been a problem during the present study because the level of ATPase activity at 30°C in the presence of Mg^{2+}ATP alone agreed closely with that in the presence of Mg^{2+}, Na^+, K^+ + ATP + 1 mM ouabain.

The fact that Mg^{2+}ATPase and Na^+, K^+-ATPase have different temperature characteristics (BOWLER and DUNCAN 1968) and, as demonstrated by the present study, the Mg^{2+}ATPase was not inhibited by ouabain shows that the two enzymes are different and may have different cellular roles. It is suggested by BOWLER and DUNCAN (1968a,b) that Mg^{2+}ATPase may be involved in regulation of the permeability of the cell membrane. However, since the function Mg^{2+}ATPase is not yet fully understood it has not formed a major part in the present study.

The high levels of Na^+, K^+-ATPase activity demonstrated in Malpighian tubules preparations during the present study strongly

indicate that this enzyme is actively involved in ion and water transport across the Malpighian tubule epithelia.

The Na^+, K^+-ATPase in Malpighian tubule preparations is similar to that described in SKOU (1965). It hydrolysed ATP and its maximum activity was measured in the presence of Mg^{2+}, Na^+, K^+ and ATP. However, it should be pointed out that because this enzyme has already been characterised by ANSTEE and BELL (1975, 1978), during the present study, the ATPase assay was not carried out in the presence of Mg^{2+}, Na^+ or Mg^{2+} + K^+ alone as recommended by ANSTEE and BOWLER (1984) for an uncharacterised enzyme.

Some workers have failed to demonstrate Na^+, K^+-ATPase activity in some insect tissue preparations (e.g. in cockroach muscle, WAREHAM et al., 1968: Calliphora rectum. BERRIDGE and GUPTA, 1968; Hyalophora cecropia and Manduca sexta rectum Harvey et al., 1983). JUNGREIS and VAUGHAN (1977) suggest that in some insect epithelia such as Lepidopteran midgut, Dipteran salivary gland and many Malpighian tubules K^+ transport does not involve Na^+, K^+-ATPase. But some of their reasoning has been disputed (ANSTEE and BOWLER, 1979). HARVEY et al., (1983) also report that in Lepidopteran insect epithelia transport of K^+ is associated with an ouabain-insensitive K^+-stimulated ATPase and that Na^+K^+ pump is, for example, absent in the tobacco hornwork (Manduca sexta) larva. HARVEY et al., (1983) also concluded that Malpighian tubules of Locusta possess both a Na^+, K^+-stimulated and a ouabain-insensitive ATPase. This conclusion was based on ANSTEE and BELL (1975) results. It is apparent that HARVEY et al., (1983) made a mistake in their calculations of the data and obtained a false picture of high ATPase activity in the presence of K^+ alone which is not the case in ANSTEE and BELL (1975) original data.

The results from the present study have also clearly shown that ouabain, over a wide range of concentrations, inhibited Na^+, K^+-ATPase activity in Locusta Malpighian tubule microsomal preparations giving a pI_{50} of 6.2 (see Figure 3.2). The exact pI_{50} could be slightly higher as indicated by the linear transformation of the data shown in Figure 3.2 (see Figure 3.3). In Figure 3.3, the line fitted by least squares regression analysis (SNEDECOR and COCHRAN, 1967) indicates the pI_{50} and 6.6 The pI_{50} value of 6.2 is in good agreement with 6.1 reported for Locusta Malpighian tubules (ANSTEE and BELL, 1975); 6.2 for Periplaneta americana antennal microsomal preparations (NORRIS and CARY, 1982) and several other insect tissues shown in Table 3.1. However, the pI_{50} value of 6.6 shown in Figure 3.3 is higher than 5.8 reported for Locusta Malpighian tubule microsomal preparations (DONKIN, 1981) and 3.9 for rat liver microsomal preparations (BAKKEREN and BONTING, 1968).

The higher pI_{50} value indicates that Locusta Malpighian tubule microsomal preparations ouabain-sensitivity might have been underestimated by some workers (e.g. DONKIN, 1981). The results presented here, show that Locusta Malpighian tubule Na^+, K^+-ATPase was even more sensitive to ouabain than some mammalian tissue (e.g. pI_{50} value of 5.7 for rabbit kidney, WHITTAM and WHEELER, 1961, and 6.0 for mammalian kidney, SKOU, 1962) (see Table 3.2). It should, however, not be generalised that insect tissues are more sensitive to ouabain than mammalian tissue. The differences in pI_{50} values reported by different investigators may be due to the differences in methods used to analyse the data. A regression analysis is vital for the determination of pI_{50} values but this has not been carried out by some workers. The varying pI_{50} values also reflect the fact that there is a difference in ouabain-sensitivity in different animal species as

has been demonstrated in mammals (PERIYASAMY et al., 1983). But often the variation in the pI_{50} values reported by different workers is due to the differences in preincubation assay procedure designed to circumvent the problems of slow equilibration with ouabain and effects of K^+ in the incubation medium. pI_{50} values are known to be lower when ouabain is present in incubation medium only (AKERA, 1971) and inhibition is dependent on assay time (SCHWARTZ et al., 1975).

AKERA (1971) and TOBIN and BRODY (1972) have shown that allowing a preincubation period for the enzyme in the presence of Na^+,Mg^{2+} and ATP and starting the reaction with K^+, the amount of ouabain necessary to effect 50% inhibition of the ATPase activity was only 23.7% of that needed by the more conventional method. There was no preincubation period allowed during the present study, but in support of results presented here, ALLEN and SCHWARTZ (1970) report that the amount of ouabain bound in the presence of ATP, Mg^{2+} and Na^+ equals that bound in the presence of ATP, Mg^{2+}, Na^+ and K^+ as long as the reaction is carried out over a long period of at least 30 minutes. As will be discussed, later, the incubation temperature also affects ouabain inhibition of Na^+, K^+-ATPase activity. At temperatures below 30°C a longer equilibration period would be required (AKERA, 1971). Incubation at 30°C for a period of 30 minutes during the present study and other similar studies (e.g.ANSTEE and BELL,1975,1978; DONKIN,1981) was considered adequate for maximum ouabain-binding and inhibition. The dose-response sigmoid shape curves shown in Figure 3.2a,b is expected of a simple uncompetitive inhibition (WOLF and PETER,1972) and is typical of insect Na^+, K^+-ATPase. The decay from 95% to 5% activity spans over 2 orders of magnitude of inhibitor concentration. Some mammalian Na^+, K^+-ATPase preparations show a biphasic effect of ouabain and stimulation may occur at very low concentrations

with inhibition at high concentrations. LEWIS (1978) points out that the relatively higher ouabain-insensitivity reported for some rat tissue Na^+, K^+-ATPase (TOBIN and BRODY, 1972; AKERA et al., 1974) may be due to the apparent anomalous behaviour at low concentrations of ouabain.

The results obtained in the present study are in agreement with suggestions of ANSTEE and BOWLER (1979). They point out that temperature and potassium are two important factors which modify ouabain inhibition of Na^+, K^+-ATPase. Figure 3.14a shows that the level of ouabain-inhibition increased as the incubation temperature increased. Similar results are reported for rat brain preparations by JUDAH and AHMED (1965) who suggested that decreased effectiveness of ouabain at low temperatures was due to an increase in the affinity of the enzyme for potassium. Similar observations were reported for Locusta rectal and Malpighian tubule preparations, PEACOCK (1981) and ANSTEE and DONKIN (1980) respectively. However, it has been found that in some tissues, ouabain-sensitivity at low temperatures may depend on the temperature the animal is adapted to, e.g. SORENSEN (1981) reports ouabain-sensitivity in preparations of Platichthys flexus at 0°C. The results presented here show that there was no significant inhibition of Na^+, K^+-ATPase at 10°C and the level of inhibition rose from 57% at 20°C, 79% at 30°C to 85% at 40°C. This effect of temperature on ouabain-sensitivity of Na^+, K^+-ATPase is comparable with that reported by DONKIN and ANSTEE (1980). However, as different concentrations of ouabain were used the values of levels of inhibition cannot be directly compared. The temperature sensitivity of the Na^+, K^+-ATPase from Malpighian tubules of Locusta agrees well with data presented by PEACOCK (1981) and DONKIN and ANSTEE (1980). Over most of the temperature range used the Q_{10} value calculated is close

to 2 (Figure 3.13). This implies a typical temperature sensitivity characteristic for this enzyme.

The present study has also demonstrated that ouabain inhibition of Na^+, K^+-ATPase was inversely related to concentration of potassium in the incubation medium. High K^+ concentrations reduced the effectiveness of ouabain. This is consistent with previous reports of SKOU (1965) and AHMED and JUDAH (1965) for mammalian Na^+, K^+-ATPase; and PEACOCK (1981) for Locusta rectal preparations. SZAMEL and RESCH (1981) also demonstrated that high concentrations of K^+ prevented the dose-dependent binding of labelled ouabain to lymphocytes. This antagonism of ouabain inhibition partly explains why ouabain is not an efficient inhibitor at low temperatures when the affinity of the enzyme for K^+ increases. Ouabain binds to the phosphorylated form of the enzyme (E_2-P). Adding K^+ discharges the E_2-P intermediate and reduces both binding and inhibition by ouabain (MATSUI and SCHWARTZ, 1966; LINDENMAYER and SCHWARTZ, 1973).

The present study has also shown that vanadate is a potent inhibitor of Na^+, K^+-ATPase from Locusta Malpighian tubule preparations. This is consistent with reports from other workers who found that vanadate inhibited Na^+, K^+-ATPase activity in different mammalian tissue preparations, e.g. kidney (CANTLEY et al., 1977; MYERS et al., 1979; HANSEN et al., 1979; BOND and HUDGINS, 1981) red blood cells (BEAUGÉ and GLYNN, 1977, 1978; BEAUGÉ and DIPOLO, 1979; BOND and HUDGINS, 1981) the heart (ERDMANN et al., 1979a,b) and the brain (WU and PHILLIS, 1979).

Figure 3.4 and 3.5 show that vanadate inhibited Locusta Malpighian tubule Na^+, K^+-ATPase in a dose-responsive manner with a pI_{50} of 5.9 (Figure 3.6). Using red cell ghost Na^+, K^+-ATPase, CANTLEY et al., (1978) obtained a higher pI_{50} value of 7 when he used

4 mM Mg^{2+} and 8 when he used 25 mM Mg^{2+} in the incubation media. QUIST and HOKIN (1978) reports a pI_{50} value of 6 for Scarcolemmal Na^+, K^+-ATPase in the presence of 6 mM Mg^{2+}. The differences in pI_{50} values may be due to the fact that the binding of vanadate depends on Mg^{2+} : ATP ratio (NEIDER et al., 1979). The latter found that increasing the ratio of Mg^{2+} : ATP facilitated vanadate inhibition of Na^+, K^+-ATPase. They obtained 20% vanadate inhibition of the enzyme in the absence of Mg^{2+} and 55% inhibition in the presence of 1 - 5 mM Mg^{2+}. The relatively low concentration of Mg^{2+} (4 mM) used during the present study may be the cause for the low pI_{50} value reported in the present study. The pI_{50} value may also depend on the source of the enzyme. For example, the human red cell Na^+, K^+-ATPase was less sensitive to vanadate than the dog kidney Na^+, K^+-ATPase (BOND and HUDGINS, 1981).

Results presented here show that lower concentrations of vanadate were required to inhibit the isolated Na^+, K^+-ATPase from Locusta Malpighian tubules than those required to inhibit the in vitro Malpighian tubule fluid secretion (see Chapter 5). This demonstrates, as previously discussed, that vanadate is an efficient inhibitor only when applied on the cytoplasmic side of the cell membrane (CANTLEY et al., 1978 and others). In the case of intact tissue, vanadate has a long diffusion path before reaching the cytoplasmic side; during which process it may be oxidised to the less inhibiting form (CANTLEY and AISEN, 1979).

The results presented here have also confirmed several other workers' reports that high concentrations of external K^+ facilitate vanadate inhibition of Na^+, K^+-ATPase, (e.g. BEAUGE and DI POLO,1978; CANTLEY et al., 1978; DLOUHA, 1981). GRANTHAM (1980) who reviews some of these workers reports shows a curve very similar to that in

Figure 3.10 and 3.11. The only difference is that in his study K^+ concentrations as low as 2 - 4 mM enhanced vanadate inhibition. In the present study, the effect of K^+ was not apparent at concentrations lower than 10 mM K^+. This difference may be due to the Mg^{2+} : ATP radio used. GRANTHAM (1980) used 1.5 ATP while 3 mM ATP was used throughout the present study.

The fact that vanadate inhibited the Malpighian tubule Na^+, K^+-ATPase in the presence of K^+ indicate that as vanadate occurs naturally in some tissues it may play a physiological role during the hydrolysis of ATP by Na^+, K^+-ATPase. KARLISH et al., (1979) suggested that vanadate-inhibits Na^+, K^+-ATPase by blocking E_2- E_1 conformational change which is thought to follow hydrolysis of the phosphoenzyme when Na^+, K^+-ATPase catalyses the hydrolysis of ATP. In the absence of K^+, the limiting step is thought to be the hydrolytic step and not the subsequent conformational change. In the absence of K^+, therefore, the hydrolytic activity of Na^+, K^+-ATPase is expected to be very much less sensitive to vanadate.

The present study has also demonstrated temperature as another factor which influences the effect of vanadate on Na^+, K^+-ATPase. The results in Figure 3.13b show that inhibition of the Na^+, K^+-ATPase activity by vanadate decreased with increasing temperature. This is expected because as previously discussed the enzyme affinity for K^+ increases at low temperature and K^+ facilitates vanadate inhibition of Na^+, K^+-ATPase. Figure 3.13a and b show that at high temperatures (30°C and above) ouabain is a more effective inhibitor of Locusta Malpighian tubule Na^+, K^+ -ATPase than vanadate which is more effective at temperatures below 30°C. Therefore in assessing ouabain- or vanadate-sensitivity in different tissues the two important factors, external K^+ concentration and incubation temperature should be considered.

And as pointed out by PEACOCK (1981) a comparison of results from studies of ouabain-sensitivity in different tissues would not be possible unless these two factors are controlled. As demonstrated by the present study this would also apply to studies of vanadate-sensitivity.

The results presented in Figure 3.15 show that furosemide did not inhibit Na^+, K^+-ATPase activity in Locusta Malpighian tubule microsomal preparations. The results are in agreement with reports for mammalian Na^+, K^+-ATPase (e.g. pig kidney preparations, PEREZ-GONZALEZ DE LA MANNA, 1980). However, the latter was able to demonstrate that furosemide inhibits the ouabain-insensitive ATPase activity. AITON et al., (1981) also demonstrated that furosemide inhibits a proportion of the total passive, ouabain-insensitive K^+ influx was independent of the (Na^+ + K^+) pump. The effect of furosemide on other ATPases isolated from Locusta Malpighian tubules is discussed in Chapter 4.

CHAPTER 4

BIOCHEMICAL DEMONSTRATION OF THE PRESENCE OF A Mg^{2+}, HCO_3^--ATPase IN MALPIGHIAN TUBULES OF LOCUSTA MIGRATORIA L.

Introduction

KASBEKAR and DURBIN (1965) reported the presence of a Mg^{2+}, HCO_3^--ATPase in microsomal preparations of frog gastric mucosa. They found that this enzyme was inhibited by SCN^- but was not affected by ouabain. Subsequently a variety of tissues, most of which are secretory, have been shown to possess relatively high levels of this Mg^{2+}, HCO_3^--ATPase. A list of such tissues is given in Table 4.1. This enzyme has been implicated in a variety of transport mechanisms involving HCO_3. It has been suggested to be involved in HCO_3 transport by gastric mucosa (BLUM et al., 1971; WIEBELHAUS et al., 1971; SACHS et al., 1972b) rat kidney brush border (KINNE-SAFFAREN and KINNE, 1980) mammalian pancreas (SIMON and THOMAS, 1972). It has also been implicated in H^+/HCO_3 transport by rabbit submandibular gland (SIMON et al., 1972a) and it is also suggested to be involved in HCO_3 transport and Na^+/H^+ exchange in rat kidney brush border (LIANG and SACKTOR, 1976).

To date, little is known about the occurrence and function of Mg^{2+}, HCO_3^--ATPase in insect tissues. The first extensive study of Mg^{2+} ATPase, was carried out by TURBECK et al., (1968) using the K^+-transporting midgut of larval Hyalophora cecropia. Later HERRERA et al., (1978) isolated Mg^{2+}, HCO_3^--ATPase from Schistocerca gregaria rectum. Subsequently, ANSTEE and FATHPOUR (1979, 1981) and FATHPOUR (1980) investigated the presence of the Mg^{2+},HCO_3^--ATPase in microsomal preparations of Malpighian tubules of Locusta. They demonstrated enzyme activity but pointed out that owing to the

TABLE 4.1 : Presence of Mg^{2+}, HCO_3^--ATPase in different tissues

Species	Tissue with Mg^{2+}, HCO_3^- -ATPase	Reference
Frog	gastric mucosa	Kasbekar & Durbin (1965)
Lizard	gastric mucosa	De Pont et al., (1972)
Lizard	gastric mucosa	Isutsu & Siegel (1972)
Rat	gastric mucosa	Narumi & Kanno (1973)
Rat	fundus mucosa	Soumarmon et al., (1974)
Mouse	kidney	Suzuki (1978)
Rabbit	kidney	Liang & Sacktor (1976)
Rat	proximal tubule	Kinne-Saffran & Kinne (1980)
Rainbow trout	gill	Kerstetter & Kirschner(1974)
Carassius auratus	gill	De Renzis & Bornancin (1977)
Rabbit	erythrocyte	Duncan * (1975)
Bos primigenius taurus	Rumen	Hegner & Anika (1975)
Hyalophora cecropia	Midgut	Turbeck et al. (1968)
Locusta migratoria	Malpighian tubules	Anstee & Fathpour (1979), Anstee & Fathpour (1981) Present Study
Schistocerca gregaria	rectum	Herrera et al. (1978)
Manduca sexta	Midgut & integument	Deaton (1984)

* All workers in Table 4.1 reported inhibition of Mg^{2+}, HCO_3^--ATPase by SCN^- except Duncan (1975) for rabbit erythrocyte.

uncertainty of its cellular localization it was difficult to conclude with confidence the nature of its role in anion transport across epithelia. The most recent report of the presence of Mg^{2+}, HCO_3^- -ATPase in insect tissue is from DEATON (1984) who demonstrated its activity in microsomal preparations of the midgut and integument of the tobacco hornworm, Manduca sexta. He suggested that K^+ transport through these epithelia may be accompanied by HCO_3 transport. Like ANSTEE and FATHPOUR (1979) he provided strong evidence for the existence of a non-mitochondrial HCO_3 - stimulated ATPase in plasma membrane but pointed out that more research was required before the role of these anion-specific ATPases could be fully assessed. Thus, some controversy exists over the interpretation of the cellular origin and role of the Mg^{2+}, HCO_3^--ATPase. Some workers suggest that this enzyme is present in microsomal fractions merely as a result of contamination by broken mitochondria, where HCO_3 - stimulated ATPase is known to be situated in the outer membrane. This view is favoured by KIMELBERG and BOURKE (1973), GRISOLIA and MENDELSON (1974), NARUMI and KANNO (1973) from work on gastric mucosa; IZUTSU and SIEGEL (1972) from work on submandibular gland; KIMELBERG and BOURKE (1973) from work on rat brain. On the other hand LIANG and SACKTOR (1976) suggested that mitochondrial contamination could account for only 15% of the activity in renal brush border preparations. Furthermore, several workers have differentiated the plasma membrane Mg^{2+}, HCO_3^--ATPase from the mitochondrial enzyme by using various membrane marker enzymes (e.g. the mitochondrial marker enzyme, succinate dehydrogenase) to identify the subcellular localization of particular membrane fractions (e.g. in rabbit submandibular gland, SIMON et al., 1972a; midgut and integuments of Manduca sexta, DEATON, 1984; and Locusta Malpighian tubules, ANSTEE and FATHPOUR, 1979, 1981; FATHPOUR 1980). Such studies are usually

carried out in association with electron microscopy. Other workers (VAN AMELSVOORT et al., 1977a,b, 1978b; KIMELBERG and BOURKE, 1973) sought to identify the enzyme source on the basis of different responses to certain inhibitors. For example, LIANG and SACKTOR (1976) reported that mitochonrial HCO_3 - ATPase is almost totally (95%) inhibited by oligomycin and quercetin while only 36% of the microsomal HCO_3 - stimulated ATPase is inhibited by these drugs.

DUNCAN (1975) reported HCO_3 - stimulated ATPase activity in rabbit erythrocyte ghosts where there is no doubt that the mitochondria are absent. However, the erythrocyte HCO_3 - stimulated ATPase has been reported to be different from HCO_3 - ATPase from other tissues. DUNCAN (1975) found that HCO_3 - stimulated ATPase from rabbit erythrocyte was insensitive to 5 mM SCN^-. VAN AMELSVOORT et al., (1978a) also cast further doubt over the intracellular distribution Mg^{2+}, HCO_3^- - ATPase when they suggested that the rabbit erythrocyte enzyme is different from the anion-sensitive Mg^{2+} - dependent ATPases of other tissues. They conclude from the effects of chloropromazine and ruthenium red, which are both thought to be specific inhibitors of erythrocyte ($Ca^{2+} + Mg^{2+}$)-ATPase, that the anion-stimulated ATPase does not represent a separate enzyme but is part of the ($Ca^{2+} + Mg^{2+}$)-ATPase system of the erythrocyte membrane.

IZUTSU and SIEGEL (1975) using rat liver microsomal preparations found that the level of mitochondrial contamination depended on the method of homogenisation and fractionation procedure used. ANSTEE and FATHPOUR, (1979) found that in Locusta Malpighian tubules microsomal preparations, the 20,000 g fraction, which had some mitochondrial contamination, showed very high levels of Mg^{2+}, HCO_3^- - ATPase activity. Preliminary experiments in the present study gave similar results.

However, it is important that ANSTEE and FATHPOUR (1979, 1981) and the present study demonstrated that there was substantial Mg^{2+}, HCO_3^- - ATPase activity in the 100,000 g fraction of Locusta Malpighian tubule microsomal preparation. Microscopic studies showed that this fraction had no obvious mitochondrial fragments and very little mitrochondrial contamination was indicated as measured by succinate dehydrogenase (SDH) activity (ANSTEE and FATHPOUR, 1979, 1981; FATHPOUR 1980). They recorded maximum activity of this enzyme in Locusta Malpithian tubules in the presence of 2 mM Mg^{2+}, 20 mM $NaHCO_3^-$ and 3 mM ATP. The same concentration of HCO_3 was used to obtain maximal stimulation of the Mg^{2+} - dependent ATPase activity in frog gastric mucosa (KASBEKAR and DURBIN, 1965), rabbit erythrocyte, (DUNCAN, 1975) mammalian pancreas (SIMON and THOMAS, 1972) rat cerebral cortex (KIMELBERG and BOURKE, 1972) and gills of Carassius auratus (De RENZIS and BORNANCIN, 1977). ANSTEE and FATHPOUR (1979, 1981) and FATHPOUR, (1980) found that concentrations below or above 20 mM HCO_3 caused a decline in the level of stimulation. Several other workers report maximal activity in the presence of higher HCO_3 concentrations, e.g. (de PONT et al., 1972) for lizard gastric mucosa and SANTIAGO et al., (1977) for rat liver and heart recorded maximal activity of Mg^{2+}-ATPase in the presence of 24 mM HCO_3. Other workers used higher concentrations of HCO_3 still to effect maximal stimulation (SUZUKI 1978; KATZ and EPSTEIN, 1971). To give maximal stimulation the highest concentration (70 mM HCO_3) is reported by KERSTETTER and KIRSCHNER (1974) for rainbow trout ATPase and the lowest concentration (10 mM HCO_3) is reported for dog submandibular gland ATPase by IZUTSU and SIEGEL (1972).

Some variation in the Mg^{2+}:ATP ratio required to provide maximal stimulation is reported in several different studies. For

example, a ratio of 1:2 is reported for the frog gastric mucosa preparation (SUZUKI, 1978); 1:1.5 for the Locusta Malpighian tubules preparation (FATHPOUR, 1980). TURBECK et al., (1968), SIMON et al., (1972a) and WIZEMAN et al., (1974) used a 1:1 ratio whilst DUNCAN (1975) used a 3:2 ratio for rabbit erythrocyte preparations. In contrast, VAN AMELSVOORT et al., (1977a) obtained maximal stimulation of rabbit gastric mucosa Mg^{2+} - ATPase in the presence of 0.6 mM Mg^{2+} and 0.3 mM ATP. Despite this observation, they used a 2:2 ratio throughout their experiments.

The optimum pH for maximum Mg^{2+}, HCO_3^- - ATPase activity reported by different workers ranged from pH 7.1 to pH 9.0 (KASBEKAR and DURBIN, 1965; SACHS et al., 1965; TURBECK et al., 1968; KIMELBERG and BOURKE, 1973; De RENZIS and BORNANCIN, 1977, SIMON and THOMAS, 1972; and several others). The optimum pH for Locusta Malpighian tubules (ANSTEE and FATHPOUR, 1979, 1981; FATHPOUR, 1980), dog gastric mucosa (BLUM et al., 1971) and rabbit red cell ghost (IZUTSU et al., 1977) Mg^{2+} ATPase activity is 7.5.

The study of Mg^{2+}, HCO_3^- - ATPase activity is complicated by its lack of specificity shown to this anion (HCO_3^-). HCO_3 is the most prominent anion which stimulates this enzyme but, because it is sensitive to several other anions, VAN AMELSVOORT et al., (1977a) suggested that the term anion-sensitive ATPase is more appropriate. Similarly, there is lack of a specific inhibitor for this enzyme. However, KASBEKAR and DURBIN (1965) and several other workers shown in Table 4.1 suggest that SCN^- may serve as a specific inhibitor of the Mg^{2+}, HCO_3^- - ATPase from several tissues except for rabbit erythrocyte ATPase (DUNCAN 1975).

Results presented in Chapter 5 show that SCN^- inhibited the

in vitro Malpighian tubule fluid secretion. ANSTEE and FATHPOUR (1979, 1981) in studies of Locusta Malpighian tubule Mg^{2+}, HCO_3^- -ATPase showed that this enzyme was sensitive to a variety of anions and was inhibited by SCN^- and other inhibitors (e.g. Sodium azide).

The objective of the present study is to investigate further the occurrence of Mg^{2+}, HCO_3^- -ATPase in microsomal preparations of Locusta Malpighian tubules. Its sensitivity to various inhibitors of transport ATPases (Ouabain, Vanadate, SITS and Furosemide) was examined in an attempt to establish the role of the Mg^{2+}, HCO_3^- -ATPase in the transport of anions through the epithelium. The results here, therefore, are to be compared with results showing the effects of similar inhibitors on the in vitro fluid secretion by Malpighian tubules (see Chapter 5).

MATERIALS AND METHODS

4.1 Preparation of the membrane microsomal fraction with Mg^{2+}-dependent HCO_3 -stimulated ATPase from Malpighian tubules of Locusta migratoria L.

Reagents (final concentration) :

All mydia were buffered at pH 7.5 unless otherwise stated.

Homogenising medium :

20 mM imidazole|HCl.

Washing medium I : 20 mM imidazole|HCl;

4 mM $MgCl_2$; 1 mM EDTA

Washing medium II :

20 mM imidazole|HCl

2 mM $MgCl_2$

1 mM EDTA

Reaction media :

1. For Mg^{2+} ATPase activity:

   2 mM $MgCl_2$,

   20 mM imidazole|HCl.

2. To measure effect of NaCl on Mg^{2+} ATPase activity :

   2 mM $MgCl_2$

   20 mM NaCl

   20 mM imidazole|HCl

3. For Mg^{2+}, HCO_3^- -ATPase:

   2 mM $MgCl_2$

   20 mM $NaHCO_3^-$

   20 mM imidazole|HCl.

Each medium contained 3 mM Tris ATP.

4.2 Preparation of the microsomal fraction with $Mg^{2+}HCO_3^-$-ATPase activity

The method is based on that described by DUNCAN (1975) and FATHPOUR (1980). Mature adult locusts were killed and the Malpighian tubules quickly dissected (as previously described) in ice cold homogenisation medium. The tubules were then homogenised in 10 mls of fresh ice-cold homogenisation medium in the same way as described in the previous chapter. The resulting homogenate was centrifuged at 600g for 10 minutes. The pellet was discarded and the supernatant centrifuged at 20,000g for 30 minutes at 0°C. The second pellet was also discarded and the supernatant centrifuged at 100,000g for 60 minutes. This third pellet was resuspended in 10 mls of washing medium I and centrifuged at 100,000g for 40 minutes. The pellet was resuspended in washing medium II and centrifuged at 100,000g for 40 minutes. This washing procedure was repeated twice. The final pellet was resuspended

in an appropriate volume of ice-cold deionised water. The tubes containing the microsomal preparation were stored on ice until it was required for the ATPase assay.

4.3 Assay of ATPase activity

Unless otherwise stated in the text, all incubations were carried out at 30°C for a period of 30 minutes. 0.5 ml of ATP plus 1 ml of appropriate reaction medium was thermo-equilibrated for 15 minutes in pairs in Pyrex boiling tubes in a water bath.

The reaction was started by the addition of 0.5 ml of the microsomal preparation to one of each of the pairs of tubes and was stopped by adding 4 mls of freshly prepared cirrasol solution, (see Chapter 3 and ATKINSON et al., 1973). The microsomal fraction was added to the control tubes after the addition of the cirrasol solution.

The tubes were left to stand for 10 minutes at room temperature for the yellow colour to develop. Any protein that precipitated was removed by centrifugation at 1,000 g for 15 minutes at 4°C. The tubes were immediately stored on ice. The supernatant of each tube was transferred to a cuvette and the absorbance, which was proportional to the amount of inorganic phosphate present, was measured at 390 mM as previously described (Chapter 3).

4.4 Estimation of the ATPase Activity

The HCO_3 -stimulation of the Mg^{2+} - ATPase was calculated as the difference between the activity in the presence of $MgCl_2$ + NaCl and the activity in the presence of $MgCl_2$ + $NaHCO_3$. The Mg^{2+}-ATPase activity was estimated by subtracting the values for the control

tubes from those of the tubes containing $MgCl_2$ alone. All results are expressed as nmoles Pi liberated/mg Protein/min.

RESULTS

4.6 Effect of HCO_3 and Cl^- on ATPase activity

The results presented in Table 4.2 show that there was a Mg^{2+} - ATPase present in the (100,000g fraction) microsomal preparations of Malpighian tubules of Locusta migratoria. This ATPase activity was determined in the presence of Mg^{2+}; Mg^{2+} + NaCl; and Mg^{2+} + $NaHCO_3$. The results show that the inclusion of NaCl did not significantly stimulate the Mg^{2+}-dependent ATPase activity. However, when $NaHCO_3$ was substituted for NaCl there was a marked (40%) stimulation of the Mg^{2+}-ATPase activity.

4.7 Effect of different inhibitors on ATPase activity

Mg^{2+}-dependent ATPase activity (in the presence and absence of NaCl) and Mg^{2+}, HCO_3 - ATPase activity were assayed in reaction media containing 1 mM of each of the following inhibitors : Ouabain, vanadate, furosemide, and SITS. The results are summarised in Tables 4.3-4.6.

(a) Ouabain

Results in Table 4.3 clearly show that ouabain (1 mM) did not significantly inhibit the Mg^{2+} - ATPase activity in the presence of either NaCl or $NaHCO_3$. The values in Table 4.3 show that the level of stimulation due to inclusion of HCO_3 remained the same both in the presence and absence of ouabain.

(b) Vanadate

The results presented in Table 4.3 show that Vanadate (1 mM) inhibited the Mg^{2+}-dependent ATPase in both the presence of or

TABLE 4.2 : Mg^{2+}-ATPase Activity in microsomal preparations of Malpighian tubules of Locusta migratoria L.

ATPase activity in presence of:			Stimulation due to NaCl	Stimulation due to $NaHCO_3$	Stimulation due to $NaHCO_3$ instead of NaCl
Mg^{2+} (A)	Mg^{2+} + NaCl (B)	Mg^{2+} + $NaHCO_3$ (C)	B-A	C-A	C-B
96.0 ± 10.57	100.3 ± 11.14	135.3 ± 14.0	4.3 ± 1.17	35.0 ± 6.90	39.3 ± 7.02

Activity in nmoles P_i liberated/mg Protein/min.

Condition: (A) 2 mM Mg^{2+}; (B) 2 mM Mg^{2+} + 20 mM NaCl; (C) 2 mM Mg^{2+} + 20 mM $NaHCO_3$ in 20 mM imadazole buffer pH 7.5. Temperature 30°C.

Each ionic medium contained 3 mM Tris ATP (final concentration).

Each value represents the average of 11 experiments ± SEM.

TABLE 4.3 : The effect of Inhibitors on Mg^{2+}-ATPase activity (n = 3)

Inhibitor	% ATPase activity in presence of :			% Stimulation due to $NaHCO_3$ instead of NaCl
	Mg^{2+} (A)	Mg^{2+} + NaCl (B)	Mg^{2+} + $NaHCO_3$ (C)	(C - B)
Furosemide (1mM)	97.48 ± 0.88	97.15 ± 0.33	88.64 ± 0.57	69.61 ± 0.36
Ouabain (1mM)	94.36 ± 1.45	93.33 ± 0.66	92.97 ± 0.57	92.07 ± 3.91
Vanadate (1mM)	58.05 ± 4.10	59.74 ± 4.00	54.54 ± 0.88	42.79 ± 5.53

Conditions as shown in Table 4.2

The 100% activity in nmole Pi/mg Protein/min was: 70.68 (Mg^{2+} alone)

70.37 ± 7.04 (Mg^{2+} + NaCl) 101.54 5.57 (Mg^{2+} + $NaHCO_3$)

31.17 ± 1.49 (Stimulation due to inclusion of $NaHCO_3$ instead of NaCl.

Activity is expressed as a percentage of that in the medium without inhibitor.

absence of NaCl by 40.3 and 42.0% respectively. When $NaHCO_3$ was substituted for NaCl vanadate inhibited the enzyme activity by 45.5%. In Table 4.3 the value (C-B) shows that stimulation due to HCO_3 was reduced by 57.2% in the presence of vanadate (1 mM).

(c) Furosemide

The results in Table 4.3 indicates that furosemide (1 mM) did not significantly inhibit the Mg^{2+}-dependent ATPase activity in either the presence or absence of NaCl. Some 30% of the enzyme activity owing to the stimulation by HCO_3 (C-B), was sensitive to 1 mM furosemide

In view of the inhibition of Mg^{2+}, HCO_3^- -ATPase activity by furosemide reported in Table 4.3, it was decided necessary to explore further this inhibition. ATPase activity was assayed, therefore, in the presence of a range of furosemide concentrations (10^{-9}- 10^{-3}M). Results obtained from two independent determinations are presented in Table 4.4. It can be seen that on both occasions, Mg^{2+} ATPase in absence or presence of NaCl was not significantly inhibited by furosemide. The results show that the small percentage (3-14%) in absence of NaCl and (10-24%) in presence of NaCl was possible only in presence of high concentrations (10^{-4}- 10^{-3}M) of furosemide.

The results further show that the Mg^{2+},HCO_3^- -ATPase was inhibited by furosemide; and that all concentrations of furosemide used in these experiments (10^{-3}- 10^{-9}M) the level of inhibition of the HCO_3^- - stimulated ATPase activity remained between 30-40%.

