studies on fluid and ion secretion by the malpighian tubules of locusta with particular reference to the role played by ATPase enzymes

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STUDIES ON FLUID AND ION SECRETION BY THE MALPIGHIAN TUBULES OF *LOCUSTA* WITH PARTICULAR REFERENCE TO THE ROLE PLAYED BY ATPase ENZYMES

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TO MY WIFE

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A Mg^{2+}-dependent HCO_3^--stimulated ATPase has been demonstrated in mitochondrial and microsomal preparation of the Malpighian tubules of Locusta migratoria L.. $[Mg^{2+}]$ and $[HCO_3^-]$ yielding maximal activity with the microsomal enzyme were 2mM and 20mM respectively. The enzyme was not inhibited by sodium acetazolamide nor by ouabain. The K_m of HCO_3^--stimulated ATPase was 0.26 ± 0.03mM and the value for V_{max} was 381.1 ± 43 nmoles Pi liberated/mg protein/min. Other anions affected Mg^{2+}-dependent ATPase activity; SO_3^{2-}, $B_4O_7^{2-}$, SeO_3^{2-} were stimulatory, SCN^-, NO_3^-, N_3^- were inhibitory, whilst Br^- and Cl^- did not significantly alter activity. Oligomycin strongly inhibited ATPase activity in different subcellular fractions, viz. 5,000g, 20,000g, and 100,000g; the pI50 values for the Mg^{2+}-dependent ATPase (+NaCl) being 4.22 ± 0.11, 4.39 ± 0.04, and 4.72 ± 0.12 respectively. Corresponding pI50 values for HCO_3^--ATPase were 4.20 ± 0.09, 4.35 ± 0.05, and 4.67 ± 0.11. Amiloride and ethacrynic acid also inhibited this ATPase activity only slightly. The distribution of HCO_3^--ATPase activity and SDH activity in various subcellular fractions suggest that HCO_3^--ATPase is not exclusively mitochondrial in origin. This is further supported by electron microscopy. The HCO_3^--ATPase was distinguishable from Na^+-K^+-ATPase; the total replacement of Cl^- by HCO_3^- inhibited the activity of Na^+-K^+ ATPase of Locusta Malpighian tubules. Whilst SCN^- and NO_3^- did not significantly alter Na^+-K^+ ATPase activity.

In vitro preparations of Locusta Malpighian tubules are able to transport K^+ against chemical and electrical gradients, indicating active transport. Whilst K^+ appears to be the 'prime mover' in

the fluid secretory mechanism, an appropriate anion is clearly important. Fluid secretion was substantially reduced when Cl^- replaced NO_3^- in the bathing medium. No such reduction was observed with Br^-.

The effect of various inhibitors on fluid secretion, oxygen consumption, and transepithelial potential of Locusta Malpighian tubules have been studied. The presence of acetazolamide in, and/or the absence of HCO_3^- from, the bathing medium significantly reduced 'urine' production, oxygen consumption and the transepithelial potential. Similar effects were observed by including SCN^- in the bathing medium. These results are discussed in relation to carbonic anhydrase and HCO_3^--ATPase involvement in fluid and ion secretion. Both ouabain and amiloride markedly inhibited in vitro 'urine' secretion, oxygen consumption, and P.D. In addition, ouabain markedly increased Na^+/K^+ ratio of the secreted fluid, suggesting that Na^+-K^+ ATPase is also involved in the secretory mechanisms of Locusta Malpighian tubules.

GLOSSARY

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin (Fraction V, Sigma)
Cyclic AMP or c.AMP	cyclic adenosine 3',5'-monophosphate
DNP	2,4-dinitrophenol
EDTA	ethylene diamine tetra-acetic acid
HCO_3^--ATPase	magnesium dependent, bicarbonate stimulated adenosine triphosphatase
Hepes	N-2 hydroxylpiperazine-N'-2 ethanesulfuric acid
Isc	short-circuit current
Mg^{2+}-ATPase	magnesium dependent adenosine triphosphatase
mV	millivolts
Na^+-K^+ ATPase	magnesium dependent, sodium-potassium stimulated adenosine triphosphatase
O.S.C.P.	olicomycin-sensitivity-conferring factor
P.D.	transepithelial potential difference
Pi	inorganic phosphate
r.p.m.	revolutions per minute
SDH	succinate dehydrogenase
S.E.M.	standard error of mean
Tris	tris [hydroxymethyl] aminomethane
5-HT	5-hydroxytryptamine

CHAPTER 1

Introduction

The mechanism of ion and water transport across the Malpighian tubules of insects was first investigated in detail using *in vitro* preparations of *Carausius* tubules (RAMSAY, 1953, 1954, 1955, 1956, 1958). Subsequently, studies have been carried out on a variety of different species of insects (e.g. *Calliphora*, BERRIDGE, 1968, 1969; *Rhodnius*, MADDRELL, 1969; *Locusta*, ANSTEE and BELL, 1975, 1978; *Glossina*, GEE, 1975, 1976A,B). On the basis of such studies, it is suggested that; (i) water movements are a secondary consequence of ion movements (MADDRELL, 1977); (ii) active K^+ transport appears to be the 'prime mover' in the majority of insect species with the exception of *Rhodnius*, which can produce 'urine' in the presence of Na^+ or K^+ (MADDRELL, 1969), and *Glossina* in which Na^+ is the 'prime mover' (GEE, 1975, 1976A,B); and (iii) that the fluid secreted by nearly all Malpighian tubules is 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 flow produced by Malpighian tubules are in fairly close inverse relationship to the osmotic concentration of the bathing solution. In other words, the rate of solute movement is approximately constant but water movements change so that the fluid produced is slightly hypertonic (MADDRELL, 1977). However, the mechanism whereby solute movements give rise to water movements is not yet clear. A number of theories have been proposed.

One such theory is based on co-diffusion and was originally proposed by KEDEM (1965) and later applied to gall bladder by DIAMOND (1965). Co-diffusion is a rather vague concept (HILL, 1977), but it seems that whenever a molecule of solute diffuses across a membrane, through channels shared by water, the solute molecule is accompanied by several hundred molecules of water. It is suggested that this effect is probably due to frictional drag exerted on water by the solute molecule moving through it. BELL (1977) discussed the application of this theory for ion and water movements across Malpighian tubules and has concluded that co-diffusion would seem unlikely to play an important role in secretion by these tubules.

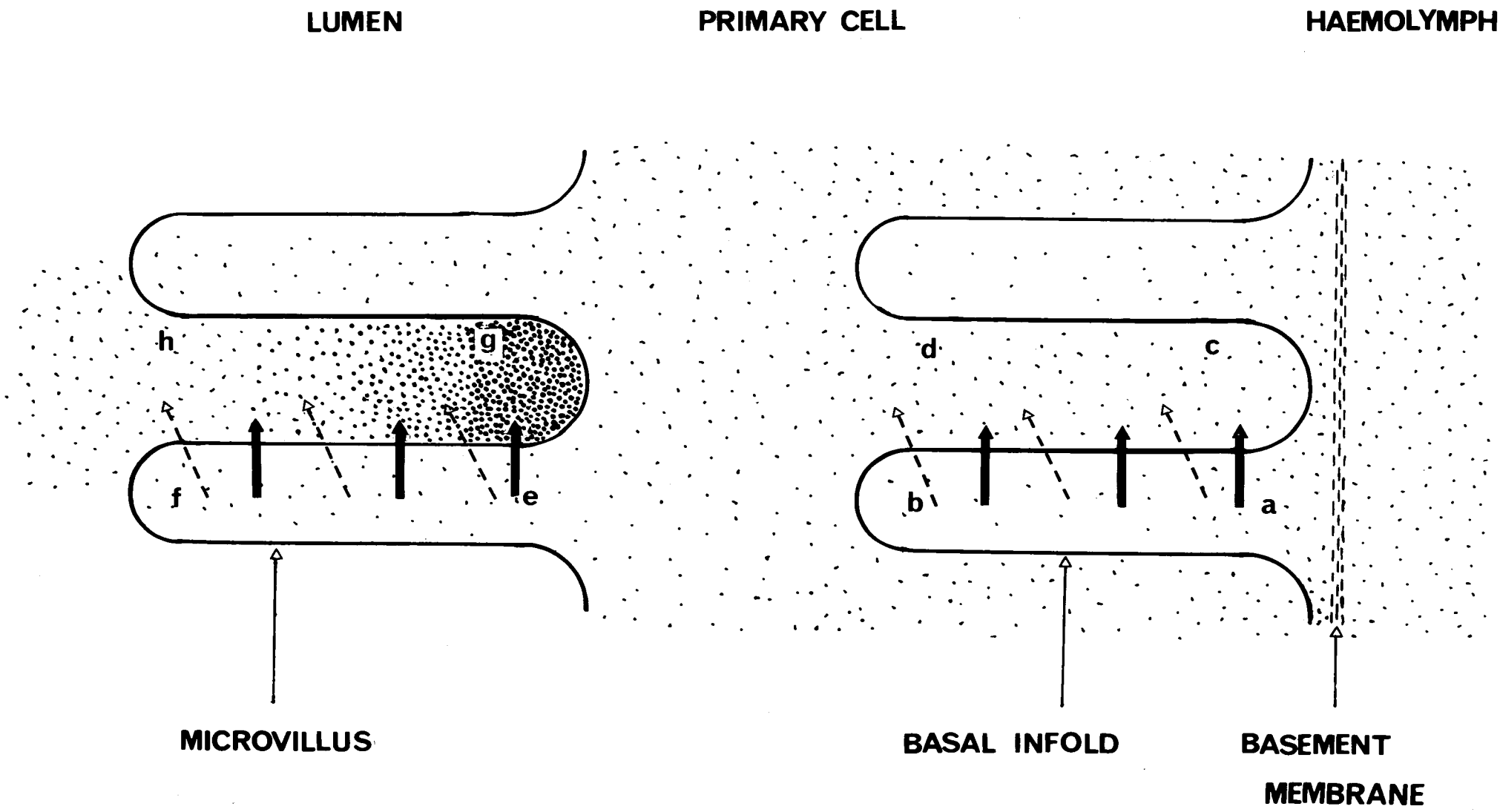
The most widely held theory is that of standing-gradient osmotic flow proposed by DIAMOND and BOSSERT (1967, 1968). This model is based on a functional geometry within fluid transporting epithelia. It depends upon channels which are structurally or functionally closed at one end. Solute is pumped into the closed ends of the spaces from the adjacent cytoplasm, making the region hyperosmotic to the cell. Water moves into the space from the adjacent cytoplasm so that towards the open end of the space, the fluid is isosmotic to the cytoplasm. In effect a standing-gradient is established along the length of the channel at equilibrium (see Figure 1.1 for detailed description). Some investigators have applied this model to insect Malpighian tubule secretion (BERRIDGE, 1968; DIAMOND and BOSSERT, 1968; BERRIDGE and OSCHMAN, 1969; MADDRELL, 1971; OSCHMAN and BERRIDGE, 1971). However, TAYLOR (1971) and MADDRELL (1977) argue that there are difficulties in applying this

Figure 1.1

Application of the standing-gradient hypothesis of solute-linked water transport to provide a model for 'urine' formation by Malpighian tubules (DIAMOND and BOSSERT, 1968). The density of dots indicates the solute concentration. Solid lines indicate the active transport of solutes and the broken lines passive water movements.

Basal surface. Solute is actively transported out of the channel (a-b) across its walls, making the channel fluid hypotonic. As 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 the 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. As solute diffuses across its concentration gradients towards the open mouth, more and more water enters the channel across its walls (i.e. from the cytoplasm) due to the osmotic gradient. In the steady state a standing osmotic gradient will be maintained in the channel (g-h) by active solute transport, with the osmolarity decreasing progressively from the closed end to the open end; and a fluid of fixed osmolarity will constantly emerge from the mouth.


LUMEN
PRIMARY CELL
HAEMOLYMPH
h
g
f
e
d
c
b
a
MICROVILLUS
BASAL INFOLD
BASEMENT
MEMBRANE

model to insect Malpighian tubules because the infoldings and microvilli are shorter than in such fluid transporting tissues as the gall bladder and the proximal tubules of vertebrate kidneys for which the model was originally developed. HILL'S (1975 A,B) criticism of this model is rather more fundamental. He argues that biological membranes do not have a sufficiently high osmotic permeability to permit isotonic flow in epithelia to occur by a process of local osmosis, or, at the most, osmotic equilibration must play little part in the process under normal physiological conditions. A further objection to the standing-gradient osmotic flow arises from the studies of GUPTA et al. (1976, 1977). These workers determined the concentrations of ions across the Malpighian tubules of Rhodnius (GUPTA et al., 1976) and Calliphora (GUPTA et al., 1977) by using electron probe X-ray microanalysis. They show that in Rhodnius tubules the ion concentrations between the microvilli conflict with what one might expect on the basis of the standing-gradient model; Na^+, K^+ and Cl^- being more concentrated towards the lumen than at the closed end of the channels. Similar results have been reported for K^+ on the basal membrane of Calliphora (GUPTA et al., 1977).

A third theory which has been proposed to explain the mechanism of ion and water movements across epithelia is based on electro-osmosis (HILL, 1975B, 1977). MADDRELL (1977) has discussed the implication of this electro-osmotic theory for insect Malpighian tubules. He suggests that electrogenic cation 'pumps' situated on the apical microvilli of tubules produces an electrical potential difference across this membrane. The resulting electrochemical gradient would

draw Cl^- out from the cell through the membrane, and in crossing the membrane the Cl^- would frictionally interact with water molecules and cause them also to move out of the cell. This mechanism relies on the maintenance of a potential gradient across this cell membrane so that it would be important that the apical wall should be so arranged that it is not bathed by fluid other than its own secretion. MADDRELL suggests that the apical membrane infoldings of the Malpighian tubule cells would serve such a purpose and would also allow a greater density of 'pump' sites because of the increase in membrane area.

Finally, it is possible that as the 'urine' of Locusta is always slightly hypertonic to the bathing medium (BELL, 1977; ANSTEE et al., 1979), simple 'local osmosis' (DIAMOND, 1964) may be responsible for water movements across the tubule. This theory proposes that the cytoplasm is marginally hypertonic to the bathing medium as a result of solute pumping across the basal membrane, and similarly the lumen becomes marginally hypertonic to the cytoplasm. Water flows passively as a result of these small osmotic pressure differences, their magnitude being determined by the rate of solute transport and the osmotic permeability of the membrane. This theory has been favoured by MADDRELL (1972) and TAYLOR (1971) for insect Malpighian tubules. They suggest that the folding of basal and apical cell membranes is primarily a device for increasing the effective passive permeability of the cells to solute and that the driving force for water movements is in fact the overall osmotic pressure difference between the lumen and bathing solution.

Whatever the exact mechanism for coupling of ions and water movements, the various models agree, that fluid secretion by insect

Malpighian tubules is a consequence of solute transport. Furthermore, whilst active cation transport has been established in insect Malpighian tubules (RAMSAY, 1953, 1955; BERRIDGE, 1967, 1968; PILCHER, 1970; MADDRELL, 1971, 1977; BELL, 1977; ANSTEE et al., 1979), the nature of ion 'pumps' remains obscure. In most of the models which have been proposed, it is generally considered that an electrogenic cation 'pump' is situated on the apical cell membrane (e.g. BERRIDGE, 1967; BERRIDGE and OSCHMAN, 1969; MADDRELL, 1977). The controversy concerns solute transport across the basal cell membrane. BERRIDGE (1967) and BERRIDGE and OSCHMAN (1969) proposed that cation movements across the basal membrane takes place by a coupled Na^+-K^+ exchange 'pump'. This 'pump' is energy dependent; ATP being required for its activity, and the simultaneous presence of Na^+ and K^+ stimulates its activity. In the majority of tissues which have been studied Na^+-K^+ exchange 'pumping' is effected by a Na^+-K^+ ATPase enzyme (e.g. 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, 1968A,B). One of the main objections concerning the involvement of Na^+-K^+ ATPase in Malpighian tubules secretion has arisen from the failure by some investigators to demonstrate that 'urine' production by isolated Malpighian tubules is sensitive to ouabain, a specific inhibitor of this enzyme (SKOU, 1969). Due to the apparent lack of ouabain sensitivity of the Malpighian tubules of Calliphora and Carausius, MADDRELL (1971) proposed an alternative hypothetical scheme to explain the mechanism of Malpighian tubules function in these insects. He proposed that K^+ is actively transported into the cell by an electrogenic 'pump'

which is stimulated by Na^+, 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 considered to be passive. In the case of Rhodnius a somewhat different model is suggested (MADDRELL, 1971, 1972). Here it is proposed that, electrogenic K^+ and Na^+ 'pumps' are situated in the basal cell membrane, each 'pump' is stimulated by another cation, as well (i.e. Na^+ 'pump' is stimulated by K^+ and vice versa). Cl^- enters the cell passively. He proposed that in this way Na^+, K^+, and Cl^- are made available to three electrogenic 'pumps' (for Na^+, K^+, and Cl^-) situated on the apical cell membrane which are responsible for transporting these ions into the lumen. More recently, in an attempt to produce a model which might apply to all Malpighian tubules whether they 'pump' Na^+ or K^+, MADDRELL (1977) proposed an alternative model. This hypothetical model will be described in detail later (Chapter 7). However, in essence what is suggested is that Na^+, K^+, and Cl^- enter the cell passively. An electrogenic cation 'pump', on the membrane facing the lumen, which has a higher affinity for Na^+ than for K^+, transfers the cations into the lumen. This model contrasts markedly with the earlier suggestion of MADDRELL (1971) that " it is difficult to envisage net passive movement of K^+ into the cell from an external concentration of, say, 5 m mol.l^{-1} when it is known that most cells have a intracellular concentration of K^+ of between 50 and 200 m mol.l^{-1} even taking into account that it is likely that the interior of the cell is at a potential which is negative with respect to the external medium".

Although K^+ has been shown to be the major cation transported across the tubules of most insects (see above), anions clearly play

an important role in 'urine' production. BERRIDGE (1969) and MADDRELL (1969, 1971) have shown that K^+ transport alone will not induce water transport unless it is accompanied by an appropriate anion. For example, if the Cl^- bathing the isolated tubules of Calliphora is replaced with a non-transporting anion such as SO^{2-}_4, fluid transport ceases (BERRIDGE, 1969). It is generally considered that Cl^- transport across the basal membrane is passive. Similarly, it is suggested that Cl^- follows K^+ out of the cell on the luminal side (MADDRELL, 1977). However, it appears that active Cl^- transport occurs across the Malpighian tubules of Rhodnius (MADDRELL, 1971, 1977). Whilst, it was initially thought the Cl^- 'pump' was located on the apical membrane, recently MADDRELL (1977) has suggested that it is in fact present on the basal cell surface.

More recently, SZIBBO and SCUDDER (1979) suggested that HCO_3^- might be excreted by the Malpighian tubules of the water boatman, Cenocorixa bifida, in order to regulate the haemolymph pH. These animals live in saline lakes with high HCO_3^- concentrations. The nature of the HCO_3^- transport in Cenocorixa bifida is unknown. These workers suggested that whilst it may be passive, active HCO_3^- transport via a lumen-directed HCO_3^- 'pump' can not be excluded.

A HCO_3^-- stimulated ATPase has been demonstrated in a variety of different tissues from numerous species where it has been implicated in a variety 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 fish gills (DE RENZIS and BORNANCIN, 1977); 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 the Na^+-K^+ ATPase, referred to above, this enzyme is apparently insensitive to ouabain but is inhibited by SCN^-.

In view of the lack of information concerning the nature of the ion 'pumps' in insect Malpighian tubules, the present study has been carried out to determine whether a Mg^{2+}-dependent HCO_3^--stimulated ATPase is present in the Malpighian tubules of Locusta migratoria L., to characterize this enzyme and to investigate its role in Malpighian tubule secretion. In addition, the role of Na^+-K^+ ATPase in tubule secretion has been examined.

CHAPTER 2

General Materials and Methods

1. Maintenance of Insects

A stock population of Locusta migratoria L., phase gregaria were maintained in an insectary at 28±0.5°C and relative humidity ca. 60%. 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. The humidity of the insectary was controlled by three humidifers (Lumatic, Humidifier Group, Bromley, Kent, England). The photoperiod was 12 hours light and 12 hours dark. Insects were reared in cages (41cm x 41cm x 60cm) supplied by Philip Harris Biological Ltd (Oldmixon, Weston-super-Mare, Avon). Each cage had a single 40w bulb for illumination. Temperature within the cage varied with the distance from the bulb and with the photoperiod. The humidity inside the cage also varied due to the addition of fresh food and water. Insects were fed daily on a diet of fresh grass, water and Bemax.

Animals were reared at sufficiently high population density to prevent reversion to the solitaria phase (JOLY and JOLY, 1953).

2. Glassware

All glassware was soaked overnight in a 2% (w/v) solution of quadralene (laboratory detergent), then rinsed at least six times with tap water and four times in distilled water. Glassware was dried in a drying oven at 100°C. In ion-analysis experiments, glassware was soaked in an acid-bath (1 vol. nitric acid: 1 vol.

hydrochloric acid: 2 vol. distilled water) for 2-3 hours following the normal washing procedure. It was then rinsed in distilled and deionized water.

3. Reagents

All chemicals were AnalaR grade or the purest commercially available. ATP (sodium salt), bovine serum albumin (BSA, Fraction V), imidazole, Hepes (N-2 hydroxylpiperazine - N'-2 ethanesulfonic acid), Tris (Tris [hydroxymethyl] aminomethane),ouabain, histidine, Bis-Tris propane (1,3-bis [tris (hydroxymethyl)-methylamino] - propane) and oligomycin (a mixture consisting of 65% oligomycin A, 20% oligomycin B and 15% oligomycin C (average molecular weight 428)) were obtained from Sigma Chemical Company (Poole, Dorset, England). Sodium acetazolamide (Diamox) was obtained from Lederle (American Cyanamid Company, Pearl River, N.Y., U.S.A.) Amiloride was a gift from Merck Sharp & Dohme Research Laboratories (Heddesdon, Hertfordshire, England) and Cirrasol ALN-WF was a gift from ICI Dyestuffs Division.

4. Insect Ringer solution

A Ringer solution buffered with Hepes (pH 7.2) was used throughout unless otherwise stated. The composition of this Ringer solution was (mM): NaCl 100; KCl 8.6; $CaCl_2$ 2; $MgCl_2$ 8.5; NaH_2PO_4 4; $NaHCO_3$ 4; glucose 34; Hepes 25; NaOH 11.

5. Statistical analysis

The statistical tables of FISHER and YATES (1963) were used. Statistical comparisons of data were performed using methods explained by SNEDECOR and COCHRAN (1967). An Olivetti Programma 101 Programmable calculator was used for calculation of Means, S.E.M., application of 't' tests and regression analyses. Probability values less than 0.05 were taken as significant.

CHAPTER 3

Biochemical studies on the Mg^{2+}-dependent HCO_3^--stimulated ATPase from the Malpighian tubules of *Locusta migratoria* L.

INTRODUCTION

In 1965, KASBEKAR and DURBIN, and SACHS *et al.*, reported the presence of a microsomal ATPase from gastric mucosa. This ATPase was dependent on the presence of Mg^{2+} and was stimulated by the presence of HCO_3^- and inhibited by SCN^- but not by ouabain. Since this time, Mg^{2+}-dependent HCO_3^--stimulated ATPase has been reported in a variety of different tissues from a number of different species. These include the gastric mucosae of various species (BLUM *et al.*, 1971; WIEBELHAUS *et al.*, 1971; DE PONT *et al.*, 1972; SACHS *et al.*, 1972B; SPENNEY *et al.*, 1973; VAN AMELSVOORT *et al.*, 1977A) where it has been implicated in acid secretion and transcellular HCO_3^- transport. IZUTSU and SIEGEL (1972) and SIMON *et al.* (1972A) reported the presence of such an enzyme in dog and rabbit submandibular gland where it may be involved in H^+/HCO_3^- transport. It has also been reported in mammalian pancreas (SIMON and THOMAS, 1972; SIMON *et al.*, 1972B; VAN AMELSVOORT *et al.*, 1978B) where it may have a role in HCO_3^- transport (SIMON and THOMAS, 1972; SIMON *et al.*, 1972B). The presence of this enzyme has also been demonstrated in brush border membranes of renal proximal tubules where it may be involved in HCO_3^- transport and in Na^+/H^+ exchange (KINNE-SAFFAREN and KINNE, 1974; LIANG and SACKTOR, 1976). DE RENZIS and BORNANCIN (1977) reported the presence of a HCO_3^-- stimulated ATPase in fish

gills where it may have a role in the Cl^-/HCO_3^- transport system. In addition, the presence of a Mg^{2+}-dependent HCO_3^--stimulated ATPase has been demonstrated in erythrocytes (DUNCAN, 1975; IZUTSU et al., 1977; VAN AMELSVOORT et al., 1978A).

If such an enzyme is to be involved in cellular transport, it should clearly be located in the plasma membrane fraction. Whilst such a localization has been proposed by certain workers (SACHS et al., 1972B; SIMON and THOMAS, 1972; DE RENZIS and BORNANCIN, 1977) others suggest that anion-sensitive ATPase activity in microsomes is derived from mitochondrial contamination (SOUMARMON et al., 1974; IZUTSU and SIEGEL, 1975; IZUTSU et al., 1978).

In an attempt to settle this problem, a number of researchers (IZUTSU and SIEGEL, 1972; SPENNEY et al., 1973; SUZUKI, 1978; COLE, 1979) have employed various membrane marker enzymes (e.g. the mitochondrial marker enzyme succinate dehydrogenase) to permit identification of the subcellular localization of their particular membrane fractions. Such studies are frequently carried out in association with electron microscopy. In addition, other workers (VAN AMELSVOORT et al., 1977A, B and 1978B; KIMELBERG and BOURKE, 1973) have sought to identify the enzymes source on the basis of different responses to certain inhibitors. For example, LIANG and SACKTOR (1976) reported that mitochondrial HCO_3^--ATPase is almost totally (95%) inhibited by oligomycin and quercetin, while microsomal HCO_3^--ATPase is not (only 36%).

To date, little information is available concerning the presence of Mg^{2+}-dependent HCO_3^--stimulated ATPase in insect tissues; notable exceptions being the demonstration of such an enzyme in the K^+- transporting midgut of larval Hyalophora cecropia (TURBECK et al., 1968), and in the rectum of Schistocerca gregaria (HERRERA et al., 1978) where it may be involved in Cl^- transport. In view of the suggested role of this HCO_3^--stimulated ATPase in ion transport, the present study was carried out to ascertain whether such an enzyme is present in the Malpighian tubules of Locusta migratoria and if so, to determine its subcellular localization and its properties.

In addition to the effects of HCO_3^- on Mg^{2+}-dependent ATPase activity, a variety of other anions are reported to modify its activity (TURBECK et al., 1968; BLUM et al., 1971; LIANG and SACKTOR, 1976; VAN AMELSVOORT et al., 1978A). This has led several authors to suggest that the enzyme is more accurately described as an anion-sensitive ATPase (WIEBELHAUS et al., 1971; DE PONT et al., 1972; VAN AMELSVOORT et al., 1977A, B). In order to permit comparison between Locusta Malpighian tubule ATPase and ATPases from other tissues the present study has been extended to determine the sensitivity of the locust enzyme to a variety of anions rather than HCO_3^-.

The inclusion of carbonic anhydrase in the reaction medium was reported to stimulate the Mg^{2+}-dependent HCO_3^--stimulated ATPase in rabbit brush-border (LIANG and SACKTOR, 1976) and erythrocyte

membranes (VAN AMELSVOORT et al., 1978A). WIEBELHAUS et al. (1971) have proposed that the HCO_3^--stimulated, SCN^- inhibited ATPase and carbonic anhydrase are likely to be functionally linked together. Similar linkage between these two enzymes has also been suggested by other workers (SACHS et al., 1972B; HEGNER and ANIKA, 1975; SIMON et al., 1972B). In the present study (Chapter 4), the carbonic anhydrase inhibitor, sodium acetazolamide (HOUSTON and Mc CARTY, 1978) was shown to inhibit fluid secretion in locust Malpighian tubules. Similar effects, on anion exchange, have been reported with cat pancreas (CASE et al., 1979) where carbonic anhydrase has been implicated in the secretory process. It has also been reported that sodium acetazolamide inhibits HCO_3^- transport (secretion and absorption) in rabbit and rat kidney (Mc KINNEY and BURG, 1978A,B; LUCCI et al., 1979). This being so, the presence of carbonic anhydrase activity in Locusta tubules has been examined together with the effects of sodium acetazolamide on the Mg^{2+}-dependent HCO_3^--stimulated ATPase. In addition, a study has been made on the effects of certain other pharmacological agents, known to inhibit fluid secretion by insect Malpighian tubules (GEE, 1976B; present study), on the Mg^{2+}-dependent HCO_3^--stimulated ATPase of Locusta Malpighian tubules.

MATERIALS AND METHODS

1. Preparation of the membrane microsomal fraction

The method employed was essentially the same as that described by DUNCAN (1975) with slight modifications.

Approximately equal numbers of mature adult male and female locusts were used throughout. Animals were killed by decapitation and their alimentary tracts dissected free and quickly transferred to an ice-cold homogenization medium consisting of 20mM imidazole-HCl, pH 7.5. The Malpighian tubules were dissected from the rest of the alimentary canal and placed in an homogenization tube containing $10cm^3$ of fresh ice-cold homogenization medium.

Homogenization was carried out in a glass homogenizer with a Teflon pestle (clearance 0.1-0.15mm) with 20 passes of the plunger at 1,000 rev/min. The homogenization medium was surrounded by ice throughout this procedure. The resulting homogenate was then centrifuged at 600g (2,000 rpm) for 10 min at 0-4°C in an MSE 2L. The pellet was discarded and the supernatant was transferred to thin-walled polypropylene tubes and centrifuged at 20,000g (17,000 rpm) for 30 min at 0-4°C using an MSE Automatic Superspeed 40, head number 2409; or Prepspin 50, head number 43114-125. Once again, the pellet was discarded and the supernatant was retained and centrifuged at 100,000g (38,000 rpm) for 60 min at 0-4°C. The supernatant was discarded and the pellet was resuspended in $10cm^3$ of washing medium I (20mM imidazole, 4mM $MgCl_2$ and 1mM EDTA, pH 7.5 with HCl). The preparation was centrifuged for 45 min at 100,000g. The pellet was resuspended

in washing medium II (20mM imidazole and 2mM $MgCl_2$, pH 7.5 with HCl) and reseparated by centrifugation for 45 min at 100,000g twice more. The final pellet was resuspended in ice-cold deionized water and was homogenized to ensure an even suspension. The final volume varied depending on the size of the experiment; in all cases care was taken to ensure that the final protein concentration did not fall below 70µg/cm^3.

In some experiments the 600g and 20,000g pellets were collected, washed in washing media I and II (as above) and re-centrifuged at the appropriate speed for 30 min and resuspended in deionized water as described for the 100,000g pellet above.

In other experiments a mitochondrial preparation was used for comparison with the preparations referred to above. The method of mitochondrial separation was based on that described by MINKS (1967) with some modifications. The Malpighian tubules were removed and placed in 5cm^3 of the ice-cold homogenization medium described above. They were gently pounded with a pestle. The resulting mass was filtered through four layers of muslin, which had been previously boiled in deionized water and saturated with cold homogenization medium. The residue was washed with 5cm^3 of homogenization medium, and the combined filtrates were centrifuged for 10 min at 150g (600 rpm). The pellet was discarded and the supernatant was recentrifuged for 10 min in an MSE High Speed 18, head number 69182 at 5,000g (6,500 rpm). The resulting pellet was washed in washing media I and II (as described above), recentrifuged at 5,000g for 10 min and finally resuspended in deionized water.

2. Experimental Procedure

Incubations were carried out in a water bath at 30 ± 0.1°C for 30 min. The temperature of the water bath was controlled by a 1100 watt immersion heater (TE-7 Tempette, Techne (Cambridge) Limited, Duxford, Cambridge, England).

Appropriate reaction media were thermoequilibrated for 15 min in Pyrex test tubes in the water bath. These consisted of 1cm^3 of ionic medium and 0.5cm^3 of 12 mM ATP (sodium salt), unless otherwise stated. Three ionic media having the following final concentrations of ions were used: (1) 2mM $MgCl_2$ in 20mM imidazole; (2) 2mM $MgCl_2$ and 20 mM NaCl in 20mM imidazole; (3) 2mM $MgCl_2$ and 20mM $NaHCO_3$ in 20mM imidazole (pH 7.5), unless otherwise stated. Reactions were started by adding 0.5cm^3 homogenate and they were stopped by adding 4cm^3 of 1:1 mixture of 1% cirrasol ALN-WF and 1% ammonium molybdate in 0.9M sulphuric acid (ATKINSON et al., 1973). Controls were used in each experiment to determine the extent of non-enzymatic hydrolysis of ATP.

3. Analysis of inorganic phosphate

Following centrifugation to remove any protein which precipitated, tubes were kept at room temperature for exactly 10 min to allow the yellow colour to develop. The contents of each tube was then transferred to a glass cuvette (1cm light path) and the absorbancy measured at 390nm in a Pye Unicam 1800 Dual Beam Spectrophotometer with a tungsten filament light source and a 10mm slit width. The amount of phosphate (Pi) released was determined by reference to a standard calibration curve relating

absorbancy to Pi concentration (ATKINSON et al. 1973).

The standard calibration curve was provided by using a stock solution of 0.6mM Na_2HPO_4 which was serially diluted to give a concentration range of 0-0.6mM (see Appendix 3.1).

4. Estimation of the ATPase activity

The HCO_3^- stimulation of ATPase activity was calculated as the difference between the activity in the presence of $MgCl_2$ + NaCl and the activity in the presence of $MgCl_2$ + $NaHCO_3$, unless otherwise stated. The Mg^{2+}-dependent ATPase activity was calculated by subtracting the values for the control tubes from those containing $MgCl_2$ alone. All results are expressed as nmoles Pi liberated/ mg protein/min.

5. Determination of carbonic anhydrase activity

a. Preparation of homogenate

The method employed was essentially the same as that described by HOUSTON and Mc CARTY (1978) with slight modifications.

Animals were killed by decapitation and the Malpighian tubules dissected out as described above. They were then placed in an homogenization tube containing $15cm^3$ of ice-cold homogenization medium consisting of 250mM sucrose, 40mM tris-H_2SO_4 (pH 7.5). Homogenization was carried out as described above and the resulting homogenate was centrifuged at 1,000g (2,500 rpm) for 20 min at 0-4°C in an MSE 2L. The pellet was discarded and the supernatant assayed for carbonic anhydrase activity.

b. Determination of enzyme activity

The colorimetric method of WILBUR and ANDERSON (1948) was

used with some modifications.

b.1 Reagents

Veronal buffer - 4.123 mg of veronal (barbitone sodium) was dissolved in 1 litre of deionized water and adjusted to pH 8.7 with N HCl.

pH indicator - 100mg of phenol red was dissolved in 5.7cm^3 of 0.05N NaOH and made up to 100cm^3 with deionized water (BELCHER and NUTTEN, 1960). The pH was adjusted to 6.3 with NaOH.

Saturated CO_2 solution - CO_2 was bubbled through a plastic tube into fresh-distilled water (which had been boiled immediately prior to use) at 0°C for at least 45 min before use.

b.2 Procedure

Carbonic anhydrase assays were carried out in test tubes (15cm x 2.4cm) and solutions were pipetted into the tubes by Finnpipette instead of by syringe, as used by WILBUR and ANDERSON (1948). Pairs of test tubes were set up: one, to determine the catalysed rate, consisted of 2cm^3 of veronal buffer, 1cm^3 of homogenate and 1 drop of phenol red solution; the other, to determine the uncatalysed rate, consisted of the same solutions except 1cm^3 of ice-cold sucrose homogenization medium was used instead of the homogenate. The reaction was started by adding 2cm^3 of CO_2-saturated water. The time required for the pH to drop from pH 8.15 to 6.3 was determined and the activity of enzyme was calculated according to the following equation:

$$\text{Activity unit} = \frac{t_o - t}{t}$$

where t_o is the reaction time for the uncatalysed reaction and t represents the reaction time for the catalysed reaction.

6. Succinate dehydrogenase activity

The method employed was the same as that described by KING (1967) with slight modifications.

6.1 Principle

Succinate dehydrogenase can catalyse the oxidation of succinate in the presence of certain artificial electron acceptors. The determination of the activity of the enzyme is based on this property. The artificial electron acceptor which was used in this study was ferricyanide. In the presence of sodium succinate (substrate), succinate dehydrogenase and ferricyanide, the succinate is converted to fumarate, and ferricyanide to ferrocyanide. The overall reaction according to SINGER and KEARNEY (1967) is:

$$\text{Succinate} + 2\ \text{Ferricyanide} \rightarrow \text{Fumarate} + 2\text{Ferrocyanide} + 2H^+$$

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

6.2 Reagents

0.2M phosphate buffer (pH 7.8)

0.6M succinic acid (sodium salt) in water, adjusted to pH 7.8 with NaOH.

0.03M potassium ferricyanide in water (freshly prepared).

1% bovine serum albumin (BSA) in water.

6.3 Preparation of homogenate

The method was the same as that described for ATPase activity (see above). Succinate dehydrogenase was determined in three fractions of the Malpighian tubules of locust; 600g, 20,000g and 100,000g.