(d) SITS

Results presented in Table 4.5 show the effect of SITS on the Mg^{2+}, HCO_3^- -ATPase. On all occasions, over 50% of the stimulation of activity due to HCO_3 was inhibited by (1 mM) SITS. It can be seen

Table 4.4 : Effect of different concentrations of Furosemide on Mg^{2+}-ATPase activity in microsomal preparations of Locusta Malpighian tubules

Furosemide (M)	% Mg^{2+} ATPase Activity in presence of :			% Stimulation of activity due to presence of Na HCO_3 instead of NaCl
	-NaCl	+NaCl	+$NaHCO_3$	
10^{-3} a	86.98	76.40	68.84	59.18
b	94.41	83.41	74.54	58.68
10^{-4} a	87.66	76.33	73.42	69.71
b	97.50	83.41	77.69	67.45
10^{-5} a	89.26	90.45	73.17	51.00
b	99.00	93.49	77.99	50.59
10^{-6} a	88.12	90.45	71.82	47.97
b	95.71	95.00	78.05	47.55
10^{-7} a	87.44	92.22	73.42	49.39
b	98.70	95.09	79.02	50.59
10^{-8} a	89.03	88.69	73.80	54.75
b	97.70	91.70	79.32	55.65
10^{-9} a	89.03	89.13	75.03	57.00
b	98.70	92.74	80.10	57.50

Enzyme activity expressed as % of that observed in the absence of Furosemide. The 100% activity was in nmoles Pi/mg Protein/min
(a) 121.71, (b) 100.3 (-NaCl).
(a) 125.95, (b) 106.1 (+NaCl)
(a) 224.41, (b) 165.4 (+$NaHCO_3$)
(a) 87.46, (b) 59.3 (Stimulation activity due to inclusion of $NaHCO_3$ instead of NaCl).

TABLE 4.5 : The effect of SITS on Mg^{2+}, HCO_3^--ATPase activity in Locusta Malpighian tubules preparation

Mg^{2+}, HCO_3^--ATPase activity (in nmoles Pi/mg Protein/min in presence of:		Activity expressed as % of activity in medium without inhibitor	P
Mg^{2+} + $NaHCO_3$ minus SITS	Mg^{2+} + $NaHCO_3$ plus SITS (1 mM)		
73.6 ± 1.4	20.4 ± 7.5	27.7 ± 10.3	<0.001

Note Values for activity were calculated by subtracting the amount of ATPase activity in presence of Mg^{2+} + NaCl from activity in presence of Mg^{2+} + $NaHCO_3$. Each value is a mean ± SEM of 3 separate preparations.

in Table 4.5 that 72.2 ± 10.3% of the stimulation due to the inclusion of HCO_3 in the medium was inhibited by SITS.

DISCUSSION

The present study has clearly demonstrated the presence of Mg^{2+}, HCO_3 - ATPase in microsomal preparations of Locusta Malpighian tubules. This is in agreement with ANSTEE and FATHPOUR (1979, 1981) and FATHPOUR, (1980) who isolated this enzyme from similar microsomal preparations. As seen in Table 4.1, Mg^{2+}, HCO_3^- -ATPase activity has been reported by several workers using different tissues.

The cellular location, and therefore the function of this enzyme is in some dispute. If this enzyme is being implicated in cellular transport of HCO_3 and other anions (SIMON et al., 1972a,b; SIMON and THOMAS, 1972; BLUM et al., 1971; WIELBEHAUS et al., 1971; SACHS et al., 1972b; LIANG and SACKTOR, 1976; DEATON, 1984) then its location should be expected in the plasma membrane fraction. Such a localization has been proposed by several workers (e.g. SACHS et al., 1972b; SIMON and THOMAS, 1972; De RENZIS and BORNANCIN, 1972; ANSTEE and FATHPOUR, 1979, 1981; DEATON, 1984). However, other workers suggest that the anion-sensitive ATPase activity in microsomal preparations is owing to mitochondrial contamination (SOUMARMON et al., 1974; IZUTSU and SIEGEL, 1975; IZUTSU et al., 1978).

As previously mentioned, the preparation of the Malpighian tubule microsomal fraction (100,000g) used in the present study was based on FATHPOUR (1980) procedure. The latter reports that the ratio of the activity of the mitochondrial marker enzyme succinate dehydrogenase (SDH) (LIANG and SACKTOR, 1976) to Mg^{2+}, HCO_3 - ATPase activity at the 100,000g fraction was 0.16 and very low when compared

to 0.57 at 20,000g fraction. He also reports that in his microscopic studies, mitochondria were apparent at the 20,000g fraction and not at the 100,000g fraction. On the basis of FATHPOUR (1980) study it is suggested that the Mg^{2+}, HCO_3^- -ATPase identified from the Malpighian tubule preparations (100,000g fraction) was mainly of microsomal origin.

The results have also shown that the Mg^{2+}-dependent ATPase activity was not affected by Cl^- anions. The Mg^{2+}-dependent ATPase activity however, was markedly stimulated by HCO_3, and this stimulation was found to vary between about 40-50% (Tables 4.2 and 4.4). This level of stimulation compares well with that obtained by ANSTEE and FATHPOUR (1979) and FATHPOUR (1980) using the same conditions and preparation. Widely different levels of stimulation are quoted for mammalian preparations. SIMON and THOMAS (1972) found a 50% stimulation by HCO_3 for the enzyme from pancreas and a similar stimulation was described for the erythrocyte enzyme by DUNCAN (1975) and kidney preparations by KINNE-SAFFAREN and KINNE (1974). Much higher levels of stimulation are described for the enzyme from brain (GRISOLIA and MANDELSON, 1974) and pancreatric islet cells (KIMELBERG and BOURKE, 1973), but lower levels of stimulation are described by SUZUKI (1978) for mouse kidney preparations.

The results clearly show that unlike Na^+, K^+-ATPase, reported in insect Malpighian tubules and hindgut (ANSTEE and BELL, 1975, 1978; PEACOCK et al., 1972, 1976; ANSTEE and BOWLER, 1979; DONKIN, 1981) the Mg^{2+}, HCO_3^--ATPase of Locusta Malpighian tubules is not inhibited by the cardiac glycoside ouabain. The results are in agreement with results obtained by Cole (1979) in rat renal cortex, SACKS et al., (1965) in frog gastric mucosa, ANSTEE and FATHPOUR, (1979) and

FATHPOUR (1980) in Locusta Malpighian tubules. In contrast, IZUTSU and SIEGEL (1972) reported that the Mg^{2+}, HCO_3^- -ATPase from dog submandibular gland is sensitive to ouabain and that the presence of 0.1 - 1 mM ouabain in the reaction medium reduced the enzyme activity by 30%.

The present study has demonstrated that unlike ouabain which is a specific inhibitor of Na^+, K^+ - ATPase (SKOU, 1965), vanadate is not a specific inhibitor and would, perhaps, be more difficult to apply in the study of the involvement of ATPases in fluid transport.

Whilst vanadate has been shown to have no effect on some enzymes such as Ca^{2+}ATPase, Actomyosin ATPase and the mitochondrial coupling factor (CANTLEY et al., 1977) the present study has demonstrated that vanadate is a potent inhibitor of Na^+, K^+-ATPase, (see Chapter 3), Mg^{2+}ATPase and Mg^{2+}, HCO_3^- ATPase activity. It is suggested that vanadate interacts with the anion selective pathways or carriers in the basolateral membrane to retard ion transport (EHRENSPEC and BRODSKY, 1975; BRODSKY et al., 1979). After entering via the anion pathway, vanadate inhibits transport by binding to the Na^+, K^+-ATPase (SOLINGER et al., 1968) in a manner analogous to that described by CANTLEY et al., (1978) in red cells. As previously discussed, Mg^{2+}, HCO_3^- -ATPase is strongly implicated in the transport of anions through the epithelia and the results presented here are also supported by EHRENSPECK (1980) who reported inhibition of Cl^- and HCO_3 transport by vanadate.

Results presented in Tables 4.3 and 4.4 show that furosemide did not significantly inhibit the Mg^{2+}-ATPase activity in either the presence or absence of NaCl. Furosemide is known to be an inhibitor of Na^+ coupled Cl^- transport (BURG, 1976) (also see Figure 1.3). It

is also almost certain that Na^+ coupled Cl^- transport is not a feature of the Mg^{2+} - ATPase. Therefore, furosemide is not expected to inhibit this enzyme's activity. However, it should not be assumed that Na^+ and Cl^- cotransport is not involved in fluid production by Locusta Malpighian tubules. Indeed, results from the present study (Chapter 5) show that furosemide (1 mM) inhibited in vitro Locusta Malpighian tubule fluid secretion by some 50-60% and a higher concentration (5×10^{-3}M) furosemide inhibited in vitro fluid secretion by 85%. Although it is difficult to interpret the results, it is also interesting that (1 mM) furosemide inhibited Mg^{2+}, HCO_3^- - ATPase by some 30%. (see Table 4.3).

Results presented in Table 4.5 show that SITS is a very effective inhibitor of the Mg^{2+}, HCO_3^- -ATPase activity. It is seen that (1 mM) SITS inhibited the enzyme activity by 72.2 ± 10.3%, Like most stilbene derivatives SITS is classically associated with inhibition of anion-exchange mechanisms in transporting epithelia of different tissues (e.g. red cell membranes, CABANTCHIK and ROTHSTEIN, 1972; turtle bladder, EHRENSPRECK and BRODSKY, 1976). It is suggested that in vertebrates the Cl^-/HCO_3 coupled transport mechanism is either inhibited either by SITS or indirectly by the carbonic anhydrase (c.a) inhibitor, acetazolamide (HANRAHAN and PHILLIPS, 1983) (see Figure 1.3). The inhibition of Mg^+HCO_3 ATPase activity by SITS together with the fact that acetazolamide inhibited the in vitro Locusta Malpighian tubule fluid secretion (Chapter 5) is a fair indication that Mg^{2+},HCO_3^- - ATPase in Locusta Malpighian tubules is involved in fluid transport and is perhaps functionally linked with the carbonic anhydrase (c.a) system. The two enzymes may be the source of energy for the Cl^-/HCO_3^- exchange in Locusta Malpighian tubule fluid production. However, the source of energy for Cl^-/HCO_3 exchange in several

transporting epithelia is still unknown (see review by Hanrahan and Phillips, 1983).

The interpretation of the present study results is further complicated by ANSTEE and FATHPOUR (1979) finding that Mg^{2+}, HCO_3^- - ATPase from Locusta Malpighian tubules (in both the 20,000 and 100,000 g fraction) was insensitive to sodium acetazolamide. Similar reports are given by other workers (KASBEKAR and DURBIN, 1965; WIEBELHAUS et al., 1971; SIMON et al., 1972b; KIMELBERG and BOURKE, 1973; DUNCAN 1975; IZUTSU and SIEGEL, 1975). In contrast, IZUTSU and SIEGEL (1972) reported that HCO_3 - stimulated ATPase from dog sub-mandibular gland is inhibited up to 40% by 1 mM sodium acetazolamide.

Whilst FATHPOUR (1980) reports the presence of carbonic anydrase (c.a) in Locusta Malpighian tubule preparations, EDWARDS and PATTON (1967) failed to demonstrate the presence of a carbonic anhydrase in Malpighian tubules of Acheta domesticus. The results from the present study were also further complicated by the fact that SITS (1 mM) did not inhibit in vitro Locusta Malpighian fluid tubule fluid secretion (results discussed in Chapter 5).

The present study like that of ANSTEE and FATHPOUR (1981) has clearly demonstrated the presence of Mg^{2+}, HCO_3 - ATPase in Locusta migratoria Malpighian tubule microsomal preparations; and that this enzyme is sensitive to a variety of inhibitors.

However, none of the inhibitors investigated by the present study is specific to Mg^{2+}, HCO_3 - ATPase which makes it difficult to assess this enzyme's involvement in the mechanism of ion and water transport across the Malpighian tubules. However, there is some evidence from the present study pointing to the fact that this enzyme plays an important role during fluid production by Locusta Malpighian tubules.

CHAPTER 5

IN VITRO STUDIES ON FLUID SECRETION BY MALPIGHIAN TUBULES OF LOCUSTA

Introduction

As previously discussed in Chapter 1, there is substantial evidence in literature suggesting that fluid transport across insect Malpighian tubules involves osmotic coupling of ion and water movements (see MADDRELL, 1980). However, it is not yet fully established as to how this is achieved. Some of the models proposed by various workers to explain the mechanism of solute and water transport have been discussed in Chapter 1.

MADDRELL (1971) summarises the points which should be incorporated by any model which attempts to account for the mode of operation of insect Malpighian tubules as follows:

(1) The concentration of ions in the secreted fluid is different from that in the haemolymph. The potassium and phosphate, in particular, are elevated and sodium, calcium and magnesium are lower.

(2) Fluid is secreted at a rate which is dependent on the potassium concentration of the bathing medium and is faster still in the presence of small concentrations of sodium (as low as 2 mmol l^{-1}). This is especially so at lower potassium concentrations. Insects which do not use K^+ as the prime mover have been mentioned above.

(3) The fluid produced by the tubules is nearly isosomotic with the bathing fluid over a wide range of concentrations of the latter. Fluid is secreted at a rate inversely proportional to the osmotic pressure of the bathing medium.

(4) A barrier accessible from the basal side is much more permeable to K^+ than to Na^+.

(5) Potassium ions move against their electrochemical gradient

and under some circumstances so do sodium ions.

(6) Sodium and potassium, each seem not to compete with each other.

(7) For prolonged secretion, Malpighian tubules require an energy source, e.g. sucrose, present in the bathing medium.

(8) Anions (except phosphates) support secretion at a rate which is related to their hydrated size. The smaller the anion, the faster is the rate of secretion supported. Phosphate ions support a higher rate of secretion than do other anions.

(9) Fluid secretion is insensitive to ouabain even when stimulation by sodium is maximal. As will be discussed later, this failure to demonstrate ouabain-sensitivity in Malpighian tubules of some insect species reported by some workers has given rise to a lot of controversy.

Several subsequent studies have provided further information on some of these points. For example, it is now known that Malpighian tubules may require different ions depending on the rate of fluid production. NICHOLSON (1980a) found that in the desert beetle, Onymacris plana, K^+ concentration rises during dehydration and returns to normal after rehydration; whilst Na^+ concentration rises during dehydration and drops dramatically after drinking. NICHOLSON (1980b) also reports that in the butterflies Acrea horta, Danaus chrysippus and Papilio demodocus stimulation of Malpighian tubules by the cAMP leads to extensive diuresis which results in an increase in Na^+ concentration and total osmolarity. MADDRELL (1980) similarly reports that in Rhodnius the tubules produce fluid much richer in Na^+ than when they secrete fluid slowly. He explains that the accelerated fluid secretion is to eliminate most of the Na^+ - rich plasma from the blood meal. After diuresis, as the blood meal is digested, K^+ from the blood cells has to be excreted and now the

tubules slowly secrete urine rich in K^+. However, in some insects such as Carausius (PILCHER, 1970) and the cabbage butterfly, Pieris brassicae (NICHOLSON, 1976a,b) the ionic dependencies of stimulated and unstimulated tubules have been compared and found that in both cases the prime mover was K^+. Such a study is lacking for Malpighian tubules of Locusta.

The most important question that has aroused several studies is that of ouabain-sensitivity in insect Malpighian tubule fluid production. As previously discussed, BERRIDGE and OSCHMAN (1969) devised a model to explain fluid secretion by Malpighian tubules of Calliphora. They suggest that the Malpighian tubule basal cell membranes possess a coupled Na^+/K^+ exchange pump, extruding Na^+ from the cell into the haemolymph in exchange for K^+, whilst on the apical surface, there is an electrogenic pump transporting K^+ into the lumen of the tubule. If this model is correct, it would be expected that the cardiac glycoside, ouabain, a specific inhibitor of Na^+, K^+-ATPase activity (SKOU, 1965) would also inhibit the in vitro Malpighian tubule fluid secretion. Therefore, one of the main objections to this model has arisen from the failure by a number of workers to demonstrate that fluid secretion was inhibited by ouabain (BERRIDGE, 1968; MADDRELL, 1969; PILCHER, 1970; GEE, 1976a,b; RAFAELI-BERNSTEIN and MORDUE, 1978). These reports have cast doubt over the involvement of Na^+, K^+-ATPase in fluid secretion.

However, other workers have shown that Malpighian tubule function of a variety of species is inhibited by ouabain, (e.g. in Drosophila hydei, ATZBACHER et al., 1974; Locusta, ANSTEE and BELL, 1975; ANSTEE et al., 1979, 1980, 1983; DONKIN, 1981; present study). Although they reported ouabain-insensitivity in their early work, MORDUE and RAFAELI-BERNSTEIN (1978) found that ouabain affected the

intra-cellular Na^+ concentrations in Locusta Malpighian tubules. More recently, ANSTEE et al., (1980, 1983) reported that ouabain reduced the P.D. across the Malpighian tubule of Locusta. In contrast, GEE (1976b) reported that ouabain did not affect the Na^+ concentration of secreted fluid in Malpighian tubules of Glossina morsitans, and FARQUHARSON (1974) found no change in Na^+ and K^+ concentrations in the urine of the pill millipede, Glomeris marginata, although fluid secretion was inhibited by 5×10^{-6} - 10^{-3}M ouabain. In their recent study, DALTON and WINDMILL (1980) too, report that (1 mM) ouabain does not inhibit fluid secretion by Malpighian tubules of Musca domestica.

It is difficult to understand the causes of these conflicting reports on ouabain-sensitivity in different animal species. It may be owing to a species variation in ouabain-sensitivity or dependence of fluid secretion on Na^+,K^+ - ATPase. ANSTEE and BOWLER (1979) discuss possible explanations for the cause of these differing results. They may arise owing to differences in experimental procedure used in different laboratories. Of these, the temperature at which experiments were carried out and the composition of the Ringer solution used could be more critical.

Therefore, in view of the data presented in Chapter 3, where ouabain-sensitivity of the Na^+,K^+ - ATPase is shown to be very temperature-sensitive, the lack of ouabain-sensitivity in fluid production reported by MADDRELL (1969), GEE (1976), RAFAELI-BERNSTEIN and MORDUE (1978), and DALTON and WINDMILL (1980) could be because they carried out their measurements at temperatures below 30°C. DALTON and WINDMILL, (1980) incubated the tubules at 22°C. In other cases, the temperature at which fluid secretion was measured and

ouabain-sensitivity tested is not precisely stated, which questions the validity of ouabain-insensitivity reported. Workers who carried out their experiments at 30°C report ouabain-sensitivity in Malpighian tubule fluid secretion (e.g. ANSTEE and BELL, 1975; ANSTEE et al.,1979; ANSTEE et al., 1979, 1980; DONKIN and ANSTEE 1980; FATHPOUR 1980; DONKIN, 1981; FATHPOUR et al., 1983 and present study).

Results presented in Chapter 3 also show that high K^+ concentrations antagonise ouabain inhibition of Na^+, K^+ - ATPase activity in Locusta migratoria Malpighian tubule microsomal preparations. This supports earlier reports from JUNGREIS (1977) who correctly pointed out that high K^+ concentrations in the bathing media used by several workers, in the study of fluid secretion, in a variety of epithelia, may not be suitable for ouabain to manifest its inhibitory effect. Other workers (KINSOLVING et al., 1963; JUDAH and AHMED, 1964; MATSUI and SCHWARTZ, 1968; AKERA, 1971) have also found that high K^+ concentration antagonises ouabain inhibition of the Na^+, K^+ - ATPase activity.

However, JUNGREIS (1977) assumed that insect tissues known to be sensitive to ouabain, have Na^+, K^+ - ATPases which are maximally stimulated by 5 mM K^+ (JUNGREIS and VAUGHAN, 1977) whereas, it has been shown in a variety of insect tissues that Na^+, K^+ - ATPase is maximally stimulated by 20 mM K^+, but is still inhibited by ouabain (GRASSO 1967; PEACOCK et al., 1976; TOLMAN and STEELE, 1976; PICCIONE and BAUST, 1977; ANSTEE and BELL, 1975, 1978; DONKIN 1981; FATHPOUR, 1980; present study). DONKIN and ANSTEE, 1980, demonstrated that K^+ concentrations up to 40 mM did not antagonise ouabain inhibition of fluid production by Locusta Malpighian tubules. Therefore, workers, like DALTON and WINDMILL (1980) may not be correct in suggesting that high K^+ concentration antagonised ouabain inhibition of fluid

production by Musca domestica. They used 20 mM K^+ in their bathing medium.

In view of the foregoing discussion, the present study investigates further the effect of ouabain on in vitro rate of fluid secretion by Locusta Malpighian tubules. In addition effects of some other inhibitors namely sodium orthovanadate, SCN^-, furosemide, sodium acetazolamide (Diamox) and SITS (4 acetamide-4' isothiocyanostilbene-2'2' disulfonic acid) were investigated. The purpose being to compare their effects on in vitro fluid secretion with their effects on the isolated ATPases from Malpighian tubule microsomal preparations (reported in Chapters 3 and 4) in order to determine the role of these ATPases in 'urine' production. The modes of action of the various inhibitors used have been previously discussed (see Chapters 3 and 4).

In an attempt to explain why some workers report ouabain-insensitivity in insect Malpighian tubule fluid production, the effects of temperature and K^+ concentrations on the inhibitory action of ouabain and vanadate have been emphasised in the present study.

MATERIALS AND METHODS

5.1 To determine the rate of fluid secretion by the Malpighian tubules of Locusta

Mature adult locusts (Locusta migratoria L.) were used throughout this study. The technique used to measure the rate of fluid secretion was essentially the same as that described by MADDRELL and KLUNSUWAN (1973) with a few modifications which will be mentioned in the text.

The animals were killed and the gut bearing the Malpighian

tubules dissected as previously described in Chapter 3. The gut was quickly washed with 'normal' Ringer solution before being placed in a fresh Ringer solution in the experimental chamber. The head was kept outside the experimental chamber to avoid contamination by regurgitated gut contents and the whole preparation (apart from the head) was then covered with liquid paraffin to avoid dessication. Individual Malpighian tubules were drawn out of the Ringer solution into the liquid paraffin and looped around small stainless steel pegs on either side of the Ringer chamber (see Figure 5.1). Care was taken to ensure that the pegs had smooth heads to avoid puncturing the tubules. As many as ten tubules could be so arranged on a single gut preparation. Each tubule was then partially severed using a very fine tungsten needle. The preparation was checked to ensure that no other cuts were present along the length of the tubule. Fresh Ringer solution was added to the experimental chamber at regular intervals of 5 minutes. Following an equilibration period of 15 minutes, at the end of which any secreted fluid was removed, the rate of 'urine' secretion was determined by measuring the increase in diameter of the droplets secreted from the partially severed tubules every 5 minutes for a period of 30 minutes. This was referred to as (Rate 1). The 'normal' Ringer solution in the experimental chamber, was then replaced with either fresh 'normal' Ringer solution (the controls) or an experimental Ringer solution before redetermining the rate of fluid secretion over a second 30 minutes period (Rate 2). A twenty minutes equilibration period was allowed before Rate 2 was redetermined. The Ringer solution in the experimental chamger was replaced with fresh Ringer solution every 5 minutes. The volume of fluid secreted was calculated by assuming the droplet to be a sphere and expressed in nls/min. The effect of the particular treatment was determined by

FIGURE 5.1

The experimental arrangement involved in setting up *in vitro* preparations of Malpighian tubules of *Locusta migratoria* L. The alimentary canal of the insect and the adherent Malpighian tubules (i.e. in a pool of Ringer solution in a trough cut in the thick floor of the perspex Petri dish (i.e. experimental chamber). The head of the insect remains outside the dish. The Malpighian tubules are looped around the stainless smooth steel pegs or fix on either side of the experimental chamber. The entire preparation is covered with liquid paraffin to prevent evaporation.

Largely drawn from MADDRELL and KLUNSUWAN (1973) the only difference being that in the present preparation, the head of the experimental animal remained outside the Petri dish.

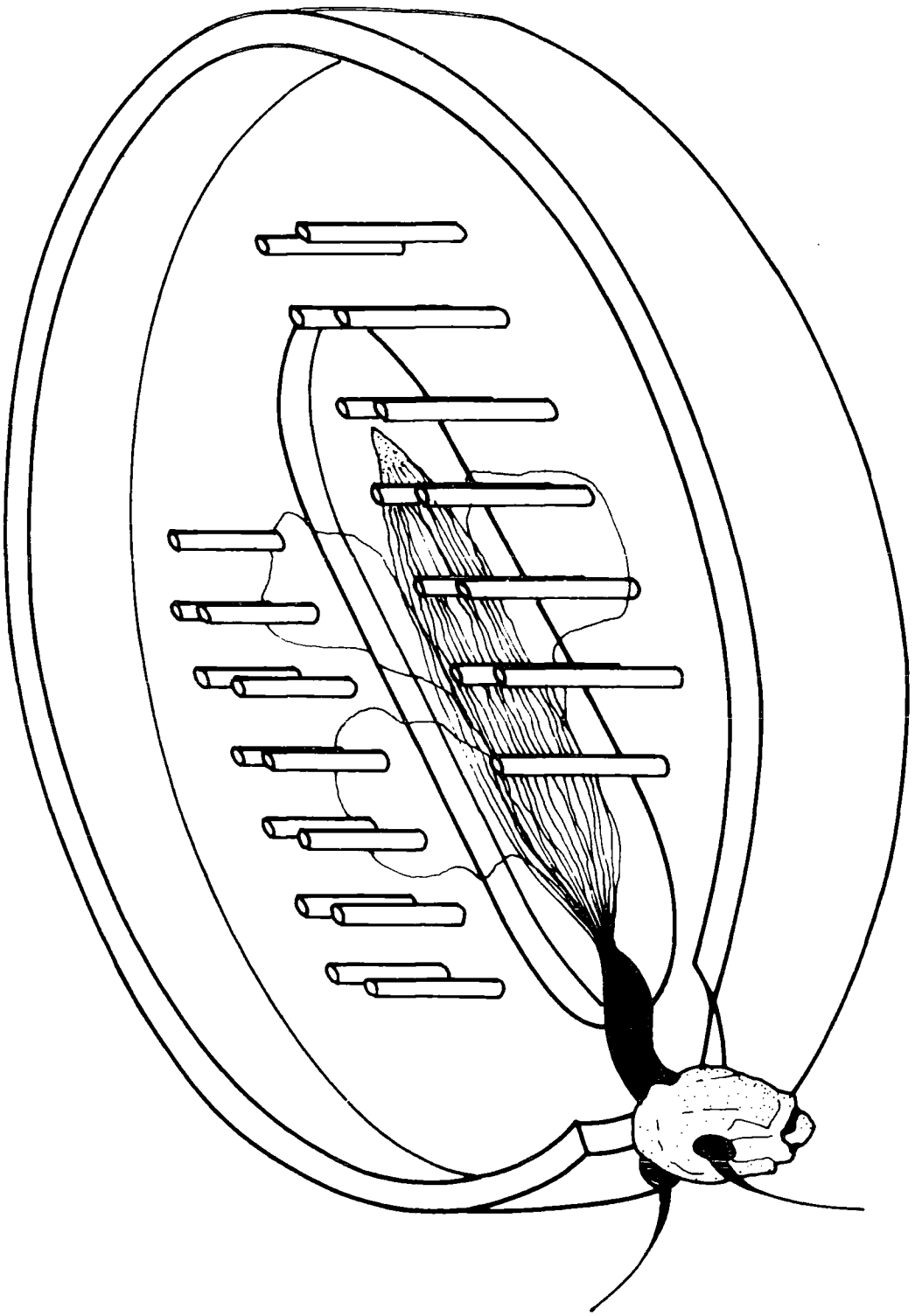


Fig. 5.1

comparing the rates, (Rate 1 and Rate 2) over the two 30 minutes periods. In this way, each tubule acts as its own control. This is necessary as the rate of secretion varies considerably between tubules. Paired 't' tests were carried out to compare the two rates. Unless otherwise stated in the text, all experiments were carried out at 30°C ± 0.1°C at pH 7.2.

5.2 To determine the effect of Temperature on fluid secretion

Two water baths were set up one at 30°C and another at 10, 15, 20, or 40°C. Rate 1 of fluid secretion was determined at 30°C in a manner described above for 30 minutes. The preparation was then quickly transferred to the experimental temperature. The tubules were allowed to equilibrate at the new temperature for 15 minutes before Rate 2 was redetermined at intervals of 5 minutes for a further 30 minutes. At high temperature (40°C) a continual flow of oxygen was bubbled through the bathing medium throughout the experimental period to ensure that oxygen depletion did not become rate-limiting. Ringer solution buffered with HEPES at pH 7.2 was used throughout, (see Table 5.1). The pH of HEPES buffered Ringers is not very temperature dependent (GOOD et al., 1966). Other compositions of Ringers used to demonstrate the effect of K^+ concentration on vanadate inhibition of fluid production are shown in Table 5.2.

5.3 Other treatments

The effect of various inhibitors and other treatments investigated during the present study of fluid secretion by Malpighian tubules are described under the appropriate 'results' sections.

TABLE 5.1 : Compositions of different Ringer solutions used in the study of in vitro Locusta migratoria L. Malpighian tubule fluid secretion

Values shown in (mM) pH 7.2

	'Normal'	K^+ free	Na^+ free
NaCl	100	108.6	0
KCl	8.6	0	108.6
$MgCl_2 6H_2O$	8.5	8.5	8.5
$CaCl_2$	2	2	2
$NaHCO_3$	4	4	0
$KHCO_3$	0	0	4
NaH_2PO_4	4	4	0
KH_2PO_4	0	0	4
NaOH	11	11	0
KOH	0	0	11
Glucose	34	34	34
H E P E S	25	25	25

TABLE 5.2 : Different Ringer Solutions made up by mixing Na^+-free and K^+-free Ringer Solutions in varying ratios (pH 7.2)

	Ratios in %				
	$5K^+ : 95Na^+$	$25K^+ : 75Na^+$	$50K^+ : 50Na^+$	$75K^+ : 25Na^+$	$95K^+ : 5Na^+$
K^+(mM)	6.38	31.9	63.8	95.7	121.22
Na^+(nM)	121.22	95.7	63.8	31.9	6.38

Note: Compositions of the stock (K^+-free and Na^+-free) Ringer Solutions are given in Table 5.1.

RESULTS

5.4 'Normal' Rate of Fluid Secretions

Figure 5.2 shows a typical example of control experiment in which the rate of fluid secretion by an individual Malpighian tubule was measured over 2 consecutive 30 minute periods in 'normal' Ringer solution (pH 7.2) at 30°C. It is clear that, in both cases, fluid secretion increased linearly with time. The actual rates of fluid secretion varied considerably with different individual animals and indeed with different tubules from the same animal. Such variation was in part due to the precise age of the experimental animal and its physiological condition. Data from the various control experiments showed that at 30°C, the mean rate of fluid secretion ranged between 2 and 4 nl/min. However, rates as high as 5-7 nl/min and as low as 1 nl/min were occasionally recorded during the present study.

5.5 Effect of Temperature on fluid secretion

Figure 5.3 and Table 5.3 show that between 10 and 40°C, the rate of fluid secretion increased with increasing temperature. At 40°C, the effect of temperature became very erratic with many tubules secreting very rapidly initially but failing to maintain fluid production for more than approximately 20 minutes. The physical movements of the tubules was also greater at the high temperatures and tubules easily snapped. It appears that 40°C is the maximum temperature for efficient tubule function under these conditions. Below 30°C, the rate of fluid secretion was low compared to the rate at 30°C. Table 5.3 shows that the average Rate 1 was 3.2 ± 0.41 and 1.2 ± 0.25 nl/min at 30 and 10°C respectively. The same Table 5.3

FIGURE 5.2

A typical example of a 'control' experiment showing the 'normal' rate of the *in vitro* *Locusta* Malpighian tubule fluid secretion.

Ordinate : Volume of fluid secreted in nl/min

Abscissa : Time in minutes.

Rate 1 in 'normal' Ringer solution at 30°C pH 7.2

Rate 2 redetermined under the same conditions as Rate 1. A period of 15 minutes is allowed for equilibration before Rate 2 is measured. See Table 5.1 for 'normal' Ringer solution composition.

Results shown in Figure 5.2 are from a single determination.

Fig. 5.2

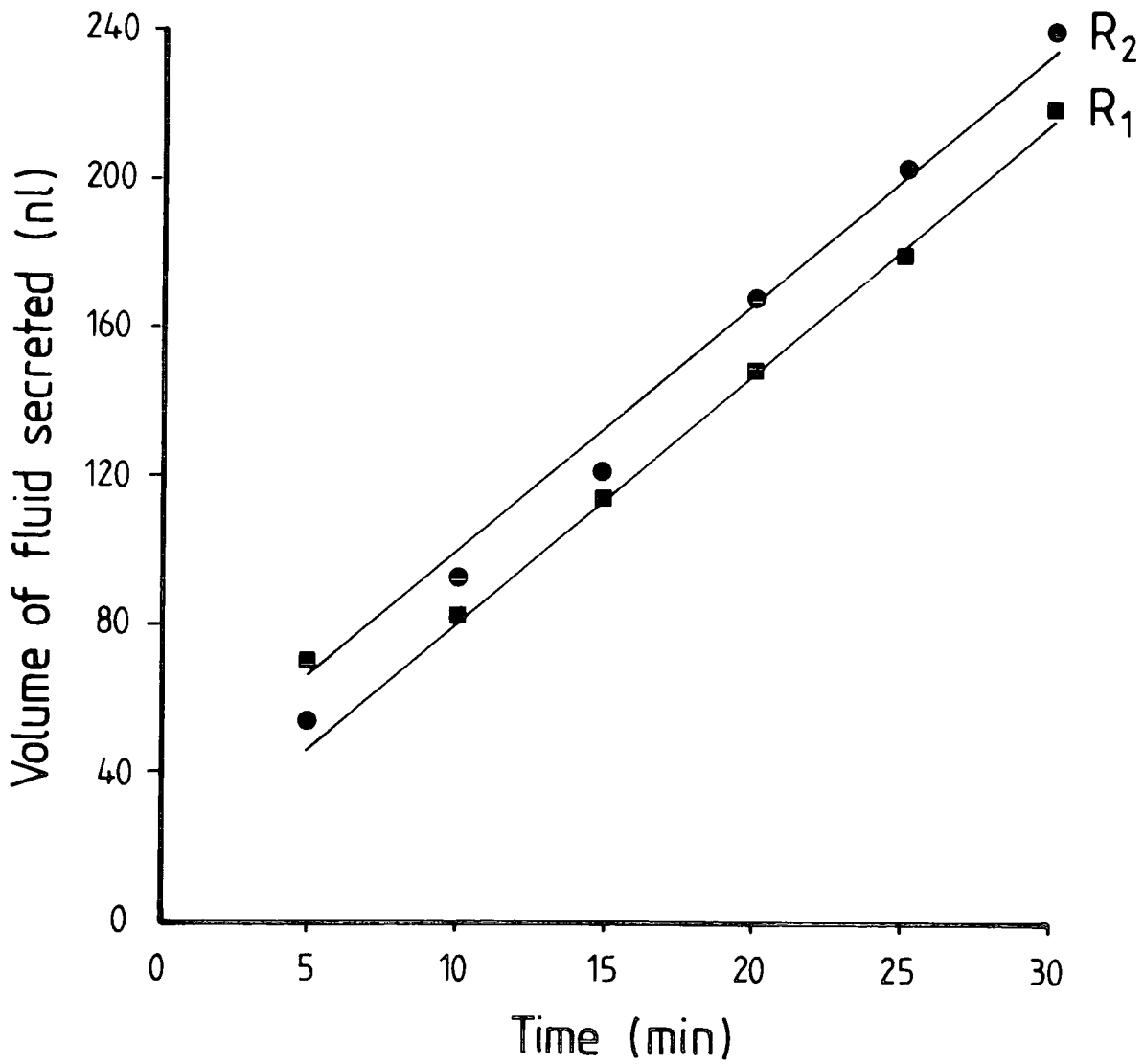

240
200
160
120
80
40
0
Volume of fluid secreted (nl)
0
5
10
15
20
25
30
Time (min)
R2
R1

FIGURE 5.3

Arrhenius plot showing the effect of varying temperature on rate of *in vitro* *Locusta* Malpighian tubule fluid production.

Ordinate : Log_{10} K (i.e. rate at T°A expressed as a percentage of rate at 30°C (= 100%)).

Abscissa : $\frac{1}{T°A} \times 10^3$

Experimental procedure (see Table 5.3).

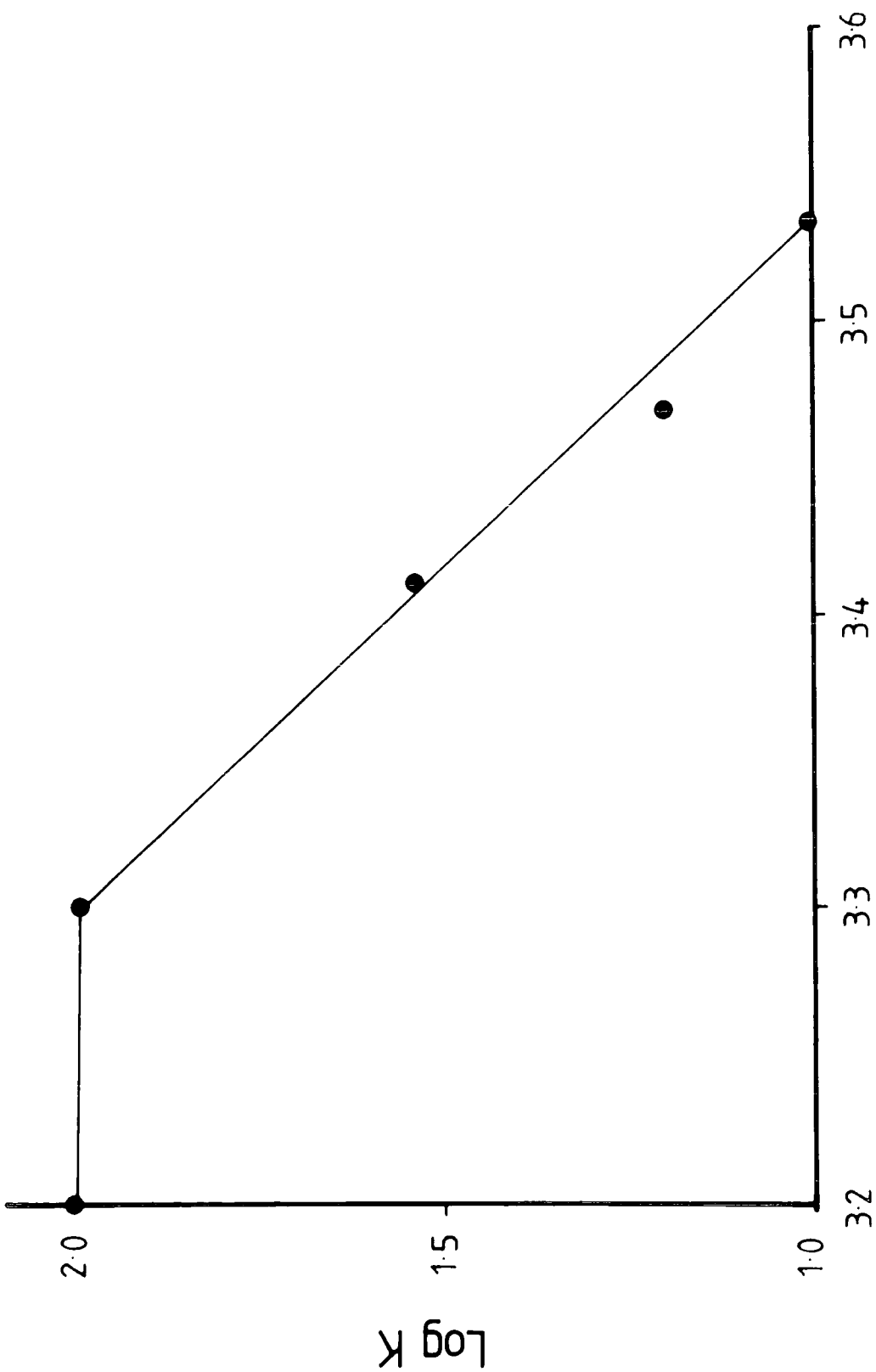


Fig. 5.3

TABLE 5.3 :

Conditions: 'Normal' Ringer solution was used (see Table 5.1).

Experimental procedure: Rate 1 of fluid secretion was measured at 30° minutes. The tubules were then equilibrated for 15 minutes at the experimental temperatures indicated before Rate 2 was redetermined for a further 30 minutes.

Rate 2 is expressed as a percentage of Rate 1. A paired 't' test was carried out to compare the two rates of fluid secretion. All p values of <0.05 were considered significant.

TABLE 5. 3 : The effect of temperature on the in vitro rate of fluid secretion by Malpighian tubules of Locusta

	Temperature °C	Fluid secretion nl/min SEM	$\frac{R_2}{R_1} \times 100 \pm$ SEM	n	P
Rate 1	30	4.29 ± 0.51	23.07 ± 4.11	30	<0.001
Rate 2	10	0.99 ± 0.02			
Rate 1	30	5.15 ± 0.31	31.07 ± 7.60	24	<0.001
Rate 2	15	1.6 ± 0.09			
Rate 1	30	3.69 ± 0.24	33.00 ± 6.81	16	<0.001
Rate 2	20	1.22 ± 0.31			
Rate 1	30	3.20 ± 0.41	103.12 ± 11.7	30	ns
Rate 2	30	3.30 ± 0.40			
Rate 1	30	6.56 ± 0.03	100.00 ± 0.00	15	ns
Rate 2	40	6.56 ± 0.04			
Rate 1	10	1.23 ± 0.25	95.90 ± 9.14	13	ns
Rate 2	10	1.18 ± 0.19			
Rate 1	18	4.93 ± 0.33	85.30 ± 8.21	16	ns
Rate 2	18	4.18 ± 0.37			
Rate 1	22	1.55 ± 0.18	86.61 ± 6.34	19	ns
Rate 2	22	1.30 ± 0.16			
Rate 1	40	4.39 ± 0.53	57.80 ± 7.52	20	<0.001
Rate 2	40	2.50 ± 0.35			

also shows that rates as high as 5.15 ± 0.31 nl/min were recorded on some occasions. At 30°C, there was no significant difference between Rate 1 and Rate 2. It is seen that, when Rate 2 was determined at a lower temperature (10, 15 and 20°C) and expressed as a percentage of Rate 1 determined at 30°C, the difference in the rates of secretion was highly significant ($p = <0.001$). In the present study, 30°C appeared to be the optimum temperature for 'normal' fluid secretion. The Arrhenius plot shown in Figure 5.3 gives a straight line between 10° and 30°C. The Ea value calculated for this range of temperatures was 20.27 K cals $mole^{-1}$ and shows a normal dependency of fluid production on temperature. At 30°C, the rates of fluid secretion were similar to those at 40°C, but fluid secretion (Rate 2) could not be maintained for more than 15 minutes at 40°C.

5.6 Effect of ouabain and vanadate on in vitro Malpighian tubule fluid secretion

Experiments were carried out to compare the rate of urine secretion in 'normal' Ringer with that of tubules bathed with 'normal' Ringer solution containing ouabain or vanadate at concentrations ranging from 10^{-7} to 10^{-3}M.

(a) Ouabain

Results presented in Table 5.4 and Figure 5.4 show that *in vitro* fluid secretion of *Locusta* Malpighian tubules was inhibited by ouabain in a dose-dependent manner at concentrations between 10^{-6}M and 10^{-3}M. A typical example of the graph showing the effect of ouabain on Malpighian tubule fluid secretion, *in vitro*, is shown in Fig. 5.4. The threshold for response was between 10^{-6} and 10^{-4}M ouabain (Fig.5.4). The physical movements of the tubules were also reduced by ouabain

TABLE 5.4 : The effect of different concentrations of ouabain on the in vitro Locusta migratoria L. Malpighian tubule fluid secretion

Rate 1 measured in 'normal' Ringer solution.

Rate 2 in 'normal' Ringer solution containing different concentration of ouabain.

Conditions: Temperature 30°C, pH 7.2.

The 'normal' Ringer solution composition is shown in Table 5.1.

A paired 't' test was carried out to compare Rate 1 and Rate 2

p values of <0.05 are considered as significant.

TABLE 5.4 : Effect of different concentrations of ouabain on in vitro Locusta migratoria L. Malpighian tubules fluid secretion

	Ouabain (M)	Rate of fluid secretion nl/min SEM	$\frac{R_2}{\bar{R}_1} \times 100 \pm SEM$	n	P
Rate 1	0	3.30 ± 0.25	33.86 ± 4.82	30	<0.001
Rate 2	10^{-3}	0.92 ± 0.12			
Rate 1	0	3.97 ± 0.31	35.80 ± 6.54	8	<0.001
Rate 2	10^{-4}	1.13 ± 0.24			
Rate 1	0	4.11 ± 0.49	79.80 ± 6.73	21	<0.01
Rate 2	10^{-5}	3.01 ± 0.35			
Rate 1	0	2.30 ± 0.21	95.40 ± 3.66	20	ns
Rate 2	10^{-6}	2.26 ± 0.23			
Rate 1	0	3.21 ± 0.41	108.80 ± 3.72	30	ns
Rate 2	0	3.36 ± 0.40			

FIGURE 5.4

Effect of different concentrations of ouabain on *in vitro* *Locusta* Malpighian tubule fluid secretion.

Ordinate : Rate 2 expressed as a percentage of original Rate 1.

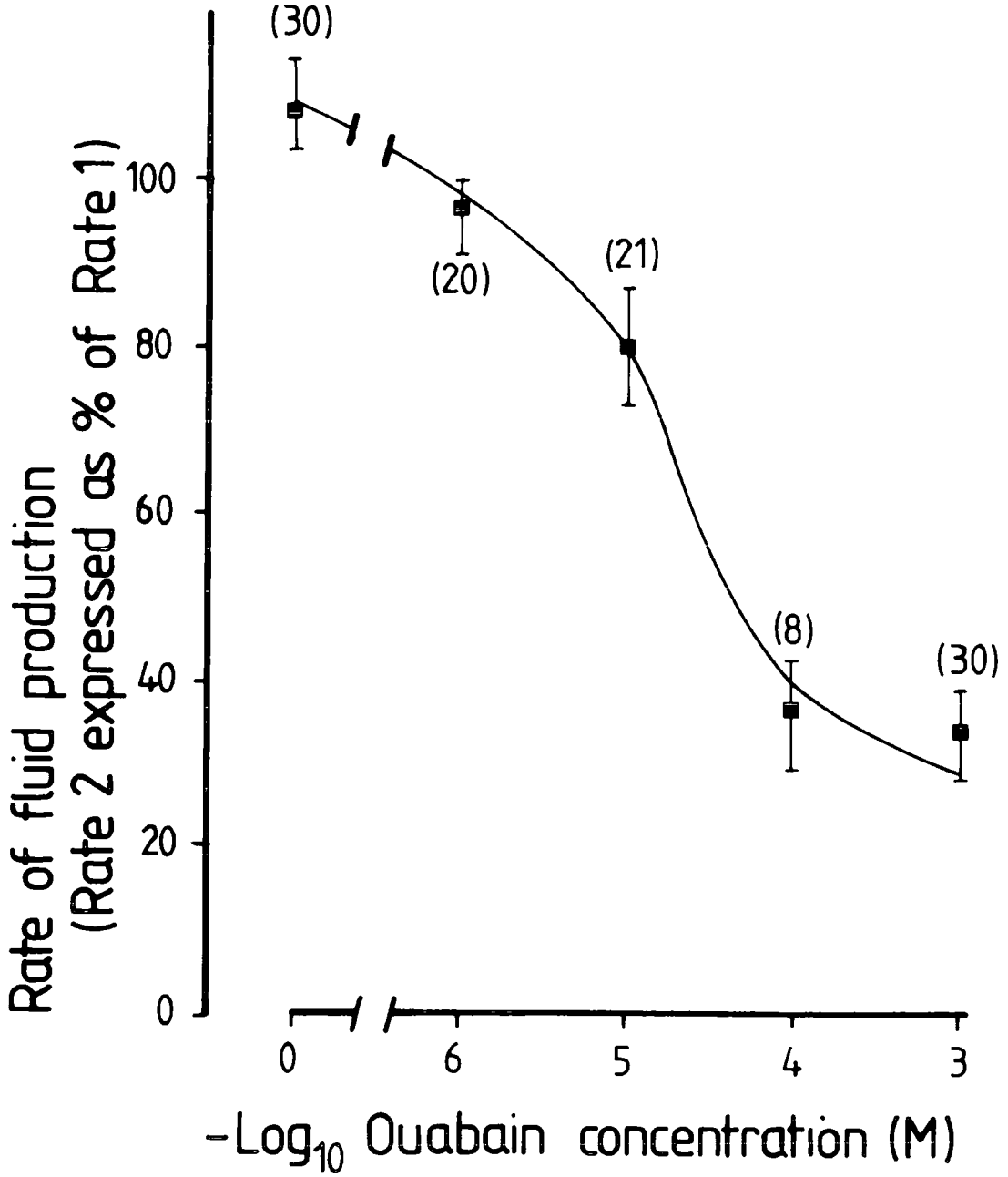
Abscissa : Negative logarithm of ouabain concentration (M)

Conditions: Rate 1 determined in 'normal' Ringer solution at 30°C for a period of 30 minutes.

Rate 2 redetermined after 20 minutes equilibration in normal Ringer containing different concentrations of ouabain at 30°C for a further 30 minutes.

Vertical lines represent 1 SEM and the figures in brackets indicate the number of determinations.

Fig. 5.4


(30)
(20)
(21)
(8)
(30)
Rate of fluid production
(Rate 2 expressed as % of Rate 1)
100
80
60
40
20
0
0
6
5
4
3
-Log10 Ouabain concentration (M)

concentrations higher than 10^{-6}M. At 30°C, high concentrations (10^{-3} - 10^{-4}M) ouabain caused between 65 and 67% inhibition when Rate 2 is expressed as a percentage of Rate 1 ($p = <0.001$ (Table 5.4). As previously shown, the results from the control experiments showed that Rate 1 and Rate 2 were not significantly different ($p > 0.05$). In Figure 5.4 the pI_{50} value can be determined as 4.3.

(b) Vanadate

Results presented in Figure 5.5 and Table 5.5 show that vanadate inhibited fluid secretion in a dose-response manner. Inhibition of 'urine' production increased with increasing concentration between 10^{-7} and 10^{-2}M vanadate. In the presence of 10^{-3}M vanadate, approximately 80% of fluid production was inhibited ($p = <0.001$). When the dose-response sigmoid curve in Figure 5.5 is transformed into Probit straight line in Figure 5.6, 4.8 can be determined as the pI_{50} value.

The results which are presented later in Table 5.12, show that when (10^{-3}M) vanadate was combined with (10^{-3}M) ouabain, the level of inhibition remained around 80% which is the same as that caused by (10^{-3}M) vanadate alone and was higher than the 66.1% inhibition caused by (10^{-3}M) ouabain alone (see Table 5.4).

5.7 The effect of temperature on Ouabain and Vanadate inhibition of fluid secretion

The effect of ouabain and vanadate on Malpighian tubule fluid secretion was investigated at different temperatures, below and above 30°C, namely 10, 15, 18, 20, 22 and 40°C. As controls to these experiments, a set of experiments where in the absence of the inhibitor, Rate 1 and Rate 2 were determined at the same temperature. These

TABLE 5.5 : The effect of different concentrations of vanadate on the in vitro Locusta Malpighian tubule fluid secretion

	Vanadate concentration (M)	Fluid secretion nl/min ± SEM	$\frac{R_2}{R_1} \times 100$ ± SEM	n	P
Rate 1	0	3.10 ± 0.34	27.0 ± 4.82	14	< 0.001
Rate 2	10^{-2}	1.00 ± 0.17			
Rate 1	0	3.38 ± 0.38	23.0 ± 4.21	30	< 0.001
Rate 2	10^{-3}	0.68 ± 0.13			
Rate 1	0	3.10 ± 0.51	44.82± 8.51	13	< 0.01
Rate 2	10^{-4}	1.36 ± 0.26			
Rate 1	0	2.20 ± 0.15	68.88± 6.38	17	< 0.02
Rate 2	10^{-5}	1.40 ± 0.15			
Rate 1	0	2.20 ± 0.27	78.00± 7.74	15	< 0.05
Rate 2	10^{-6}	1.54 ± 0.12			
Rate 1	0	2.20 ± 0.44	84.7 ± 4.38	7	< 0.05
Rate 2	10^{-7}	1.91 ± 0.39			
Rate 1	0	2.07 ± 0.22	95.3 6.60	16	ns
Rate 2	0	1.80 ± 0.18			

FIGURE 5.5

The effect of varying concentrations of vanadate on *Locusta* Malpighian tubule *in vitro* fluid secretion

Ordinate : Rate of fluid production Rate 2 expressed as % of original Rate 1 in absence of vanadate.

Abscissa : Negative Log_{10} of vanadate concentration (M).

Conditions : Rate 1 was determined in 'normal' Ringer solution at 30°C pH 7.2, and Rate 2 was redetermined in normal Ringer solution containing varying concentrations of vanadate (10^{-7} - 10^{-2}M).

Vertical lines represent 1 SEM and the figures in brackets indicate the number of determinations.

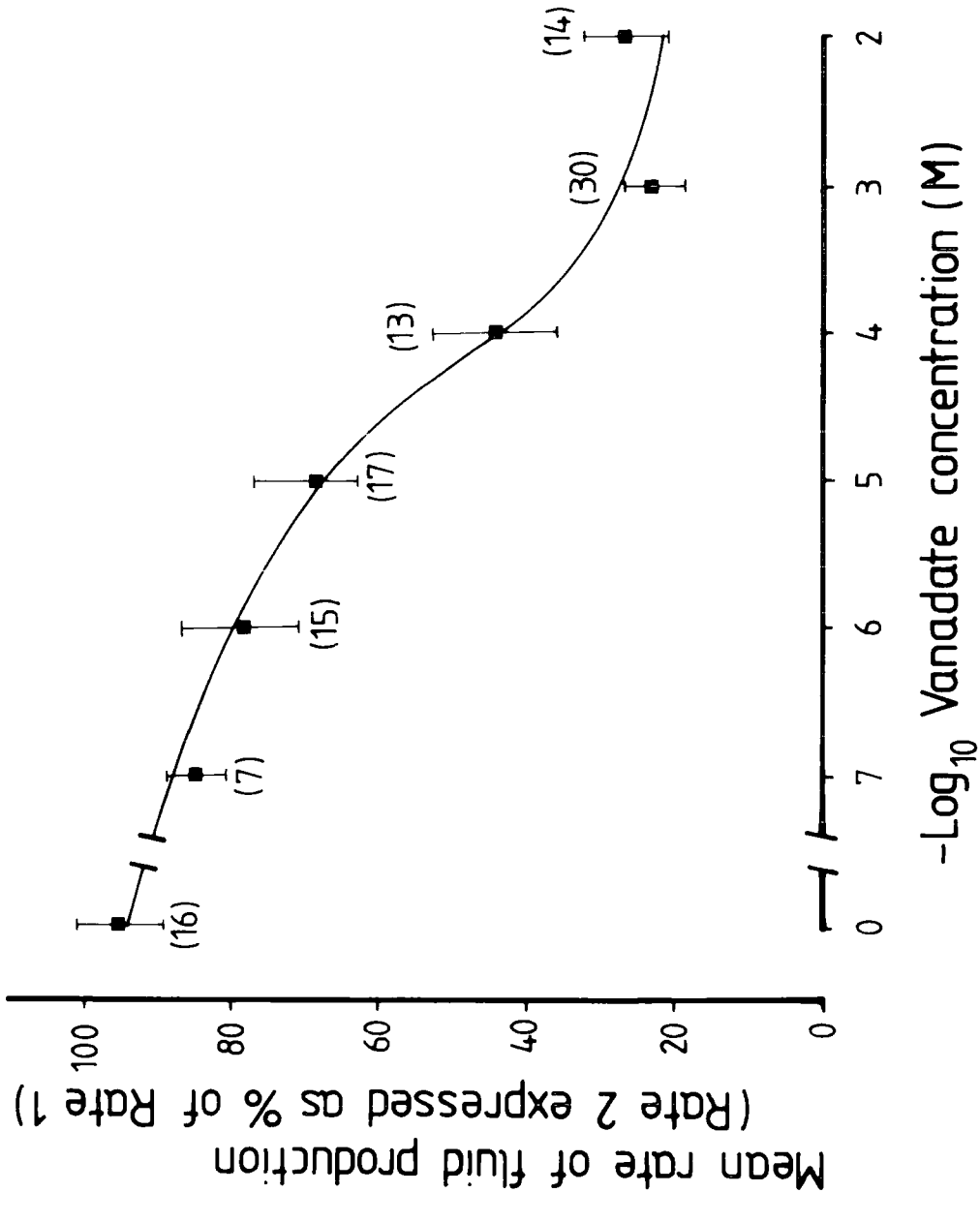

Mean rate of fluid production
(Rate 2 expressed as % of Rate 1)
100
80
60
40
20
0
(16)
(7)
(15)
(17)
(13)
(30)
(14)
0
7
6
5
4
3
2
-Log10 Vanadate concentration (M)

Fig. 5.5

FIGURE 5.6

Transformation of the sigmoid dosage inhibition curve (shown in Figure 5.5 for vanadate into a straight line by using Probits (Fisher and Yates, 1966).

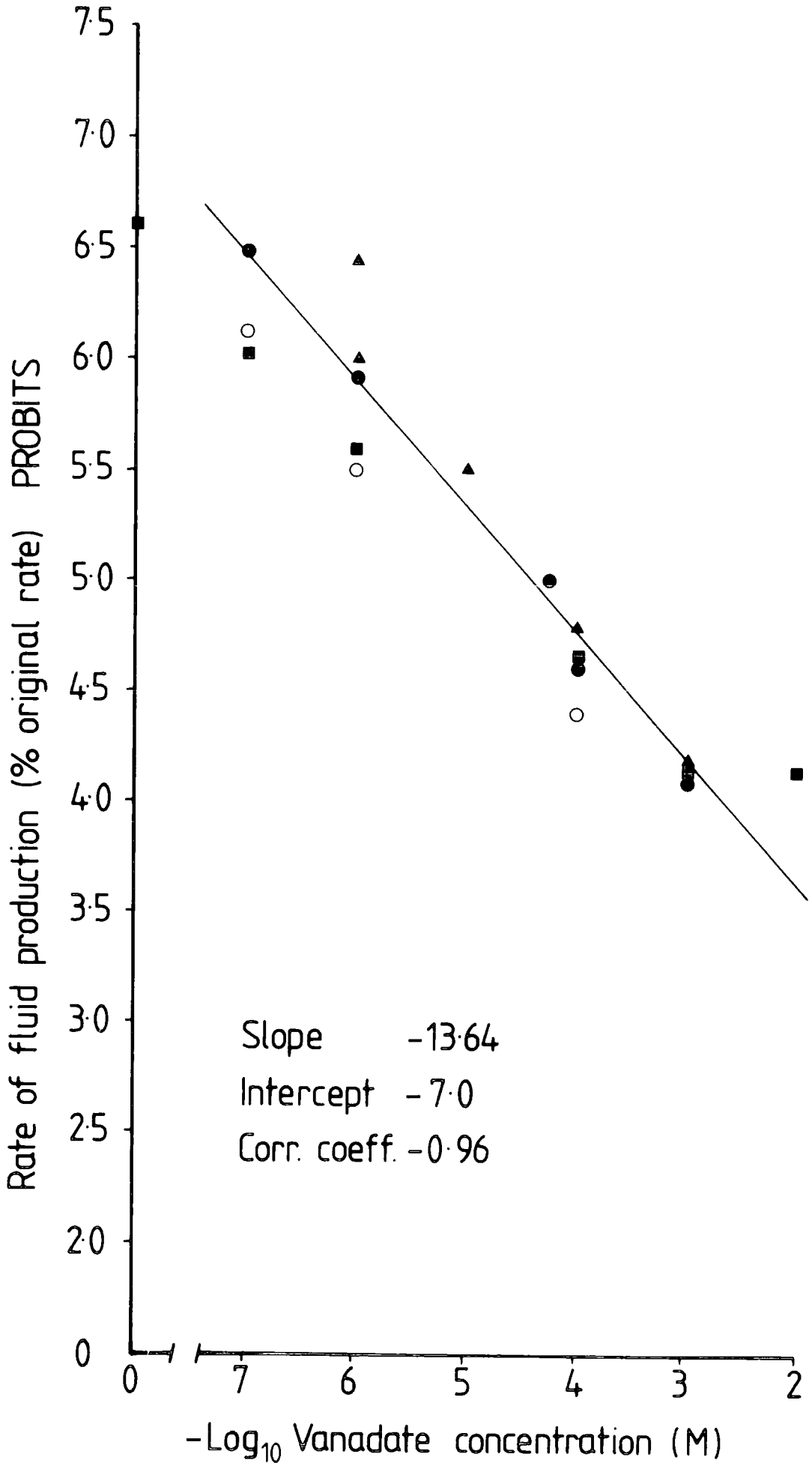
Ordinate: Volume of fluid produced nl/min transformed into Probits.

Abscissa: Negative Log_{10} of vanadate concentration (M).

Conditions as desribed for Figure 5.5

The line was fitted by a regression analysis.

Fig. 5.6


7·5
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
0
Rate of fluid production (% original rate) PROBITS
0
7
6
5
4
3
2
-Log10 Vanadate concentration (M)
Slope -13·64
Intercept -7·0
Corr. coeff. -0·96

control experiments were designed to demonstrate whether fluid secretion was maintained throughout the experimental period at each of the various temperatures. If so, any change in the rate of fluid secretion observed in the presence of an inhibitor would be due to the action of the inhibitor alone.

The results for these control experiments (Table 5.3) showed that at the temperatures investigated, Rate 1 and Rate 2 were not significantly different, except at 40°C where the preparation gets strained and Rate 2 cannot be maintained for more than 10-15 minutes.

(a) Ouabain

Results showing the effects of ouabain on fluid secretion at different temperatures are summarised in Table 5.6. These results and results previously presented show that at 30°C the presence of 10^{-3}M ouabain in the bathing Ringer solution, caused between 60-70% inhibition (see Fig. 5.7a). Results in Table 5.6 show that below 30°C, ouabain is less effective than at 30°C. Although the results show that there was 27.5% inhibition of fluid secretion by 10^{-3}M ouabain at 22°C, this drop in fluid secretion is not significantly different from the 22.5% drop observed in the absence of the inhibitor (control) at the same temperature.

The results also clearly show that at lower temperatures (22, 18 and 10°C) 10^{-3}M ouabain had no significant effect on 'urine' production. The curve showing the effect of ouabain at temperatures below 30°C shown in Figure 5.7b is almost identical with the control curve in the absence of ouabain.

TABLE 5.6 : The effect of temperature on ouabain inhibition of in vitro Locusta Malpighian tubule fluid secretion

Temperature °C	Treatment	Mean Rate 1 nl/min±SEM	Mean Rate 2 nl/min±SEM	$\frac{R_2}{R_1} \times 100 \pm SEM$	n	p
30	Control	3.21 ± 0.41	3.30 ± 0.40	103.12 ± 9.11	30	ns
	Ouabain (1mM)	2.60 ± 0.36	1.00 ± 0.17	38.46 ± 4.52	25	<0.001
22	Control	1.55 ± 0.18	1.20 ± 0.16	77.42 ± 9.11	19	ns
	Ouabain (1mM)	2.08 ± 0.32	1.57 ± 0.26	72.48 ± 10.32	20	ns
18	Control	5.10 ± 0.61	4.80 ± 0.52	94.11 ± 11.76	16	ns
	Ouabain (1mM)	5.11 ± 0.60	5.30 ± 0.53	103.9 ± 8.95	16	ns
10	Control	1.23 ± 0.27	1.18 ± 0.31	95.93 ± 10.11	13	ns
	Ouabain (1mM)	1.35 ± 0.03	1.20 ± 0.04	88.89 ± 12.25	10	ns

Experimental Procedure : As previously described in section 5.3

FIGURE 5.7a and b

Typical example of the effect of temperature on ouabain inhibition of the *in vitro* *Locusta* Malpighian tubule fluid secretion.

Rate 1 in 'normal' Ringer solution at 30°C (5.7a) and 18°C (Figure 5.7b) pH 7.2

Rate 2 in 'normal' Ringer solution containing ouabain (1 mM) at 30°C(Figure 5.7a) and 18°C (Figure 5.7b).

Ordinate: Volume of fluid produced in nl/min

Abscissa: Time in minutes.

Each point on the graph represents a single determination.

Fig. 5.7a

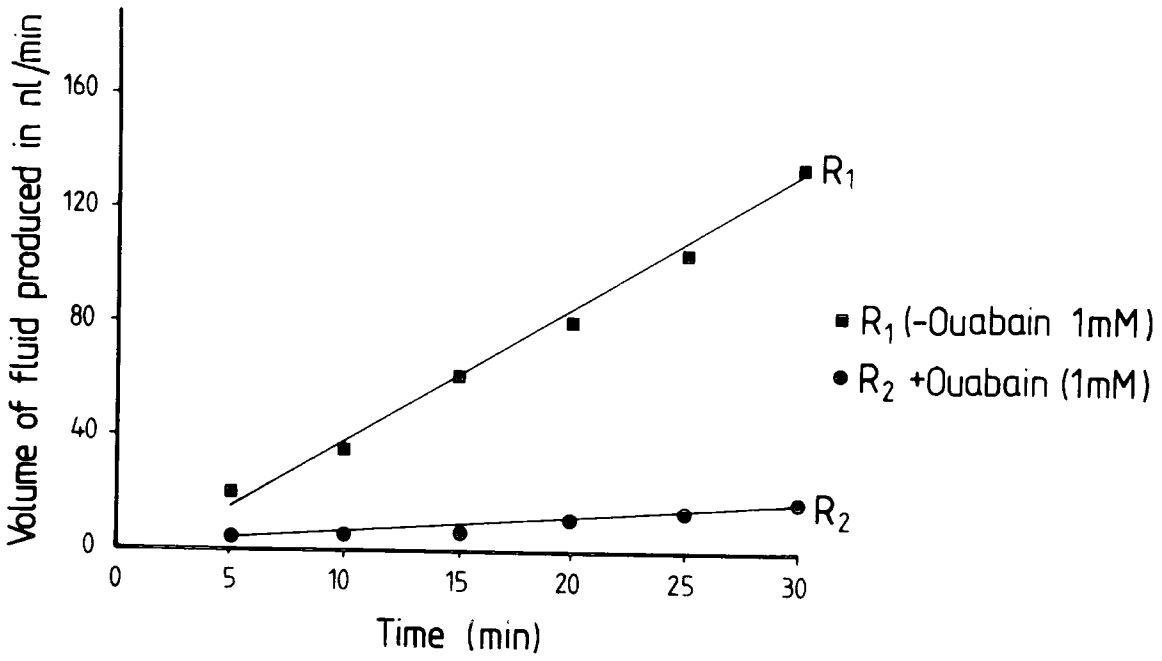
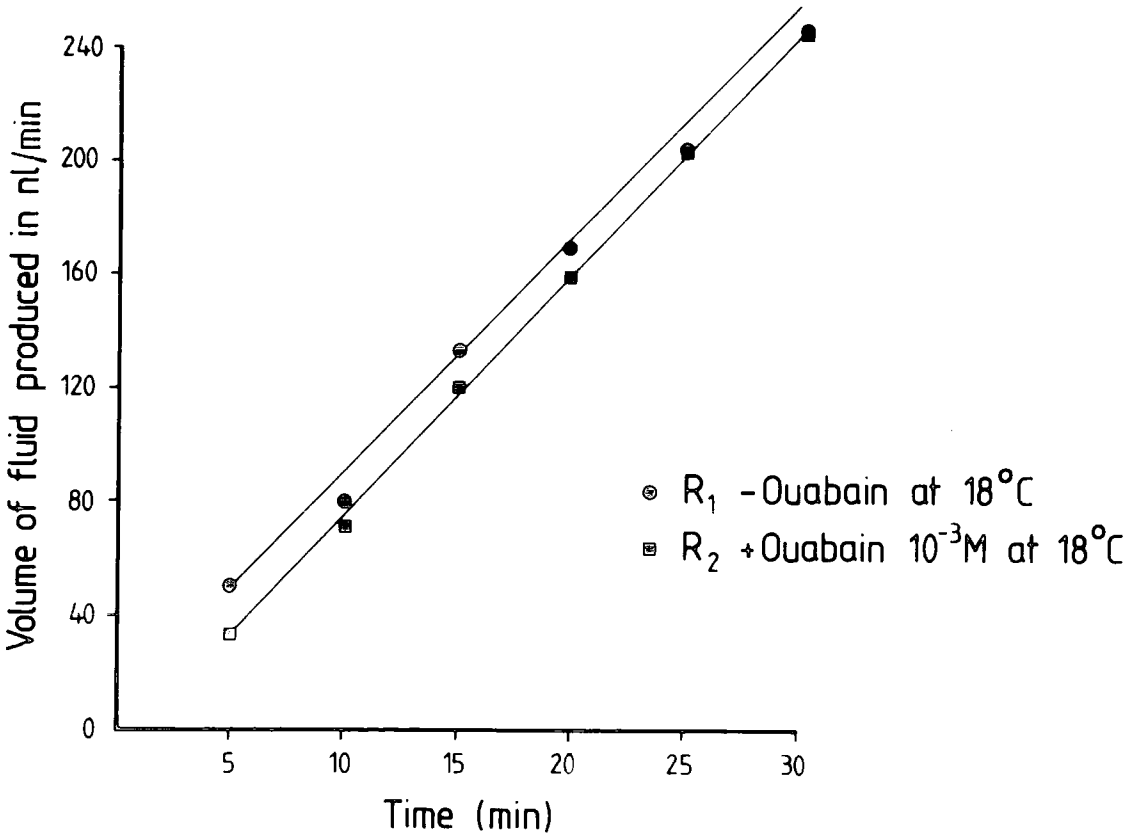


Fig 5.7b



(b) Vanadate

Results presented in Table 5.7 and Fig. 5.8 a,b show that, at 30°C, vanadate (10^{-3}M) inhibited fluid secretion by 79.5%. At a lower temperature (18°C), a high level of inhibition (71.2%) by vanadate was maintained. However, at 10°C, the level of inhibition by (10^{-3}M) vanadate dropped to only 25.2%, ($p < 0.05$) when controls (-vanadate) are compared with experiments (+vanadate). Similarly, at 40°C, vanadate has no significant effect on the *in vitro* Malpighian tubule fluid production. The optimum temperature for vanadate inhibition appears to be between 18 and 30°C. Figure 5.9 shows that between 10 and 30°C the level of vanadate inhibition of fluid secretion increased with the increase in temperature, but at 40°C only 6% inhibition was possible.