6.4 Experimental procedure

Reaction media were thermoequilibrated in Spectronic 20 tubes in a water bath at 30 $\pm$ 0.1°C for 15 minutes. These consisted of 1.5cm^3 buffer, 0.2cm^3 sodium succinate, 0.3cm^3 BSA, 0.1cm^3 potassium ferricyanide and 0.5cm^3 water. The reaction was started by the addition of 1cm^3 diluted homogenate. Changes in absorbancy, at 420nm, were measured at 2 min intervals over a 20 min period using a Spectronic 20 spectrophotometer. Control tubes were run in parallel in which 1cm^3 water replaced the 1cm^3 of homogenate.

6.5 Calculation

The observed changes in absorbancy were converted to units of mM succinate oxidized as described by KING (1967) viz. 0.485 x Δ420nm. The specific activity is expressed as µmoles of succinate oxidised/mg protein/min.

7. Na^+-K^+ATPase activity

7.1 Preparation of the membrane microsomal fraction

The method employed was essentially the same as that described by PEACOCK et al. (1972).

The Malpighian tubules were dissected out as described before (see above) and they were placed in an homogenization tube containing

10cm^3 of ice-cold homogenization medium consisting of 250mM mannitol, 5mM EDTA and 0.1% sodium deoxycholate in 30mM histidine - HCl, pH 7.2. Homogenization was carried out as described before and the resulting homogenate was extracted using sodium iodide solution consisting of 4M NaI, 5mM $MgCl_2$ and 10mM EDTA pH 7.2. The homogenate was transferred to a 100cm^3 Pyrex conical flask and 10cm^3 of NaI solution was mixed with it. The mixture was allowed to stand for 30 min on ice. After 30 min it was diluted by adding 30cm^3 of deionized water (giving a ratio of 1 NaI : 1.5H_2O). This gives a final NaI concentration of 0.8M.

The homogenate was transferred to thin-walled polypropylene tubes and centrifuged at 50,000g (27,000 rpm) for 30 min at 0-4^oC using MSE Automatic Superspeed 40. The pellet was discarded and the supernatant retained and centrifuged at 100,000g (38,000 rpm) for 60 min at 0-4^oC. The supernatant was discarded and the pellet was resuspended in 10cm^3 of washing medium consisting of 5mM NaCl and 5mM EDTA pH 7.2. The preparation was centrifuged for 45 min at 100,000g, and then the complete washing procedure was repeated. The final pellet was resuspended in ice-cold deionized water and was homogenized to ensure an even suspension.

7.2 Experimental procedure

Appropriate reaction media were thermoequilibrated for 15 min in Pyrex test tube in the water bath. These consisted of 1cm^3 of ionic media, 0.5cm^3 of 12mM Tris ATP (see Appendix 3.3 for method of preparation). Two ionic media, having the following final concentrations of ions were generally used: (1) 4mM $MgCl_2$ in

50mM histidine - HCl pH 7.2; (2) 4mM $MgCl_2$, 20mM KCl and 100mM NaCl in 50mM histidine - HCl pH 7.2, unless otherwise stated. Reactions were started by adding 0.5cm^3 homogenate and they were stopped by adding 4cm^3 of 1:1 mixture of 1% cirrasol ALN-WF and 1% ammonium molybdate in 0.9M sulphuric acid (ATKINSON et al., 1973) as described previously. Controls were used in each experiment to determine the extent of non-enzyme hydrolysis of ATP.

Analyses of inorganic phosphate were carried out as described above.

7.3 Estimation of the Na^+-K^+ATPase

The Na^+-K^+ ATPase activity was calculated as the difference between the activity in the presence of Mg^{2+} alone and the activity in the presence of Mg^{2+}, Na^+ and K^+. The Mg^{2+}-dependent ATPase activity was calculated by subtracting the values for the control tubes from those containing Mg^{2+} alone. All results are expressed as nmoles Pi liberated/mg protein/min.

8. Protein estimation

Protein determination was made by the method of LOWRY et al. (1951) using bovine serum albumin (BSA) Fraction V (Sigma Chemical Co.) as standard (see Appendix 3.2 for typical calibration curve).

9. Treatments of saturation kinetics data

The results were presented graphically using the Lineweaver-Burk (1934) plot which modifies the Michaelis-Menton equation, $\frac{1}{[s]}$ was plotted against $\frac{1}{[v]}$ (s = substrate concentration, v = reaction velocity). V_{max} was calculated from the intercept on the y axis

$(1/V_{max})$ and K_m from the intercept on the x axis $(\frac{-1}{K_m})$. The slopes were calculated by regression analysis (SNEDECOR and COCHRAN, 1967).

10. Electron microscopy of membrane fractions

(i) Preparation of membrane fractions

Preparations were obtained from the Malpighian tubules of locust as described before (see (1) above). Three fractions: 600g, 20,000g and 100,000g were examined.

(ii) Fixation, embedding and staining of the 600g, 20,000g and 100,000g pellets was carried out according to the following schedule:

1. Fix pellets in 5% gluteraldehyde in 0.1M sodium cacodylate buffer (pH 7.3) overnight.
2. Wash in 0.1M sodium cacodylate buffer (2-3 hours).
3. Post fix in 1% osmium tetroxide in 0.1M sodium cacodylate (pH 7.3) for 2 hours.
4. Wash in 0.1M cacodylate buffer for 30 min.
5. Dehydration through a series of graded alcohols (50%, 70%, 95%) 10 min each, 2 x 30 min in absolute alcohol.
6. 2 x 10 min in propylene oxide.
7. Infiltrate in 50:50 Epon resin (see below) and Epoxypropane overnight.
8. Infiltrate in Epon for 8 hours.
9. Embedded in fresh Epon. Polymerisation was effected at 60°C for 48 hours.

Epon resin:

Mix equal volumes of

A { Epon 812 (62 volumes)
  { DDSA (100 volumes)

and

B { Epon 812 (100 volumes)
  { MNA (89 volumes)

Silver/silver gold sections were cut on a Reichart NK ultratome, expanded with diethyl ether vapour and mounted on uncoated copper grids. Sections were stained in uranyl acetate and lead citrate (REYNOLDS, 1963) and were examined in an AEI 801 electron microscope.

RESULTS

3.1 Effect of HCO_3^- and Cl^- concentration on ATPase activity

Mg^{2+}-dependent ATPase activity was determined over a range of concentrations of NaCl and $NaHCO_3$. The results are shown in Figure 3.1 and Table 3.1. It can be seen that different concentrations of Cl^- did not markedly affect the Mg^{2+}-dependent ATPase activity, although in some experiments slight inhibition of activity was observed. In contrast, when $NaHCO_3$ was substituted for NaCl there was a significant stimulation of the basic Mg^{2+}-dependent ATPase activity, maximum stimulation being observed at 20mM HCO_3^-. Further increases in $NaHCO_3$ concentration resulted in a somewhat reduced level of stimulation. With 40mM $NaHCO_3$, the stimulation of Mg^{2+}-dependent ATPase decreased to 63.8 $\pm$ 12.3% compared with that obtained with 20mM $NaHCO_3$.

In a series of experiments (n = 61) mean activity rose from 304 $\pm$ 13 (Mg^{2+} = 2mM) and 298 $\pm$ 13 nmoles Pi liberated/mg protein/min (Mg^{2+} = 2mM, NaCl = 20mM) to 448 $\pm$ 19 nmoles Pi liberated/mg protein/min when 20mM $NaHCO_3$ was substituted for the NaCl. A significant rise to 149 $\pm$ 2% of the rate in the presence of Mg^{2+} alone and 152 $\pm$ 2% of rate in the presence of Mg^{2+} and NaCl ($P<0.001$).

3.2 Effect of pH on ATPase activity

A 30mM bis-tris propane buffer system, was used to produce a stable pH range from pH 7.0 to 9.0. Reaction media containing 3mM

TABLE 3.1

Effect of Cl^-/HCO_3^- on ATPase activity

Concentration of Cl^-/HCO_3^- (mM)	Mg^{2+}-dependent ATPase activity		% stimulation of activity due to the presence of HCO_3^- instead of Cl^-
	+ NaCl	+ $NaHCO_3$	
10	227.0 ± 42.9	331.0 ± 58.6	147.2 ± 2.7
15	227.0 ± 42.9	342.6 ± 62.5	151.8 ± 3.1
20	227.6 ± 42.8	348.2 ± 65.6	153.2 ± 2.2
25	230.8 ± 42.1	339.4 ± 66.0	146.2 ± 3.4
30	229.0 ± 43.5	338.6 ± 65.6	148.2 ± 3.5
40	231.4 ± 42.2	317.6 ± 55.3	138.4 ± 3.7

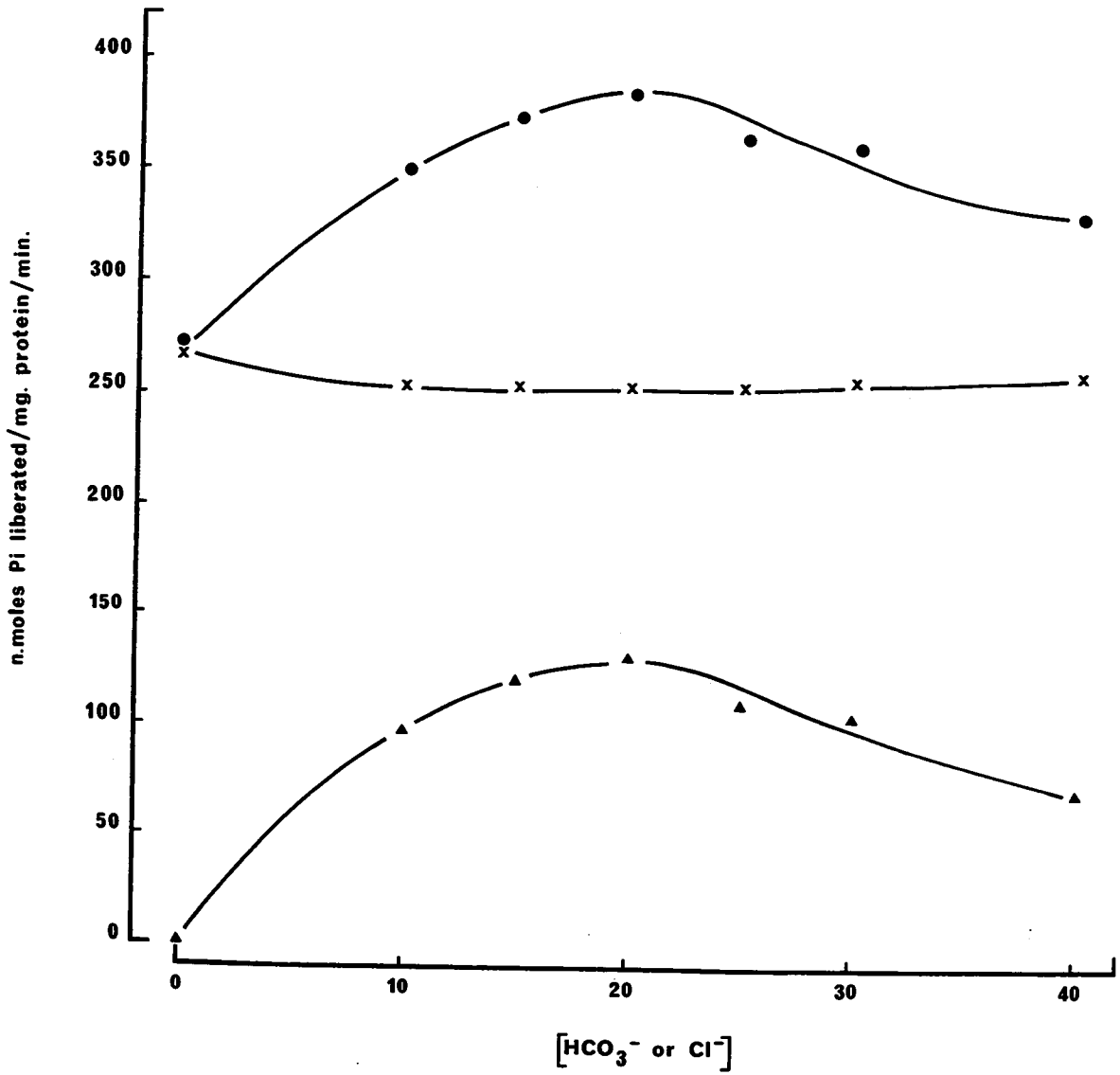
Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M. The Mg^{2+}-dependent ATPase activity in the absence of Cl^- and HCO_3^- was 237.6 ± 38.1 nmoles Pi liberated/mg protein/min.

Figure 3.1

Effect of HCO_3^-/Cl^- concentration on the ATPase activity in the presence of 2mM Mg^{2+} + $NaHCO_3$ (●); 2mM Mg^{2+} + NaCl (x); stimulation due to the presence of HCO_3^- instead of Cl^- (▲). Typical experiment representative for five experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: HCO_3^- or Cl^- concentration (mM).


n.moles Pi liberated/mg. protein/min.
400
350
300
250
200
150
100
50
0
0
10
20
30
40
[HCO3- or Cl-]

ATP were adjusted to the required pH with HCl. Solutions were stored at -20°C until ready to be used. $1cm^3$ of reaction medium (ionic medium + ATP) was pipetted into a test tube and the reaction started by the addition of $1cm^3$ homogenate. Figure 3.2 shows the changes observed in Mg^{2+}-dependent ATPase activity in relation to pH. The pH optimum was 7.5 in the absence of NaCl and $NaHCO_3$. However, this value was somewhat modified by the inclusion of NaCl and $NaHCO_3$ in the reaction media. In the presence of NaCl maximal activity was observed at pH 7.75 $\pm$ 0.1 whilst in the presence of $NaHCO_3$ the pH optimum was 7.58 $\pm$ 0.1. The stimulation due to HCO_3^- was also maximal at pH 7.58 $\pm$ 0.1 (n = 6) (see Appendix 3, Table 1).

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

Mg^{2+}-dependent ATPase and Mg^{2+}-dependent HCO_3^--stimulated ATPase were assayed in reaction media in which the Mg^{2+} concentration varied from 0 to 20mM. Figure 3.3 shows the relationship between ATPase activity and Mg^{2+} concentration. In the absence of Mg^{2+} there was, of course, no activity. A marked increase in activity was observed with increasing Mg^{2+} concentration up to 2mM for Mg^{2+}-dependent HCO_3^--stimulated ATPase (Table 3.2). Increasing the Mg^{2+} concentration above 2mM resulted in a decline in ATPase activity. This decline was rapid initially but thereafter, increased Mg^{2+} had a lesser effect (Figure 3.3). Maximal stimulation due to the presence of HCO_3^- was observed at 2mM Mg^{2+}.

TABLE 3.2

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

Concentration of Mg^{2+} (mM)	Mg^{2+}-dependent ATPase activity		% stimulation of activity due to the presence of HCO_3^-
	$-NaHCO_3$	$+NaHCO_3$	
0	0	0	0
1	125.6 ± 12.2	165.0 ± 17.4	131.2 ± 6.4
2	153.0 ± 16.9	210.0 ± 28.3	136.6 ± 7.3
3	144.3 ± 13.9	190.0 ± 24.0	130.8 ± 4.5
4	133.0 ± 12.0	176.3 ± 22.7	131.6 ± 5.7
6	132.3 ± 12.3	158.6 ± 20.7	119.0 ± 4.8
8	132.6 ± 12.0	143.0 ± 20.0	109.8 ± 2.8
10	126.3 ± 17.0	147.3 ± 18.9	116.8 ± 1.4
15	130.0 ± 13.4	131.0 ± 19.6	107.1 ± 2.5
20	124.6 ± 10.0	122.0 ± 16.8	103.7 ± 1.1

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

Figure 3.2

Effect of pH on ATPase activity in the presence of 2mM Mg^{2+} (o); 2mM Mg^{2+} + 20mM NaCl (x); 2mM Mg^{2+} + 20mM $NaHCO_3$ (●). Substrate: 3mM Na_2ATP throughout. Typical experiment representative for six experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: pH.