5.8 Effect of different concentrations of K^+ on vanadate inhibition of fluid secretion

A set of Ringer solutions of varying compositions were made as shown in Table 5.2. To obtain these Ringer solutions, stock of Na^+-free and K^+-free Ringer solutions were made up and these were mixed in varying ratios to give Ringer solutions shown in Table 5.2.

The results presented in Table 5.8 show that the inhibition of fluid secretion by vanadate was enhanced by high concentrations of potassium in the bathing medium. In the presence of 121.2mMK^+, inhibition of fluid secretion by vanadate (10^{-3}M) was almost complete. Rate 2 was only 5% of the original Rate 1 ($p = <0.001$) (Table 5.8 and Figure 5.10a and b).

The results also show that in the presence of high K^+ concentration (121 mM K^+) the rate of fluid secretion was faster than that observed

TABLE 5.7 : The effect of temperature on vanadate inhibition of the in vitro Locusta Malpighian tubule fluid secretion

Temperature °C	Treatment	Fluid secretion nl/min		$\frac{R_2}{R_1} \times 100$	n	P
		Rate 1 ±SEM	Rate 2 ±SEM			
10	Control	1.23 ± 0.25	1.18 ± 0.19	95.9	13	ns
	Vanadate	2.7 ± 0.38	1.0 ± 0.27	74.8	8	<0.05
18	Control	4.93 ± 0.70	4.18 ± 0.62	84.7	16	ns
	Vanadate	3.75 ± 0.41	1.08 ± 0.12	28.8	26	<0.001
30	Control	3.17 ± 0.52	3.16 ± 0.52	99.7	30	ns
	Vanadate	3.26 ± 0.38	0.67 ± 0.13	20.5	28	<0.001
40	Control	6.50 ± 0.31	6.56 ± 0.32	100·0	15	ns
	Vanadate	6.7 ± 0.23	6.3 ± 0.18	94.0	10	ns

Conditions and Experimental procedure are as described in Section 5.3 but using Vanadate as the inhibitor

Note Data as in Fig.5.9.

FIGURE 5.8a and b

Typical examples of the effect of temperature on vanadate inhibition of the *in vitro* *Locusta migratoria* L. Malpighian tubule fluid secretion.

Rate 1 in 'normal' Ringer solution at 30°C (Figure 5.8a) and at 40°C (Figure 5.8b) pH 7.2

Rate 2 in 'normal' Ringer solution containing vanadate (1 mM) at 30°C (Figure 5.8a) and 40°C (Figure 5.8b) pH 7.2

Ordinate: volume of fluid produced in nl/min

Abscissa: Time in minutes

Each point on the graph represents a single determination.

Fig. 5.8a

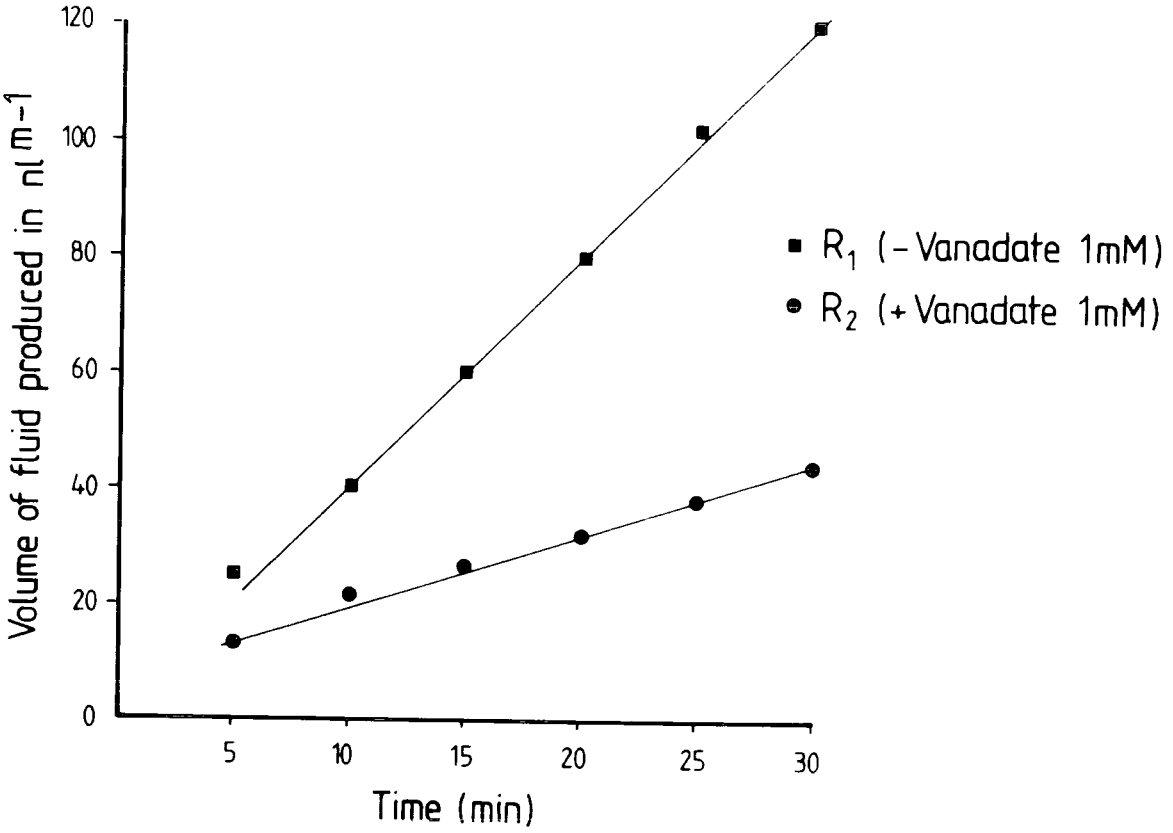


Fig. 5.8b

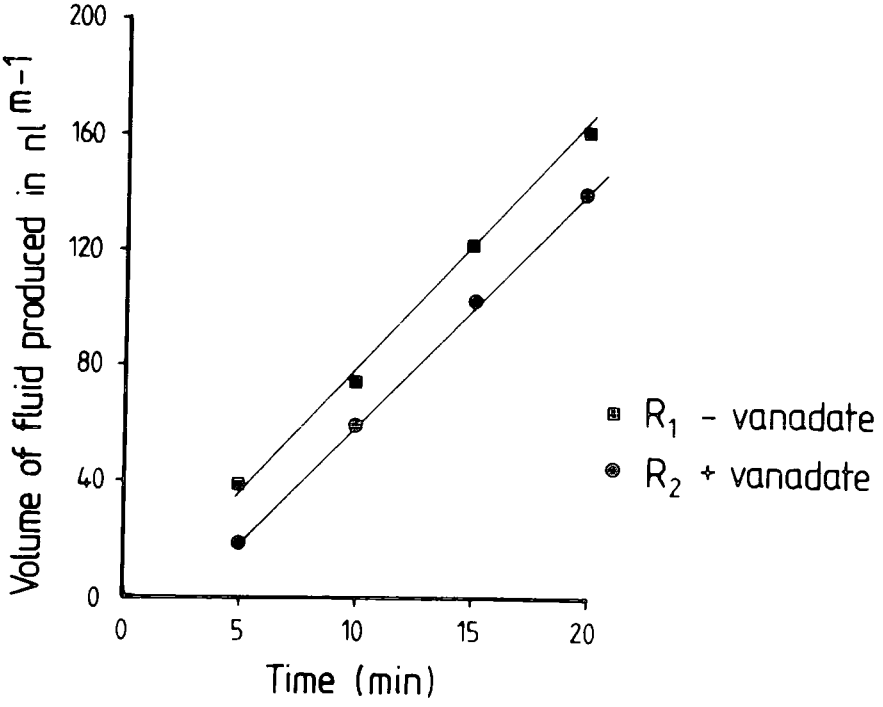


FIGURE 5.9

The effect of temperature on vanadate inhibition of the *in vitro* *Locusta migratoria* L. Malpighian tubule fluid secretion.

Ordinate : Rate of fluid secretion. Rate 2 expressed as as % of Rate 1 SEM.

Abscissa : Temperature °C.

Rate 1 in 'normal' Ringer solution at a known temperature.

Rate 2 in 'normal' Ringer solution containing 1 mM vanadate at the same temperature used for Rate 1.

Control : Rate 1 and Rate 2 measured at the same temperature in the absence of vanadate.

The vertical lines represent 2SEM of the mean and figures in brackets indicate the number of determinations.

Fig. 5.9

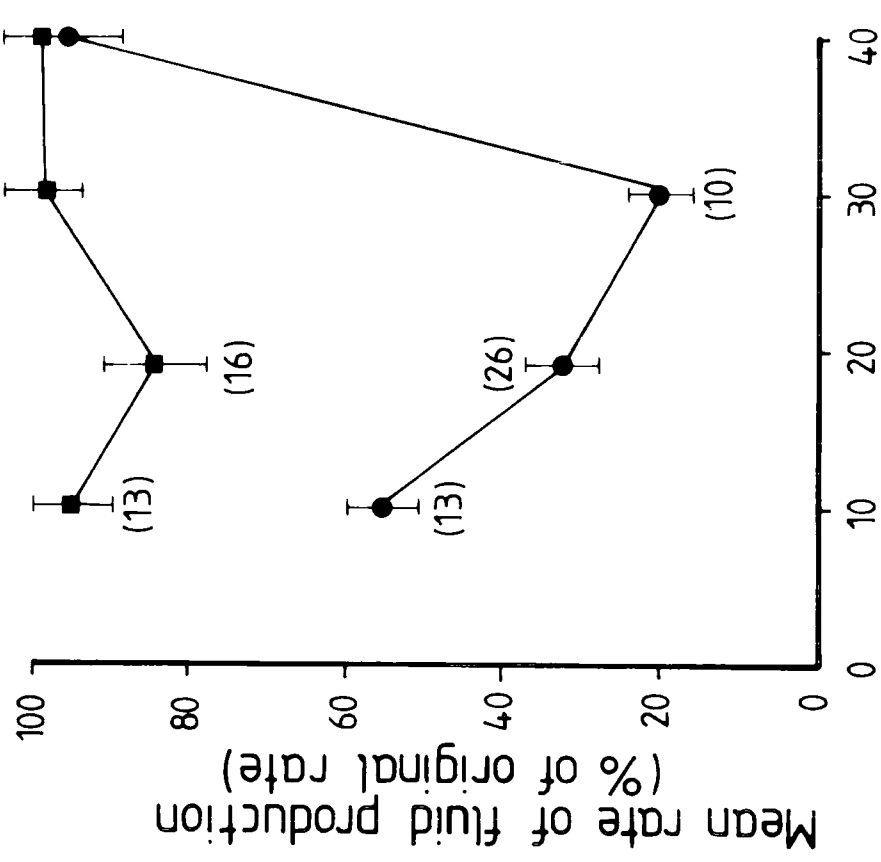

■ – Vanadate 10^{-3}M
● + Vanadate 10^{-3}M
Mean rate of fluid production
(% of original rate)
100
80
60
40
20
0
0
10
20
30
40
Temperature °C
(13)
(16)
(30)
(10)
(13)
(26)
(10)

TABLE 5.8 : Effect of different concentrations of K^+ on vanadate inhibition of the in vitro locusta migratoria L. Malpighian tubule fluid secretion

Composition of Ringer solutions used:

The concentration of K^+ and Na^+ varied according to the different ratios shown. The rest of the composition remained the same as in the 'normal' Ringer solution (see Table 5.1). Rate 1 and Rate 2 were measured in similar Ringer solutions in the absence of vanadate (control); and Rate 1 was determined in the absence of vanadate and Rate 2 redetermined in the presence of vanadate (10^{-3}M) for experiment.

All experiments were carried out at 30°C at pH 7.2

A paired 't' test was carried out to compare Rate 1 and Rate 2.

Note: Data as in Fig.5.10 a,b.

Table 5.8 : Effect of different concentrations of K^+ on vanadate inhibition of the in vitro *Locusta migratoria* L. Malpighian tubule fluid secretion

Experimental Conditions (mM)	Treatment	Rate 1 SEM	Rate 2 SEM	Rate 2 as % Rate 1	n	P
6.38 K^+, 121.22 Na^+	Control	2.98 ± 0.38	3.3 ± 0.27	110.70	20	ns
	Vanadate	2.97 ± 0.49	1.83 ± 0.27	61.62	20	<0.001
31.9 K^+, 95.7 Na^+	Control	2.98 ± 0.44	3.25 ± 0.62	109.02	20	ns
	Vanadate	4.6 ± 0.61	2.80 ± 0.41	60.83	20	<0.001
63.8 K^+, 63.8 Na^+	Control	5.94 ± 0.62	5.58 ± 0.60	94.90	10	ns
	Vanadate	6.69 ± 0.68	4.3 ± 0.34	64.24	20	<0.001
95.7 K^+, 31.9 Na^+	Control	3.8 ± 0.42	5.51 ± 0.71	144.71	20	ns
	Vanadate	7.01 ± 1.0	0.75 ± 0.18	12.50	20	<0.001
121.22 K^+, 6.38 Na^+	Control	4.3 ± 0.57	5.6 ± 0.71	130.20	20	
	Vanadate	9.1 ± 0.24	0.46 ± 0.19	5.03	20	≤0.001

Note Also see Fig.5.10a,b.

FIGURE 5.10a and b

Effect of varying concentrations of K^+ on vanadate inhibition of *in vitro* *Locusta* Malpighian tubule fluid secretion.

FIGURE 5.10a

Ordinate : Fluid production, Rate 2 expressed as % Rate 1.

Abscissa : K^+ concentration (mM).

FIGURE 5.10b

Ordinate : % inhibition.

Abscissa : K^+ concentration (mM)

Vertical lines represent 2SEM and numbers in brackets show the number of determinations (n).

Experimental procedure. Rate 1 determined in 'normal' Ringer solution containing varying K^+ concentrations and Rate 2 redetermined in similar Ringer solutions but containing 1 mM vanadate.

Fig. 5.10a

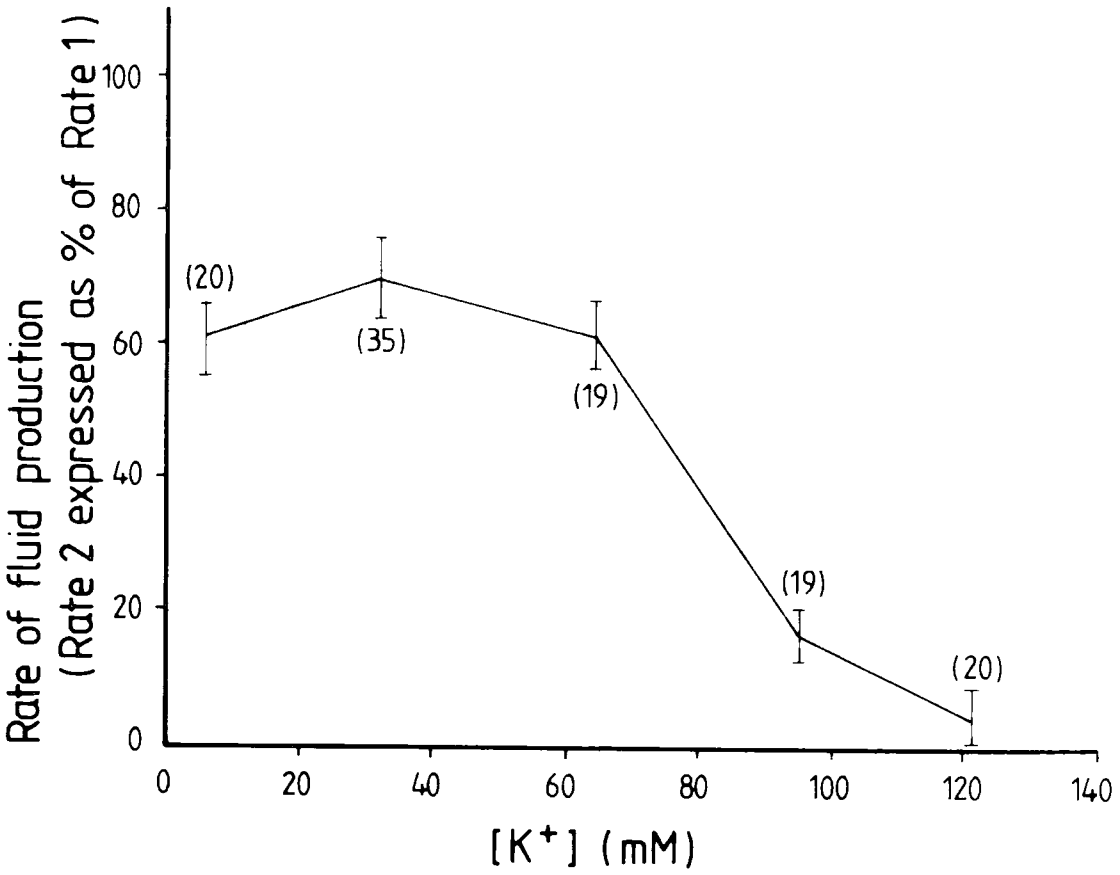
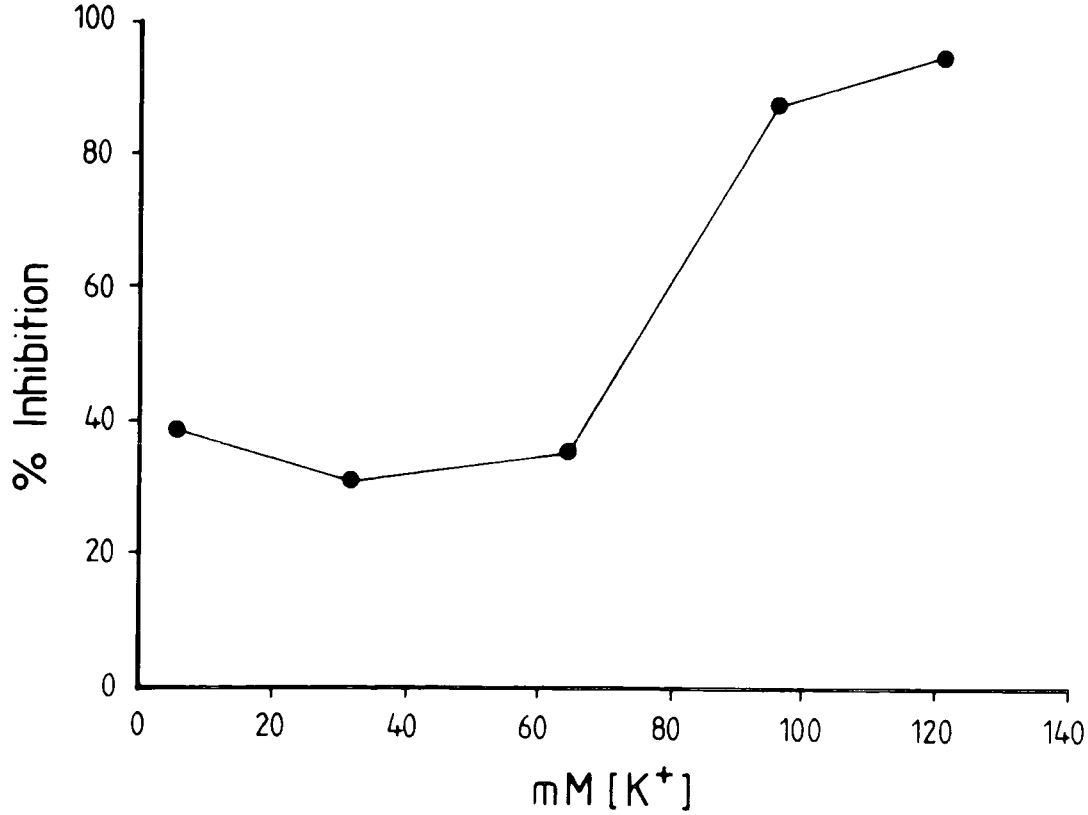


Fig. 5.10b



under 'normal' Ringer when Rate 2 was expressed as a percentage of Rate 1. At 121.22 K^+, a rate as high as 9.1 ± 0.24 nl/min was recorded. The mean rate of fluid secretion was between 4 and 5.6 ± 0.71 nl/min (Table 5.8) and this was considerably higher than 3.2 nl/min previously recorded in 'normal' Ringer solution.

5.9 Other Inhibitors

Experiments similar to those described in Section 5.6 were repeated using other inhibitors namely furosemide, sodium thiocynate (SCN), sodium acetazolamide and SITS. In these experiments, except for furosemide, only one concentration (10^{-3} M) of each inhibitor was used. The results are presented in Tables 5.9 and 5.10.

(a) Furosemide

Figure 5.11 and Table 5.10 show that furosemide inhibited urine production in a dose-dependent manner. It is, however, seen that it is only the high concentrations (10^{-4} to 5 x 10^{-3} M) which substantially inhibited fluid production. The effective dose ranged over just 2 orders of magnitude. Furosemide (5 x 10^{-3} M) inhibited some 85% of fluid secretion but at 10^{-3}M it inhibited only between 50 and 60% and at concentrations below 10^{-4}M, it did not significantly affect fluid secretion.

(b) Sodium acetazolamide

Only one concentration (10^{-3}M) acetazolamide was investigated. Acetazolamide (10^{-3}M) was found to inhibit some 68% of fluid secretion (see Table 5.9).

(c) Sodium thiocyanate (SCN^-)

Sodium thiocyanate was not a very efficient inhibitor of fluid secretion. The effective dose ranged over just 2 orders of magnitude. To inhibit fluid secretion by about 60% concentrations as high as 10 mM NaSCN were required. As a control to these experiments, 10 mM NaSCN was replaced by 10 mM NaCl in order to establish the fact that the change in fluid production was due to SCN^- and not to the increase in osmotic concentration caused by the addition of 10 mM Na^+. Results in Table 5. 9 show that the inclusion of 10 mM NaCl in the bathing medium did not affect fluid production. Rate 2 determined in the presence of 10 mM NaCl was 94.4% of Rate 1 measured in 'normal' Ringer solution.

In contrast, Rate 2 measured in the presence of 10 mM NaSCN was 38.4% of the original Rate 1 determined in 'normal' Ringer solution without the inhibitor, ($p = <0.001$) (see Table 5.9).

(e) SITS

Results presented in Table 5.9 show that SITS (10^{-3}M) did not significantly affect in vitro Malpighian tubule fluid secretion. Rate 2 determined in the presence of SITS was 85.5% of the original Rate 1 measured in normal Ringer solution without the inhibitor, ($p = >0.05$).

TABLE 5.9 : The effect of different inhibitors on Locusta migratoria L. in vitro Malpighian tubules fluid secretion

	Fluid secretion nl/min ± SEM	$\frac{R_2 \times 100}{R_1}$	n	P
Rate 1 -	3.98 ± 0.31	42.71 ± 5.71	35	<0.001
Rate 2 Sodium Acetazolamide (1mM)	1.70 ± 0.20			
Rate 1 -	2.47 ± 0.22	38.46 ± 6.94	32	<0.001
Rate 2 SCN^- (10 mM)	0.95 ± 0.08			
Rate 1 -	2.7 ± 0.30	94.4 ±11.52	12	ns
Rate 2 *NaCl (10 mM)	2.55 ± 0.10			
Rate 1 -	4.5 ± 0.61	85.5 ± 9.65	30	ns
Rate 2 SITS (1 mM)	3.85 ± 0.45			
Rate 1	3.08 ± 0.30	20.7 ± 4.11	37	<0.001
Rate 2 Vanadate (1mM) + ouabain (1mM)	0.64 ± 0.14			

Concentrations of different inhibitors used are indicated. A paired 't' test was carried out to compare Rate 1 (in the absence of inhibitor) and Rate 2 in presence of inhibitor. P values 0.05 were considered significant. *This experiment where 10 mM NaCl was included in the bathing Ringer solution was taken as the control for the SCN^- experiment. (-) minus inhibitor.

TABLE 5.10 ; The effect of different concentrations of furosemide on the in vitro Locusta Malpighian tubule fluid secretion

Furosemide Concentration	Rate 1	Rate 2	$\frac{\text{Rate 2}}{\text{Rate 1}} \times 100$ ±SEM	n	P
5×10^{-3}	3.37 ± 0.91	0.41 ± 0.18	14.9 ± 5.20	15	< 0.001
10^{-3}	3.1 ± 0.36	1.0 ± 0.15	37.9 ± 4.46	30	< 0.001
10^{-4}	2.9 ± 0.54	2.02 ± 0.34	70.0 ± 4.65	8	< 0.01
10^{-5}	2.31 ± 0.32	1.89 ± 0.28	82.7 ± 7.10	9	ns
0	2.21 ± 0.74	2.21 ± 0.74	100.0 ± 0.00	5	ns

FIGURE 5.11

The effect of different concentrations of furosemide on *Locusta* Malpighian tubule fluid secretion *in vitro*

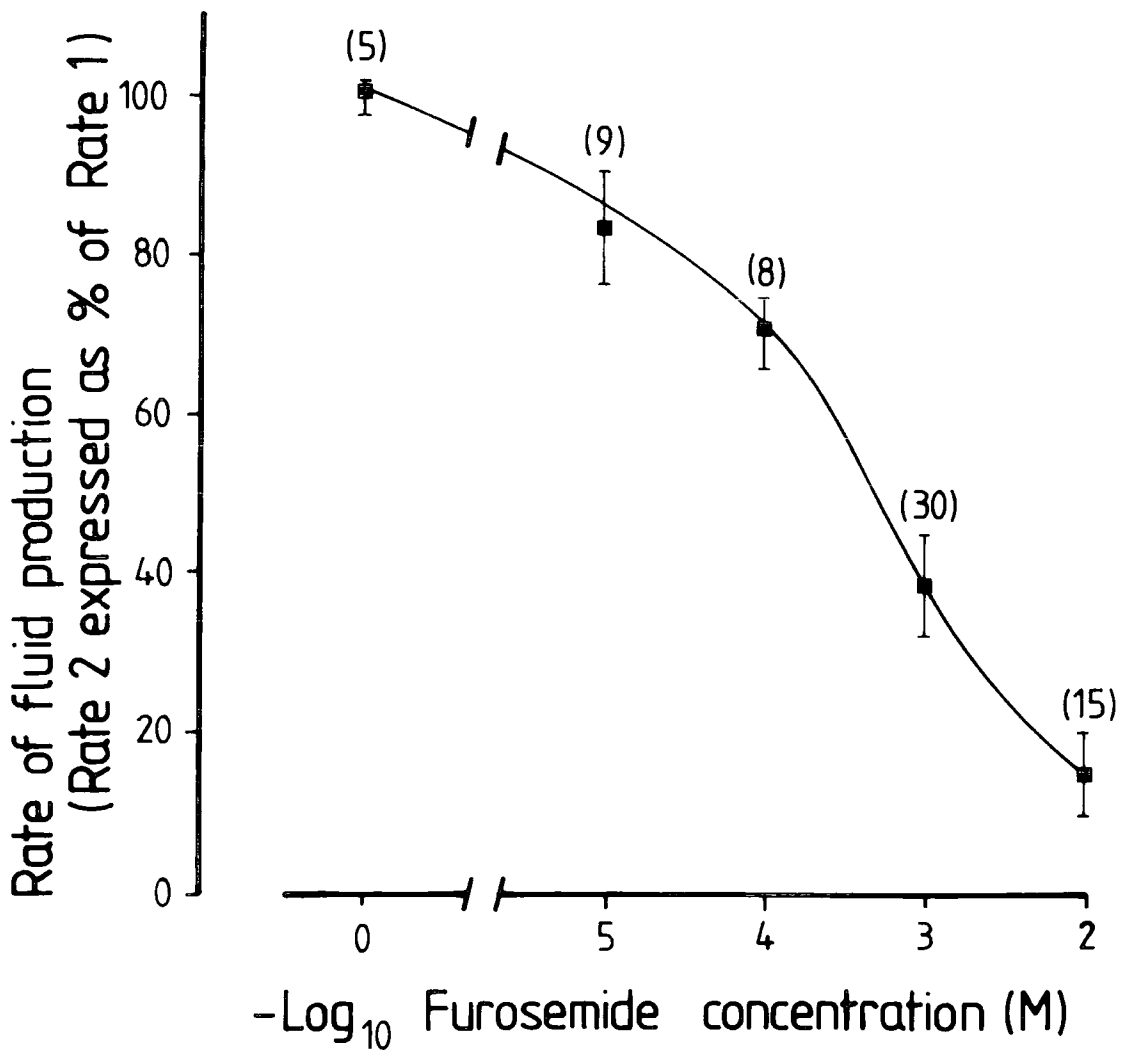
Ordinate : Fluid production. Rate 2 expressed as a percentage of the original Rate 1.

Abscissa : Negative logarithm of furosemide concentration (M).

Procedure Rate 1 determined in 'normal' Ringer solution at 30°C pH 7.2.
Rate 2 determined under similar conditions in the presence of different concentrations of furosemide.

Vertical lines represent 1 SEM and the figures in brackets indicate the number of determinations.

Fig. 5.11


Rate of fluid production
(Rate 2 expressed as % of Rate 1)
100
80
60
40
20
0
(5)
(9)
(8)
(30)
(15)
0
5
4
3
2
-Log10 Furosemide concentration (M)

DISCUSSION

The results presented here show that at 30°C, individual Malpighian tubules of Locusta were able to maintain fluid secretion at a constant rate for more than 1 hour when bathed with 'normal' Ringer solution. This confirms BERRIDGE's (1966) discovery that Malpighian tubules of Calliphora function for long periods of time in artificial media, containing an energy source, a fact which has made insect Malpighian tubules very accessible to study. Under optimal conditions, the rate of fluid secretion increased linearly with time (see Figure 5.2). This linear relationship makes it possible to attribute any dramatic changes in rate, following alterations in conditions to the experimental treatment.

The mean rate of fluid secretion in 'normal' Ringer at 30°C was 3.2± 0.41 nl/min (n = 30). This rate is higher than values of 1-2 nl/min reported by MADDRELL and KLUNSUWAN (1973) for Schistocerca gregaria; but compares favourably with 3.1 nl/min for Locusta observed under the same conditions (DONKIN, 1981). Fluid secretion was inhibited by ouabain over a wide concentration range (10^{-6} - 10^{-3}M). The results agree with those reported by ANSTEE and BELL (1975, BELL (1977), ANSTEE et al., (1979, 1980), FATHPOUR (1980), DONKIN and ANSTEE (1980), DONKIN (1981), and FATHPOUR et al., (1983) who studied fluid secretion in Locusta Malpighian tubules under similar conditions.

Results discussed in Chapter 3 have demonstrated that Na^+, K^+-ATPase from Locusta Malpighian tubules was inhibited by similar concentrations of ouabain. This has been taken to imply that in Locusta Malpighian tubules, Na^+, K^+-ATPase is involved in ion and water transport (ANSTEE and BELL 1975, 1978; ANSTEE et al., 1979, 1980; FATHPOUR et al., 1983). The latter workers also report that the PD across Locusta

Malpighian tubule was reduced by ouabain and they concluded that the active transport of Na^+ and K^+ maintained the PD, and that Na^+, K^+-ATPase is involved in the process of ion and water transport through the Malpighian tubule. Ouabain has been shown to inhibit active Na^+ and K^+ transport in a variety of tissues (GLYNN, 1964; SKOU, 1965; PODEVIN and BOUMENDIL-PODEVIN, 1972). FARQUHARSON (1974) also showed that fluid secretion by the pill millipede, Glomeris marginata was sensitive to ouabain at concentrations as low as 5×10^{-6}M. Further evidence of ouabain inhibition of Malpighian tubule function comes from in vivo experiments carried out by ATZBACHER et al., (1974), and HAVERT (1975). They showed that the rate of excretion of two dyes, azocarmine and indigo carmine was significantly reduced if 0.001 mls of 3×10^{-4}M ouabain was injected into the haemolymph of Drosophila hydei. In Ixodid ticks, salivary glands produce fluid secretions and Na^+ and K^+ are essential for maximal secretory rates in this tissue too. KAUFMAN and PHILLIPS (1973) have shown that such fluid production in Dermacentor andersoni was completely inhibited by 10^{-6}M ouabain. The inhibition of fluid secretion by ouabain, demonstrated in a variety of species suggests that at least one of the ion 'pumps' involved in 'urine' production is Na^+, K^+-ATPase. However, this pump has been shown to be absent in some transporting epithelia. For example, active K^+ transport in the midgut of the Lepidopteran, Manduca sexta does not require Na^+ (HARVEY et al., 1983). The latter workers also demonstrated that the short-circuit current in isolated midgut of M.sexta was not affected by ouabain (10^{-3}M) during a 2 hour incubation period under conditions (80 mM Na^+ 8 mM K^+ at 30°C) favouring ouabain inhibition of Na^+, K^+-ATPase activity.

Several workers have also objected to the idea of a Na^+/K^+ pump due to their failure to show that tubule secretion was inhibited by

ouabain (BERRIDGE, 1968; MADDRELL, 1969; PILCHER, 1970; GEE, 1976a,b; RAFAELI-BERNSTEIN and MORDUE, 1978). It is difficult to explain the cause of this reported ouabain-insensitivity. It may be that ouabain was not reaching the enzyme site in sufficient quantities to bring about inhibition as has been suggested by IRVINE and PHILLIPS (1971). For Schistocerca hindgut they found that high concentrations of ouabain (10^{-2}M) were needed to inhibit water and Na^+ movements but lower concentrations (10^{-3}M) had no effect on the transport mechanism. However, their results are not in agreement with those of other subsequent studies which show that even lower concentrations of ouabain to be effective (GOH and PHILLIPS 1978). Results reported from the present study showing that relatively high concentration of ouabain (pI_{50} = 4.3) was required to inhibit fluid production as compared with (pI_{50} = 6.8) for the isolated Locusta Malpighian tubule Na^+, K^+-ATPase, can be taken to support IRVINE and PHILLIPS (1971) view. The higher concentration, it could be argued, is required to overcome diffusion barriers and perhaps non-specific binding. There is, therefore, some justification for the suggestion that ouabain may be slow to reach or may not reach at all, the enzyme sites in insects such as Calliphora (BERRIDGE, 1968) and Rhodnius (MADDRELL, 1969) where 'urine' secretion is reported to be insensitive to ouabain. Several other reasons may be given to account for the ouabain insensitivity (see HARVEY et al., 1983). For example results presented here show that below 30°C (10^{-3}M) ouabain did not significantly affect the Malpighian tubule fluid secretion. Similar results are reported by ANSTEE and BELL (1975), DONKIN and ANSTEE (1980) and DONKIN (1981). Indeed, one of the more obvious methodological differences between laboratories, is the temperature at which fluid secretion by Malpighian tubules

has been studied. RAFAELI-BERNSTEIN and MORDUE (1978) who found fluid secretion in Locusta Malpithian tubules insensitive to ouabain, carried out their experiments at room teperature (18 - 24°C). It is, however, interesting to note that MORDUE and RAFAELI-BERBSTEIN (1978) found that Na^+ concentration in the fluid secreted by Locusta Malpighian tubules was affected by ouabain even though the rate of fluid production was not. Other workers have also performed experiments at room temperature (19 - 22°C) (e.g. GEE 1976b).

Results of the present study show that ouabain inhibition of fluid secretion was temperature-sensitive. Results presented in Table 5.6 show that at 30°C, 10^{-3}M ouabain caused 71.4% inhibition and this was reduced to insignificant levels of inhibition by the same concentration of ouabain at 22, 18, 15 and 10°C. The level of inhibition (71.4%) by 10^{-3}M ouabain at 30°C is higher than 56% reported by DONKIN (1981) but compares favourably with 65% reported by ANSTEE et al., (1979) using Locusta Malpighian tubules. However, 71.4% inhibition is significantly lower than 93% inhibition reported in an earlier study by ANSTEE and BELL (1975) using a different bathing medium. In their reviews, ANSTEE and BOWLER (1979) and HARVEY et al., (1983) emphasise the importance of temperature in the study of ouabain-sensitivity, a point ignored in several of the studies mentioned.

The concentration of K^+ in the bathing medium is also critical. High K^+ concentration reduces the effectiveness of ouabain inhibition (SKOU, 1965). This effect of high K^+ concentration on ouabain inhibition might explain MORDUE's (1969) observation that fluid production by Locusta Malpighian tubules was ouabain-insensitive. His media contained 20 mM K^+.

It is pertinent that DONKIN (1981) too could not show ouabain inhibition of fluid production using Mordue's media but did so when she used a medium similar to that described in Table 5.1. RAFAELI-BERNSTEIN and MORDUE (1978) too were unable to demonstrate ouabain inhibition of fluid production in Locusta Malpighian tubules using a Ringer solution containing 20 mM K^+ and they suggested that the low concentration of K^+ (8.6) mM) used by ANSTEE and BELL (1975) (and during the present study) may account for the differences in results. JUNGREIS (1977) also pointed out that K^+ concentration in Ringer solutions used by several workers may be unsuitable for demonstrating ouabain inhibition. Results showing K^+ antagonism to ouabain inhibition of Na^+, K^+-ATPase activity have been discussed in Chapter 3. However, it appears that sometimes K^+ antagonism for ouabain action may be overestimated. The results presented in Chapter 3, show that, although its effectiveness decreased with increasing K^+ concentration, ouabain still inhibited Na^+, K^+-ATPase activity at all concentrations of K^+. Unfortunately, direct comparisons cannot be made because similar studies were not carried out on K^+ antagonism of ouabain inhibition of the in vitro fluid production during the present study. However, DONKIN, (1981) using Locusta Malpighian tubules showed that varying the K^+ concentration of the bathing medium from 10 - 40 mM K^+ had no effect on ouabain inhibition of fluid secretion at 30°C. Therefore DALTON and WINDMILL (1980) who used (20 mM K^+) should not attribute the ouabain-sensitivity in Musca domestica to be due to K^+ antagonism of ouabain inhibition.

The results obtained from the present study show that there are a number of other anti-diuretic substances other than ouabain

that could be used in studies of enzymic mechanisms involved in water and ion transport through insect Malpighian tubules.

Vanadate, which was found to be a very potent inhibitor of Locusta Malpighian tubule Na^+, K^+-ATPase (Chapter 3) and both Mg^{2+}-ATPase and Mg^{2+},HCO_3^- - stimulated ATPase (Chapter 4); and also inhibited in vitro Malpighian tubule fluid secretion. It was, however, observed that higher concentrations of vanadate were required to inhibit in vitro Malpighian tubule fluid production than those used for the isolated ATPase (Chapters 3 and 4). Figure 5.6 shows that the pI_{50} for vanadate inhibition of in vitro fluid secretion is 4.8 while the pI_{50} for the isolated Na^+, K^+-ATPase is 5.8 (see Fig.3.6). This may reflect that vanadate is effective only when applied from the cytoplasmic side of the cell membrane (CANTLEY et al., 1979; DLOUHA et al., 1981). HIGASHI and BELLO-REUSS, 1980 also found that vanadate was an efficient potent diuretic and natriuetic substance only when applied intravenously in rat kidney. As previously discussed, when vanadate is applied extracellularly, it is oxidized to a less effective state as it crosses the reducing environment of the cytoplasm.

The results presented in Table 5.11 agree with results discussed in Chapter 3 showing that vanadate inhibition is facilitated by high K^+ concentration. It is seen that in the presence of 121.22 mM K^+ and 6.38 mM Na^+, vanadate (1 mM) inhibited in vitro Malpighian tubule fluid secretion by 95%. Rate 2 in the presence of vanadate was 5.01 ± 4.66% ($p = <0.001$) of Rate 1 measured under the same Ringer in the absence of vanadate. At the lowest K^+ concentration used (6.38 mM K^+ + 121.22 mM Na^+) vanadate (1 mM) inhibited only 38.4% of fluid production, (see Figure 5.10a and b). However, it is not clear why at 8.6 mM K^+ in normal Ringer solution (1 mM) vanadate

inhibited fluid secretion by 70%. How K^+ enhanced vanadate and reduced ouabain inhibition of Na^+, K^+ - ATPase activity in Locusta Malpighian tubule preparation is already discussed in Chapter 3. In further studies it would be interesting to investigate whether K^+ also reduces ouabain inhibition of in vitro Malpighian tubule fluid secretion. In summary, the fact that vanadate, a strong inhibitor of both Na^+, K^+ - ATPase and Mg^{2+} HCO_3^- - ATPase from Locusta Malpighian tubule, also inhibited in vitro Locusta Malpighian tubule fluid secretion strongly implicates the involvement of these enzymes in the process of water and ion transport through the Malpighian tubule epithelia.

Results presented in Tables 5.10 and Figure 5.11 show that furosemide inhibited in vitro Locusta Malpighian tubule fluid production in a dose-dependent manner. Furosemide appears to be a weak inhibitor when applied topically because very high concentrations were needed to inhibit fluid secretion. The results show that the effective threshold ranged over only 2 orders of magnitude (5×10^{-3} - 10^{-4}M). 5×10^{-3}M inhibited about 85% ($p < 0.001$) but the difference between rate of fluid production in absence of furosemide and presence of 10^{-5}M furosemide was statistically insignificant ($p = > 0.05$). Results discussed in Chapter 4 also show that furosemide is a weak inhibitor of the isolated $Mg^{2+}HCO_3^-$ - ATPase from Locusta Malpighian tubule preparations.

As previously discussed (Chapter 4) in vertebrates furosemide is thought to inhibit the Na^+ coupled Cl^- transport through a variety of transporting epithelia. Using locust rectal epithelia HANRAHAN and PHILLIPS (1983) investigated the Na^+ dependence of rectal Cl^- transport. Among their several experiments they used (1 mM) furosemide as an inhibitor and report that furosemide has no effect on cAMP-stimulated short circuit current (Isc) or the net Cl^- flux in locust

rectal epithelia. They also pointed out that if Cl^- transport was driven indirectly by Na^+ recycling involving a Na^+, K^+-ATPase (as suggested in NaCl cotransport models, Figure 1.3) at either serosal or mucosal membrane, it should be inhibited by ouabain. They report that ouabain had no effect on the Cl^--dependent Isc across locust rectum. Their study gave no evidence of NaCl cotransport occurring in locust rectal epithelia. The results from the present study show that both furosemide and ouabain inhibited in vitro fluid secretion and Mg^{2+}, HCO_3^--ATPase and Na^+, K^+-ATPase, respectively which indicates the possibility of Na^+ coupled Cl^- transport in Locusta Malpighian tubule epithelia.

Results presented in Table 5.9 show that sodium acetazolamide (1 mM) inhibited in vitro Malpighian tubule fluid production by some 57.3% when control (Rate 1) is compared with experimental (Rate 2) ($p < 0.001$). Although the percentage of inhibition reported here is higher than 40% reported by FATHPOUR (1980) using the same acetazolamide concentration (1 mM), the results can favourably be compared. The results are in contrast with reports from BERRIDGE (1968) and MADDRELL (1969) who found that acetazolamide did not inhibit fluid secretion by Malpighian tubules of Calliphora or Rhodnius. However, GOODING (1975) found that acetazolamide inhibited diuresis in Glossina morsitans which agrees with results of the present study.

In Schistocerca gregaria, transepithelial potential across the rectum (HERRERA et al., 1977, 1978; WILLIAMS et al., 1978) was inhibited by acetazolamide. Similarly, acetazolamide inhibited the transepithelial potential across the Malpighian tubules of Locusta (FATHPOUR 1980). Several other workers report inhibitory effects of acetazolamide on transport processes in vertebrate animals e.g. McKINNEY and BURG (1977); BURG and GREEN (1977) have shown that fluid

absorption by rabbit renal proximal tubules is inhibited by acetazolamide by 22 - 40%; and CHEUNG et al., (1977) also reports that acetazolamide caused a dramatic fall in the secretory rate to about 10% of the 'control' value in isolated seminiferous tubules of the rat. In studies different but relevant to the present study, acetazolamide inhibited net secretion of HCO_3^- in human sweat glands (SLEGERS and MOONS, 1968) turtle bladder (GONZALES, 1969; GONZALES and SCHILB, 1969) dog renal tubules (KLEINMAN, 1978; MATHISEN et al., 1978) rabbit cortical collecting tubules (McKINNEY and BURG 1978a,b) and rat renal tubules (HOPPE et al., 1976; LUCCI et al., 1979). Others report acetazolamide inhibition of secretion in dog gastric mucosa (JANOWITZ et al., 1952a,b; POWELL et al., 1962) human jejunum (TURNBERG et al., 1970a) cat pancreas (CASE et al., 1979) and rabbit corneal endothelium (HULL et al., 1977).

As previously pointed out acetazolamide is known to inhibit carbonic anhydrase, an enzyme which is thought to be functionally linked with the Mg^{2+}, HCO_3^- -ATPase (BURG, 1976). According to the proposed Cl^- transport (Figure 1.3 A-D) acetazolamide is thought to inhibit the Cl^-/HCO_3^- exchange mechanism. FATHPOUR (1980) reports that application of 1 mM acetazolamide in either HCO_3^- -free Ringer or 'normal' Hepes Ringer, caused similar inhibition levels (55%). His results and those of the present study indicate the involvement of Mg^{2+}, HCO_3^- -ATPase and carbonic anhydrase in fluid transport through Locusta Malpighian tubule fluid production and may also support the existence of Cl^-/HCO_3 exchange mechanism in fluid transport.

The results of the present study are further supported by the fact that SITS inhibited the Mg^{2+}, HCO_3^- -ATPase activity in Locusta

Malpighian tubule preparations (results discussed in Chapter 4) but further complicated by the fact that SITS (1 mM) did not significantly inhibit *in vitro* Malpighian tubule fluid secretion. It is important to recall that FATHPOUR (1980) isolated a carbonic anhydrase and Mg^{2+}, HCO_3^- -ATPase from *Locusta* Malpighian tubule preparation and strongly implicated these enzymes involvement in fluid transport through the Malpighian tubule epithelia. The present study did not investigate the effects of acetazolamide on the ATPases from *Locusta* Malpighian tubules which does not allow direct comparisons to be made. However, FATHPOUR (1980) points out that the conflicting reports on the effects of acetazolamide in different studies may be due to the differences in time allowed for preincubation in presence of the inhibitor which was found to be an important factor by some workers (POWELL *et al.*, 1962; and BERKOWITZ and JANOWITZ, 1967, working on dog gastric mucosa). They observed that acetazolamide manifested its maximum effect on water secretion and ion flux 30 minutes after initial application. An equilibration period of 20 minutes was allowed before Rate 2 in presence of acetazolamide was redetermined during the present study.

As previously discussed (Chapter 4) Cl^-/HCO_3^- is either inhibited by acetazolamide or SITS (KRISTEINSEN, 1972). However, results of the present study show that SITS does not inhibit *in vitro* Malpighian tubule fluid secretion but very effectively inhibited Mg^{2+}, HCO_3^- -ATPase in *Locusta* Malpighian tubule preparations (Chapter 4). In their investigation of the possible existence of Cl^-/HCO_3^- exchange mechanism in locust rectal epithelia, HANRAHAN and PHILLIPS (1983) found that SITS 1 mM did not affect the Cl^- -dependent short circuit (Isc) across a cAMP-stimulated locust recta. More recently GERENCSER (1984)

reported that SITS had no effect on Cl^- transport in apical plasma vesicles isolated from bovine tracheal epithelium. They concluded that it is the membrane permeability that determines the rate of Cl^- uptake and that Cl^- transport is not specifically coupled with either Na^+ or K^+ but that Cl^- crosses the tracheal membrane via an electrogenic transport mechanism. A view shared by HANRAHAN and PHILLIPS (1983) for locust rectum. But there is no evidence for or against such reasoning from the present study.

In contrast, PERRY and RANDALL (1981) report that SITS significantly reduced Cl^- influx in the rainbow trout, Salmo gairdneri, gill. They concluded that the effect of SITS on contralateral exchange processes is secondary in nature resulting from altered epithelial cell pH due to primary inhibitory action of these drugs.

The only possible explanation why SITS does not inhibit in vitro Malpighian tubule fluid secretion is the fact that SITS does not penetrate the membrane. It reacts with small sites on the membrane which are associated with anion permeability and cannot reach sites for cation permeability (and this gives it its specificity) (CABANTCHIK and ROTHSTEIN, 1972).

The effect of SCN^- in vitro Malpighian tubule fluid secretion was also investigated during the present study. SCN^- is so far, the most well known inhibitor of Mg^+, HCO_3^- -stimulated ATPase. ANSTEE and FATHPOUR (1979), and FATHPOUR, (1980) demonstrated that SCN^- inhibited both in vitro Locusta Malpighian tubule fluid secretion and the Mg^{2+}, HCO_3^- -ATPase activity in Locusta Malpighian tubule in preparations (see Chapter 4). The results presented here (Table 5.12) show that sodium thiocyanate (10 mM) inhibited in vitro Locusta

Malpighian tubule fluid secretion by 61.6% when control Rate 1 in absence of inhibitor was compared with Rate 2 in presence of 10 mM SCN^- ($p < 0.001$). In one set of control experiments 10 mM NaSCN was replaced by 10 mM NaCl and results show that this inclusion of 10 mM NaCl in the bathing medium has no effect on the rate of urine production. This showed that the change in urine production observed in presence of 10 mM NaSCN was due to the inhibitor action and not to the increase in osmotic concentration caused by 10 mM Na^+. The results are in agreement with those obtained by FATHPOUR (1980) who reports that 1 mM SCN^- and 10 mM SCN^- reduced the rate of fluid secretion by Locusta Malpighian tubule, by 33 and 45% respectively. BERRIDGE (1969) working with Calliphora Malpighian tubules observed that replacement of Cl^- by SCN^- inhibited rate of fluid secretion by 25%. Inhibitory effects of SCN^- have been reported elsewhere, e.g. SACHS et al., (1972a) reported that gastric mucosal acid secretion was completely inhibited by 12 mM SCN^-, and FORTE and DAVIES (1964) report that acid secretion by bullfrog gastric mucosa is inhibited by 1-10 mM SCN^- by 70 - 90%. SCN^- is, so far, the most well known inhibitor of Mg^{2+}, HCO_3^--ATPase. Since ANSTEE and FATHPOUR (1979, 1981) and FATHPOUR (1980) have demonstrated SCN^- inhibition of Mg^{2+}, HCO_3^--ATPase in Locusta Malpighian tubule preparations its inhibition of the in vitro Malpighian tubule fluid secretion implicates the involvement of the Mg^{2+}-dependent HCO_3^--stimulated ATPase in the process of water and ion transport through the Malpighian tubule epithelia.

In contrast, more recently DALTON and WINDMILL (1980) report that the presence of 10 mM NaSCN in the bathing medium, stimulated fluid production by Malpighian tubules of Musca domestica. They interpreted their results in relation to the proposed existence of

NaCl cotransport pump in secreting epithelia. Musca domestica Malpighian tubules secrete a fluid constantly hyperosmotic to the bathing medium, by active transport of K^+ and Na^+ (with K^+ as the prime mover) with Cl^- moving passively down the electrochemical gradient as the major anion, and Na^+ and Cl^- being actively reabsorbed from the luminal fluid via a sodium-chloride linked pump, thus reducing the osmolarity of the tubule fluid to near that of the bathing medium. It is postulated that SCN^- abolishes the Na^+ and Cl^- linked reabsorption, thus raising the concentration of Na^+ and Cl^- in the tubule fluid. If Cl^- movement into the lumen during secretion is passive as suggested (see Chapter 1) then the equilibrium with the medium Cl^- concentration observed by DALTON and WINDMILL (1980) is expected. They suggested that the increase in fluid secretion in the presence of SCN^- may reflect the flow of water down the increased osmotic gradient and their observation that K^+ concentration remained constant suggests that water movement is intimately linked and limited by K^+ transport and K^+ transport pathway, and hence fluid movement normally operates to maximal capacity. (K^+ being a prime mover of fluid transport in several insects was discussed earlier in this Chapter). SCN^- inhibition of NaCl coupled transport is also reported elsewhere (e.g. fish gill, EPSTEIN et al., 1973, and amphibian cornea, ZADUNAISKY, et al., 1971).

CHAPTER 6

SECTION I

CYTOCHEMICAL LOCALIZATION OF POTASSIUM-DEPENDENT, OUABAIN-SENSITIVE P-NITROPHENYL PHOSPHATASE IN MALPIGHIAN TUBULES OF LOCUSTA MIGRATORIA L.

Introduction

It is clear from the previous Chapters that the membrane bound Na^+, K^+-ATPase (E.C.3.6.13. SKOU, 1965) and its involvement in fluid and ion transport has been extensively studied (see reviews by GLYNN and KARLISH, 1975, ROBINSON and FLASHNER, 1979; SKOU, 1975; JØRGENSEN, 1982; STEKHOVEN and BONTING, 1981; HANRAHAN and PHILLIPS, 1983; ANSTEE and BOWLER, 1979; 1984). Several models have been proposed to explain the mechanism of fluid and solute transport through various epithelia (see Chapters 1 and 5) but a definite localization of the cytological structures primarily responsible for active transport is largely lacking.

The cytological location of the active transport step is of prime concern to any theory of fluid transport across the epithelia. Subcellular localization of magnesium-activated adenosine. triphosphatase activity (Mg^{2+}-ATPase) by modification of the Gomor-Type heavy metal precipitation reaction (GOMORI, 1952) was first introduced by WACHSTEIN and MEISEL (1957) this technique has subsequently been applied to a variety of tissues specialised in active transport by various investigators (e.g. NOVIKOFF et al., 1962; BARRNETT, 1964; GOLDFISCHER et al., 1964; WACHSTEIN and BESEN, 1964, FARQUHAR and PALADE, 1966). But because the functional significance of the Mg^{2+}-ATPase and its relationship to the transport system remain uncertain, further interest in ATPase cytochemistry

has been centred around attempts to localize the ouabain-sensitive Na^+, K^+ - ATPase. With the exception of work with the red blood cells (MARCHESI and PALADE, 1967), repeated attempts to localize Na^+, K^+ - ATPase using various modifications of the WACHSTEIN and MEISEL (1957) technique, have met with little success (ABEL, 1969; FARQUHAR and PALADE, 1966; JACOBSEN and JORGENSEN, 1969; TORMEY, 1966; NOVIKOFF et al., 1961). Their failure has prompted a critical assessment of the procedure itself (ABEL, 1969; ERNST and PHILPOTT, 1970; GANOTE et al., 1969; MOSES and ROSENTHAL, 1968; JACOBSEN and JORGENSEN, 1969; TORMEY, 1966; TICE, 1969) and has led to much controversy regarding the validity of this technique for demonstration of sites of enzymatic activity (MOSES and ROSENTHAL, 1967; NOVIKOFF, 1967, 1970). These investigators have shown several problems encountered in applying the WACHSTEIN-MEISEL (1957) technique. Some of these problems are summarised by ERNST (1972a,b) as follows:

(a) Lead strongly inhibits Mg^+-ATPase and particularly, Na^+, K^+ - ATPase.

(b) Lead catalyzes a nonenzymatic hydrolysis of ATP which may contribute, as a source of Pi, to artificial deposits but does not in itself account for all of the reaction product localized.

(c) Reaction product contains precipitated nucleotide as well as lead and Pi.

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

(e) The pattern of nonenzymatically produced deposits may mirror the distribution of enzymatically produced precipitates.

In view of the problems inherent with the WACHSTEIN-MEISEL (1957) technique, it has been necessary to examine alternative procedures for localization of Na^+, K^+-ATPase. The partial reactions involved in the hydrolysis of ATP by Na^+, K^+-ATPase provided a good basis for new techniques to be developed. As previously discussed in Chapter 3, the hydrolysis of ATP occurs in at least two steps: one of a Mg^{2+}, Na^+ dependent phosphorylation of the enzyme and the other a K^+-dependent hydrolysis of the resulting acylphosphate or conformational change (E ~ P) (ANSTEE and BOWLER, 1984). Several studies have indicated that the K^+-dependent hydrolysis of some simple organic phosphates such as acetyl-phosphate and p-nitrophenyl phosphate by Na^+, K^+-ATPase is equivalent to the K^+-dependent hydrolysis of E ~ P within the ATPase complex (ASKARI and KOYAL, 1968; GARRAHAN et al., 1970; ROBINSON, 1970). Using the avian salt gland as a model transport tissue, ERNST (1972a) indicated that a cytochemical procedure for K^+-dependent phosphatase activity might provide an alternative method of localizing the transport enzyme complex. ERNST (1972a) used strontium as the capture ion for the hydrolyzed phosphate because it appeared to be less inhibitory to the transport enzyme than other heavy metals such as lead used in the WACHSTEIN-MEISEL technique. p-nitrophenyl phosphate provided a good substrate since its hydrolysis yields a yellow coloured nitrophenol as well as an inorganic phosphate. The phosphate is precipitated by strontium and the amount of p-nitrophenol released can be measured spectrophotometrically to determine enzymatic activity quantitatively under cytochemical conditions (ERNST (1972a) also found that strontium does not

catalyze non-enzymatic hydrolysis as would lead in ATP containing medium. In the WACHSTEIN-MEISEL type of medium, the Mg^{+}-ATPase activity would form 40% of the total ATPase activity and this would mask the Na^{+},K^{+}-ATPase activity. In contrast, K^{+}-activated NPPase represents 80% of the total NPPase activity. However, ERNST (1972a) pointed out that as much as 87% of the K^{+}-activated NPPase activity could be inhibited by 20 mM of strontium, (a concentration used in his technique). This percentage of inhibition due to strontium could be lowered by altering the pH from alkaline to a neutral pH.

Fixation of the tissues for cytochemical studies may also cause problems. The activity of Na^{+}, K^{+}-ATPase is markedly sensitive to aldehyde fixation, although not all aldehydes are equally inhibitory. ERNST and PHILPOTT (1970) demonstrated that formaldehyde was less inhibitory than glutaraldehyde even when used in much higher concentrations. But fixation with formaldehyde alone often does not adequately preserve the tissue fine structure. Recently ERNST (1975) showed that a combination of fixative consisting of 1% formaldehyde and 0.25% glutaraldehyde would allow acceptable preservation of tissue morphology while preserving enzyme activity adequate for cytochemical localization.

Several workers have subsequently utilized this kinetically-based cytochemical procedure for Na^{+}, K^{+}-ATPase localization for a variety of epithelia (e.g. not kidney, ERNST, 1975; avian salt gland, ERNST, 1972b; ERNST and MILLS, 1977; alimentary tract of Locusta, PEACOCK, 1976; Lachrymal salt gland of Malaclemys, THOMPSON and COWAN, 1976; rectal gland of spiny Dogfish, GOERTEMILLER and ELLIS, 1976; gills of Euryhaline teleost, HOOTMAN and PHILPOTT, 1979;

Larval rectum of Aeshna cyanea, KOMNICK and ACHENBACH, 1979).

Reports from these different studies show that ERNST (1972a) technique has been successfully applied to localise Na^+, K^+-ATPase in a variety of tissues (DIBONA and MILLS, 1979). However, application of this technique, in insect studies has been unsuccessful and the results inconclusive (PEACOCK, 1976b; KOMNICH and ACHENBACH, 1979; present study). In these studies a K^+-stimulated component of NPPase activity was apparent and distinct from alkaline phosphatase but it was inconsistently inhibited by 1 mM ouabain. Similar results were also reported by ERNST (1975) for rat kidney cortex. Possible explanations for the inconsistent results obtained during various studies are briefly given by ANSTEE and BOWLER (1984). ERNST (1972a) showed that strontium alters the sensitivity of the K^+-stimulated NPPase to ouabain. He suggested that a 50-fold increase in ouabain concentration was required to cause 50% inhibition of Na^+, K^+-ATPase in the presence of strontium. The fixative, 3% paraformaldehyde used, also inhibits the K^+ - activated NPPase activity. It appears that the localization technique for ERNST (1972b) may be suitable only where a tissue is rich in Na^+, K^+-ATPase (ANSTEE and BOWLER, 1984). A modification of the ERNST (1972b) technique has been suggested by GUTH and ALBERS (1974). They recommended the use of dimethyl sulfoxide instead of strontium in the incubation medium. Here the Pi released is thought to be captured by Mg^{2+} and subsequently it is converted to a coloured precipitate by treatment with cobalt chloride (ERNST et al., 1980). However, this modification was found useful in light microscopy only and was successfully applied to kidney tubules where the staining was K^+-dependent and ouabain-sensitive (GUTH and ALBERS, 1974).

The present study is the first attempt to use ERNST (1972b) technique for localization of Na^+, K^+ - ATPase in Malpighian tubules of Locusta migratoria. In addition to the cytochemical experiments parallel comparative measurements of K^+-stimulated NPPase activity were also carried out, the purpose being to investigate the effect of fixation and histochemical incubation (20 mM $SrCl_2$, pH 9.0) on the K^+-dependent NPPase activity, and assess the validity of this technique in localizing Na^+, K^+-ATPase in Malpighian tubules.

MATERIALS AND METHODS

The procedure used to localize K^+-NPPase during the present study is essentially the same as that used by ERNST (1972a,b) in localization of K^+-dependent phosphate activity in the avian salt gland. Small modifications made to suit particular experiments will be mentioned in the text. Mature adult locusts of both sexes were used throughout these experiments.

6.1 Preparation of tissue for electron microscopic cytochemistry

The Malpighian tubules were dissected in a similar way as described previously in ice-cold (ca 4°C) paraformaldehyde in 0.1M cacodylate buffer pH 7.5 containing 0.25 m sucrose. The material was fixed in the same but fresh solution for 30 minutes at ca 4°C.

After paraformaldehyde fixation the tubules were incubated for 60 minutes at room temperature. But in view of the fact that ouabain inhibition is temperature-sensitive (ANSTEE and BOWLER, 1979; DONKIN and ANSTEE, 1980; present study), in one set of experiments, both the experimental and control tubules were incubated at 30°C instead of room temperature. The standard incubation medium was composed of : (final concentrations)

5 mM NPP

20 mM Sr_2Cl

10 mM KCl

100 mM Tris-HCl Buffer, pH 9.0

10 mM MgCl

Control incubation media

The standard medium was varied in three different ways:

(1) 10 mM KCl was replaced by 10 mM choline chloride.

(2) 5 mM NPP was replaced by 5 mM β-glycerophosphate

(3) 1 mM or 10 mM ouabain was added to the standard incubation medium.

Karnovsky's fixative:

Solution A

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

Solution B

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

Note: Solutions A and B are mixed in 1:1 ratio just before use.

Soft Araldite mixture for Malpighian tubules:

Araldite	10 ml
DDSA	10 ml
Dibutyl	2 ml
D.M.P. 30	1 ml

Post-incubation procedure

Following the 1 hour incubation period in the various media shown above, the tubules were rinsed in three changes of 0.1 Tris-HCl buffer, pH 9.0 and were treated with two 5 minutes rinses with 2% $Pb(NO_3)_2$. This latter step was necessary to convert the precipitated strontium phosphate to lead phosphate for visualisation in the electron microscope (ERNST, 1972b). His preliminary experiments had shown that strontium phosphate precipitates do not give enough density for easy viewing in transmission electron microscopy, and that strontium phosphate precipitates are soluble at neutral pH and in osmium solutions even when the post-fixation was carried out at alkaline pH.

After the lead nitrate solution, the tissue was washed in 0.25M sucrose to remove any free lead, rinsed in 0.1M Tris and cacodylate buffer, pH 7.5 and post-fixed for 30 minutes 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.5.

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

(1) Dehydration through a series of graded alcohols. 15 minutes in 70% alcohol, i.e. 3 changes at intervals of 5 minutes.

(2) 15 minutes in 95% alcohold with 3 changes, one after every 5 minutes.

(3) 15 minutes in Absolute alcohol, 3 changes.

(4) 20 minutes in Acetone, 3 changes.

(5) 1-2 hours in Acetone mixed with Araldite in 1:1 ratio at room temperature.

In other tissues propylene oxide is normally used at this stage, but for Malpighian tubules Acetone was preferred

to propylene oxide (see composition of soft Araldite mixture, page 190).

(6) 30 minutes in Araldite alone at 45°C.

(7) Embedded in fresh Araldite alone for polymerisation at 45°C overnight.

(8) Preparation moved from 45°C to 60°C for a further 48 hours.

Dilver/gold sections were cut on a Reichart NK ultratome using glass knives. The sections were 'expanded' with Diethyl ether vapour and mounted on uncoated copper grids (3 mm in diameter). The sections were stained in uranyl acetate and lead citrate (REYNOLDS, 1963) and were examined in an AEI 801 electron microscope.

6.2 Biochemical Assay of K^+-NPPase activity

In addition to the cytochemical experiments, comparative biochemical measurements of K^+-NPPase activity were carried out on both fixed and unfixed material. The purpose of the biochemical experiments was to investigate the effects of fixation and incubation in the histochemical reaction medium on the activity of K^+-NPPase in Malpighian tubule preparations of Locusta.

Malpighian tubules of sexually mature adult locusts were used. The tubules were isolated under ice-cold (ca 4°C) 200 mM sucrose solution containing 5 mM Tris-HCl buffer at either pH 7.2 or 9.0. The tubules were washed in six changes of the same solution. The material was then dried on a filter paper. Malpighian tubules from equal numbers of locusts were used to study enzyme activity in fixed and unfixed tissue. This would minimise delays in handling the material.

The unfixed portion was then homogenized in about 10 mls of the isolation medium. The second portion was fixed with 3%

paraformaldehyde in 100 mM cacodylate buffer and 0.25 mM sucrose in the same way as in the cytochemical procedure. The fixed portion was subsequently washed in 3 changes of Tris-HCl buffer and homogenised in the isolation medium.

Samples of both homogenates were added to the various incubation media in a 1:1 ratio. The incubation media were prepared at twice the final concentration shown below.

Biochemical incubation media

These were basically the same as the cytochemical incubation media but without the strontium and at both pH 7.2 and pH 9.0.

Composition of the final concentration of the biochemical media was:

(1) Standard incubation medium.

(a) 5 mM NPP
10 mM KCl
10 mM Mgcl
100 mM sucrose
100 mM Tris-HCl pH 7.2 or 9.0

Control biochemical incubation media:

(b) plus 5 mM ouabain

(c) The same as the standard incubation medium (a) but excluding 10 mM KCl.

(2) Histological Incubation media.

(a) The same as the standard incubation medium (a) plus 20 mM $SrCl_2$.

(b) The same as standard medium (a) plus 5 mM ouabain and 20 mM $SrCl_2$.

(c) The same as standard incubation medium excluding 10 mM KCl and adding 20 mM $SrCl_2$.

All media control, and experimental were made at twice the final concentrations given above.

Experimental procedure

(1) 1 ml reaction medium plus 1 ml of the homogenate were incubated for 30 minutes at 30°C. A 'Blank' was set up for each reaction medium.

(2) The reaction was stopped by the addition of 2 mls of ice-cold (ca 4°C) 20% Trichloro-acetic acid (T.C.A.).

(3) 3 mls of 2M Tris solution were added to each preparation.

(4) The tubes were allowed to stand at room temperature for 10 minutes for the yellow colour to develop and kept on ice before centrifugation.

(5) The preparations were centrifuged at 1,000g for 15 minutes at 0°C.

(6) The tubes were returned to ice and using the supernatant from each tube, the extinction was measured spectrophotometrically at 420 nm.

A standard p-nitrophenol calibration curve (Figure 6.1) was prepared and the protein concentrations were determined according to the method of LOWRY et al., (1951). The results were calculated and expressed as the amount of p-nitrophenol released in nmoles/mg protein/min.

6.3 p-nitrophenol standard calibration curve

Solutions of varying p-nitrophenol concentration were prepared by a serial dilution of stock of 2 mM p-nitrophenol (NP) to give 0, 0.05 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 mM (NP). To 2 mls of each solution, 2 ml of 20% T.C.A. (trichloro-acetic acid) was added. Then 3 ml of a 2M Tris solution was added to each mixture. The tubes were allowed to stand for 10 minutes at room temperature for the yellow colour to develop. The amount of NP in each tube was measured photospectrometrically at 420 nm.

As standard calibration curve was prepared by plotting the amount of NP in nmoles/ml against absorbance (Figure 6.1).

FIGURE 6.1

Standard curve for p-nitrophenol concentration against absorbancy

Ordinate: Optical density 420 nm

Abscissa: p-nitrophenol concentrations in nmoles

Line fitted by eye

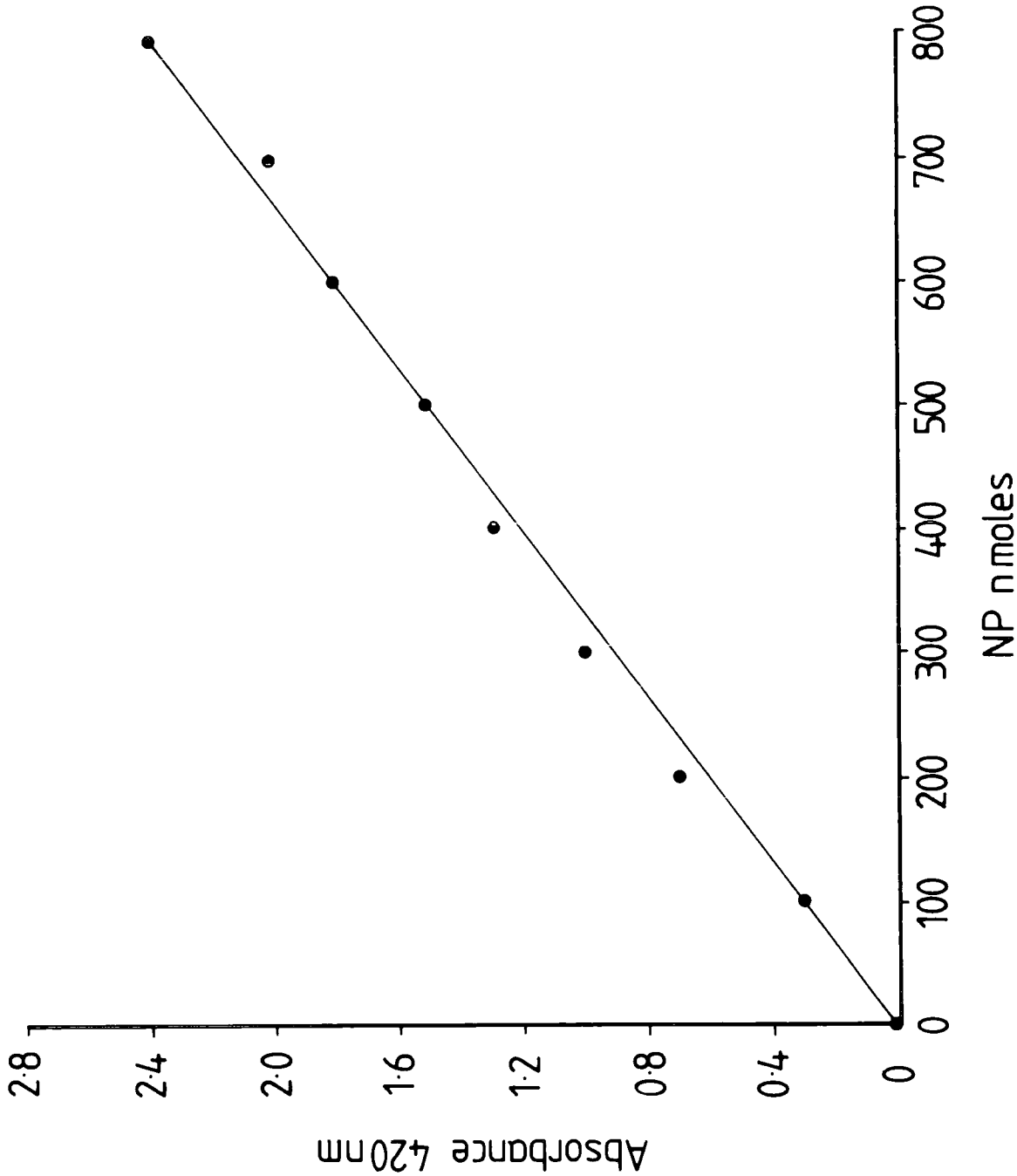

Absorbance 420nm
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
0
100
200
300
400
500
600
700
800
NP n moles

Fig. 6.1

RESULTS

Like other secretory epithelia, it is seen that the cells of the Malpighian tubule, primary cells in particular, are characterised by extensive infoldings of the basal cell membrane which form complex intracellular and extracellular compartments (see Plate 1). The preservation of the fine structure during the cytochemical NPPase localization experiments was on some occasions unsatisfactory, a problem encountered by other workers too (e.g. KOMNICK and ACHENBACH, 1979).