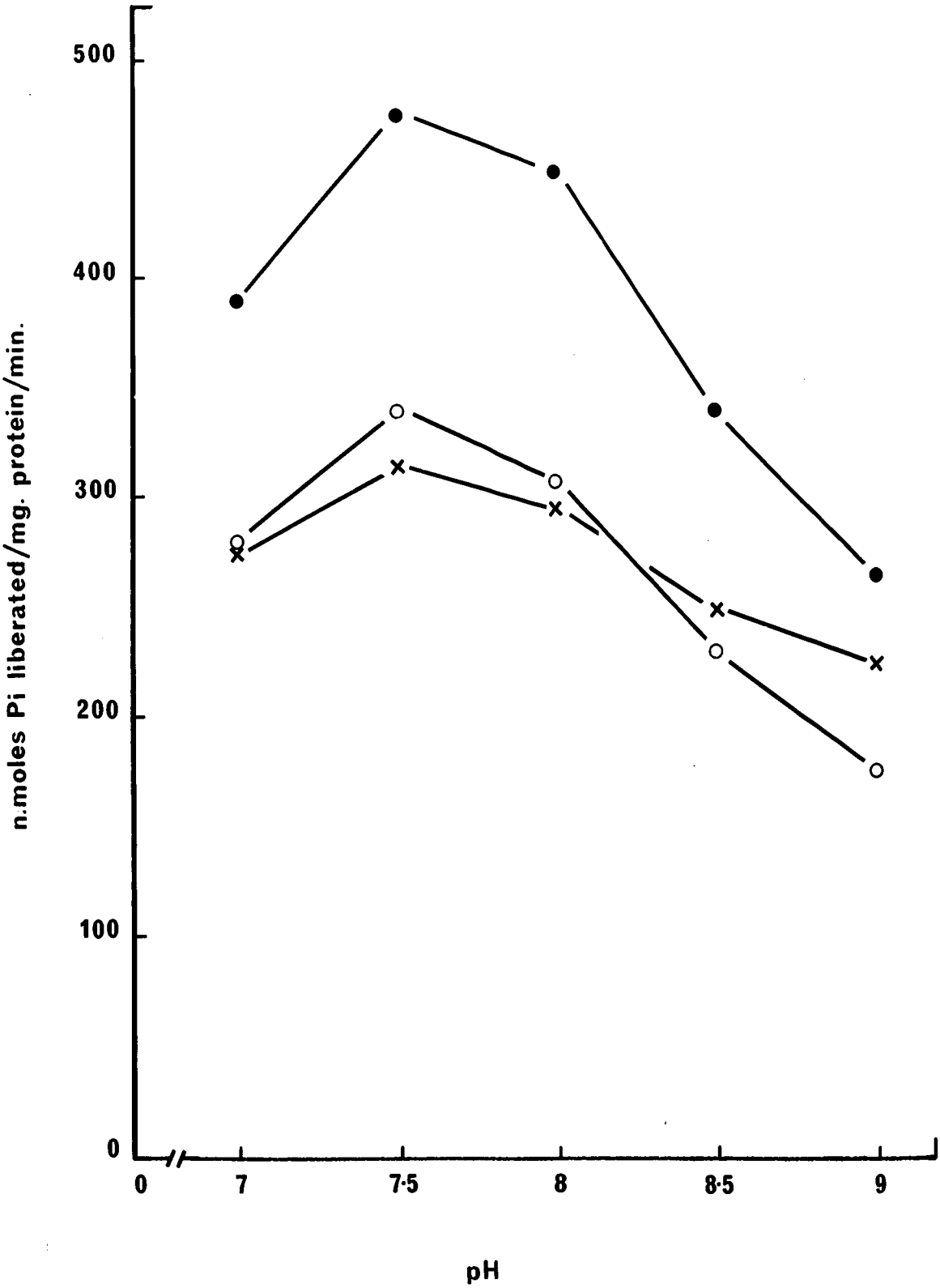
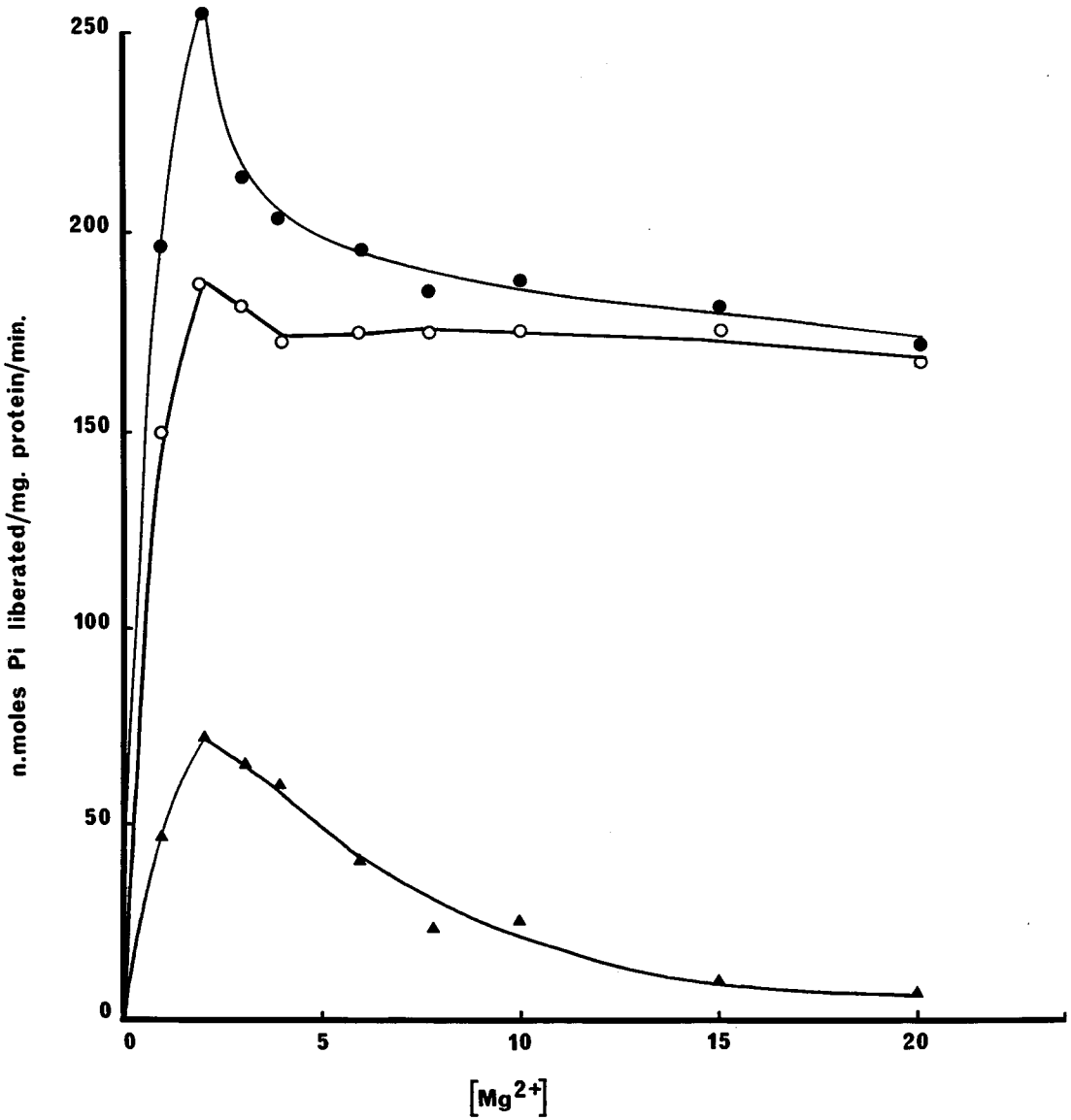

500
400
300
200
100
0
n.moles Pi liberated/mg. protein/min.
0
7
7·5
8
8·5
9
pH

Figure 3.3

Effect of Mg^{2+} concentration on ATPase activity in the presence of (●) and absence (o) of 20mM $NaHCO_3$. The stimulation due to HCO_3 (▲) represents the difference between (●) and (o). Typical experiment representative for three experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: Mg^{2+} concentration (mM).


250
200
150
100
50
0
n.moles Pi liberated/mg. protein/min.
0
5
10
15
20
[Mg2+]

3.4 Effect of sodium acetazolamide on ATPase activity

Mg^{2+}-dependent ATPase and Mg^{2+}-dependent HCO_3^--stimulated ATPase activity from Malpighian tubules (both 20,000g and 100,000g fractions) were assayed in reaction media containing concentrations of sodium acetazolamide from 0 to 10^{-3}M. Sodium acetazolamide had no effect on either the Mg^{2+}-dependent ATPase (in the presence or absence of NaCl) or the Mg^{2+}-dependent HCO_3^--stimulated ATPase activity of the 20,000g or the 100,000g fractions, over the above concentration range (Tables 3.3 and 3.4; Appendix 3, Tables 2 and 3).

3.5 Effect of ouabain on ATPase activity

The presence of 1mM ouabain in the reaction media did not inhibit either the Mg^{2+}-dependent ATPase (in the presence or absence of NaCl) or the Mg^{2+}-dependent HCO_3^--stimulated ATPase from the 20,000g or 100,000g fractions (Table 3.5).

3.6 Effect of sodium azide on ATPase activity

Mg^{2+}-dependent ATPase and Mg^{2+}-dependent HCO_3^--stimulated ATPase were assayed in reaction media containing 1mM sodium azide. Sodium azide almost completely inhibited the activity of both the Mg^{2+}-dependent ATPase and the Mg^{2+}-dependent HCO_3^--stimulated ATPase in both the 20,000g and 100,000g fractions ($P<0.001$) (Table 3.6).

3.7 Effect of NaSCN on ATPase activity

Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and Mg^{2+}-dependent HCO_3^--stimulated ATPase were assayed in reaction media in which NaSCN was present in the concentration range 0-10mM. The relationship between ATPase activity and SCN^- concentration is shown in Figure 3.4. It can be seen that SCN^- inhibited ATPase

TABLE 3.3

Effect of sodium acetazolamide on ATPase activity (100,000g fraction)

Concentration of acetazolamide (M)	Mg^{2+}-dependent ATPase activity			Stimulation due to the presence of HCO_3^- instead of Cl^-
	-NaCl	+NaCl	+$NaHCO_3$	
0	100	100	100	100
10^{-7}	96.8 ± 2.2	99.4 ± 1.3	100.1 ± 1.6	106.1 ± 9.5
10^{-6}	97.3 ± 1.8	97.8 ± 1.5	99.4 ± 2.2	104.8 ± 4.9
10^{-5}	98.5 ± 2.5	98.1 ± 1.1	98.6 ± 1.4	103.6 ± 8.2
10^{-4}	100.8 ± 2.2	98.4 ± 1.0	99.4 ± 1.9	107.8 ± 14.3
10^{-3}	104.6 ± 2.3	96.9 ± 0.6	99.4 ± 2.7	113.6 ± 16.8

Activity is expressed as a % of that observed in the absence of sodium acetazolamide. The 100% activity was 231 ± 12 (-NaCl), 233 ± 18 (+ NaCl), 297 ± 15 (+ $NaHCO_3$) and 73.3 ± 15 nmoles Pi liberated/mg protein/min (HCO_3^- stimulation). Each value represents the average of three experiments ± S.E.M.

TABLE 3.4

Effect of sodium acetazolamide on ATPase activity (20,000g fraction)

Concentration of acetazolamide (M)	Mg^{2+}-dependent ATPase activity			Stimulation due to the presence of HCO_3^- instead of Cl^-
	-NaCl	+NaCl	$+NaHCO_3$	
0	100	100	100	100
10^{-7}	101.3 ± 2.6	99.6 ± 0.7	102.0 ± 0.9	112.1 ± 4.2
10^{-6}	100.3 ± 2.9	100.8 ± 1.9	100.7 ± 0.8	98.9 ± 9.6
10^{-5}	100.6 ± 1.8	99.8 ± 0.6	101.6 ± 0.3	108.5 ± 4.7
10^{-4}	101.2 ± 1.9	101.1 ± 0.4	102.8 ± 0.4	108.9 ± 5.8
10^{-3}	104.3 ± 0.7	98.2 ± 1.6	102.2 ± 2.4	118.0 ± 20.5

Activity is expressed as a % of that observed in the absence of sodium acetazolamide. The 100% activity was 453.0 ± 42.8 (-NaCl), 476.6 ± 47.7 (+ NaCl), 607.0 ± 97.2 ($+NaHCO_3$) and 138.6 ± 47.8 nmoles Pi liberated/mg protein/min (HCO_3^- stimulation). Each value represents the average of three experiments ± S.E.M.

TABLE 3.5

Effect of ouabain on ATPase activity

	Enzyme activity			
	20,000g fraction		100,000g fraction	
	-ouabain	+ ouabain	- ouabain	+ ouabain
Mg^{2+}-dependent ATPase (in the absence of NaCl)	460.0 ± 11.7	453.0 ± 10.2	315.6 ± 21.4	308.6 ± 16.5
Mg^{2+}-dependent ATPase (in the presence of NaCl)	503.3 ± 9.0	491.6 ± 8.4	322.0 ± 20.0	310.0 ± 19.6
Mg^{2+}-dependent HCO_3^--stimulated ATPase	849.3 ± 17.3	809.0 ± 16.0	526.3 ± 37.9	502.3 ± 37.1

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

TABLE 3.6

Effect of sodium azide on ATPase activity

	Enzyme activity			
	20,000g fraction		100,000g fraction	
	- azide	+ azide	- azide	+ azide
Mg^{2+}-dependent ATPase (in the absence of NaCl)	463.0 ± 11.7	3.5 ± 0.1	315.6 ± 21.4	2.0 ± 0.3
Mg^{2+}-dependent ATPase (in the presence of NaCl)	503.3 ± 9.0	3.9 ± 0.1	322.0 ± 20.0	2.1 ± 0.4
Mg^{2+}-dependent HCO_3^--stimulated ATPase	849.0 ± 17.3	7.7 ± 0.1	526.3 ± 37.9	4.2 ± 0.3

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

activity irrespective of whether HCO_3^- or Cl^- were present or not. Whilst there was an overall reduction in ATPase activity due to the presence of SCN^-, the stimulation due to HCO_3^- was less sensitive to SCN^- than the Mg^{2+}-dependent ATPase (in the absence and presence of NaCl) (Table 3.7). Thus 5mM SCN^- effected a 79% inhibition of Mg^{2+}-dependent ATPase (72% in the presence of 20mM NaCl) and 63% inhibition of the Mg^{2+}-dependent HCO_3^--stimulated ATPase. In contrast, the stimulation of the Mg^{2+}-dependent ATPase, due to the presence of HCO_3^- instead of Cl^-, in the reaction medium was inhibited by only 45% over the same concentration range. At 1mM SCN^- the difference in sensitivity was even more marked, whereas the Mg^{2+}-dependent ATPase was inhibited by 54%, the stimulation of the Mg^{2+}-dependent ATPase due to HCO_3^- was inhibited by only 14% (Table 3.7; Appendix 3, Table 4).

3.8 Effect of ATP concentration on ATPase activity

Enzyme assays were carried out in reaction media in which the ATP concentration varied between 0 and 3mM. $1cm^3$ aliquots of these reaction media were incubated with $1cm^3$ homogenate for 30 min. Care was taken to ensure that substrate availability did not become rate limiting during the course of the reactions; in no case was more than 10% of the available ATP split. Figure 3.5 shows the results obtained in a typical experiment. Figure 3.6 is a Lineweaver-Burk plot, using the same data. From such plots the apparent Michaelis constant (K_m) and V_{max} for the Mg^{2+}-dependent ATPase (in the presence of NaCl) and the Mg^{2+}-dependent

TABLE 3.7

Effect of sodium thiocyanate on ATPase activity

Concentration of NaSCN (mM)	Mg^{2+}-dependent ATPase activity			Stimulation of activity due to the presence of $NaHCO_3$ instead of NaCl
	-NaCl	+NaCl	+$NaHCO_3$	
0	100	100	100	100
0.5	60.5 ± 2.0	67.9 ± 3.0	78.8 ± 0.7	100.2 ± 4.1
1	46.2 ± 1.9	52.6 ± 3.4	63.5 ± 2.8	86.1 ± 11.0
5	20.9 ± 0.6	28.3 ± 3.8	37.0 ± 1.0	55.1 ± 7.4
10	16.5 ± 2.7	16.7 ± 4.6	22.5 ± 2.4	33.4 ± 3.2

Activity is expressed as a % of that observed in the absence of NaSCN. The 100% activity was 209.6±61.3 (-NaCl), 212.6±62.1 (+NaCl), 318±92.7 (+$NaHCO_3$) and 105.7 ± 30.1nmoles Pi/mg protein/min (HCO_3^- stimulation). Each value represents the average of three experiments ± S.E.M.

Figure 3.4

Effect of SCN^- concentration on ATPase activity in the presence of 2mM Mg^{2+} (o); 2mM Mg^{2+} + 20mM NaCl (x); 2mM Mg^{2+} + 20mM $NaHCO_3$ (●). Typical experiment representative for three experiments.

Ordinate: ATPase activity (nmoles Pi liberated/ mg protein/min).

Abscissa: SCN^- concentration (mM).

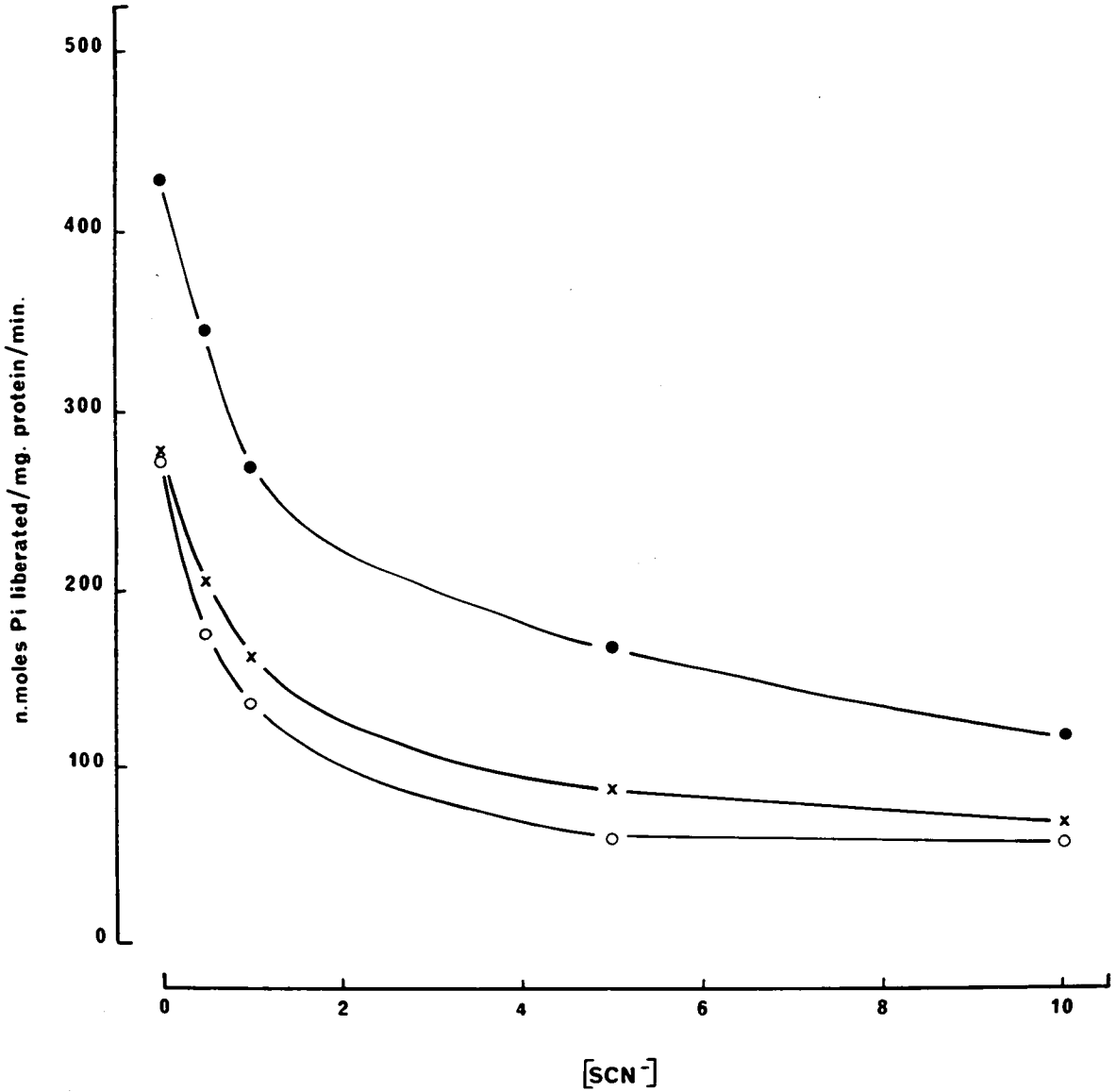

500
400
300
200
100
0
n.moles Pi liberated/mg. protein/min.
0
2
4
6
8
10
[SCN⁻]

Figure 3.5

Effect of ATP concentration on ATPase activity in the presence of 2mM Mg^{2+} + 20mM NaCl (x); 2mM Mg^{2+} + 20mM $NaHCO_3$ (●); HCO_3^- stimulated Mg^{2+}-ATPase (▲). Typical experiment representative for five experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: ATP concentration (mM).