However, as the fine ultrastructure of Malpighian tubules of Locusta is already well documented (see MARTOJA, 1959, 1960; PEACOCK, 1975; CHARNLEY, 1975; BELL and ANSTEE, 1977; BELL, 1977; DONKIN, 1981) it will not be dealt with further in the present study.

6.4 K^+-NPPase cytochemistry

Preliminary studies showed that incubation of the tubules in 'normal' standard incubation medium at either 30°C or room temperature gave similar results. Therefore, for the purposes of the ouabain control experiments, all experiments were carried out at 30°C.

(a) Localization of NPPase activity

The general distribution of the reaction product is shown in Plate I. It is seen that even at low magnification, it is clear that the precipitate is restricted almost exclusively to the plasma membrane which as previously described, forms the extended basal compartments of the primary cell of the Malpighian tubule of Locusta. The basement membrane, the nucleus and mitochondria are clearly free of deposits. There are some deposits in the microvilli in the apical region but precipitates are totally absent from the lumen.

CYTOCHEMISTRY

Standard incubation medium (Plates 1-3)

Localization of NPPase activity in Locusta migratoria Malpighian tubule epithelium. Paraformaldehyde-fixed Malpighian tubules were incubated, unless otherwise noted, in the standard cytochemical medium (5 mM NPP, 20 mM $SrCl_2$, 10 mM KCl, 10 mM $MgCl_2$, 10 mM Tris/HCl buffer, pH 9.0) at 30°C for 60 minutes, rinsed briefly in 0.1M Tris/HCl buffer, pH 9.0, and then treated with 2% $Pb(NO_3)_2$ to convert precipiated SrPi to electron dense PbPi. The tissue was subsequently washed in 0.25M sucrose solution to remove any free lead, then rinsed in 0.1M Tris and cacodylate buffer, pH 7.5, post-fixed in 1% osmium in 0.1M cacodylate, pH 7.5, dehydrated in a series of ethanols and embedded for electron microscopy as described in the text.

All sections were stained with uranyl acetate and lead citrate.

Note: all ultrastructural features referred to in these cytochemistry results are described by BELL and ANSTEE, 1977; DONKIN, 1981.

PLATE 1

A high power TS through the basal region showing clearly that the deposits are on the intracellular face of the basal plasma membrane infoldings (Arrows). There are no apparent precipitates in the mitochondria and the surrounding cytoplasmic area. The basement membrane (BM) is also free from precipitates.

Scale: Plate 1 = 0.25 μm

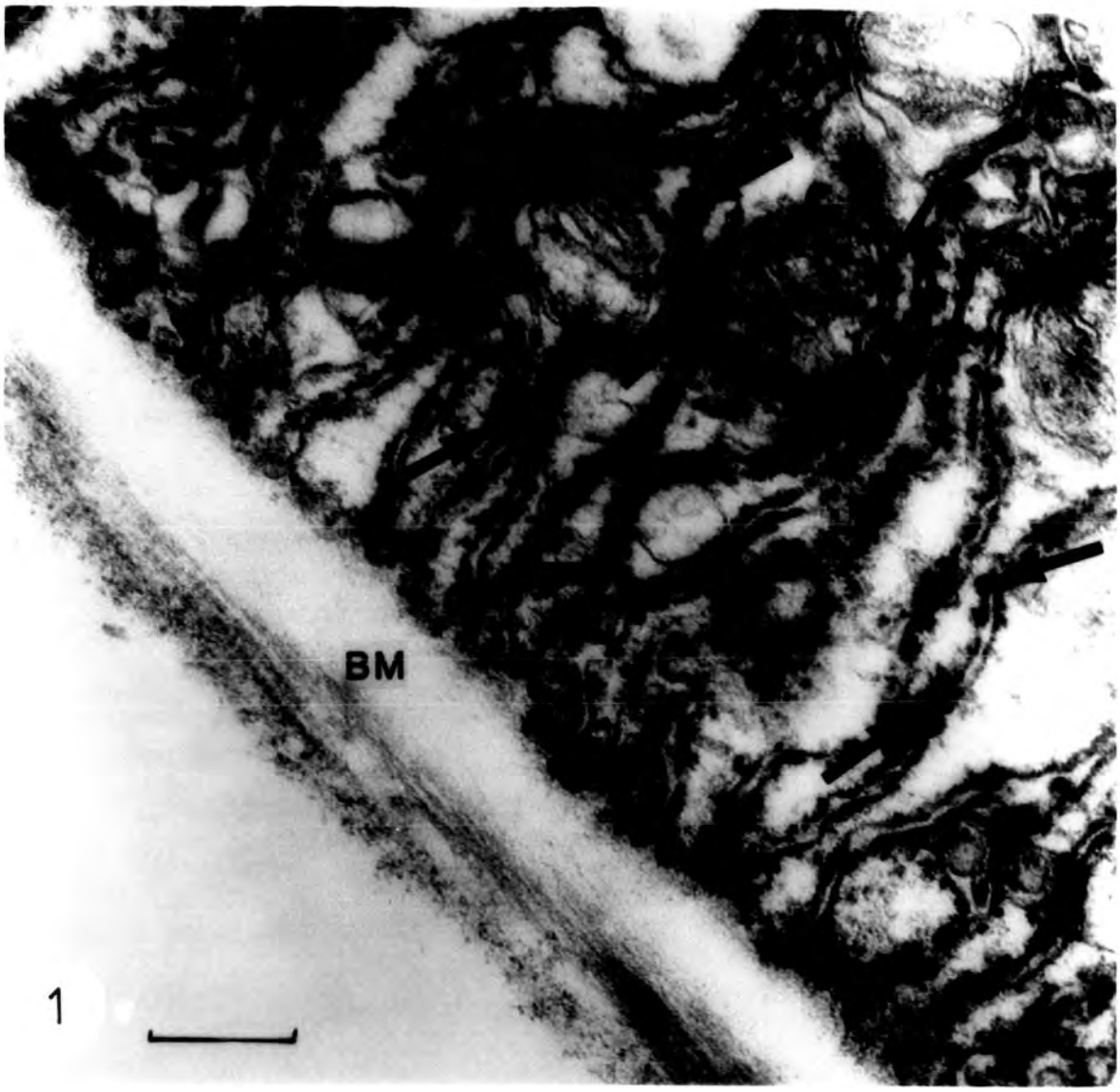

BM
1

PLATE 2

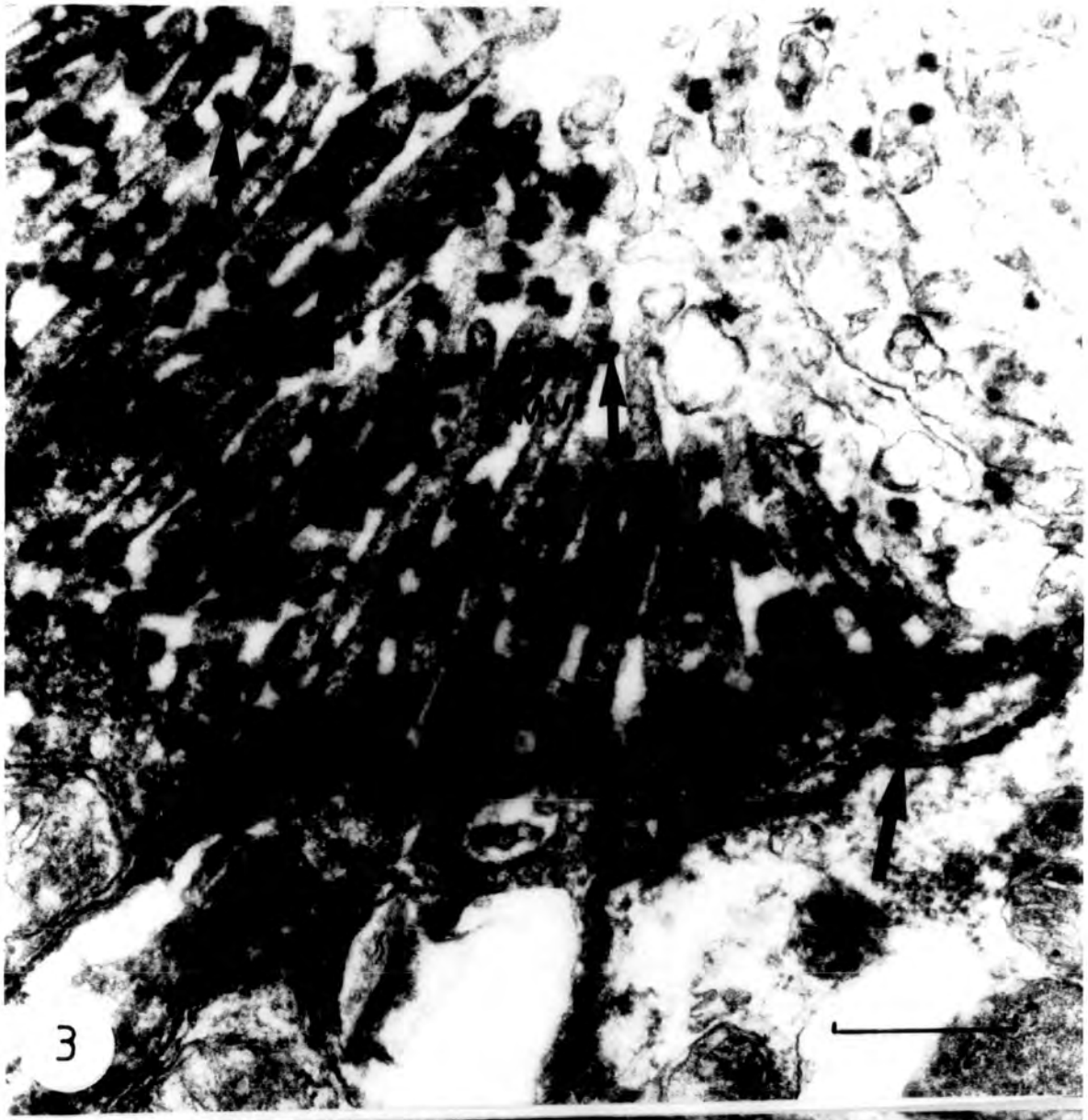
High magnification micrograph showing part of the intercellular space filled with dense precipitates. The surrounding cytoplasmic area is free from precipitates.

Scale = 0.25 μm.

PLATE 3

A high power magnification micrograph showing a TS through the apical region of the cell. A substantial amount of deposits are seen on the apical cell membrane and along the outer membranes of the microvilli. Some deposits are also seen in association with the septate desmosome (SD).

Scale = 0.5μm.



3

2

Close examination of a higher magnification of the basal region of the primary cell in Plate I shows that the deposits are located on the inner surface or cytoplasmic side of the plasma membrane in some areas the deposits are so dense that they occlude portions of the intracellular compartments where the cytoplasmic 'leaflets' are quite narrow. The surrounding cytoplasmic areas are, otherwise free of deposits. As previously mentioned, Plate I also shows that mitochondria, too, which are closely associated with the plasma membrane infoldings have no deposits. In some regions, the intercellular spaces are filled with reaction products (Plate 2).

A high magnification electron micrograph of the apical region (Plate 3) shows a substantial amount of deposits around the microvilli. However, although the deposits are in close association with the microvilli, they appear to be only on the outside walls of the microvilli. On some occasions it was difficult to distinguish between artefactual deposits and the reaction product. Overstaining of the apical membranes is known to occur due to long incubation periods (30 min-1 hour) (ERNST, 1975).

The results described in Plate I is a typical example of results obtained from three independent determinations which indicates the consistency in the distribution pattern of the reaction product when tubules are incubated in the 'normal' incubation medium.

(b) Control Experiments

The purpose of these control experiments was to determine the subcellular site of K^+-NPPase activity under kinetically defined conditions (ERNST 1972a,b) and also examine the effect of variations in these kinetic parameters on the deposition of the reaction

product. Since results discussed in Chapter 3 show that Na^+,K^+-ATPase activity in microsomal preparations of Locusta Malpighian tubules was inhibited by ouabain, it was also important to show, cytochemically, the sensitivity of K^+-NPPase to ouabain and the dependence of this enzyme on its activating cations, K^+, in particular.

(i) Effect of Ouabain

When incubation was carried out at room temperature, the addition of 1 mM ouabain to the standard incubation medium did not affect the amount and density of the reaction product deposited. The distribution and density of the reaction product was the same as that observed in the absence of ouabain. When the tubules were incubated at 30°C in the presence of 1 mM ouabain, there was a marked reduction in the amount of reaction product deposited. However, the results were on a number of occasions, inconsistent. Plate 4 shows a marked reduction in the density of deposits along the basal cell membrane infoldings (when Plate 4 is compared with Plate 5 (control). It is again clear that the deposits are confined to the cytoplasmic side of the lateral and basal cell membrane infoldings. The cytoplasmic areas between the infoldings, the vacuoles and mitochondria are free of the reaction product.

In the presence of 1 mM ouabain at 30°C, the amount of deposits in the apical region was also markedly reduced. There are fewer deposits seen in the microvilli (Plate 6) than those seen in the absence of ouabain (Plate 3).

In another set of results, (Plate 7) shows a total inhibition of the reaction product by 1 mM ouabain when tubules were incubated at 30°C (compare Plate 7 and Plate 8) (control). Yet in another,

Effect of Ouabain (Plates 4, 6, 7, 9)

A series of electron micrographs showing varying degrees of reduction of the reaction produce when Malpighian tubules were incubated in the standard incubation medium containing either 1 mM ouabainor 10 mM ouabain at 30°C.

PLATE 4

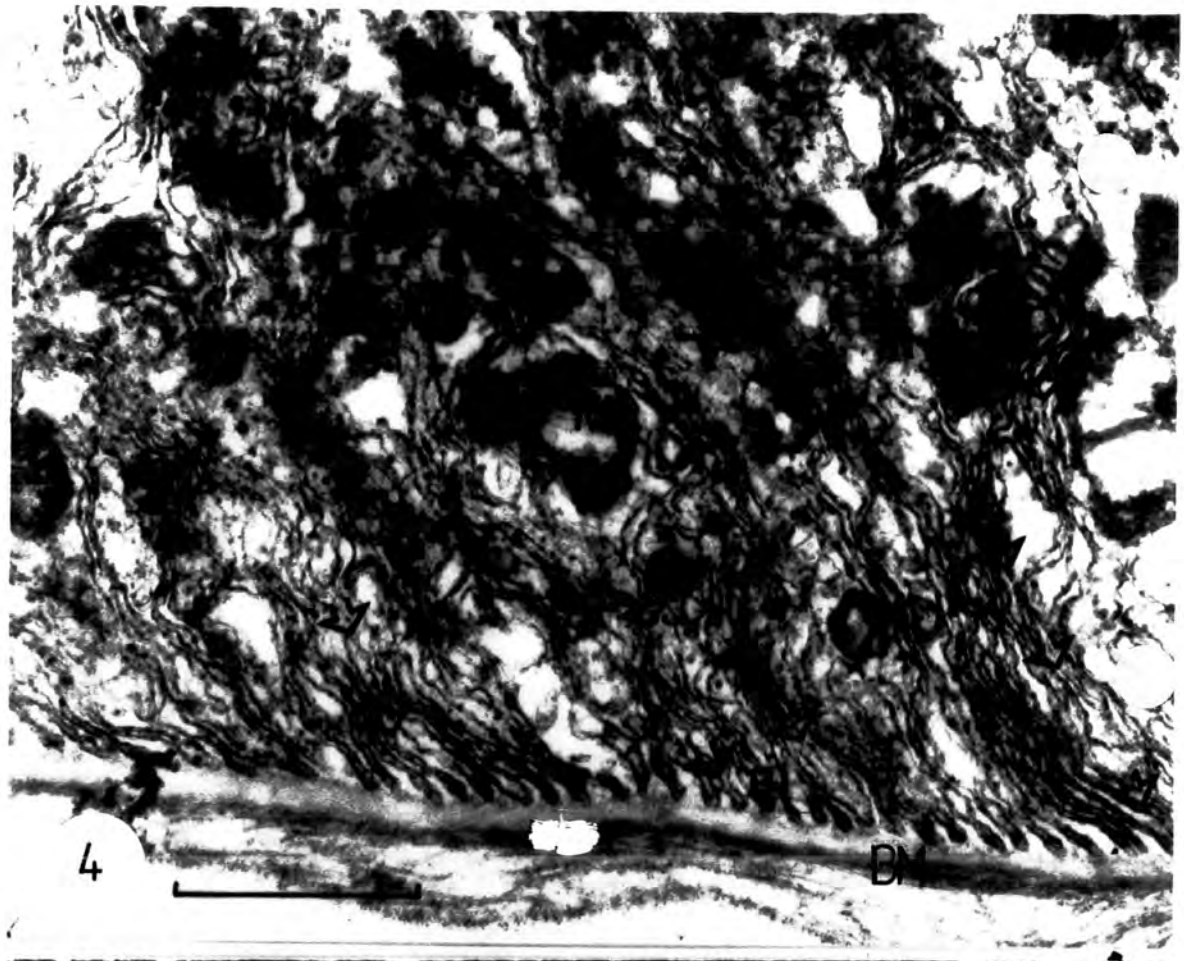
Basal region of a primary cell showing that membrane staining along the basal infoldings (open arrows) is greatly reduced in the presence of 1 mM ouabain. Compare Plate 4 with Plate 5 (control). In Plate 4 the sparse precipitates remaining are on the upper parts of the infoldings extending through the intermediate region (arrows).

Scale : Plate 4 = 1 µm.

PLATE 5

Control (i.e. in standard incubation medium without ouabain as previously described see Plate 1)

Scale : Plate 5 = 1 µm.


4
BM

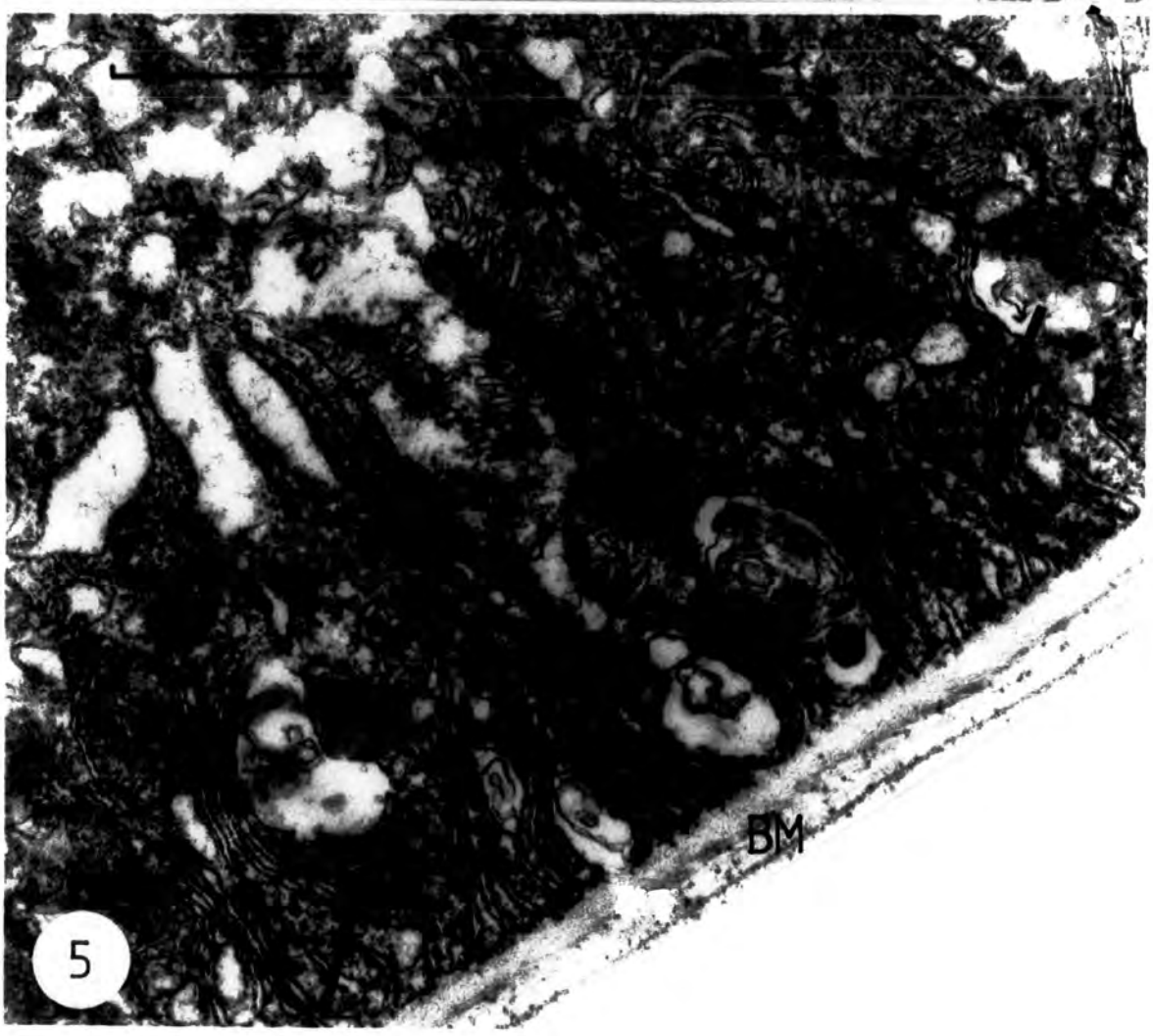

BM
5

PLATE 6

Electron micrograph showing very few precipitates in the microvilli when 1 mM ouabain is added to the standard incubation medium and incubation carried out at 30°C (arrows).

The mitochondria (M) associated with the microvilli (Mv) are also free from precipitates. The Lumen (L) too has no precipitates.

Scale = 1 μm.

Compare Plate 6 to Plate 3 as a control.


L
6

PLATE 7

Electron micrograph showing that the addition of 1mM ouabain to the standard medium, at 30°C completely abolished the reaction product deposition along at basal infoldings of the plasma cell membrane where they are heavily deposited in the absence of ouabain (Plate 8). The latter is reproduced here for quick comparison.

Bl basal infoldings

BM basement membrane

M mitochondria

Va vacuoles

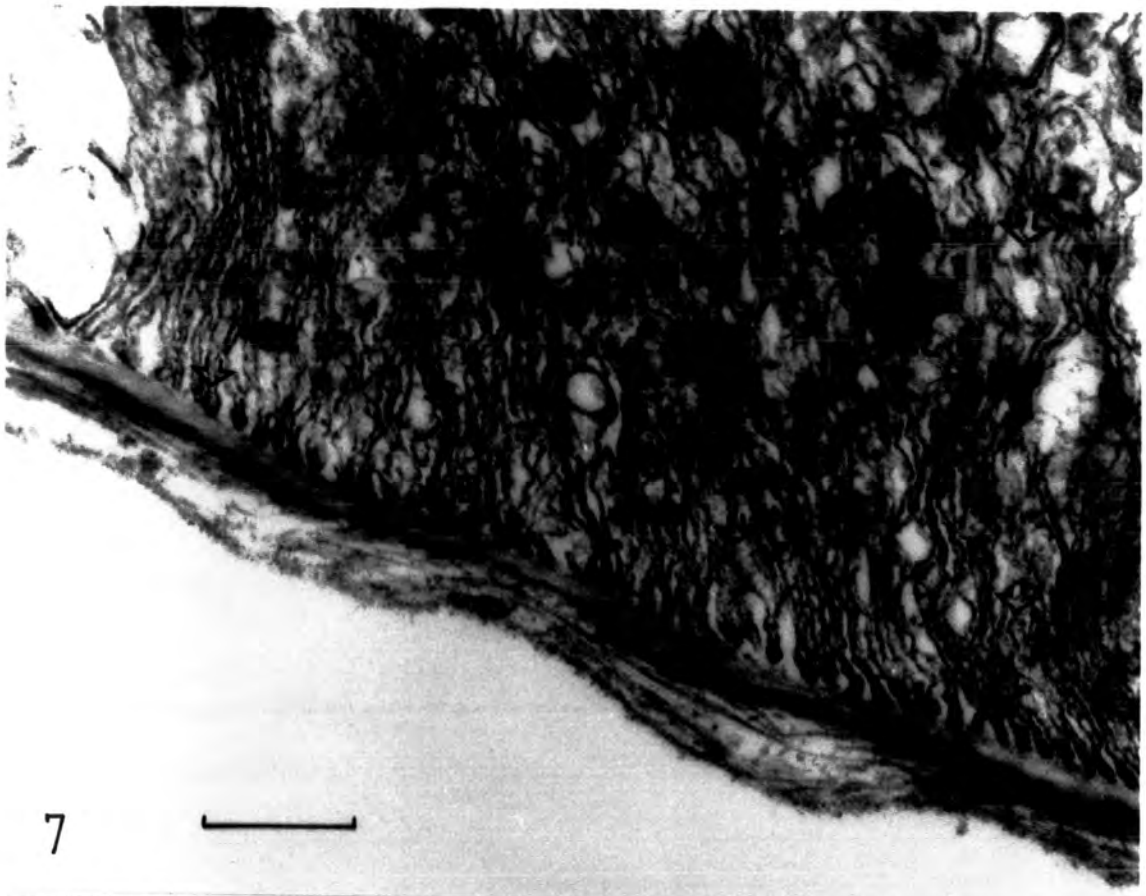
Scale: Plate 7 = 1 µm.

PLATE 8

Control : as previously described (see Plate 1)

Incubation in standard incubation medium in the absence of ouabain.

Scale: Plate 8 = 1 µm.



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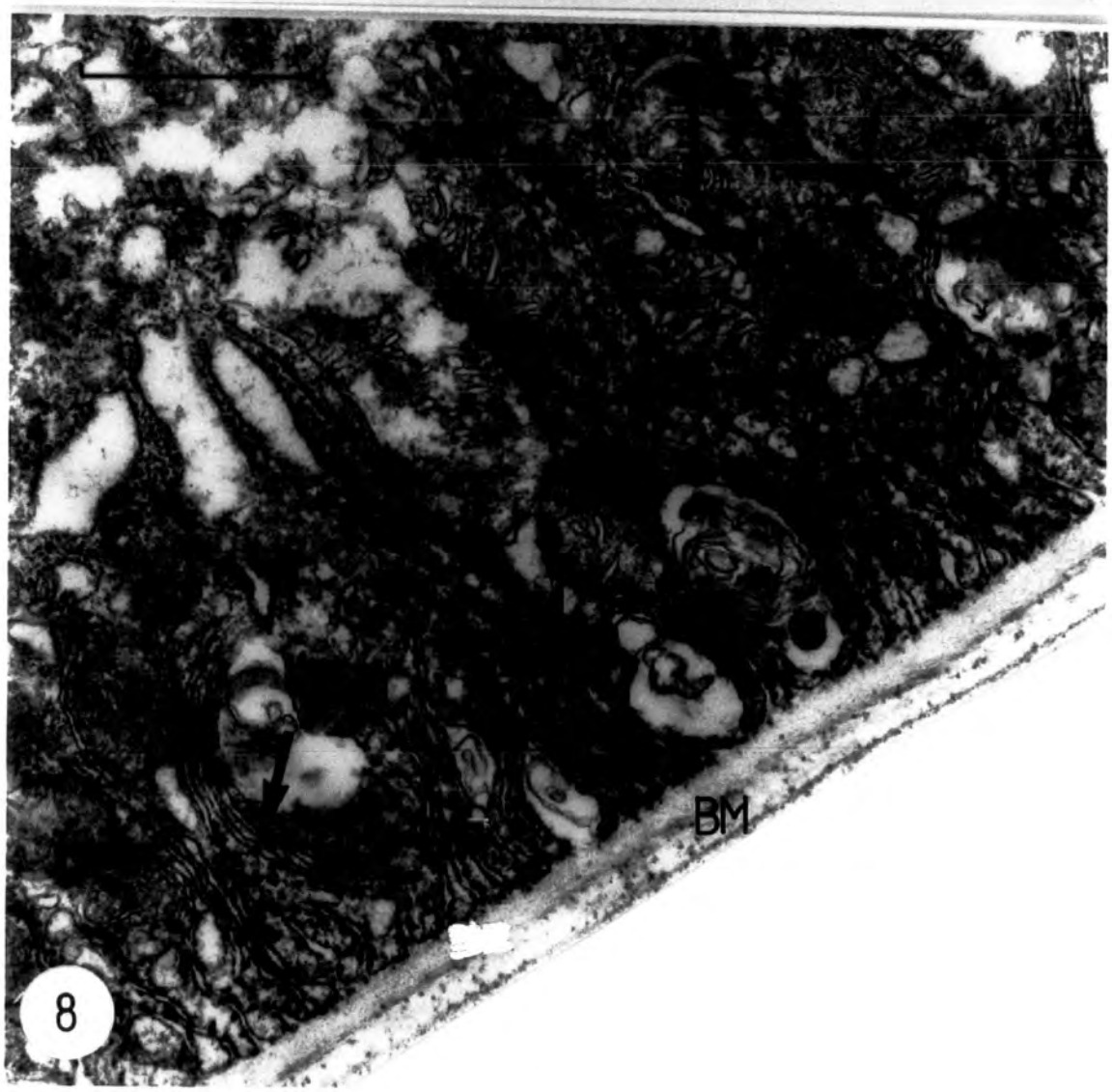


8

PLATE 9

High power magnification electron micrograph showing a TS through the basal region. This image shows a total abolition of the reaction product when 10 mM ouabain was added to the standard incubation medium and incubation carried out at 30°C.

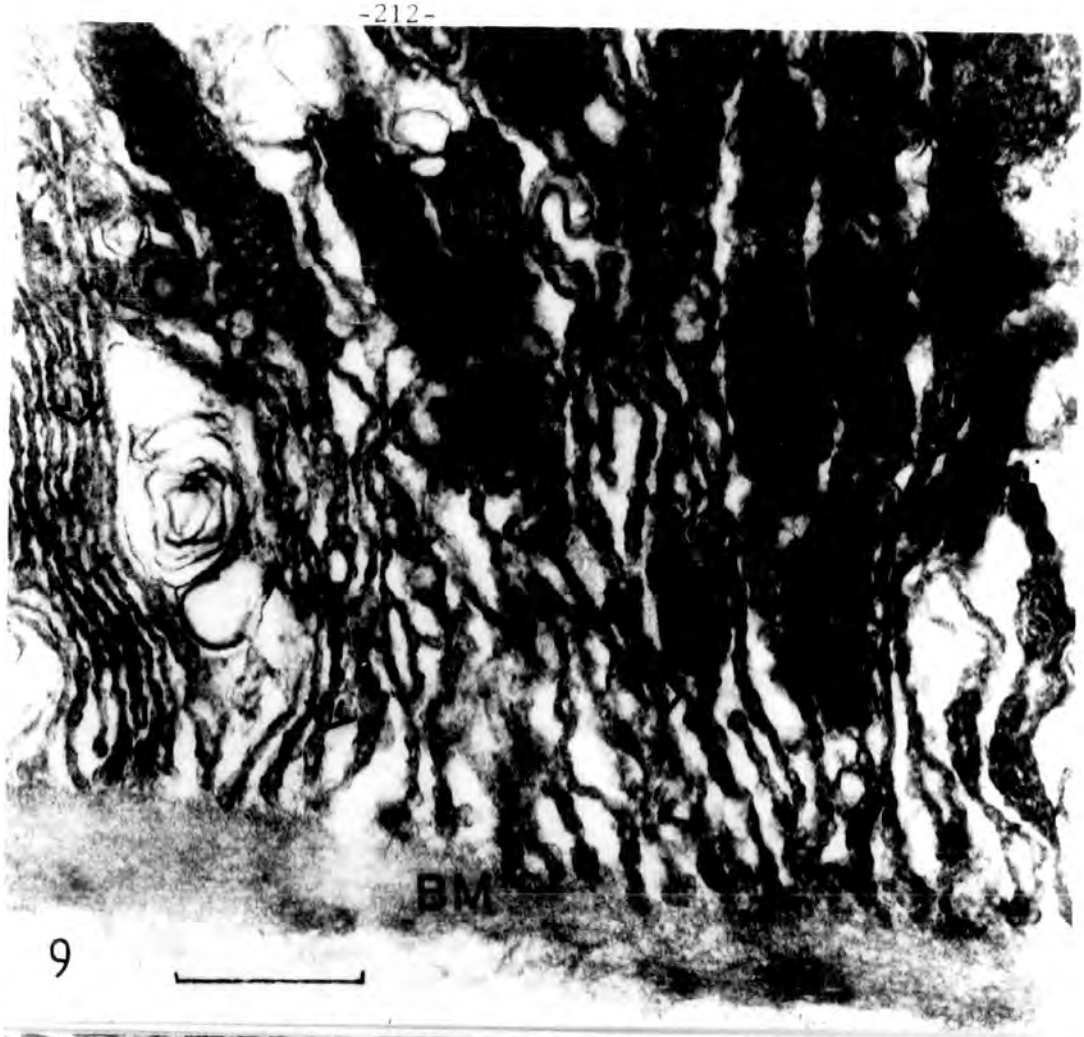
The basement membrane (BM) the basal cell membrane infoldings (Bl), the mitochondria (M) and the surrounding cytoplasmic area are free from precipitates (compare to Plate 10 (control)).

Scale = 0.5 µm.

Plate 10 Control.

Incubation in the standard incubation medium without ouabain, as previously described (see Plate I).

Scale = 0.5 µm.