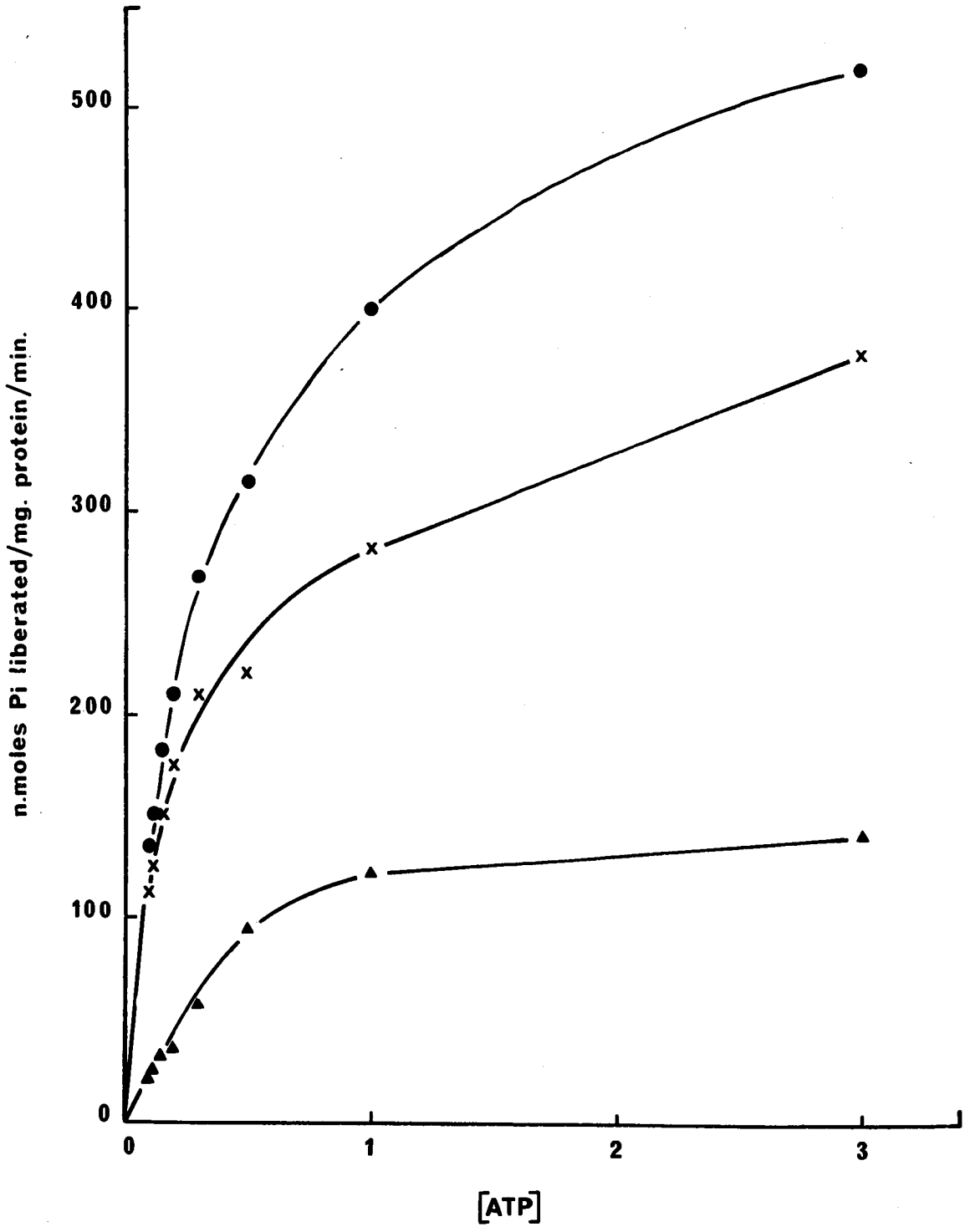
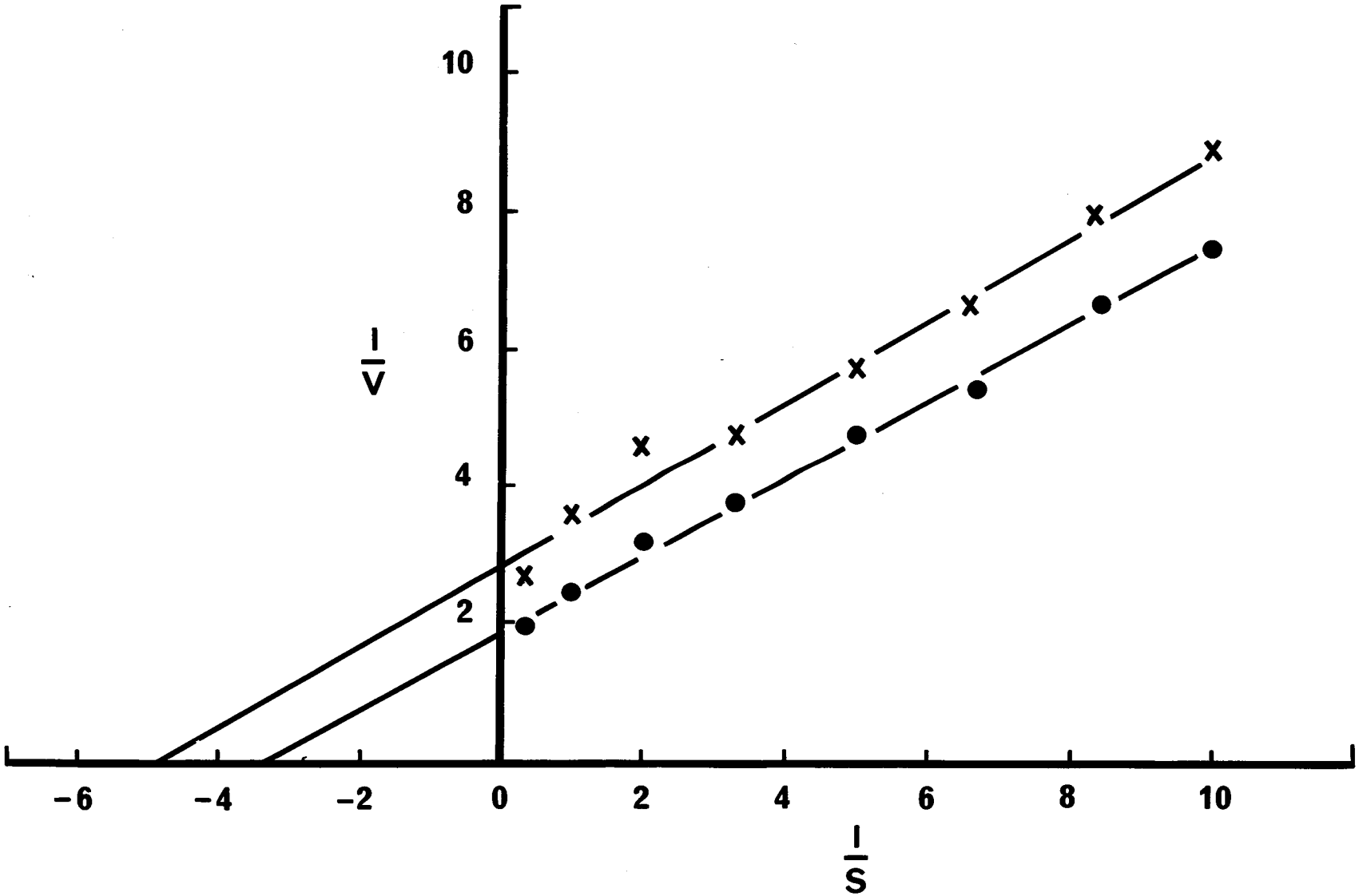

n.moles Pi liberated/mg. protein/min.
500
400
300
200
100
0
0
1
2
3
[ATP]

Figure 3.6

Lineweaver-Burk plot of ATPase activity in the presence of 2mM Mg^{2+} + 20mM $NaHCO_3$ (●); 2mM Mg^{2+} + 20mM NaCl (x). Typical experiment representative for five experiments.

Ordinate: reciprocal of ATPase activity (nmoles Pi liberated/mg protein/min) x 10^3.

Abscissa: reciprocal of ATP concentration (mM).


10
8
6
4
2
1/V
-6
-4
-2
0
2
4
6
8
10
1/S

HCO_3^--stimulated ATPase were calculated (Table 3.8). HCO_3^- increased both the K_m and the V_{max} of the Mg^{2+}-dependent ATPase. The K_m was increased from 0.16 to 0.26mM and the V_{max} from 239 to 381 nmoles Pi liberated/mg protein/min (see Table 3.8). The apparent K_m and V_{max} for the stimulation due to the presence of HCO_3^- was 0.98 mM and 214 nmoles Pi liberated/mg protein/min, respectively (see Appendix 3 Table 5).

3.9 Effect of SO_3^{2-} on ATPase activity

The effect of different concentrations of Na_2SO_3 (0-40mM) on the Mg^{2+}-dependent ATPase (in the presence and absence of 20mM NaCl) and Mg^{2+}-dependent HCO_3^--stimulated ATPase activity of locust Malpighian tubules is shown in Figure 3.7 and 3.8. Sulphite increased the activity of both Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and the Mg^{2+}-dependent HCO_3^--stimulated ATPase. Maximum activity was observed in the presence of 10mM SO_3^{2-}; the level of maximum activity being largely unaffected by the presence or absence of 20mM NaCl or $NaHCO_3$.

The activity of the Mg^{2+}-dependent ATPase was increased to 200.5 $\pm$ 16.0% of the unstimulated rate in the absence of NaCl. The presence of 20mM NaCl in the reaction medium effected a slight reduction in the percentage stimulation due to SO_3^{2-}; maximum activity in the presence of 10mM SO_3^{2-} being 176.2 $\pm$ 15.0% of the activity in its absence. An even more marked reduction in the percentage stimulation due to SO_3^{2-} was observed when 20mM HCO_3^- replaced Cl^- in the reaction media (Figure 3.8). The presence of 10mM SO_3^{2-} increased the relative activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase to only 121.3 $\pm$ 2.2% of the activity

TABLE 3.8

K_m and V_{max} values for Malpighian tubules ATPase

	n	K_m (mM ± S.E.M.)	V_{max} (nmoles Pi liberated mg protein/min ± S.E.M.)
Mg^{2+}-dependent ATPase (in presence of NaCl)	5	0.16 ± 0.01	239.0 ± 33
Mg^{2+}-dependent HCO_3^--stimulated ATPase	5	0.26 ± 0.03	381.1 ± 43
Stimulation of activity due to presence of $NaHCO_3$ instead of NaCl	5	0.98 ± 0.15	214.0 ± 32

Figure 3.7

Effect of SO_3^{2-} concentration on ATPase activity in the presence of 2mM Mg^{2+} (O); 2mM Mg^{2+} + 20mM NaCl (■); 2mM Mg^{2+} + 20mM $NaHCO_3$ (◍). Typical experiment representative for four experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: SO_3^{2-} concentration (mM).

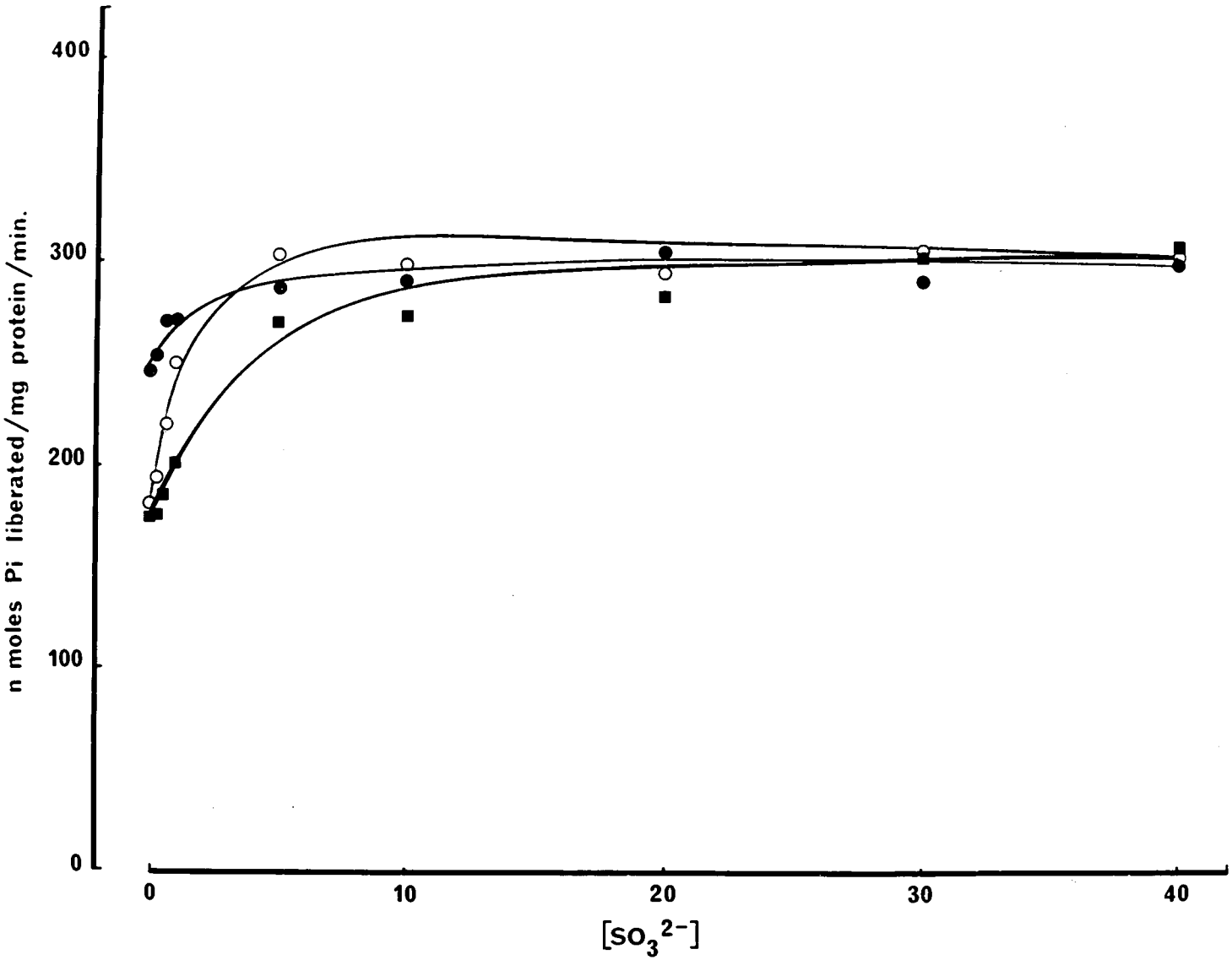

400
300
200
100
0
n moles Pi liberated/mg protein/min.
0
10
20
30
40
[SO3 2−]

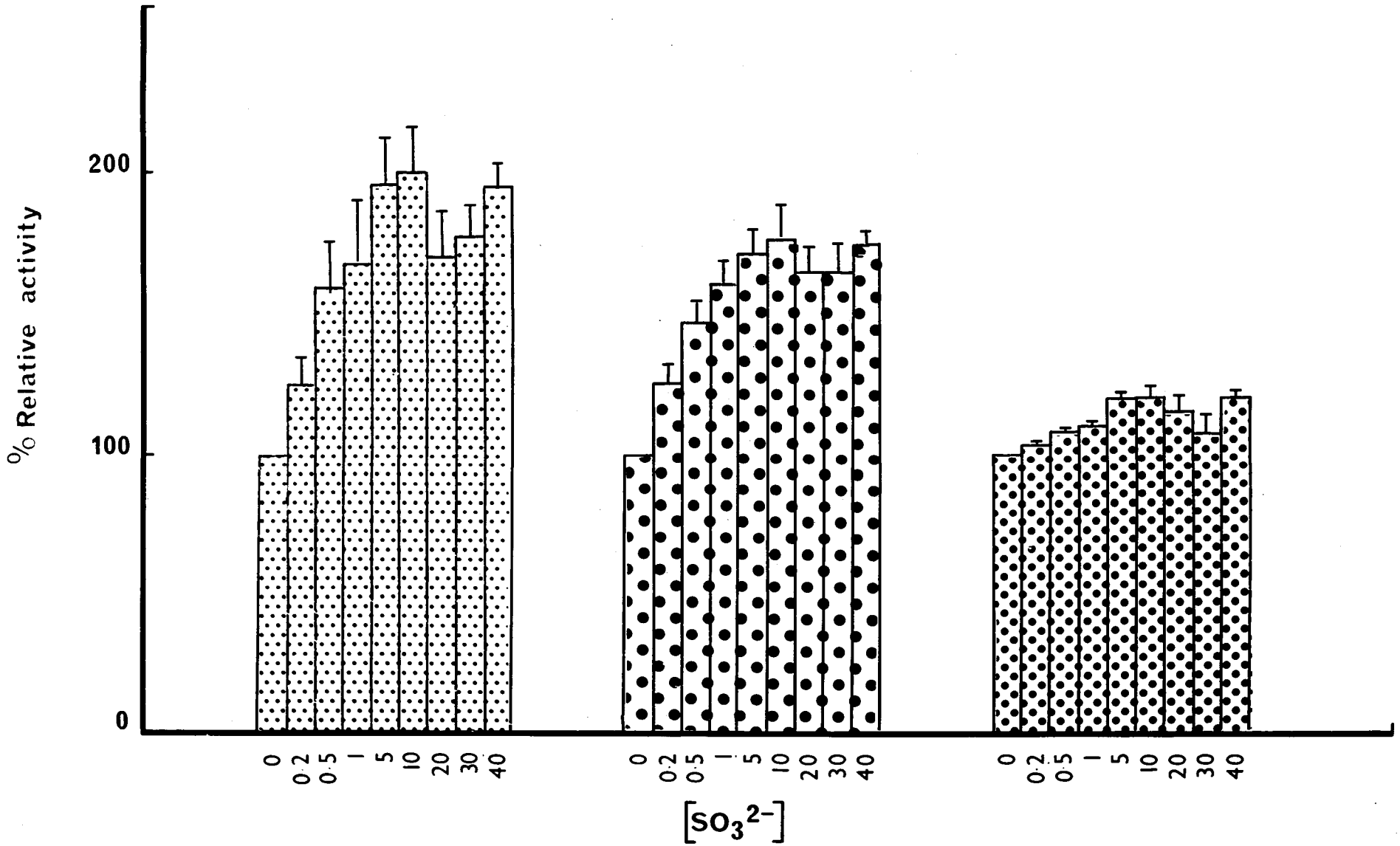
Figure 3.8

Effect of SO_3^{2-} concentration on ATPase activity in the presence of 2mM Mg^{2+} (⁘); 2mM Mg^{2+} + 20mM NaCl (⁂); 2mM $MgCl_2$ + 20mM $NaHCO_3$ (⁘). Activity is expressed as a % of that observed in the absence of SO_3^{2-}. The 100% activity was 233.2 ± 33.1 (- NaCl), 238.2 ± 29.7 (+ NaCl), 348.0 ± 52.4 (+ $NaHCO_3$) nmoles Pi liberated/mg protein/min. Each column represents the mean value of four experiments ± S.E.M.

Ordinate: % Relative ATPase activity

Abscissa: SO_3^{2-} concentration (mM).

The activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase in this series of experiments was 144.5 ± 5.8 and 148.5 ± 3.2% relative to the Mg^{2+}-dependent ATPase (in the presence and absence of NaCl respectively).


% Relative activity
200
100
0
0
0·2
0·5
1
5
10
20
30
40
[SO3 2−]

observed in its absence. As shown in Figure 3.8, further increase in SO_3^{2-} concentration, above 10mM, did not significantly increase enzyme activity (see Appendix 3, Table 6).

3.10 Effect of $B_4O_7^{2-}$ on ATPase activity

Enzyme assays were carried out in reaction media in which the concentration of $Na_2B_4O_7$ varied between 0 and 40mM. Borate increased the activity of the Mg^{2+}-dependent ATPase (in the presence and absence of 20mM NaCl) (see Figure 3.9). As shown in Figure 3.9 and 3.10 maximal activity was observed in the presence of 10mM $B_4O_7^{2-}$. Increasing the $B_4O_7^{2-}$ concentration above this, reduced the level of stimulation. In the presence of 10mM $B_4O_7^{2-}$, the Mg^{2+}-dependent ATPase activity increased to 143.6 $\pm$ 1.6% and 151.9 $\pm$ 3.5% in the presence and absence of NaCl respectively. This level of stimulation by $B_4O_7^{2-}$ is similar to that obtained with HCO_3^- (see Table 3.1). However, when $B_4O_7^{2-}$ was included in reaction medium containing 20mM HCO_3^-, the activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase was decreased (Figure 3.9 and 3.10). Indeed, as the concentration of $B_4O_7^{2-}$ increased, so the activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase decreased (Figure 3.10; Appendix 3, Table 7).

3.11 Effect of SeO_3^{2-} on ATPase activity

Mg^{2+}-dependent ATPase and Mg^{2+}-dependent HCO_3^--stimulated ATPase were assayed in reaction media in which Na_2SeO_3 was present over the concentration range 0-40mM. The results obtained are shown in Figures 3.11 and 3.12 (Appendix 3, Table 8).

SeO_3^{2-} stimulated the activity of the Mg^{2+}-dependent ATPase;

Figure 3.9

Effect of $B_4O_7^{2-}$ concentration on ATPase activity in the presence of 2mM Mg^{2+} (O); 2mM Mg^{2+} + 20mM NaCl (■); 2mM Mg^{2+} + 20mM $NaHCO_3$ (●). Typical experiment representative for four experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: $B_4O_7^{2-}$ concentration (mM).

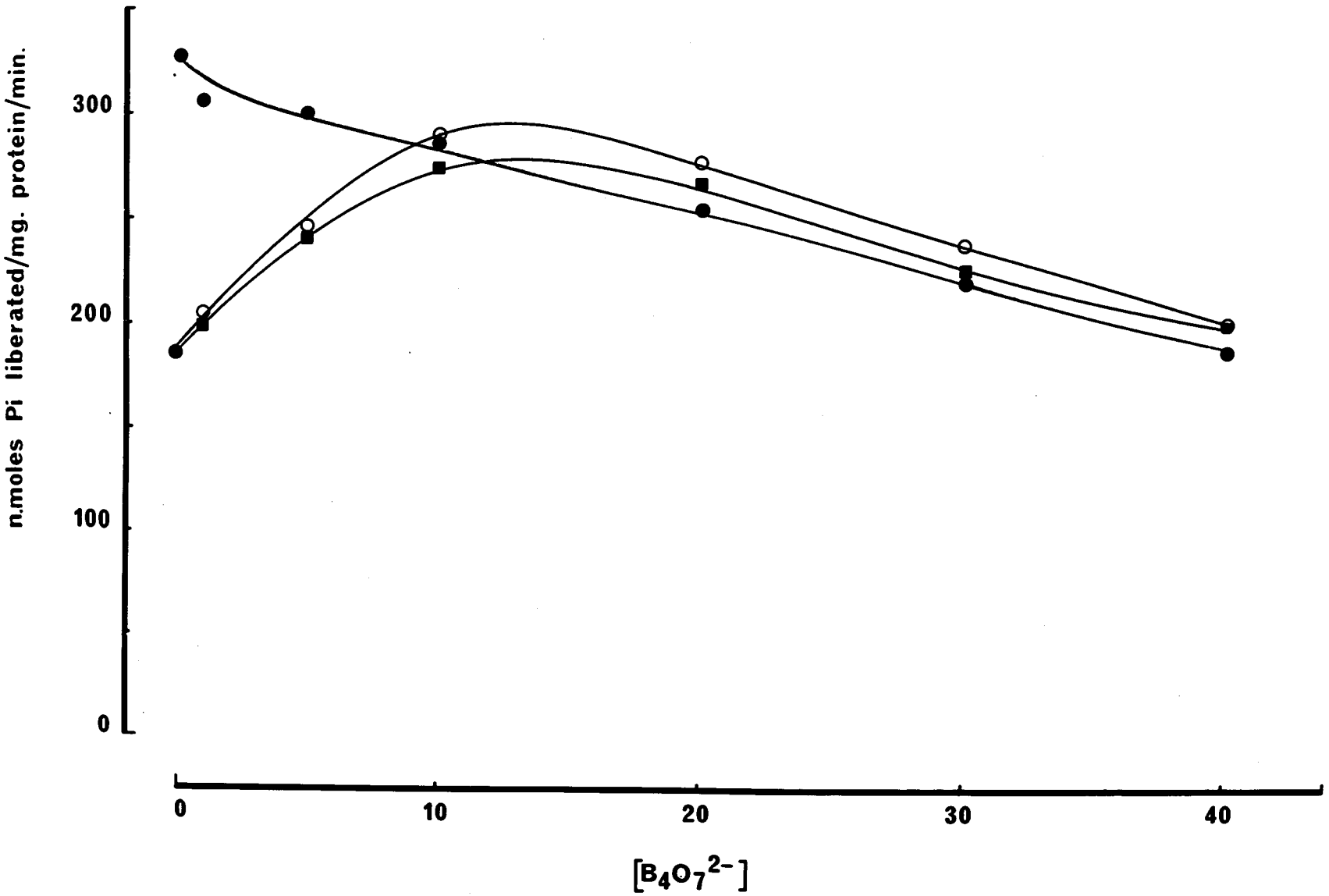
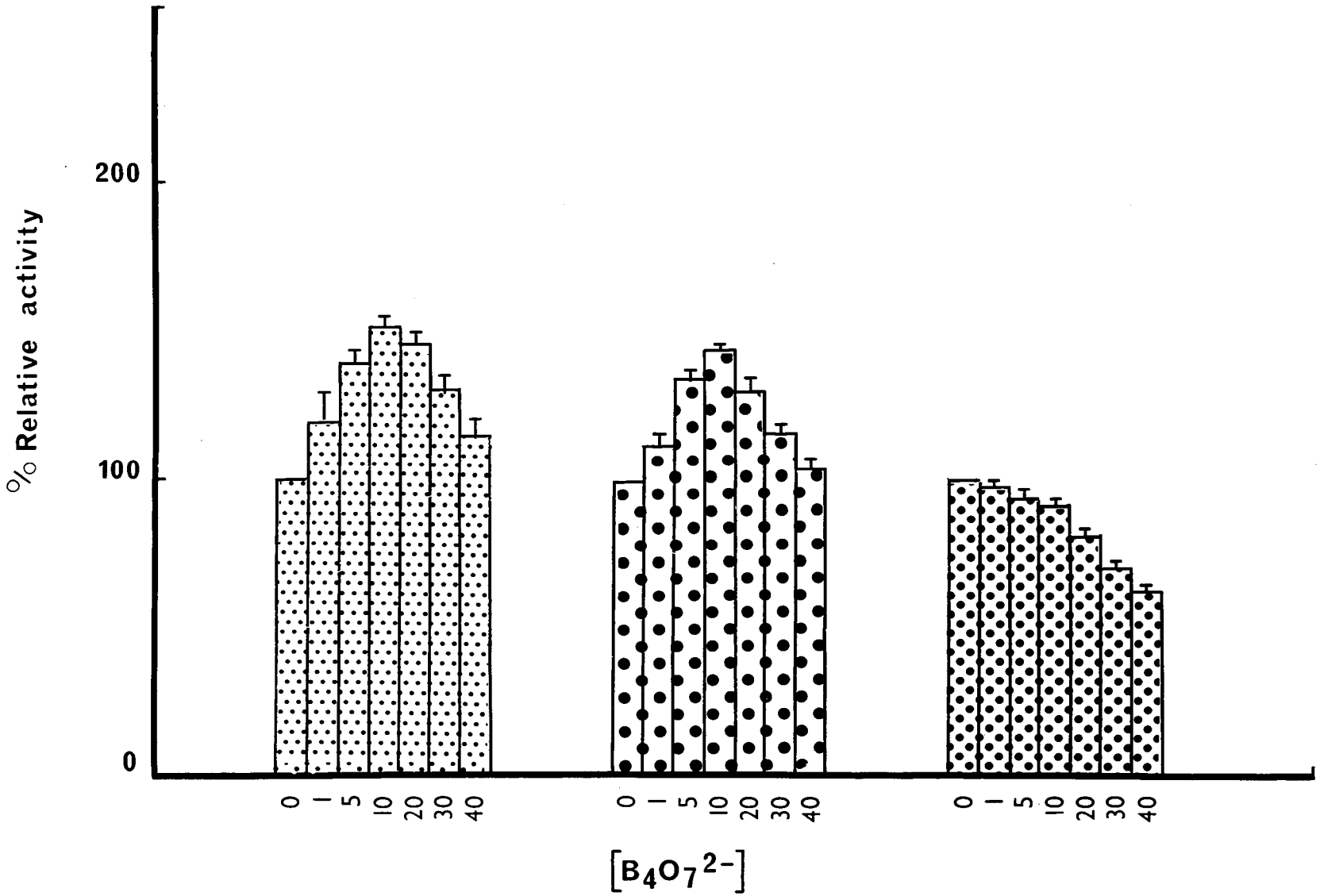

n.moles Pi liberated/mg. protein/min.
300
200
100
0
0
10
20
30
40
[B4O7 2-]

Figure 3.10

Effect of $B_4O_7^{2-}$ concentration on ATPase activity in the presence of 2mM Mg^{2+} (); 2mM Mg^{2+} + 20mM NaCl (); 2mM Mg^{2+} + 20mM $NaHCO_3$ (). Activity is expressed as a % of that observed in the absence of $B_4O_7^{2-}$. The 100% activity was 166.7 ± 34.0 (-NaCl), 170.2 ± 34.2 (+ NaCl), 274.7 ± 63.9 (+ $NaHCO_3$) nmoles Pi liberated/mg protein/min. Each column represents the mean value of four experiments ± S.E.M.

Ordinate: % Relative ATPase activity

Abscissa: $B_4O_7^{2-}$ concentration (mM).


% Relative activity
200
100
0
0 1 5 10 20 30 40
0 1 5 10 20 30 40
0 1 5 10 20 30 40
[B4O7 2-]

increased stimulation being associated with increasing concentrations of SeO_3^{2-}. Thus in the presence of 40mM SeO_3^{2-} the activity was increased to 163.1 $\pm$ 6.4%. This stimulatory effect was reduced by the inclusion of 20mM NaCl in the reaction media but nevertheless remained significant (141.0 $\pm$ 4.1% in the presence of 40mM SeO_3^{2-}). When 20mM $NaHCO_3$ replaced NaCl, a rather different response to SeO_3^{2-} was observed. Increased concentrations of this latter anion had little effect on the activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase. However, the level of activity observed in the presence of 40mM SeO_3^{2-} and 20mM HCO_3^- was somewhat lower than that observed in the absence of $NaHCO_3$ and NaCl (see Figures 3.11 and 3.12; Appendix 3, Table 8). Nevertheless, the level of activity was greater than that observed in the presence of 20mM NaCl. It would seem therefore that both HCO_3^- and Cl^- compete in someway with the stimulatory effect of SeO_3^{2-} on the Mg^{2+}-dependent ATPase. In contrast, SeO_3^{2-} seems to have little effect on the HCO_3^- stimulation of this enzyme (Figure 3.12).

3.12 Effect of Br^- and NO_3^- on ATPase activity

The effects of varying concentrations of Br^- and NO_3^- were assayed in two distinct ways:

(i) The concentration of NaCl or $NaHCO_3$ was kept constant (at 20mM) but the Br^-/NO_3^- concentration was varied between 0 and 20mM.

(ii) The anion concentration was kept constant but 20mM NaCl was reduced by substitution with NaBr or $NaNO_3$.

Figure 3.11

Effect of SeO_3^{2-} concentration on ATPase activity in the presence of 2mM Mg^{2+} (O); 2mM Mg^{2+} + 20mM NaCl (■); 2mM Mg^{2+} + 20mM $NaHCO_3$ (●). Typical experiment representative for three experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: SeO_3^{2-} concentration (mM).

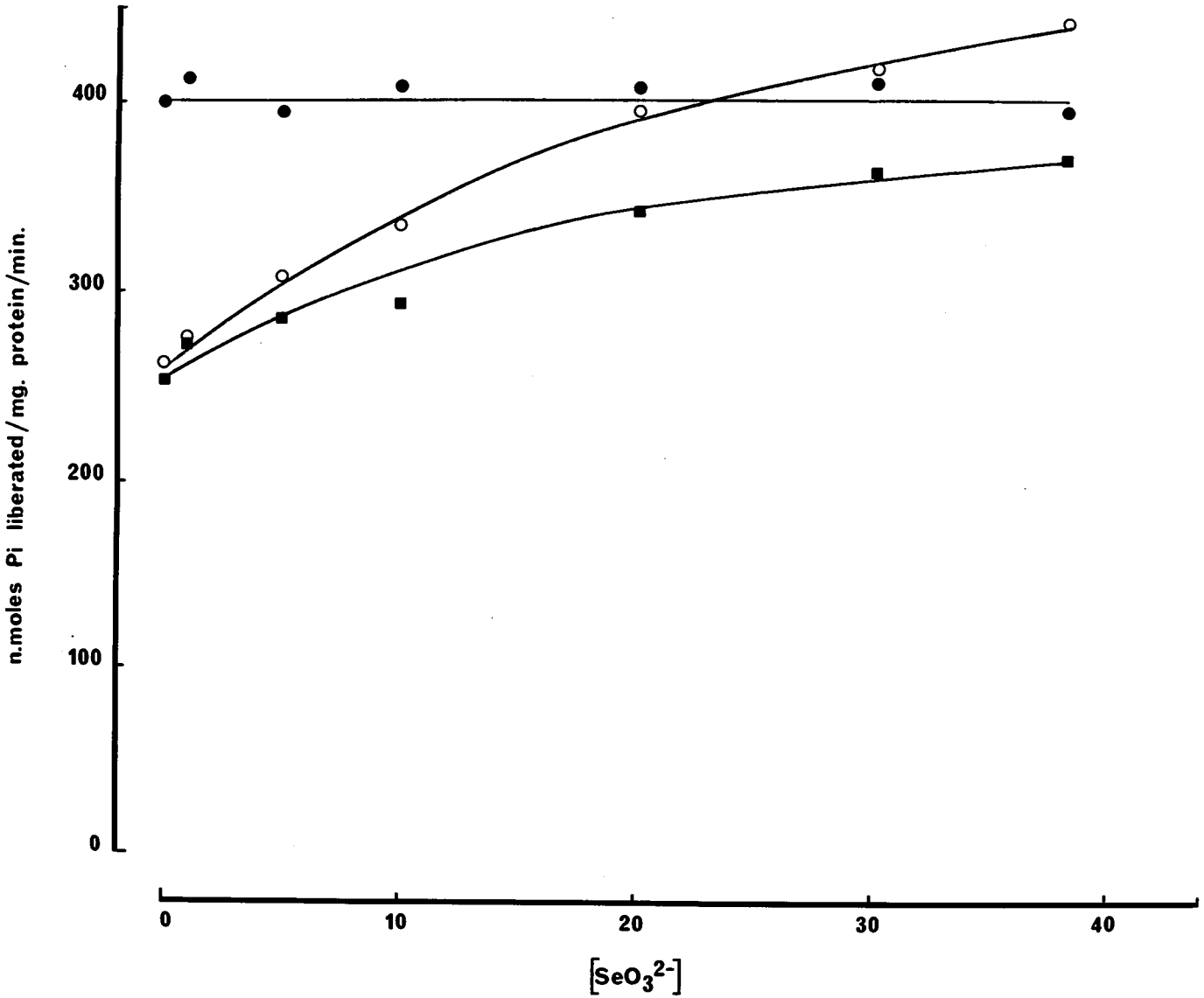
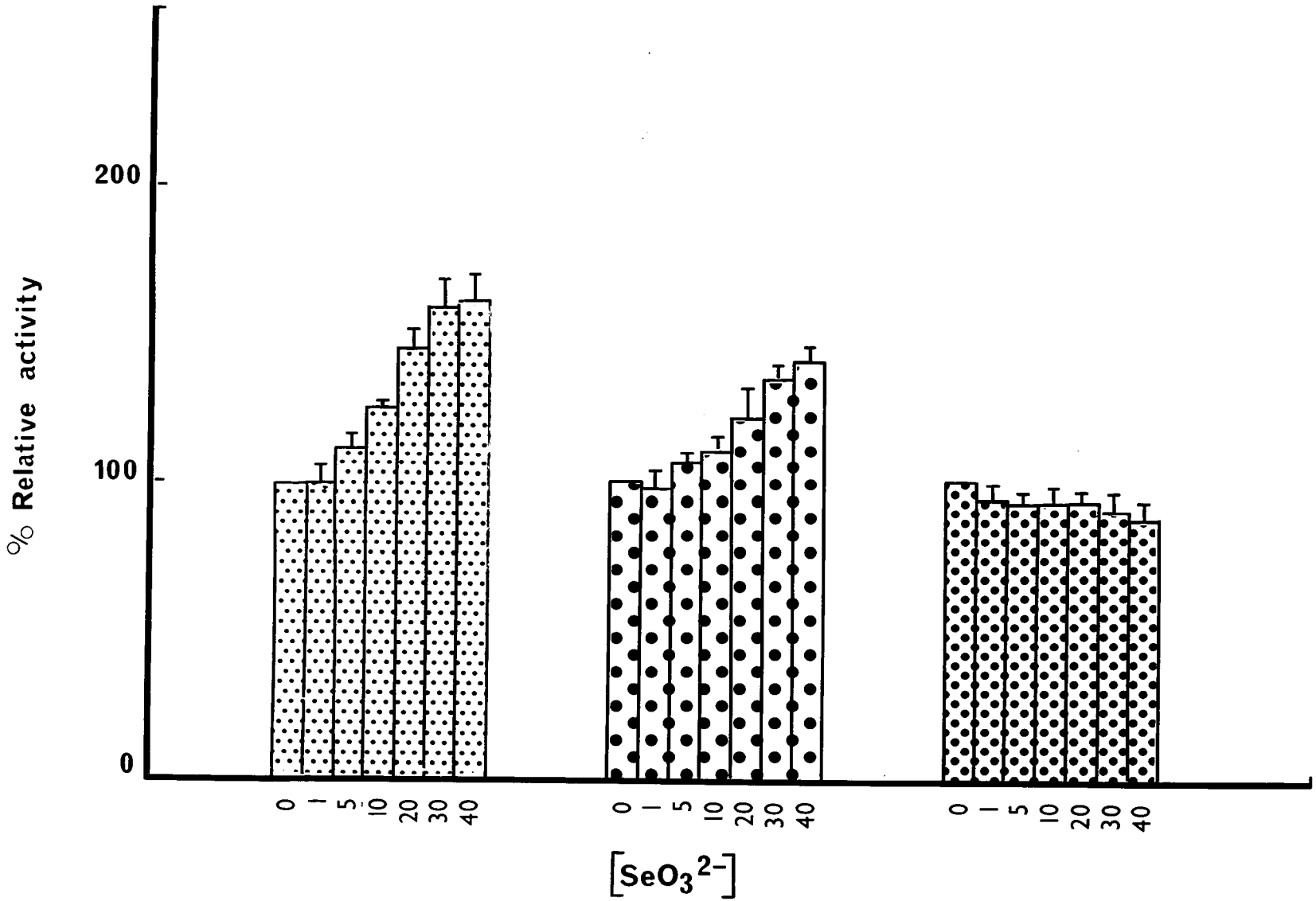

n.moles Pi liberated/mg. protein/min.
400
300
200
100
0
0
10
20
30
40
[SeO3 2-]

Figure 3.12

Effect of SeO_3^{2-} concentration on ATPase activity in the presence of 2mM Mg^{2+} (⁘); 2mM Mg^{2+} + 20mM NaCl (⁘); 2mM Mg^{2+} + 20mM $NaHCO_3$ (⁘). Activity is expressed as a % of that observed in the absence of $B_4O_7^{2-}$. The 100% activity was 214.0 ± 27.4 (- NaCl), 213.0 ± 24.8 (+ NaCl), 336.6 ± 32.2 (+ $NaHCO_3$) nmoles Pi liberated/mg protein/min. Each value represents the mean value of three experiments ± S.E.M.