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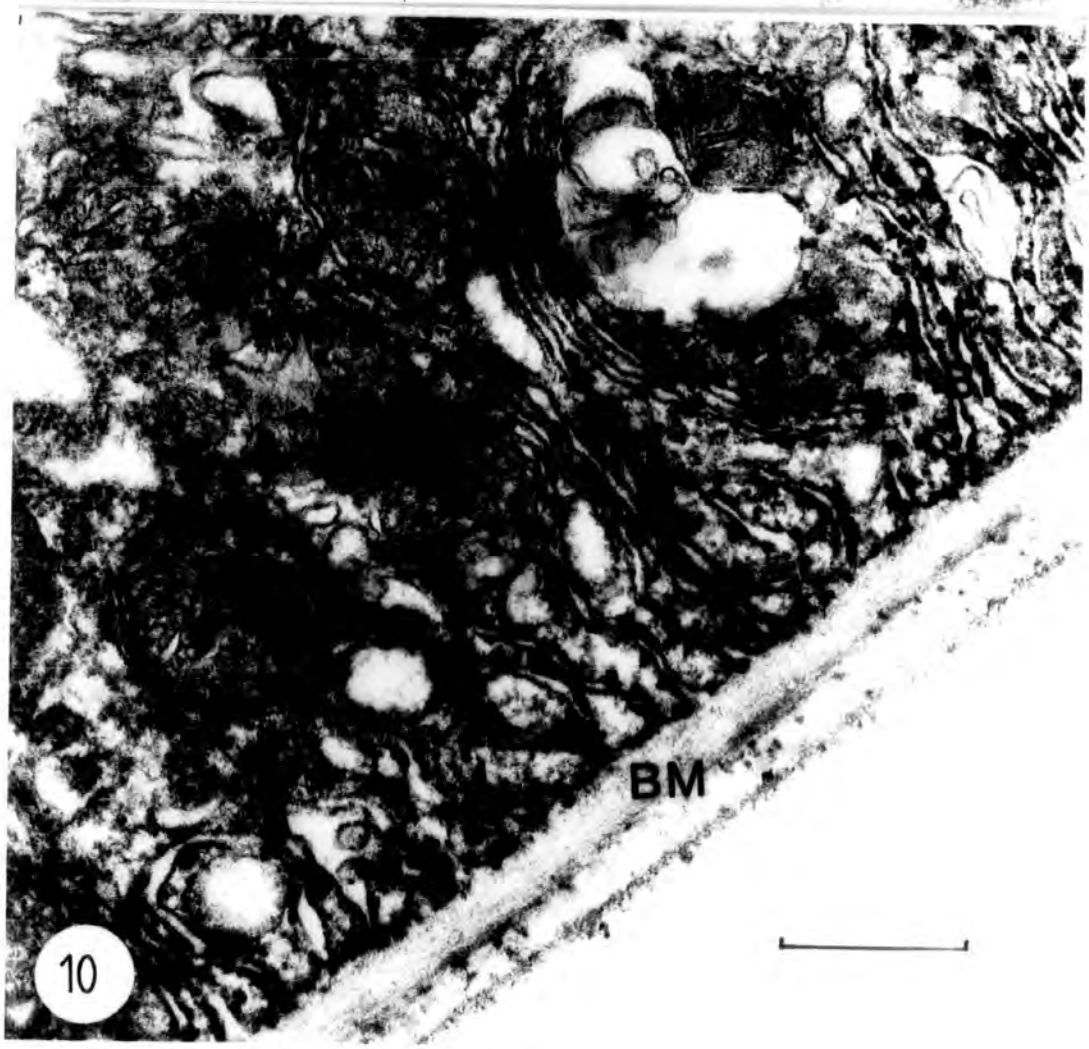


10

similar, control experiment, there was no marked reduction of the amount of reaction product deposited in the presence of 1 mM ouabain at 30°C (result not shown).

The more decisive results with ouabain as a control, were obtained when a higher concentration (10 mM) ouabain was added to the standard incubation medium. It was found that on two independent determinations, the same results shown in Plate 9 were obtained. There is a total absence of reaction product (Plate 36) even along the basal cell membrane infoldings where they are so heavily deposited in the absence of ouabain (Plate 10). In the presence of 10 mM ouabain the apical region had a few fine deposits which could either be due to K^+-independent, ouabain-insensitive NNPase activity or to over-staining caused by long incubation periods (ERNST 1975). The ultrastructure of the Malpighian tubule was not well preserved in the presence of 10 mM ouabain (Plate 9).

An attempt to increase ouabain inhibition, by modifying the technique, was unsatisfactory. In one set of experiments, the tubules were pre-incubated in 10 mM ouabain before fixation and later incubated again in the standard incubation containing 10 mM. The fine cell structure was poorly preserved after this treatment and deposits were seen along the basal cell membrane infoldings and all other regions of the cell except the mitochondria.

(ii) Effect of K^+

When choline chloride was substituted for potassium chloride in the incubation medium, there was a small reduction in the amount of reaction product deposited along the basal infoldings of the cell membrane. Results shown in Plates 11 and 12 show that the deposits in the absence of K^+ were not as dense as those in the standard

Effect of K^+ (Plates 11, 12, 14)

Electron micrographs showing a small reduction in the amount of reaction product deposited following an hour incubation period in a standard incubation medium in which 10 mM KCl were replaced by 10 mM choline chloride.

PLATE 11

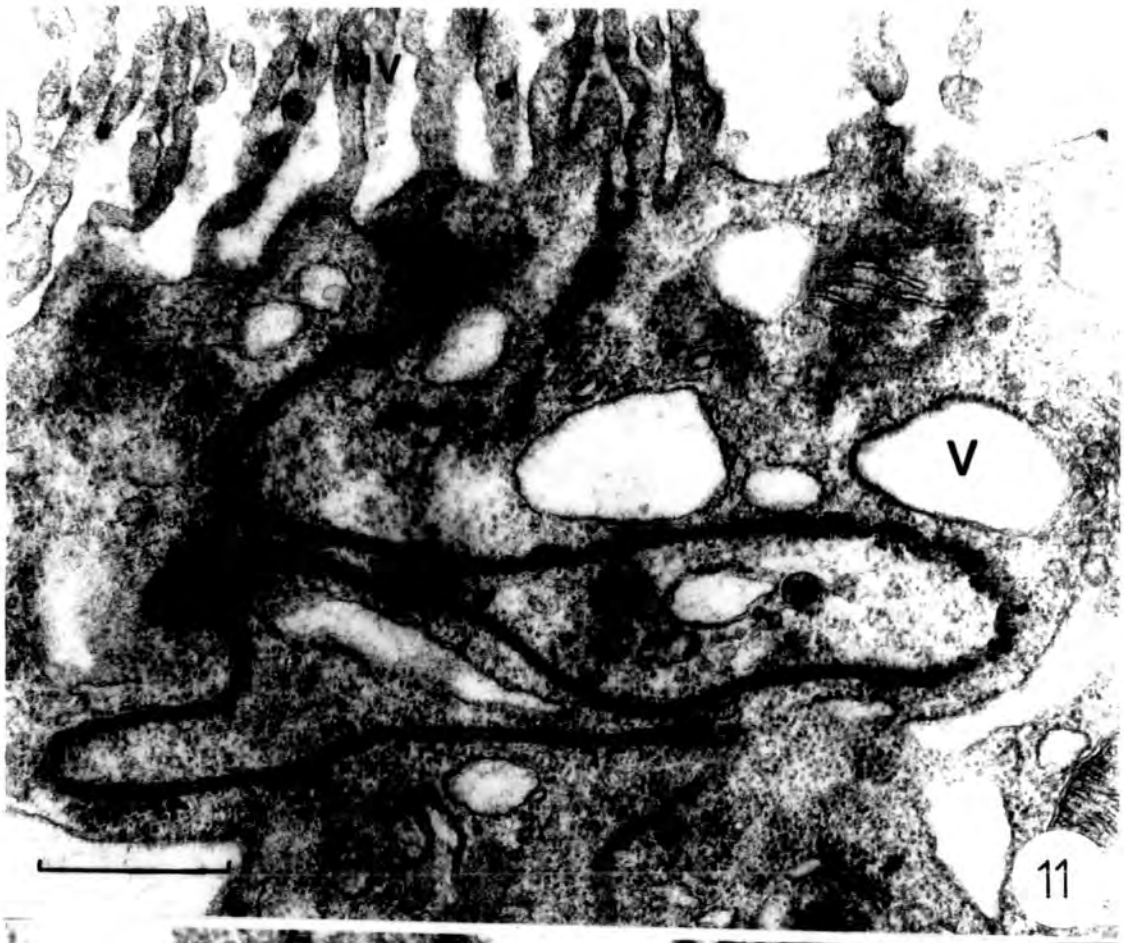
A high power magnification electron micrograph of the basal cell region showing a small reduction in some areas of the basal cell membrane infoldings (see arrows) Compare to Plate I.

Scale = 1 μm.

PLATE 12

Electron micrograph showing parts of an intercellular channel (open arrows) without precipitates. Compare Plate 12 to Plate 2. (M) mitochondira (v) vacuole (BM) Basement membrane (CE) Extracellular spaces.

Scale = 0.5 μm.


V
V
11

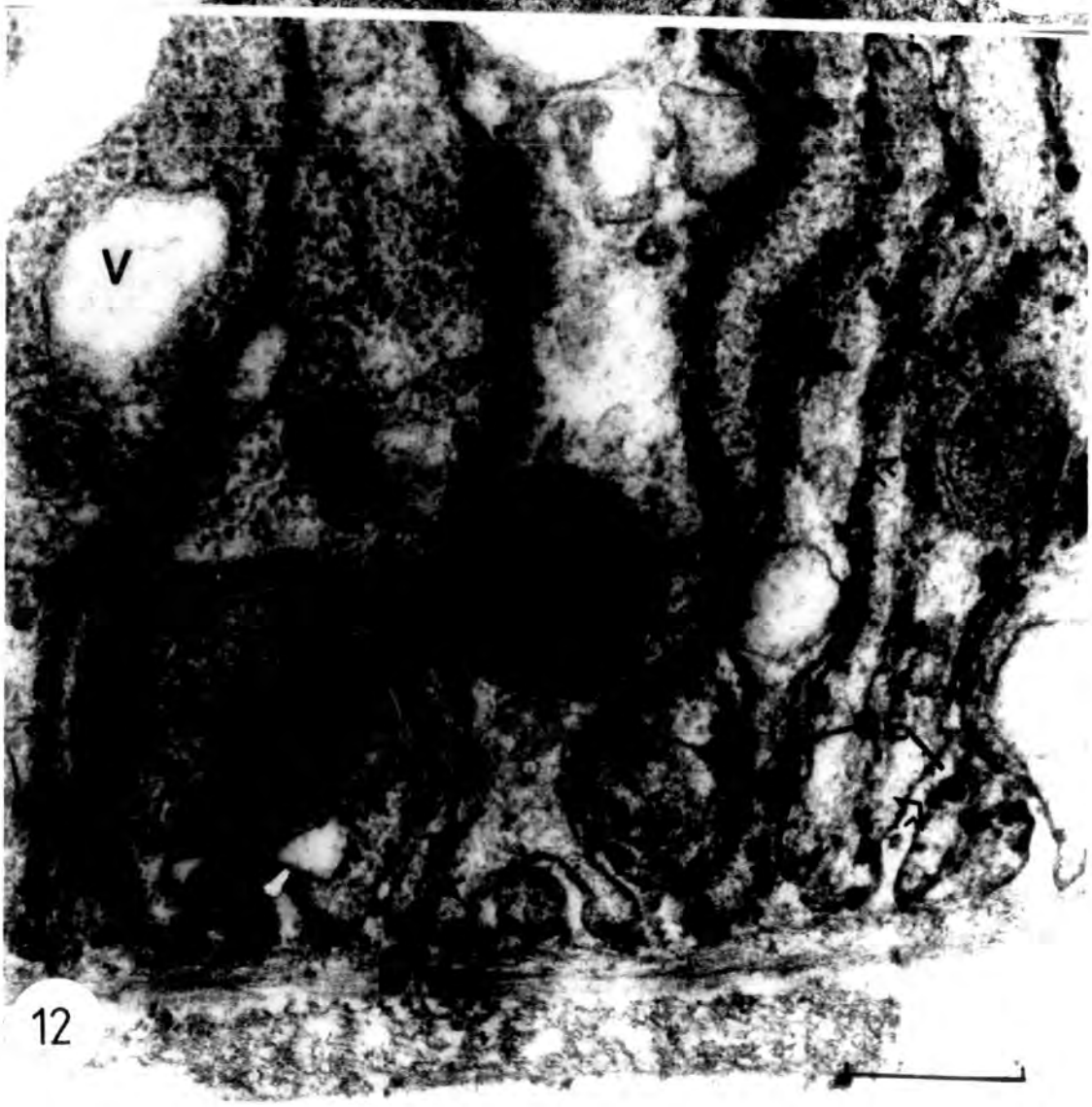

V
12

PLATE 13

Choline chloride substituted for KCl in the standard incubation medium. Micrograph showing a total absence of the reaction product deposits in the basal cell membrane infoldings (Bl) (see open arrows).
(compare to Plate 14).

Scale = 0.5 µm.

PLATE 14 (as control)

Localization of NPPase in standard incubation medium. As previously described deposits are exclusively along the basal infoldings of the cell plasma membrane (Bl) (solid arrows). No deposits in the mitochondria (M).

Scale = 0.5 µm.


13
14
M

solution. (When Plates 11 and 12 are compared with Plates I and 2). High magnifications of the basal region (shown in Plates 11 and 12), show a small reduction in deposits (when Plates 11 and 12 are compared with Plates I and 2). On this occasion, there was a total absence of deposits in the microvilli region.

In another separate experiment, the absence of K^+ in the incubation medium, totally abolished the deposition of the reaction product (Plate 13 compared with Plate 14).

(iii) Effect of the Substrate NPP

The K^+-independent, ouabain-insensitive alkaline phosphatase activity was selectively demonstrated by substituting β-glycerophosphate for NPP, as β-glycerophosphate is not a substrate for the K^+-dependent phosphate (ERNST 1972b, 1975).

Except for a few focal deposits, the results shown in Plate 15 show a total absence of the reaction product when Plate 15 is compared to Plate 16 (control). Similarly, Plate 17 shows that there were also no deposits around the microvilli when Plate 17 is compared with Plate 18. It should be noted that the substitution of β-glycerophosphate for NPP was the only control where results were consistent throughout the present study.

Effect of NPP (Plates 15,17)

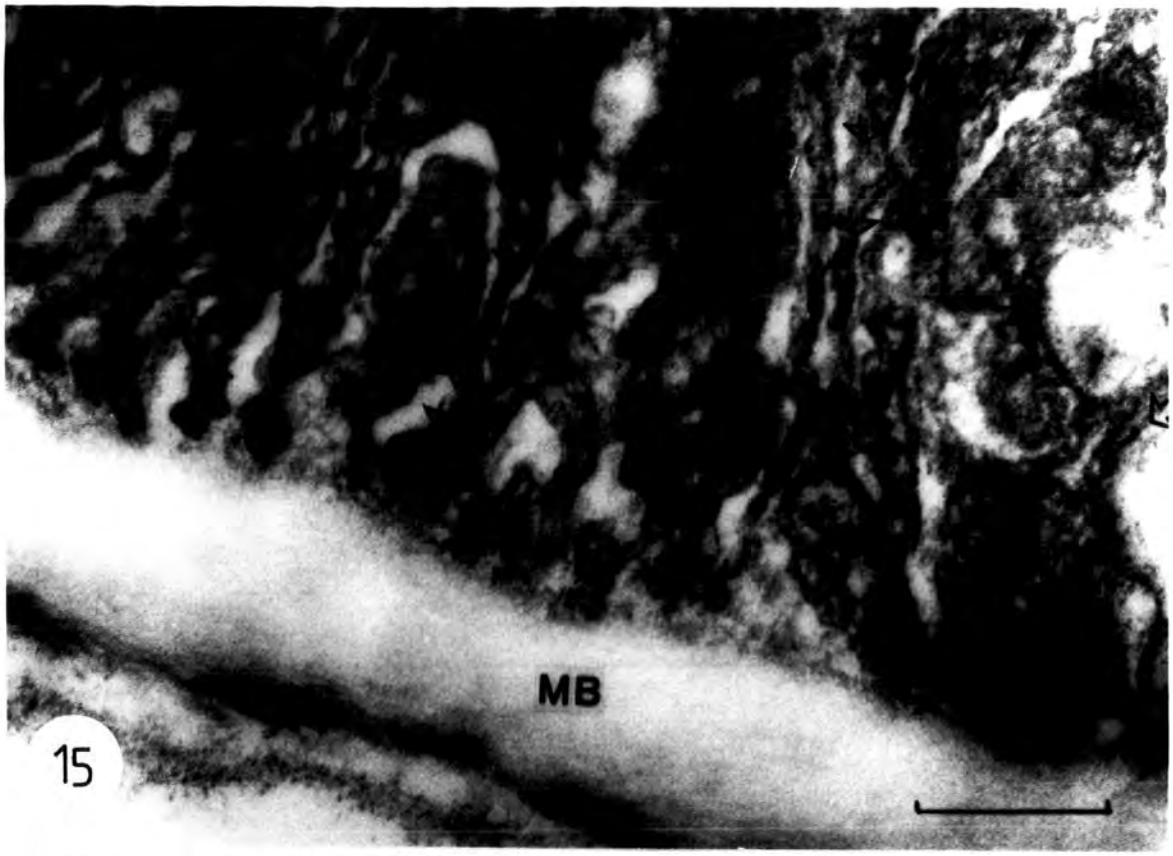
Electron micrographs showing results obtained when 5 mM NPP in the standard incubation medium was replaced by 5 mM β-glycerophosphate. Results shown here are representative of results obtained on 3 separate occasions.

Plate 15 shows a total absence of the reaction product in the basal cell membrane infoldings of the Malpighian tubule primary cell (see open arrows) and compare to Plate 16 (control). (BM) Basement membrane.

Scale = 0.5 μm.

Plate 16 Control i.e. incubation in normal standard incubation medium as described previously (see Plate 1).

Scale = 0.25 μm.


MB
15

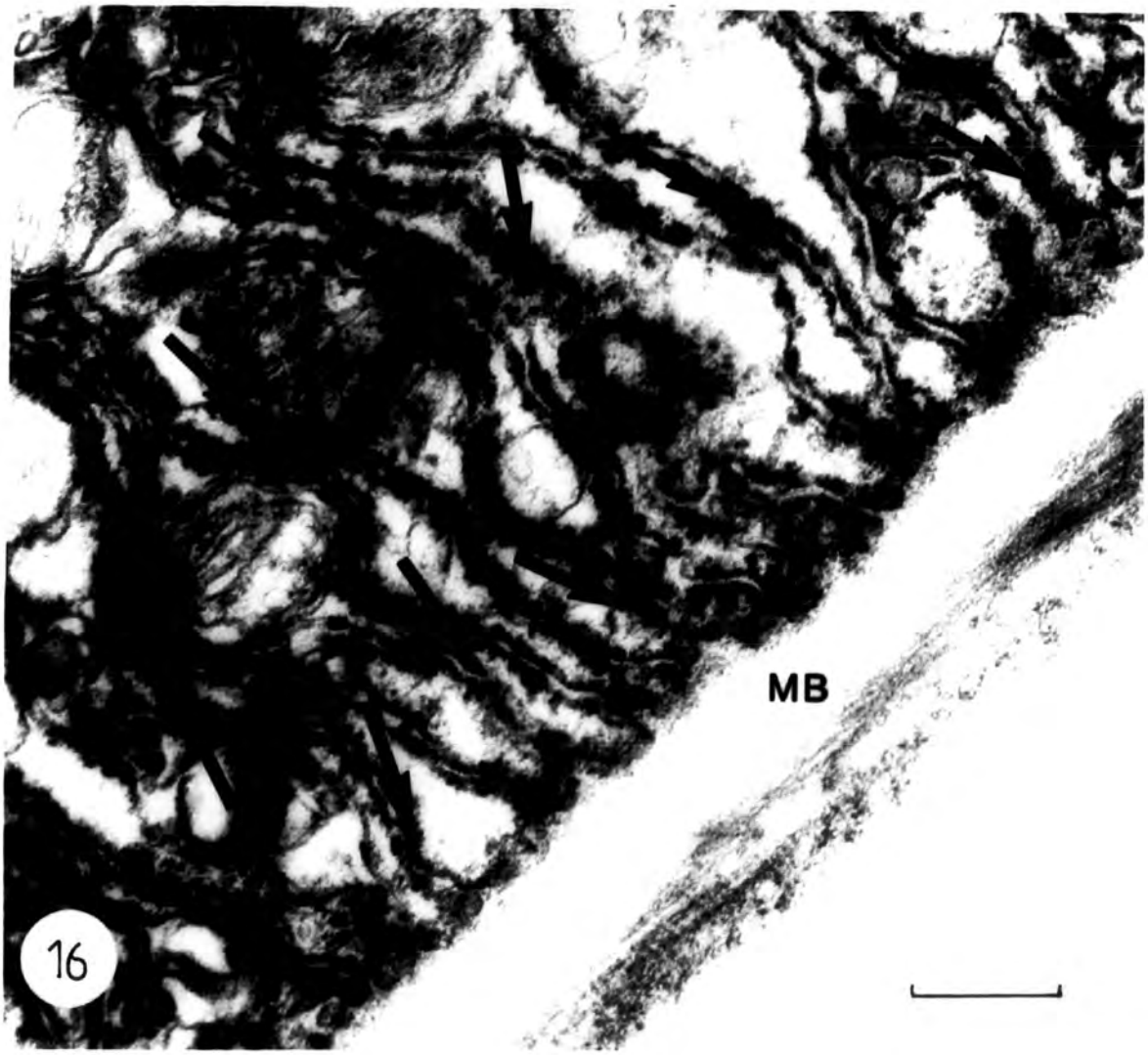

MB
16

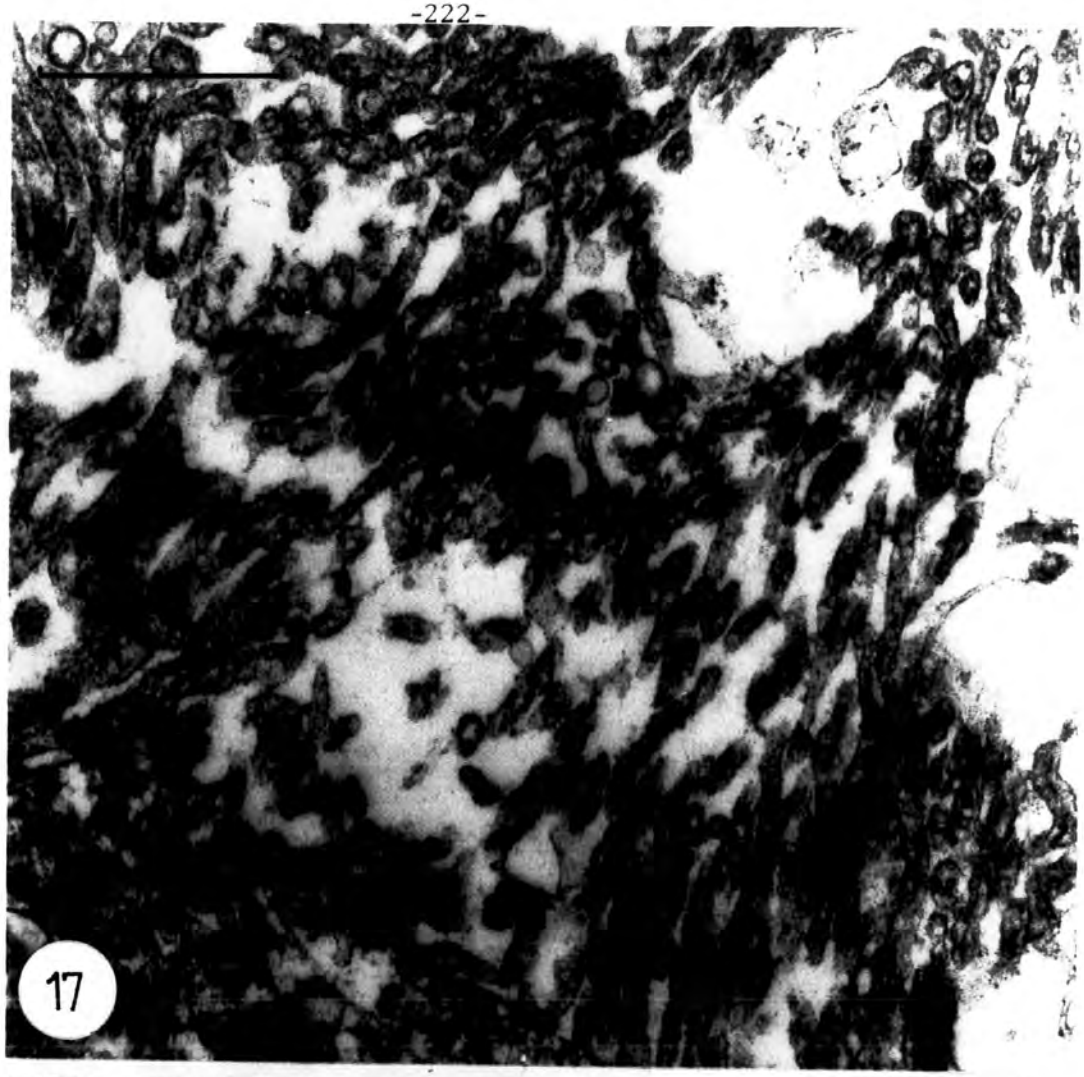
PLATE 17

High power magnification of TS through the apical region of a primary cell showing the absence of precipitates around the microvilli, (Mv) when β-glycerophosphate was substituted for NPP in the standard incubation medium. Compare Plate 17 to Plate 18.

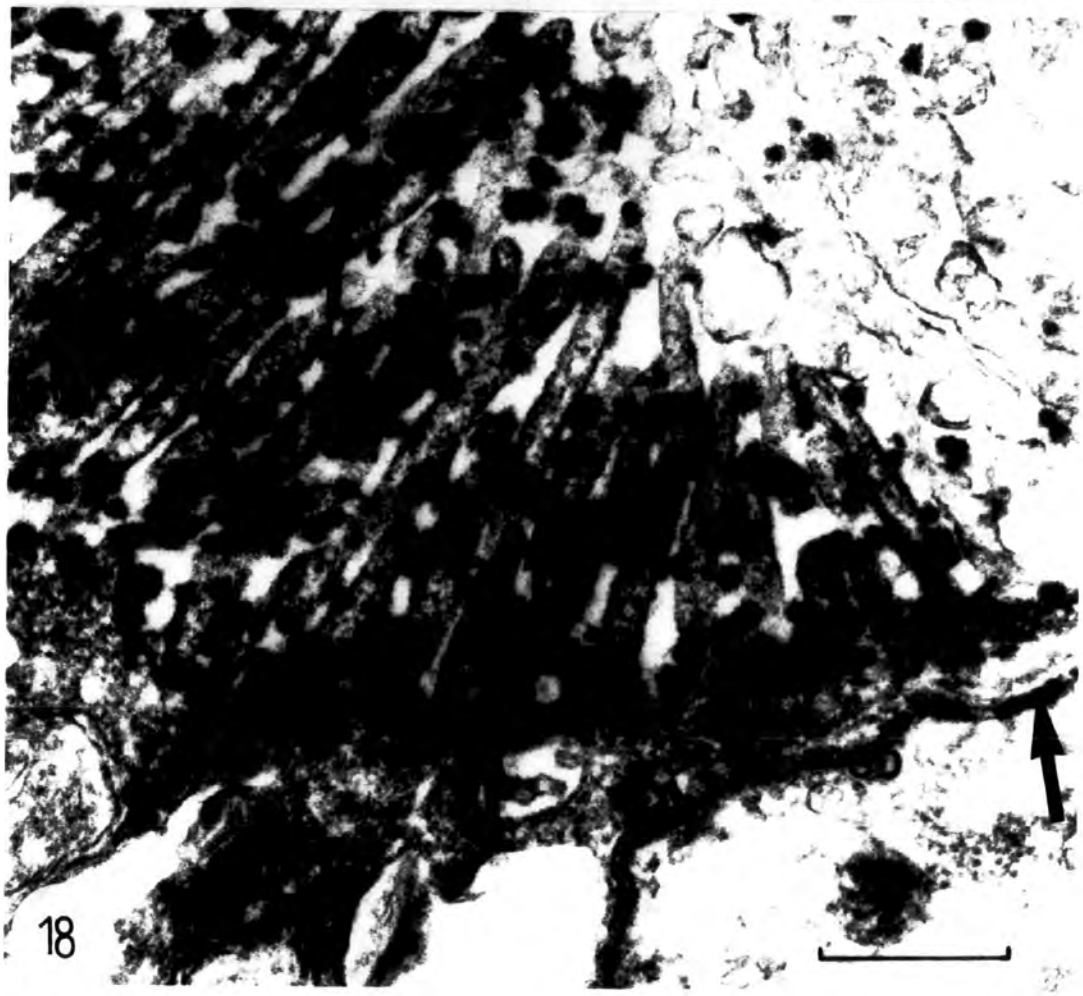
Scale = 1 μm.

PLATE 18 (control)

Control i.e. in normal standard incubation medium as previously described (see Plate 3).



17



18

PLATE 19 A-F

A summary of results obtained on different occasions when Malpighian tubules were incubated in the normal standard incubation medium for one hour at 30°C.

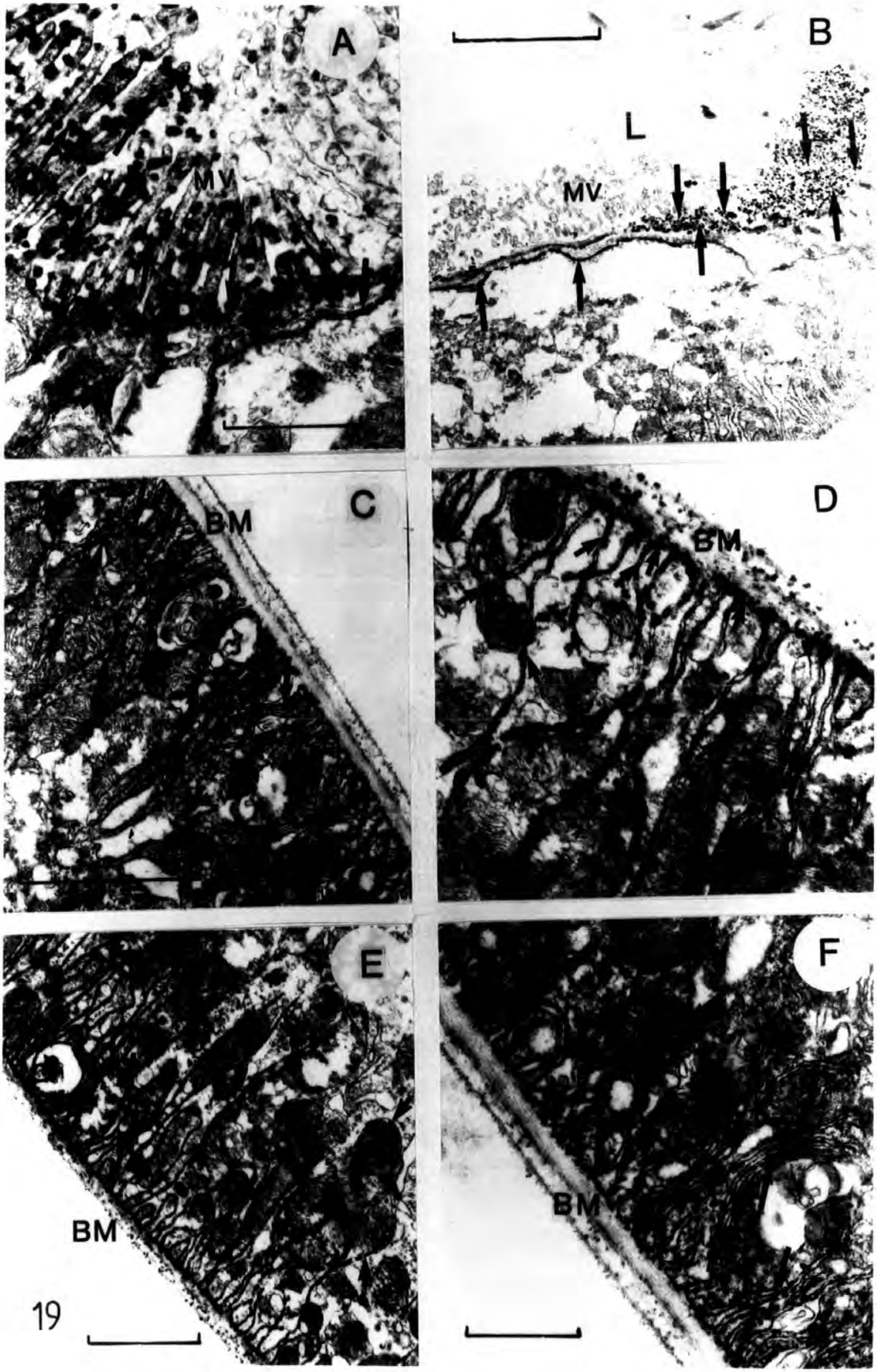
Apical region Fig. 19 A,B

In the apical region precipitates were mainly outside the microvilli.

On some occasions (Fig.19B) it was difficult to decide whether the precipitates were due to the reaction product or just due to overstaining as suggested by ERNST (1972b).

Basal region Fig. 19 C-F

Low power electron micrographs showing the typical localization of the reaction product (presumed to be due to NPPase activity) along the basal cell membrane invaginations (Bl).



19

SECTION II

COMPARATIVE BIOCHEMICAL MEASUREMENTS OF K^+-NPPASE IN FIXED AND UNFIXED HOMOGENATES OF LOCUSTA MIGRATORIA L. MALPIGHIAN TUBULES

6.5 K^+-stimulated NPPase activity

Figure 6.2 (column 1-3) shows that the addition of 10 mM K^+ to the biochemical reaction medium results in an increase in NPPase activity in homogenates of unfixed Malpighian tubules. The results show that 36.9% of the total activity measured in the homogenates of unfixed Malpighian tubules under biochemical conditions at pH 7.2 is K^+-dependent NPPase activity. Figure 6.2A column 3 shows that the presence of 5 mM ouabain in the reaction medium at pH 7.2 completely abolishes this K^+ activation of activity. This is shown by the fact that the amount of NPPase activity observed in the presence of 5 mM ouabain (Figure 6.2A column 3) is almost equal that observed when K^+ was deleted from the standard biochemical reaction medium (Figure 6.2A, column 4 and 5 being not significantly different from each other ($p = >0.05$).

6.6 Effect of paraformaldehyde fixation

When homogenates of paraformaldehyde fixed Malpighian tubules were incubated in the standard biochemical reaction medium, the total NPPase activity was reduced by 42.3% (compare Figure 6.2A Column 2 and 7).

It appears that fixation reduces the unspecific NPPase activity more than it reduces the K^+-activated NPPase activity. The latter constituted 59.7% of the total NPPase activity measured in fixed tissue (Figure 6.2A column 9) whilst it constituted about 34% of the total NPPase activity measured in unfixed tissue (Figure 6.2A column 4). It is also seen that K^+-activated NPPase in fixed tissue was less sensitive to ouabain than that in unfixed tissue. In fixed tissue 59.7% was K^+-activated NPPase activity and only 39.6% was inhibited by ouabain (Figure 6.2A column 10).