Ordinate: % Relative ATPase activity

Abscissa: SeO_3^{2-} concentration (mM).


% Relative activity
200
100
0
0 1 5 10 20 30 40
0 1 5 10 20 30 40
0 1 5 10 20 30 40
[SeO3 2-]

(a) Effect of Br^- on ATPase activity

In the case of (i) above the presence of 20mM NaBr effected a slight stimulation in the activity of the Mg^{2+}-dependent ATPase in the presence of 20mM NaCl (Table 3.9, Figure 3.13A). In contrast, when 20mM $NaHCO_3$ replaced NaCl, the presence of 20mM NaBr produced a 10% inhibition of the Mg^{2+}-dependent HCO_3^--stimulated ATPase activity (Table 3.9, Figure 3.13B).

Similarly, when the effect of Br^- on the Mg^{2+} -dependent ATPase activity was assayed as indicated in (ii) above, the substitution of Br^- for Cl^- effected only a slight stimulation in the activity of the Mg^{2+}-dependent ATPase (Figure 3.14). Thus in the presence of 20mM Br^- (zero $[Cl^-]$) the relative activity was 111.0 $\pm$ 0.3%.

(b) Effect of NO_3^- on ATPase activity

In the case of (i) above the presence of 20mM $NaNO_3$ decreased the activity of enzyme in the presence of 20mM NaCl to 71.7 $\pm$ 0.8% of the level in the absence of NO_3^- (Table 3.9, Figure 3.13A). Similarly, when NaCl was substituted by $NaHCO_3$ the activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase decreased to 65.8 $\pm$ 0.8% of the rate observed in the absence of NO_3^- (Table 3.9, Figure 3.13B).

The effect of NO_3^- on the Mg^{2+}-dependent ATPase activity, assayed as indicated in (ii) above, is shown in Table 3.10 and Figure 3.14. The replacement of Cl^- with 20mM NO_3^- significantly

TABLE 3.9

Effect of Br^- and NO_3^- on ATPase activity in the presence of 20mM Cl^-/HCO_3^-

Concentration of Br^- /NO_3^- (mM).	Mg^{2+}-dependent ATPase activity			
	NaCl + Br	NaCl + NO_3^-	$NaHCO_3$ + Br^-	$NaHCO_3$ + NO_3^-
0	100	100	100	100
1	100.2 ± 0.2	97.2 ± 0.1	97.3 ± 1.3	95.4 ± 1.4
5	102.4 ± 0.8	88.6 ± 0.4	96.3 ± 1.0	88.6 ± 1.2
10	103.7 ± 0.9	81.4 ± 1.0	94.6 ± 1.5	80.3 ± 0.7
15	104.8 ± 1.0	75.9 ± 0.2	91.1 ± 0.6	73.0 ± 1.0
20	107.3 ± 1.9	71.7 ± 0.8	90.5 ± 1.1	65.8 ± 0.8

Activity is expressed as a % of that observed in the absence of Br^- or NO_3^-. The 100% activity in the presence of Mg^{2+} (+ NaCl) was 398.6 ± 7.8 and Mg^{2+} + $NaHCO_3$ 648.0 ± 5.0 nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

TABLE 3.10

Effect of Br^- and NO_3^- on ATPase activity in the presence of different concentration of Cl^-

Concentration (mM)		Mg^{2+}-dependent ATPase activity	
Cl^-	Br^-/NO_3^-	NaCl + Br^-	NaCl + NO_3^-
20	0	100	100
19	1	100.8 ± 0.6	94.5 ± 0.4
15	5	102.3 ± 0.2	79.3 ± 0.4
10	10	105.4 ± 0.2	70.3 ± 0.3
5	15	108.9 ± 0.4	63.7 ± 0.2
0	20	111.0 ± 0.3	59.1 ± 0.5

Activity is expressed as a % of that observed in the absence of Br^- or NO_3^-. The 100% activity in the presence of Mg^{2+} (+ NaCl) was 357.2 ± 19.0 nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

Figure 3.13

Effect of Br^- (□) and NO_3^- (●) on ATPase activity in the presence of 2mM Mg^{2+} + 20mM NaCl (A); 2mM Mg^{2+} + 20mM $NaHCO_3$ (B). Typical experiment representative for three experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: Br^- or NO_3^- concentration (mM).

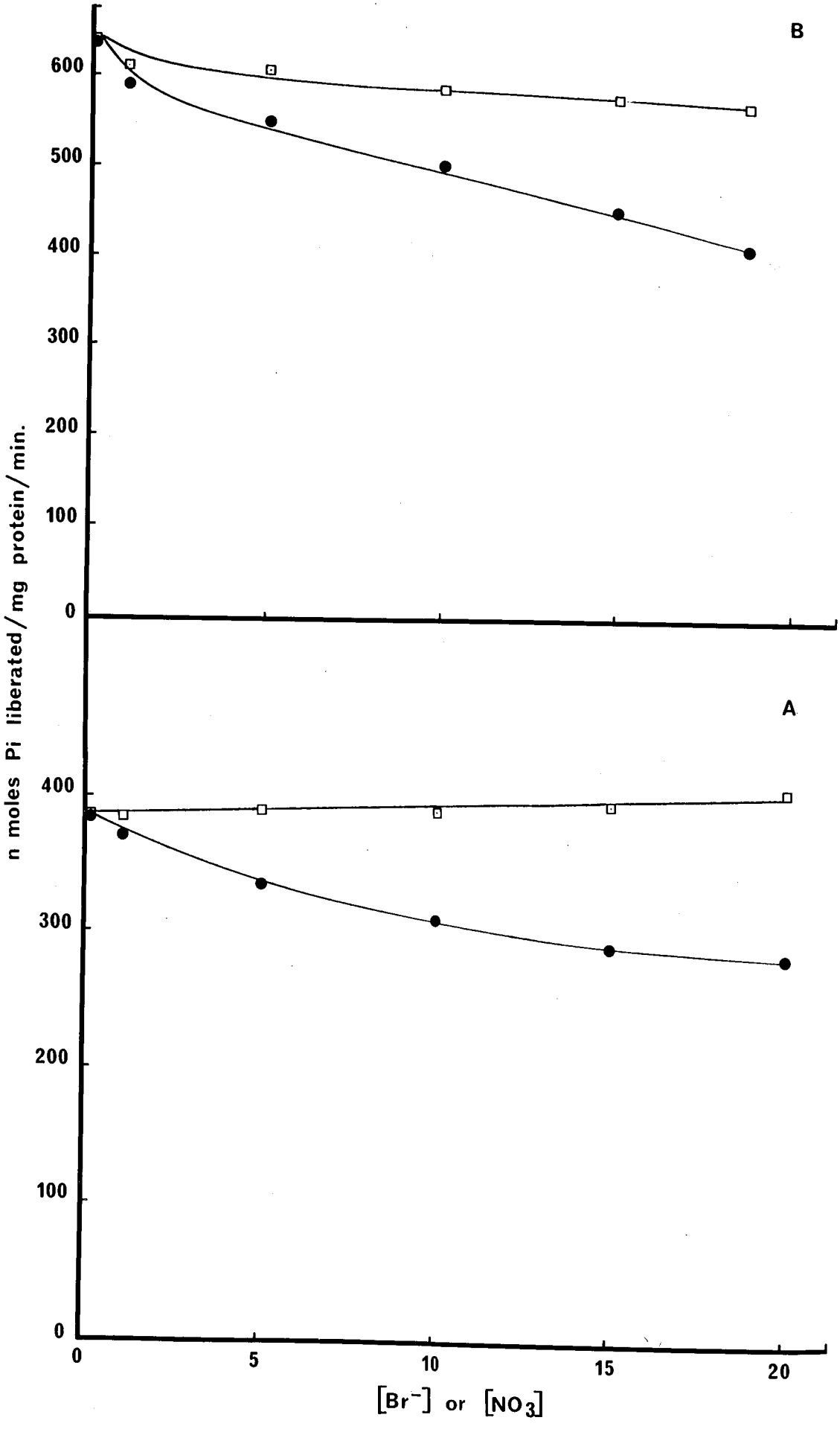
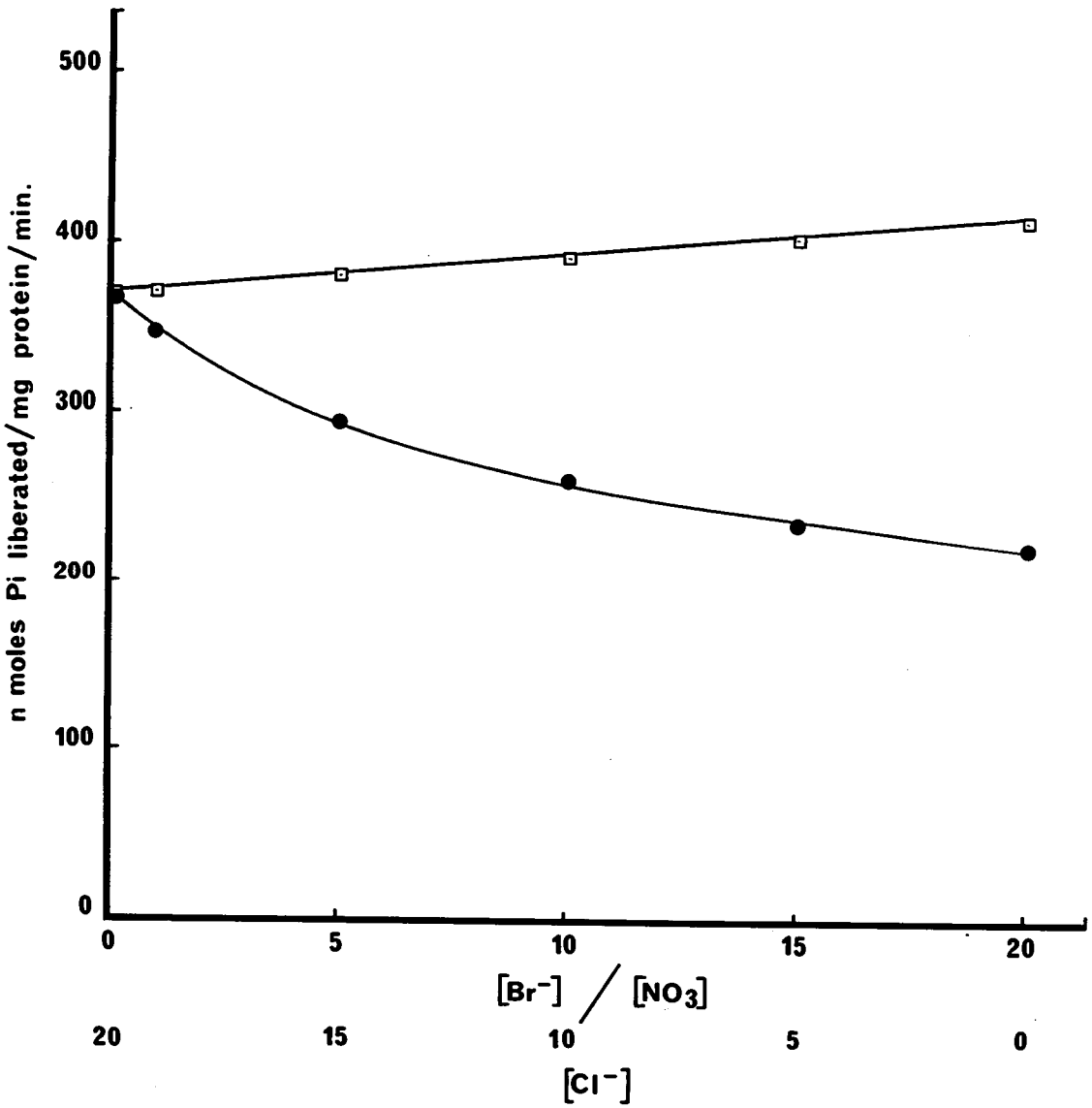

B
A
600
500
400
300
200
100
0
400
300
200
100
0
0
5
10
15
20
n moles Pi liberated/mg protein/min.
[Br⁻] or [NO3]

Figure 3.14

Effect of Br^- (□) and NO_3^- (●) on ATPase activity in the presence of 2mM Mg^{2+} and different concnetration of NaCl. Typical experiment representative for five experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: Br^-/NO_3^- and Cl^- concentration (mM).


500
400
300
200
100
0
n moles Pi liberated/mg protein/min.
0
5
10
15
20
[Br⁻] / [NO3]
20
15
10
5
0
[Cl⁻]

decreased the relative activity of the Mg^{2+}-dependent ATPase to 59.1 $\pm$ 0.5% ($P<0.001$).

The results clearly show that Cl^- can be substituted by Br^- without any marked effect on Mg^{2+}-dependent ATPase activity, whereas substitution of Cl^- by NO_3^- inhibited the activity of the Mg^{2+}-dependent ATPase. In addition, whilst Br^- has little effect on Mg^{2+}-dependent HCO_3^--stimulated ATPase, NO_3^- markedly inhibits this stimulated activity (see Appendix 3, Tables 9,10).

3.13 Effect of amiloride on ATPase activity

Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and Mg^{2+}-dependent HCO_3^--stimulated ATPase were assayed in reaction media in which amiloride was present in the concentration range 0-5 x 10^{-4}M. The results are shown in Table 3.11. It can be seen that amiloride did not markedly affect the Mg^{2+}-dependent ATPase (in the presence or absence of NaCl). However, there was some slight, but significant, inhibition of activity at the highest concentration (5 x 10^{-4}M); the inhibition of Mg^{2+}-dependent ATPase activity being 12% and 7% in the presence and absence of NaCl respectively. Similarly, the Mg^{2+}-dependent HCO_3^--stimulated ATPase was largely unaffected by amiloride over much of the concentration range. At 5 x 10^{-4}M amiloride the enzyme activity was inhibited by 15% (see Appendix 3, Table 11).

3.14 Effect of ethacrynic acid on ATPase activity

Enzyme assays were carried out in media in which the ethacrynic acid concentration varied from 0-10^{-3}M. At the lower concentrations (0-10^{-5}M) ethacrynic acid did not affect the activity of either the Mg^{2+}-dependent ATPase (in the presence or absence of NaCl) or the Mg^{2+}-dependent HCO_3^--stimulated ATPase (Figure 3.15,

TABLE 3.11

Effect of amiloride on ATPase activity

Concentration of amiloride (M)	Mg^{2+}-dependent ATPase activity		
	- NaCl	+ NaCl	+ $NaHCO_3$
0	100	100	100
5×10^{-8}	98.9 ± 1.5	100.9 ± 2.3	98.9 ± 2.3
5×10^{-7}	99.1 ± 1.1	100.5 ± 3.2	97.5 ± 1.8
5×10^{-6}	100.3 ± 1.1	98.9 ± 2.3	97.7 ± 2.3
5×10^{-5}	97.5 ± 2.8	97.1 ± 2.5	94.1 ± 1.5
5×10^{-4}	93.3 ± 2.1	88.2 ± 1.1	85.4 ± 1.0

Activity is expressed as a % of that observed in the absence of amiloride. The 100% activity was 342.2 ± 11.5 (- NaCl); 348.2 ± 15.4 (+ NaCl); 503.2 ± 12.5 (+ $NaHCO_3$) nmoles Pi liberated/ mg protein/min. Each value represents the average of four experiments ± S.E.M.

Table 3.12). Only at concentrations greater than 10^{-4}M was the enzyme activity significantly lowered. The relative activity of the Mg^{2+}-dependent ATPase was 74.8 ± 3.6% and 82.2 ± 3.8% in the presence and absence of NaCl respectively, when 10^{-3}M ethacrynic acid was present in medium. Similarly, the relative activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase was reduced to 74.7 ± 2.5% when 10^{-3}M ethacrynic acid was present in medium (Table 3.13) (see Appendix 3, Table 12).

3.15 Effect of 2,4-dinitrophenol on ATPase activity

Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and Mg^{2+}-dependent HCO_3^--stimulated ATPase were assayed in the presence of 0-5mM 2,4-dinitrophenol (DNP). As can be seen in Table 3.13, DNP stimulated the Mg^{2+}-dependent ATPase (both in the presence and absence of NaCl). In the presence of 5mM DNP, the Mg^{2+}-dependent ATPase was stimulated 55.2 ± 5.2% and 46.4 ± 5.5%, (in the presence and absence of NaCl respectively). In contrast, this concentration of DNP did not significantly affect the activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase (see Appendix 3, Table 13).