6.7 Effect of the Histochemical reaction medium

Under histochemical conditions, (pH 9.0, 20 mM $SrCl_2$ present) there was further loss of p-NPPase activity in homogenates of both unfixed and fixed Malpighian tubules. 38.6% of the total NPPase activity observed in homogenates of unfixed Malpighian tubules under biochemical conditions (Figure 6.2A, column 2) was abolished in the presence of 20 mM $SrCl_2$ (Figure 6.2B column 2).

Although the overall activity is reduced in the presence of strontium the relationship between the K^+-activated and the ouabain-sensitive fractions remain much the same as previously described for the biochemical conditions.

Results in Figure 6.2B, show that fixation of the Malpighian tubules and addition of strontium to the reaction medium caused

further drastic reduction of the total NPPase activity. Under these histochemical conditions, the total NPPase activity is only 27.3% of the original activity measured in unfixed tissue using the standard biochemical reaction medium; and only 43.0% of the total p-NPPase observed in unfixed tissue incubated in the presence of strontium.

It appears that in the presence of strontium, fixation of the tissue preserves a substantial amount of K^+-activated NPPase. K^+-activated NPPase makes up 41.6% (compare columns 17 and 19, Figure 6.2B) of the total NPPase observed in fixed tissue, in the presence of strontium, as compared to 17-22% (compare columns 12 and 14, Figure 6.2B), that it constitutes in homogenates of unfixed tissue under the same histochemical conditions. As previously shown for biochemical conditions, it is seen that even under histochemical conditions the K^+-activated NPPase in fixed tissue was less sensitive to ouabain than that of unfixed tissues.

6.8 Effect of pH of the reaction media

It was important to establish whether the differences in the amount of NPPase activity measured under the biochemical conditions (pH 7.2, minus 20 mM $SrCl_2$) and histochemical conditions (pH 9.0 + 20 mM $SrCl_2$) were due to the difference in pH. The NPPase activity in both fixed and unfixed tissue was measured at pH 7.2 and 9.0 under biochemical and histochemical conditions, as indicated in Fig. 6.2A,B.

The results presented in Figure 6.2A,B show that there was no significant difference ($p > 0.05$) in the amount of NPPase activity in unfixed tissue under biochemical reaction media at

FIGURE 6.2A,B

Comparative measurements of NPPase activity in homogenates of fixed and unfixed Malpighian tubules of Locusta incubated in

(A) Biochemical, and (B) Histochemical reaction medium.

pH 7.2/9.0 (as indicated).

Figure 6.2A Biochemical reaction medium

Columns (1-5) Homogenates of unfixed Malpighian tubules (pH 7.2).

(6-10) Homogenates of fixed Malpighian tubules (pH 7.2)

(11-15) Homogenates of unfixed Malpighian tubules (pH 9.0).

Figure 6.2B Histochemical reaction medium (+ 20 mM $SrCl_2$)

Columns (1-5) Homogenates of unfixed Malpighian tubules (pH 7.2).

(6-10) Homogenates of fixed Malpighian tubules (pH 7.2).

(11-15) Homogenates of unfixed Malpighian tubules (pH 9.0).

(16-20) Homogenates of fixed Malpighian tubules (pH 9.0).

Note: Columns (1,6,11,16) without KCl

(2,7,12,17) 10 mM KCl (for Total NPPase)

(3,8,13,18) 10 mM KCl+ 5 mM Ouabain

The 'normal' biochemical reaction medium contained:

5 mM $NPPNa_2$, 10 mM MgCl , 10 mM KCl, 100 mM Sucrose, 100 mM Tris/HCl buffer pH 7.2

The Histochemical reaction medium was the same as the biochemical reaction medium plus 20 mM $SrCl_2$, pH 9.0

Dotted colums represent K^+-activated NPPase activity and

Striated columns represent ouabain-sensitive NPPase activity.

Verticle lines represent 1 S.E.M.

(n = 4)

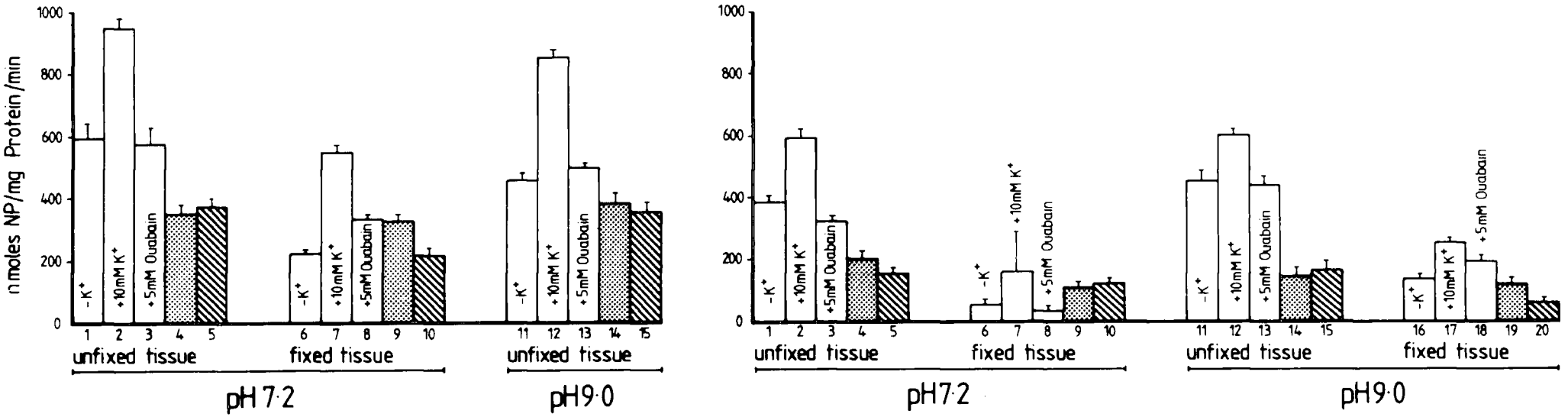
Fig. 6.2

A. Biochemical Incubation Medium

pH as indicated, ($SrCl_2$) absent

B. Histochemical Incubation Medium

pH as indicated (20mM $SrCl_2$) present



pH 7.2 and 9.0. It is however, seen that using the histochemical reaction medium containing 10 mM K^+, the activity in fixed tissue was greater at pH 9.0 than at pH 7.2 by almost 50% ($p < 0.01$).

Discussion

ERNST (1972a) demonstrated the specificity of the cytochemical reaction used in the present study and his work has been confirmed by subsequent studies (e.g. FIRTH, 1974; MILLS and ERNST, 1975a,b; ERNST and MILLS 1975; ELLIS and GOERTEMILLER, 1976). The use of NPP as a substrate has also been studied by SKOU (1975). The advantages of ERNST (1972b) technique over the WACHSTEIN-MEISEL (1957) procedure have previously been discussed. Therefore, the activity demonstrated in the present study by the use of ERNST (1972a,b) technique represents the reactive sites for the Na^+/K^+ exchange pump. The underlying assumption of this method is that K^+-dependent NPPase activity represents the terminal phosphatase step of Na^+, K^+-ATPase. The biochemical evidence supporting this is reviewed by ERNST (1972a) and there has been some correlation with independent methods for localization of Na^+, K^+-ATPase such as ^3H ouabain autoradiography (ERNST and MILLS 1977) and immunocytochemical techniques (KYTE, 1976a,b).

The cytochemical localization of NPPase activity in Locusta Malpighian tubule cell reported here (Plate 6) shows that the inorganic phosphate released by hydrolysis of NPP was precipitated by strontium at the site of hydrolysis. As previously pointed out β-glycerophosphate is not hydrolyzed by Na^+,K^+-ATPase (FUJITA et al., 1966, ERNST, 1972a). Therefore, the results showing a complete absence of the reaction product along the

basal cell infoldings when β-glycerophosphate was substituted for NPP in the standard incubation medium, suggest that the membrane associated phosphatase demonstrated during the present study is not a nonspecific alkaline phosphatase (see Plate 15). However, on some occasions there were some sparse deposits in the apical region when NPP was replaced by β-glycerophosphate. It has been suggested that this staining in the apical region may be due to nonspecific alkaline phosphatase activity (ERNST 1972b).

With the exception of the choroid plexus (QUINTON et al., 1973) where Na^+, K^+-ATPase has been localized apically, localization of Na^+, K^+-ATPase has almost exclusively been reported along the cytoplasmic side of the lateral and basal cell membrane infoldings in several secreting epithelia, e.g. (red cell membranes, SEN and POST, 1964; avian salt gland, ERNST, 1972b alimentary canal of Locusta, PEACOCK, 1976; rectum of Aeshna cyanea larvae, KOMNICK and ACHENBACH, 1979). However, in contrast to these workers and several others ERNST and MILLS, 1977; KARNAKY et al., 1976; SILVA et al., 1977) the results obtained from the present study show that although the reaction product was predominantly found in the basal cell membrane infoldings, there was also a substantial amount of deposits associated with the microvilli (Plate 3). However, the reaction product was consistently absent from the lumen itself, throughout the present study. In contrast, HOOTMAN and PHILLOT, (1979) report deposition of the reaction product in the luminal region of chloride cells from gills of euryhaline teleost.

Although the reaction product (which is thought to be due to the K^+-activated NPPase activity) was consistently localized along the lateral and basal cell membrane infoldings, the results from the present study and other studies (KOMNICK and ACHENBACH,1979;

PEACOCK, 1976) show that this fraction was inconsistently inhibited by ouabain. As recently reported by ANSTEE and BOWLER (1984) the addition of 1 mM ouabain to the standard incubation medium gave inconsistent results during the present study. This was particularly so when incubation was carried out at room temperature. On some occasions the reaction product was reduced while on others the reaction was not at all altered by the presence of 1mM ouabain. Subsequent experiments using 1 mM or 10 mM ouabain and incubating at 30°C instead of room temperature proved to be more successful (Plate 7). As previously pointed out, ouabain inhibition of Na^+, K^+-ATPase is temperature-sensitive (ANSTEE and BOWLER, 1979; Chapter 3). Therefore, temperature may be one of the causes of the ouabain insensitivity reported from KOMNICK and ACHENBACH (1979) and PEACOCK (1976) studies where incubation was carried out at 20°C and room temperature, respectively.

The most reliable results from the present study were obtained when 10 mM ouabain was used and incubation carried out at 30°C. But this concentration of ouabain is in excess of the concentration (10^{-4} - 10^{-3}M) ouabain required for complete inhibition of Locusta Malpighian tubule microsomal Na^+, K^+-ATPase (Chapter 3). ERNST and MILLS, (1977) too demonstrated that K-NPPase was completely inhibited by 10 mM ouabain. This high concentration of ouabain may be required because in agreement with ERNST (1972a), the present study has also shown that in the presence of strontium, a substantial amount of K^+-activated NPPase activity may be protected from ouabain inhibition. Furthermore, it was previously suggested that higher ouabain concentrations may be required to overcome the long diffusion path when intact tissues are used (IRVINE and PHILLIPS, 1971).

The deletion of K^+ of the incubation medium led to a small reduction in the amount of the reaction product deposited in some areas of the basal cell membrane infoldings. When choline chloride was substituted for KCl in the incubation medium similar results were obtained (Plates 11,12). This shows that the reduced staining observed in the absence of K^+ was not due to a change in the total ionic strength of the medium as TORMEY (1966) reported to be the case in the Na^+, K^+-ATPase medium proposed by McCLURKIN (1964). However, except for one occasion (see Plate 13) the deletion of K^+ or replacement of K^+ by choline chloride from the incubation medium did not cause as much reduction as that reported by ERNST (1972b) in the avian salt gland. He found that exclusion of K^+ or substitution of choline chloride for K^+, caused even greater reduction of the reaction product than that caused by 10 mM ouabain. GOERTEMILLER and ELLIS (1976b) too report great reduction of the precipitate along the basal infoldings of the plasma membrane of the spiny dogfish Squalus acanthias rectum when K^+ was replaced by choline chloride. It cannot be confidently stated that all deposits observed in the present study using the normal incubation were due to K^+-activated NPPase activity, since there was also a substantial amount of deposits in the absence of K^+ (Plates 11,12). NPPase insensitive to ouabain has been localized apically in lachrymal salt gland (THOMPSON and COWAN, 1976) and they questioned the existence of a Na^+,K^+ -pump on the basal surface of the cell. Results from the present study support the existence of a Na^+,K^+-pump on the basal cell membrane but may also suggest that there may be a second Na^+, K^+-pump on the apical cell membrane as suggested by BERRIDGE (1967) and OSCHMAN and BERRIDGE, (1971). However, as the biochemical studies further reveal, results from the cytochemical

localization should be interpreted cautiously.

The partial failure of control experiments reported in the present study and other studies (KOMNICK and ACHENBACH, 1979; PEACOCK, 1976) suggest that ERNST (1972b), technique may not be suitable for localization of Na^+,K^+-ATPase in insect epithelia. KOMNICK and ACHENBACH (1979) found that in the rectum of Aeshna cyanea larvae there were reaction products along the basal and lateral cell membrane infoldings in all their experiments including all controls and they concluded that the technique was unsuitable for localization of Na^+, K^+-ATPase in the rectum of Aeshna cyanea.

The results from the biochemical studies, which were carried out parallel to the cytochemical localization study, have also led to further questioning of the validity of ERNST (1972 b) technique in localizing Na^+, K^+-ATPase in insect epithelia. Paraformaldehyde fixation is thought to preserve a large proportion of the K^+-activated NPPase activity (ERNST, 1972a) and it is seen that K^+-activated NPPase activity constituted 46.7% of the total NPPase activity observed under histochemical conditions. Other workers (e.g. KOMNICK and ACHENBACH, 1979) found that fixation and strontium completely inhibited the enzyme activity.

Results presented in Figure 6.2A,B show that the cytochemical incubation medium used was kinetically sound for a substantial amount of NPPase activity to be measured. Under histochemical conditions (20 mM $SrCl_2$ at pH 9.0) in fixed tissue 46.7% was K^+-activated NPPase. Whilst the K^+-activated NPPase in unfixed tissue, was completely abolished by the presence of 5 mM ouabain in the biochemical incubation medium, the results show that in fixed tissue, in the presence of strontium. K^+-activated NPPase was less sensitive to

ouabain. Only 50% of the total K^+-activated NPPase activity was inhibited by 5 mM ouabain (see Figure 6.2B, column 19, 20).

However, these biochemical results have in several aspects cast further doubt over the validity of this technique for localizing Na^+, K^+-ATPase in Locusta Malpighian tubule epithelia cell. This is mainly because the results in Figure 6.2 A,B show that fixation of the tissue and presence of strontium (20 mM) in the incubation medium greatly reduced the overall NPPase activity. It is seen that 73% of the activity observed under biochemical conditions was abolished by fixation and strontium. It should be emphasised that according to results previously discussed, the differences in activity under biochemical and histochemical reaction media cannot be attributed to the differences in pH of the media. As ANSTEE and BOWLER, (1984) pointed out, this ERNST (1972b) technique is probably suitable only where a tissue is rich in Na^+, K^+-ATPase sites so that there is enough K^+-activated NPPase activity remaining uninhibited by fixation and strontium to permit histochemical localisation. Whilst ERNST (1972a) found that in the avian salt gland, 60% of the NPPase activity was due to K^+-activated NPPase activity, it is seen that only 46.7% of the total NPPase activity in Locusta fixed Malpighian tubule preparations, in the presence of strontium was due to K^+-activated NPPase activity.

CHAPTER 7

CONCLUSION

There is increasing evidence that the transport of Na^+ through several secreting epithelia involve a Na^+/K^+ pump (SKOU, 1965; WHITTAM and WHEELER, 1970). This pump requires ATP and is specifically inhibited by the cardiac glycoside, ouabain (SKOU, 1965; SCHATZMANN, 1953). This cation transport process has been correlated with the activity of Na^+, K^+-ATPase (E.C.3.6.1.3., SKOU, 1965), an enzyme synergistically stimulated by Na^+ and K^+ and also specifically inhibited by ouabain (SKOU, 1969). The involvement of Na^+, K^+-ATPase in ion and water transport mechanism is well-documented (see BONTING, 1970, STEKHOVEN and BONTING, 1981).

Results discussed in Chapter 3 show that, in common with several other transporting tissues which have been studied (SKOU, 1957, 1969; NAKAO et al., 1965; PROVERBIO et al., 1970; WHITTAM and WHEELER, 1970) the Malpighian tubules of Locusta possess a Na^+, K^+-activated ATPase. The results have confirmed earlier reports from ANSTEE and BELL, (1975), FATHPOUR (1980), DONKIN and ANSTEE (1980) and DONKIN (1981) (see also Table 3.1).

Na^+, K^+-ATPase has been implicated in the mechanism of fluid secretion by Locusta Malpighian tubules by virtue of the sensitivity of the fluid secretory process to ouabain (ANSTEE and BELL 1975, Chapter 5). However, as previously discussed in Chapters 3 and 5, some workers have failed to demonstrate ouabain-sensitivity in fluid secretion in different tissues (e.g. MADDRELL, 1969; PILCHER, 1970; GEE, 1976; RAFAELI-BERNSTEIN and MORDUE, 1978).

These conflicting reports have cast doubt over the involvement of Na^+, K^+ - ATPase in ion and water transport system and have given rise to a lot of controversy.

In agreement with ANSTEE and BELL (1975), ANSTEE et al., (1979, 1980), DONKIN and ANSTEE (1980) and DONKIN (1981), the present study has clearly shown that in vitro Locusta Malpighian tubule fluid secretion is inhibited by ouabain. The results are further supported by reports from ANSTEE et al., (1980) and FATHPOUR et al., (1983) that (1 mM) ouabain reduced the transepithelial potential across Locusta Malpighian tubules, which indicate that in Locusta Malpighian tubules, active ion transport is responsible for maintenance of the transepithelial potential and that Na^+, K^+-ATPase is involved in cation transport.

The present study has also established the fact that experimental conditions are extremely important for demonstrating the inhibitory effect of ouabain. In particular ouabain inhibition is very sensitive to temperature, a fact that has been emphasised by ANSTEE and BOWLER (1979) and DONKIN and ANSTEE (1980). Results presented in Chapter 5 show that (1 mM) ouabain does not significantly inhibit fluid secretion at temperatures below 30°C and that at room temperature (18°C), it does not at all affect fluid secretion. As previously pointed out workers who report ouabain-insensitivity in fluid secretion by various epithelia, carried out their experiments at temperatures below 30°C, (e.g. at 24-25°C, RAFAELI-BERNSTEIN and MORDUE, 1978; 19-22°C, GEE, 1976 a,b DALTON and WINDMILL, 1980) and in some cases workers do not state the precise temperature at which the experiments were carried out. The lack of ouabain-sensitivity at temperatures below 30°C is expected since ouabain inhibition of Na^+, K^+-ATPase activity has

also been shown to be extremely temperature-sensitive (Chapter 3). This confirms results reported by other workers (CHARNOCK et al., 1975; PEACOCK et al., 1976; DONKIN and ANSTEE, 1980; PEACOCK,1981). Therefore, failure to demonstrate ouabain inhibition of fluid secretion at temperatures below 30°C, should not be taken as evidence against the involvement of Na^+, K^+-ATPase in the mechanism of fluid secretion.

The present study has also demonstrated that the uninhibited activity of Na^+, K^+-ATPase of Locusta Malpighian tubule preparations (Figure 3.13) and the in vitro Locusta Malpighian tubule rate of fluid secretion (Figure 5.3) are similarly dependent on temperature. This is clearly shown by the close resemblance in the two slopes (Fig. 3.13 and 5.3). This further implicates Na^+, K^+-ATPase to be involved in the process of ion and water transport through the Malpighian tubules of Locusta.

It has also been suggested (JUNGREIS, 1977; RAFAELI-BERNSTEIN and MORDUE, 1978) that K^+ concentration in the bathing medium may influence ouabain inhibitory action. High K^+ concentration have been shown to antagonise ouabain inhibition of Na^+, K^+-ATPase (KINSOLVING et al., 1963; JUDAH and AHMED, 1964; MATSUI and SCHWARTZ, 1968; AKERA, 1971; BONTING, 1970; present study, Chapter 3). The present study has demonstrated K^+ antagonism of ouabain inhibition of Na^+, K^+-ATPase activity in microsomal preparations of Locusta Malpighian tubules. Results show that (10^{-6}M) ouabain inhibition decreased from 62% at 2 mM K^+ to ca 40% at 100 mM K^+. Similar studies were not carried out for in vitro Malpighian tubule fluid secretion during the present study. However, DONKIN and ANSTEE (1980), and DONKIN (1981) report that K^+ concentrations

from 10 - 40 mM failed to affect ouabain inhibition *in vitro* fluid secretion by *Locusta* Malpighian tubules. As it appears that K^+ concentration in the bathing medium may vary over a wide range without affecting ouabain inhibition, K^+ antagonism of ouabain inhibition may sometimes be overexaggerated. In summary, whilst it is true that the mechanism of fluid secretion may vary in different insect species, it appears that methodological differences, to a great extent, account for the conflicting reports in literature on ouabain-sensitivity of fluid secretion in different insect species studied.

The use of inhibitors of Na^+, K^+-ATPase has been a powerful tool in elucidating aspects of the reaction mechanism of the enzyme. As reflected in the present study, in studies on insect Na^+, K^+-ATPase only ouabain has been extensively used. Ouabain has been preferred because of its high solubility. Other glycosides require alcohol or organic solvents which complicates the interpretation of results because the solvents are usually inhibitors too (RUBIN *et al.*, 1980). The present study has investigated the use of vanadate, furosemide, acetazolamide, SCN^- and SITS in the study of Na^+, K^+-ATPase, and other ATPases from *Locusta* Malpighian tubules and their involvement in the process of ion and fluid transport through the epithelia.

As previously discussed, vanadate was discovered accidentally as a contaminant in certain brand ATP (CHARNEY *et al.*, 1975 and later identified by CANTLEY *et al.*, 1977) as (Vanadium V) which caused potassium-dependent inhibition of Na^+, K^+-ATPase similar to that seen in the commercial ATP. Vanadate occurs naturally in animal cells, but there is evidence to indicate that within the

cells vanadium is principally in the 4+ state (VO^{2+}) (CANTLEY and AISEN, 1979; GRANTHAM and GLYNN, 1979) which is much less effective. The mode of action of vanadate has been previously discussed (Chapter 3) and is well documented (GRANTHAM and GLYNN (1979)and GRANTHAM, 1980). The present study has demonstrated that vanadate is a potent inhibitor of Na^+, K^+-ATPase activity in Locusta Malpighian tubule preparations. This is in agreement with reports from several other workers using vertebrate tissues (e.g. QUIST and HOKIN, 1978; NECHAY and SAUNDERS, 1978; GRANTHAM and GLYNN, 1979). Results in Chapter 5 show that vanadate also inhibited the in vitro Malpighian tubule fluid secretion. This further implicates Na^+, K^+-ATPase involvement in the mechanism of ion and water transport through Locusta Malpighian tubule. However, the interpretation of results is complicated by the fact that vanadate is not a specific inhibitor. Other classes of ATPases are also inhibited by vanadate (O'NEAL et al., 1979). Results discussed in Chapter 4 show that vanadate also inhibited the Mg^{2+}-ATPase as well as the Mg^{2+},HCO_3^- -ATPase activities in Locusta Malpighian tubule preparations. This lack of specificity results from the action of vanadate as a transition-state analogue of phosphate; consequently, enzymes that hydrolyze phosphate ester bonds are likely targets for inhibition by vanadate (CANTLEY et al., 1978).

However, vanadate could be used in the study of Na^+, K^+-ATPase as it is shown to manifest its inhibitory effect on Na^+, K^+-ATPase under conditions where ouabain would be ineffective. As shown in Figure 3.14 vanadate is negatively affected by increasing temperature and high K^+ concentrations in the bathing medium enhances its inhibitory action (Fig. 3.10). These differences in their

sensitivity to $[K^+]$ and temperature demonstrated during the present study explains why vanadate and ouabain bind on different conformational changes of Na^+, K^+-ATPase during the hydrolysis of ATP (see previous discussion in Chapter 3).

Results presented in Chapter 5 show that (1 mM) ouabain inhibits Locusta Malpighian tubule in vitro fluid secretion by 60-70% whilst 1 mM vanadate inhibits ca.80% of fluid secretion. The fact that vanadate inhibition goes beyond that of ouabain indicates the involvement of a different pump(s) other than Na^+, K^+-ATPase in the production of fluid by Locusta Malpighian tubules.

The present investigation has identified a Mg^{2+},HCO_3^- -ATPase which also appear to have a role in fluid production by Locusta Malpighian tubules. The results from the present study confirm reports from ANSTEE and FATHPOUR (1981) whose studies also implicated Mg^{2+}, HCO_3^- -ATPase in the process of ion and water transport across Locusta Malpighian tubule. However, the exact origin of this enzyme remain uncertain.

Due to the lack of a specific inhibitor, the study of the role of Mg^{2+}, HCO^- -ATPase in the production of fluid in several secreting epithelia has not been easy. SCN^- is so far the only inhibitor which directly inhibits Mg^{2+}, HCO_3^- -ATPase and is considered to be a specific inhibitor of this enzyme by some workers (see Table 4.1) If this is true, the inhibition of in vitro Locusta Malpighian tubule fluid secretion by SCN^- observed during this study implicates this enzyme in the urine production mechanism. The results are supported by reports from ANSTEE and FATHPOUR (1979) and FATHPOUR (1980) who found that SCN^- inhibited

the Mg^{2+}, HCO_3^- - ATPase activity in Locusta Malpighian tubule preparations, the in vitro Malpighian fluid secretion and also reduced the transepithelial P.D. SZIBBO and SCUDDER (1979) reported that the excretion of HCO_3 by Malpighian tubules of water boatman, Cenocorixa bifida may play an important role in regulation of haemolymph pH, since these animals live in saline lakes with high HCO_3 concentrations (36.7 mM) and hence high pH. They suggest that HCO_3 might enter the lumen of tubules to maintain the electroneutrality of the secreted fluid or actively via a lumen-directed HCO_3 'pump' localized in the epithelial wall of the tubules. Alternatively, SZIBBO and SCUDDER (1979) suggested that the high pH of Segment II of Malpighian tubules could be a result of an electrogenic K^+/H^+ exchange pump localized on this segment.

As mentioned earlier, the present study has demonstrated that Mg^{2+}, HCO_3-ATPase is also inhibited by vanadate. The fact that the latter also inhibited fluid secretion by Locusta Malpighian tubules, indicates that Mg^{2+}, HCO_3 - ATPase is involved in fluid secretion. However, it is difficult to interpret the results obtained with vanadate as an inhibitor because vanadate also inhibited the Mg^{2+} - ATPase activity both in the presence and absence of NaCl.

Further evidence of other 'pumps' involved in the process of water and ion transport comes from the present work on SITS and acetazolamide. SITS appears to be a very strong inhibitor of Mg^{2+}, HCO_3^- - ATPase from Malpighian tubules of Locusta. However, in the same way as acetazolamide, SITS is known to affect Mg^{2+},HCO_3^- - ATPase indirectly through its inhibition of the c.a. which would otherwise make the HCO_3^- ions available for Mg^{2+}, HCO_3^- - ATPase (see HANRAHAN and PHILLIPS, 1983). Results show

that SITS (1 mM) inhibited Mg^{2+}, HCO_3^- - ATPase by 72.2% (Chapter 4) and that acetazolamide (1 mM) inhibited in vitro fluid secretion by Malpighian tubules of Locusta by ca 58% ($p = <0.001$) Chapter 5. The results, therefore, indicate that there may be a Cl^-/HCO_3^- exchange pump and that c.a is functionally linked by Mg^{2+},HCO_3^- - ATPase. This idea is supported by FATHPOUR (1980) who identified c.a. in Locusta Malpighian tubules preparations and also reports that either the exclusion of HCO_3 from the bathing medium or the addition of acetazolamide to the bathing medium resulted in a drop in in vitro fluid production by Malpighian tubules of Locusta.

Although results from the present study show that SITS is an efficient inhibitor of Mg^{2+}, HCO_3^- - ATPase, there is no evidence that SITS is specific for this enzyme. It has in fact, been reported elsewhere, that SITS also inhibits Na^+,K^+-ATPase in both turtle bladder and eel electric organ where it proved to be even a more potent inhibitor than ouabain (EHRENSPECK and BRODSKY, 1976). Further investigations of the effects of SITS on ATPases in Locusta Malpighian tubules need to be done.

The present study has also investigated the possible use of ERNST (1972b) technique in localization of Na^+,K^+-ATPase in the Malpighian tubule cell. The preference of this technique to the original WACHSTEIN-MEISEL (1957) procedure, which faces a lot of criticism, has been previously discussed (Chapter 6).

The underlying principle of ERNST (1972b) localization technique is that the hydrolysis of ATP by Na^+, K^+ -ATPase occurs, at least in two steps : a (Na^+ + Mg^{2+})-dependent phosphorylation of enzyme protein by ATP and a (K^+ + Mg^{2+})-dependent, ouabain-sensitive dephosphorylation of the phosphorylated intermediate

(see review by ERNST et al., 1980). The latter may be selectively demonstrated with artificial substrates such as p-nitrophenyl phosphate (NPP) (JUDAH et al., 1962).

Results presented in Chapter 6, (Section I) show that this K^+-NPPase procedure is advantageous in that it lends itself to electron microscopic analysis of Na^+, K^+-ATPase distribution and it is easily correlated with biochemical assay of enzymatic activity.

Using the standard incubation, the reaction product was consistently localized along the basal and lateral infoldings of the cell membrane (Plate 1 and Plate 19C-F). This localization of the K^+-activated NPPase along the basal plasma membrane is also true for a number of other transporting epithelia (e.g. mammalian renal tubules, ERNST, 1975; SHAVER and STIRLING, 1978; amphibian epidermis, FARQUHAR and PALADE, 1966; MILLS et al., 1977; salt avian gland, ERNST, 1972b; ERNST and MILLS, 1977; reptilian salt gland, ELLIS and GOERTEMILLER, 1976; THOMPSON and COWAN, 1976; elasmobranch rectal gland, GOERTEMILLER and ELLIS, 1976; KARNAKY et al., 1976a; teleost chloride cell, KARNAKY et al., 1976b, HOOTMAN and PHILPOTT, 1979).

The localization of Na^+, K^+-ATPase, an enzyme implicated in ion and water transport (ANSTEE and BELL, 1975, 1978; ANSTEE et al., 1979, 1980; FATHPOUR, 1980; DONKIN, 1981; FATHPOUR et al., 1983; present study) along the basal cell membrane infoldings is expected as these structures are thought to be specially modified for the transporting of ion and water through the secreting epithelia.

As previously described, in Locusta Malpighian tubule primary cell, the basal cell membrane is geometrically amplified with extensive basal and lateral infoldings giving rise to numerous

intracellular channels which would facilitate the formation of standing osmotic gradients, a theory proposed by DIAMOND and BOSSERT (1968) to explain ion and fluid transport through insect Malpighian tubules and other secreting epithelia.

Admittedly, despite this consistency in localization of the reaction product along the basolateral plasma membranes of Locusta Malpighian tubule cell, the findings of the present study are subject to a number of important limitations:

(1) The reaction product which is thought to be due to the K^+-activated NPPase activity was inconsistently inhibited by ouabain. Complete abolition of the reaction product could only be achieved by using a high concentration of ouabain (10 mM) which is in excess of the concentration required for complete inhibition of either Na^+, K^+-ATPase activity in Locusta Malpighian tubule preparations (Chapter 3) or the in vitro Malpighian tubule fluid secretion (Chapter 5). However, a high concentration is required because, according to ERNST (1972b) and results presented in Figure 6.2b, a strontium alters the sensitivity of the K^+-NPPase to ouabain. It is seen that 50% of the K^+-NPPase in homogenates of fixed Malpighian tubules was not inhibited by ouabain in the presence of 20 mM $SrCl_2$.

(2) A large percentage of NPPase activity is lost during fixation.

(3) Inclusion of 20 mM $SrCl_2$ in the standard incubation medium caused further loss of activity so that 73% of the original activity observed in unfixed tissue under biochemical conditions (i.e. minus 20 mM $SrCl_2$) was lost.

Although fixation is thought to preserve the K^+-activated NPPase activity (ERNST, 1972a) the latter was more inhibited than the nonspecific ouabain-insensitive NPPase. In fixed tissue, under histochemical incubation medium (i.e. + 20 mM $SrCl_2$), K^+-NPPase constituted only 46.7% of the total NPPase. Similar results are reported for the rectum Locusta migratoria (PEACOCK, 1976) and the dragonfly larvae Aeshna cyanea KOMNICK and ACHENBACH, 1979) and they conclude that ERNST (1972b) technique is not valid for localization of Na^+, K^+-ATPase in insect tissue. ANSTEE and BOWLER (1984) suggest that this technique is perhaps suitable only where a tissue is rich in Na^+, K^+-ATPase sites. In such a tissue there may be sufficient K^+-NPPase activity remaining uninhibited to permit cytochemical localization. Otherwise, the uninhibited activity may be too low to be resolved.

However, it is certain that the reaction product localized along the basal cell membrane was not due to a nonspecific alkaline phosphatase. This was demonstrated by the total abolition of the reaction product along the basal and lateral infoldings of the cell membrane when β-glycerophosphate was substituted for NPP in the incubation medium. It is known that β-glycerophosphate is not hydrolysed by Na^+, K^+-ATPase (ERNST, 1972; FUJITA et al., 1966).

In vertebrates, the localization of Na^+, K^+-ATPase along the basal plasma membranes, reported by several workers (e.g. avian salt gland, ERNST 1972b, rat hepatocyte, BLITZER and BOYER, 1978; lacrymal glands of marine turtles, ELLIS and GOERTEMILLER, 1976) suggests Na^+/K^+ exchange pump is located on the basal surface of the epithelia, although in many cases the possible presence of the Na^+/K^+ pump on the apical surface is not strongly excluded.

However, as previously pointed out, results from cytochemical localization studies should be interpreted with some caution.

During the present study, substantial precipitates were also deposited in the microvilli (Plate 3 and Plate 19A,B). Apical localization of Na^+, K^+-ATPase has been reported elsewhere (e.g. in frog choroid plexus, QUINTON et al., 1973; lacrymal salt gland, THOMPSON and COWAN, 1976). The latter questions the existence of Na^+/K^+ pump on the basal cell membrane surface. In Plate 19B, the precipitates seen in the apical region are clearly artefactual but this may or may not be true for Plate 19A. Therefore there is no clear evidence from the present study to suggest that Na^+, K^+-ATPase is exclusively located on the basal surface of the Malpighian tubule cell. As previously discussed, BERRIDGE (1967) and OSCHMAN and BERRIDGE (1971) suggest that Na^+/K^+ pump is present on both apical and basal surface. This would make it possible for the cell to maintain a relatively low intracellular sodium concentration. Their view is supported by recent reports by GUPTA and HALL (1983) that Na^+, K^+-ATPase may be present on both apical and basal membranes of the cockroach salivary gland cells.

Although the reaction product has been localized both on the apical and basal surface of the Locusta Malpighian tubule cell and was on a number of occasions inhibited by ouabain it is difficult to state with confidence that all reaction products observed throughout the present study were due to the specific Na^+, K^+-ATPase activity. As the present study is the first of its kind in the study of Na^+, K^+-ATPase in Locusta Malpighian tubules, further investigations are required to determine the exact location of Na^+, K^+-ATPase in Malpighian tubules of Locusta.

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