3.16 Effect of cyclic AMP on ATPase activity

Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and Mg^{2+}-dependent HCO_3^--stimulated ATPase were incubated in reaction media in which 1mM of c.AMP was present. Cyclic AMP did not affect either the activity of the Mg^{2+}-dependent ATPase or that of the Mg^{2+}-dependent HCO_3^--stimulated ATPase (Table 3.14; Appendix 3, Table 14).

TABLE 3.12

Effect of ethacrynic acid on ATPase activity

Concentration of ethacrynic acid (M)	Mg^{2+}-dependent ATPase activity		
	- NaCl	+ NaCl	+ $NaHCO_3$
0	100	100	100
10^{-7}	98.7 ± 1.7	98.4 ± 1.7	100.8 ± 1.1
10^{-6}	97.5 ± 1.5	99.0 ± 2.3	101.1 ± 0.7
10^{-5}	96.9 ± 2.2	98.6 ± 3.3	101.7 ± 1.4
10^{-4}	93.3 ± 2.6	92.9 ± 3.3	93.2 ± 2.6
10^{-3}	82.2 ± 3.8	74.8 ± 3.6	74.7 ± 2.5

Activity is expressed as a % of that observed in the absence of ethacrynic acid. The 100% activity was 319.7 ± 20.8 (- NaCl); 321.7 ± 25.4 (+ NaCl) and 470.0 ± 32.1 (+ $NaHCO_3$) nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

Figure 3.15

Effect of ethacrynic acid on ATPase activity in the presence of 2mM Mg^{2+} (O); 2mM Mg^{2+} + 20mM NaCl (■); 2mM Mg^{2+} + 20mM $NaHCO_3$ (●). Typical experiment representative for four experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: -log [ethacrynic acid] (M).

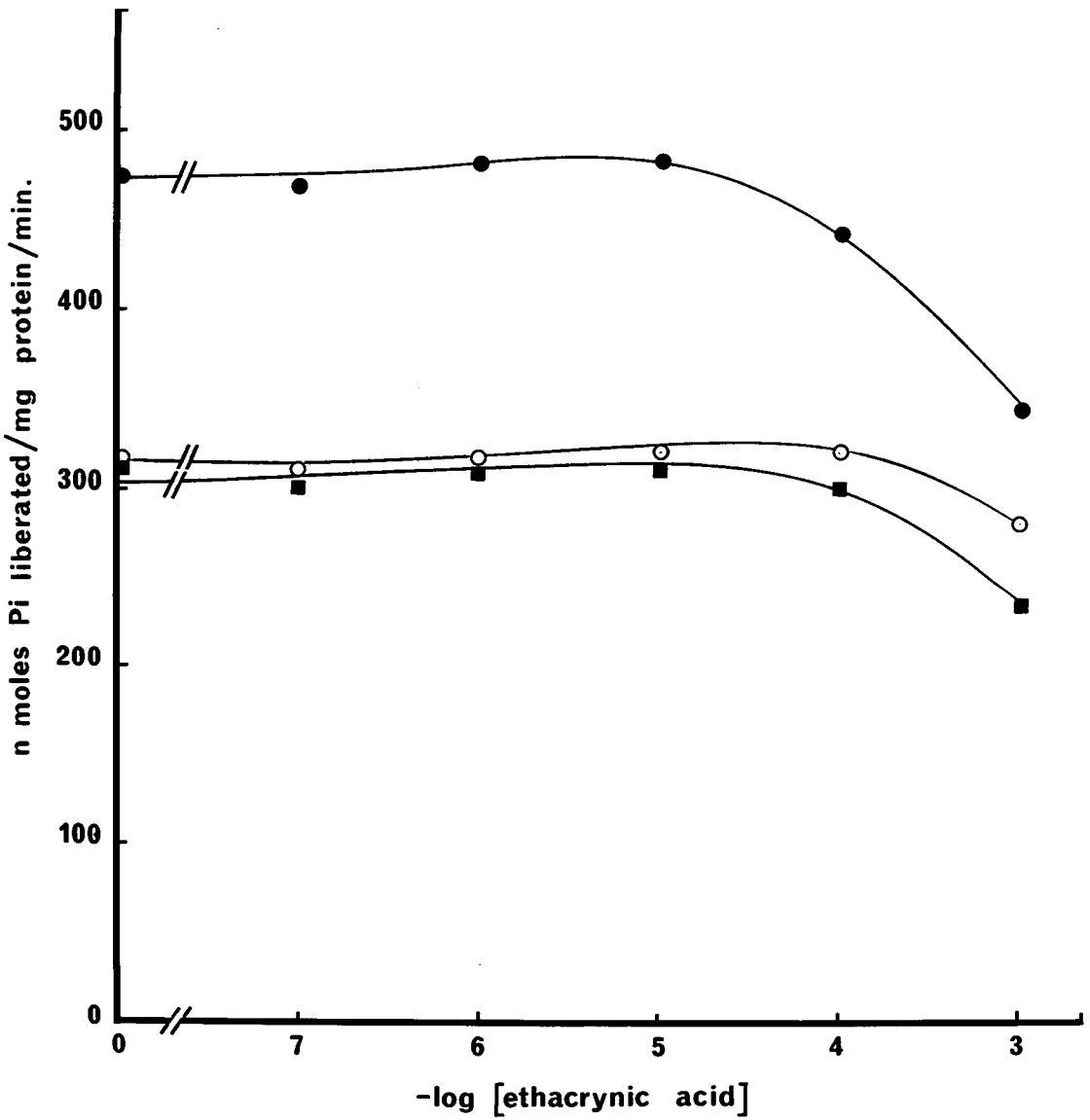

500
400
300
200
100
0
n moles Pi liberated/mg protein/min.
0
7
6
5
4
3
-log [ethacrynic acid]

TABLE 3.13

Effect of DNP on ATPase activity

Concentration of DNP (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+ NaCl	+ $NaHCO_3$
0	100	100	100
1	121.9 ± 1.4	118.5 ± 3.1	107.2 ± 5.6
5	146.4 ± 5.5	155.2 ± 5.2	111.7 ± 6.5

Activity is expressed as a % of that observed in the absence of DNP. The 100% activity was 234.0 ± 46.3 (- NaCl); 237.0 ± 48.8 (+ NaCl); 372.7 ± 70.9 (+ $NaHCO_3$) nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

TABLE 3.14

Effect of c.AMP on ATPase activity

Concentration of c.AMP (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+ NaCl	+ $NaHCO_3$
0	100	100	100
1	102.5 ± 1.4	99.6 ± 0.8	99.4 ± 1.1

Activity is expressed as % of that observed in the absence of c.AMP. The 100% activity was 332.5 ± 12.3 (- NaCl); 338.7 ± 15.1 (+ NaCl); 506.5 ± 21.8 (+ $NaHCO_3$) nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

3.17 Effect of oligomycin on ATPase activity

Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and the Mg^{2+}-dependent HCO_3^--stimulated ATPase of various subcellular fractions of locust Malpighian tubules, viz. 5,000g (mitochondrial preparation, see Materials and Methods); 20,000g and 100,000g were assayed in reaction media in which the oligomycin concentration varied between 0-3 x 10^{-3}M. Oligomycin was dissolved in 95% ethanol (INTURRISI and TITUS, 1968) and added to the ionic media before the addition of the appropriate homogenate. The effects of oligomycin on ATPase activity of the three fractions are shown in Figures 3.16,3.17 and 3.18. From such plots, one can calculate the pI50 values for each fraction (Table 3.15). The pI50 values for Mg^{2+}-dependent ATPase (in the presence and absence of NaCl), Mg^{2+}-dependent HCO_3^--stimulated ATPase and HCO_3^--stimulated activity were not significantly different for the 5,000g and 20,000g fractions ($P>0.1$). In contrast, the pI50 for the Mg^{2+}-dependent ATPase (in the presence and absence of NaCl) and the Mg^{2+}-dependent HCO_3^--stimulated ATPase of the 100,000g fraction were significantly higher than the values obtained with the 5,000g ($P<0.02$) and the 20,000g fractions ($P<0.05$) indicating that the 100,000g enzyme is more sensitive to oligomycin. However, although the pI50 for the HCO_3^--stimulated activity at 100,000g was significantly different from that of the 20,000g fraction ($P<0.05$) it was not significantly different from that observed with the 5,000g fraction ($P>0.05$).

Oligomycin inhibition was incomplete with all fractions used; maximum inhibition being some 90-93% of the total activities

TABLE 3.15

Effect of oligomycin on ATPase activity

Enzyme	5,000g		20,000g		100,000g	
	pI50	Rest activity (%)	pI50	Rest activity (%)	pI50	Rest activity (%)
Mg^{2+}-dependent ATPase (-NaCl)	4.24±0.11	11.4±0.9	4.29±0.06	6.4±0.9	4.74±0.13	6.4±0.7
Mg^{2+}-dependent ATPase (+NaCl)	4.22±0.11	14.0±0.9	4.39±0.04	7.0±0.9	4.72±0.12	6.2±0.7
Mg^{2+}-dependent HCO_3^--stimulated ATPase	4.20±0.09	12.0±0.9	4.33±0.05	8.8±0.9	4.67±0.11	8.4±1.0
HCO_3^- stimulation of ATPase activity	4.32±0.10	9.8±0.10	4.39±0.05	11.2±0.9	4.61±0.10	11.2±2.3

pI50 is the negative logarithm of the molar concentration at half-maximal inhibition.

Rest activity is % ATPase activity remaining at maximal inhibition. Each value represents the average of five experiments ± S.E.M.

Figure 3.16

Effect of oligomycin on the relative activity of 5,000g. fraction.

A Mg^{2+}-dependent ATPase (- NaCl).

B Mg^{2+}-dependent ATPase (+ NaCl)

C Mg^{2+}-dependent HCO_3^--stimulated ATPase.

D HCO_3^--stimulated ATPase activity.

The results represent the mean $\pm$ S.E.M. percentage activity relative to the activity without added oligomycin. The data is derived from five experiments in each case.

Ordinate: % Relative ATPase activity

Abscissa: -log [oligomycin] (M).

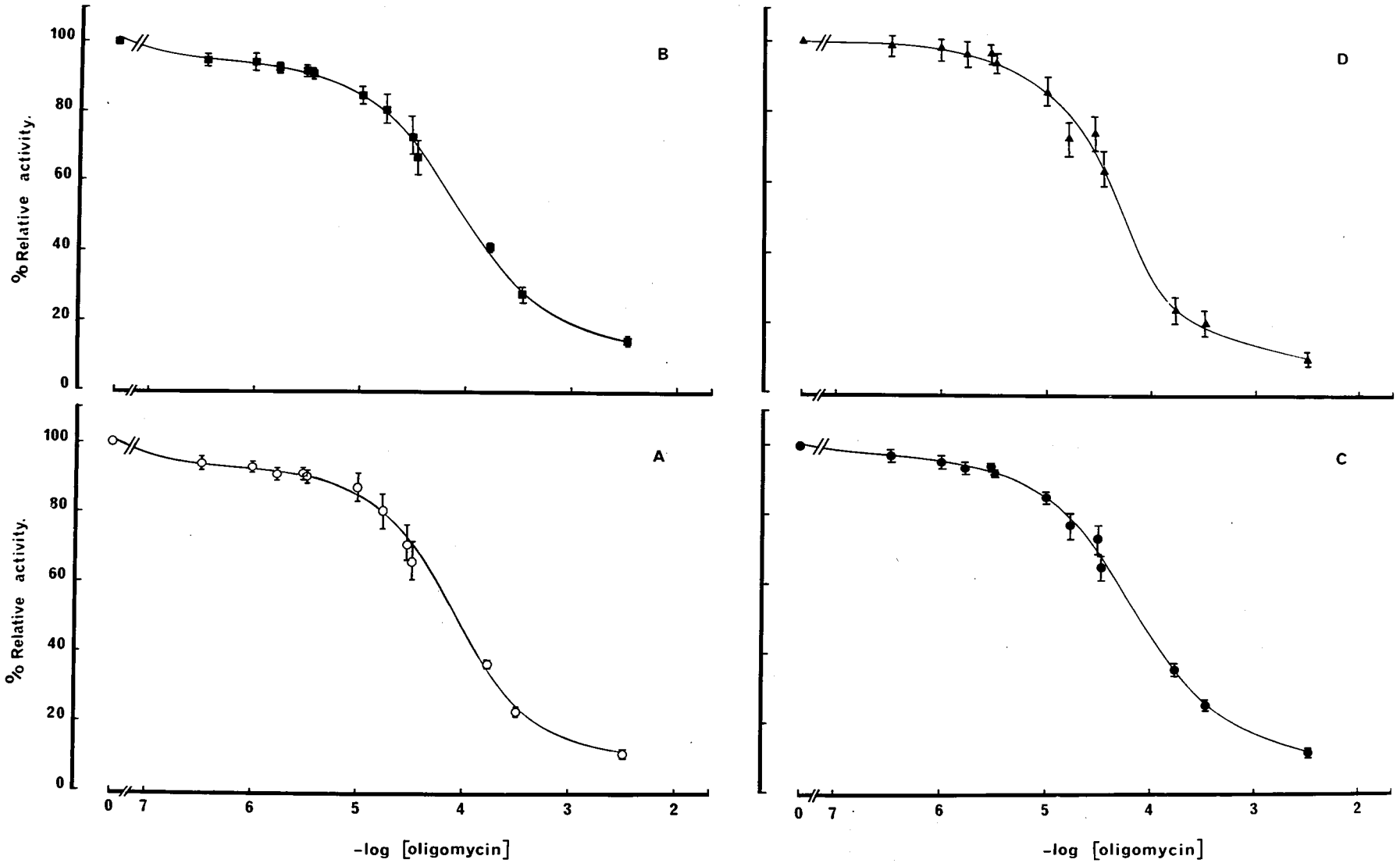

% Relative activity.
100
80
60
40
20
0
B
A
D
C
0 7
6
5
4
3
2
-log [oligomycin]

Figure 3.17

Effect of oligomycin on the relative ATPase activity of 20,000g fraction.

A Mg^{2+}-dependent ATPase (- NaCl)

B Mg^{2+}-dependent ATPase (+ NaCl)

C Mg^{2+}-dependent HCO_3^--stimulated ATPase

D HCO_3^--stimulated ATPase activity

The results represent the mean $\pm$ S.E.M. percentage activity relative to the activity without added oligomycin. The data is derived from five experiments in each case.

Ordinate: % Relative ATPase activity

Abscissa: -log [oligomycin] (M).

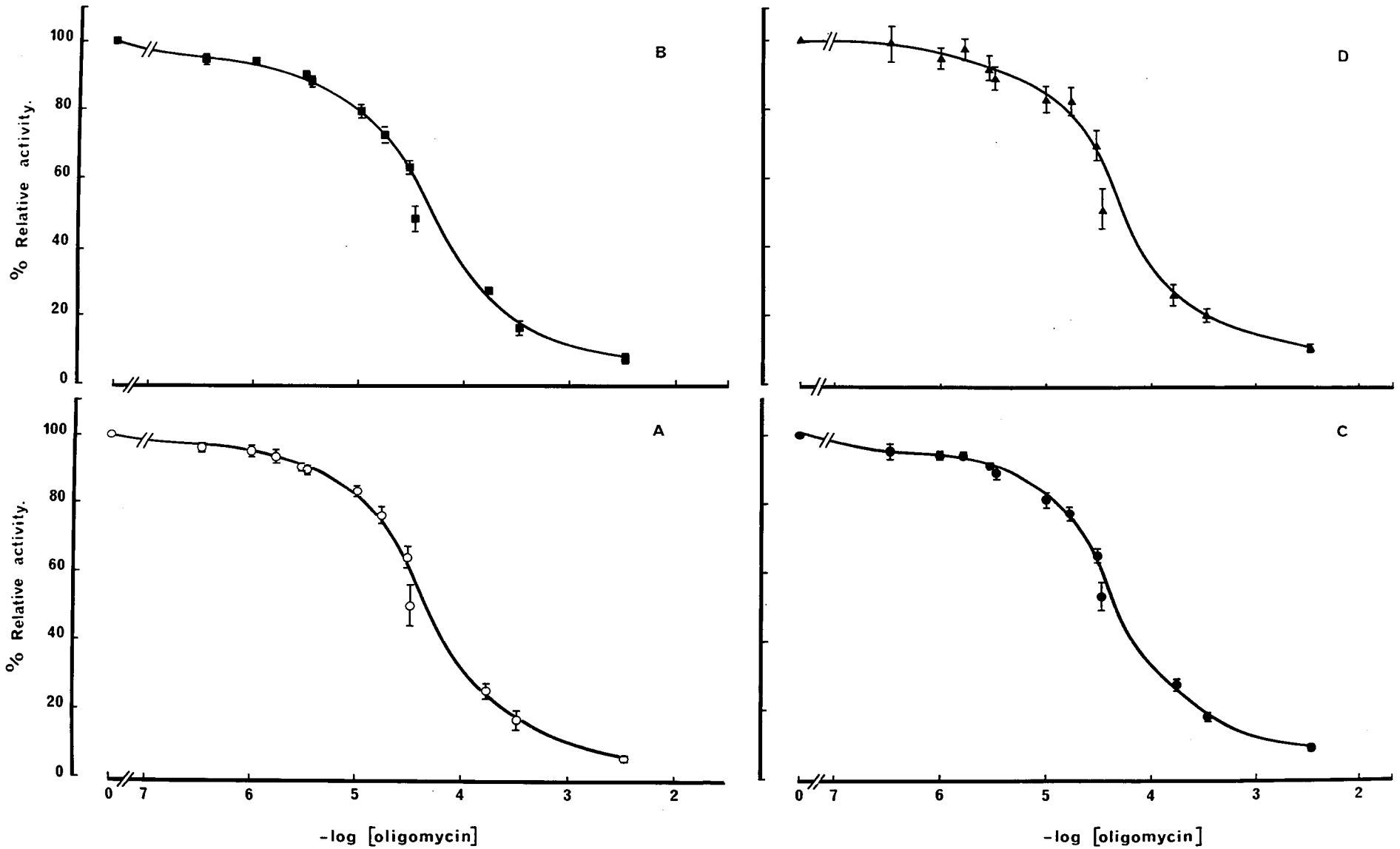

B
D
A
C
% Relative activity.
% Relative activity.
100
80
60
40
20
0
0
7
6
5
4
3
2
-log [oligomycin]
-log [oligomycin]

Figure 3.18

Effect of oligomycin on the relative ATPase activity of 100,000g fraction.

A Mg^{2+}-dependent ATPase (- NaCl)

B Mg^{2+}-dependent ATPase (+ NaCl)

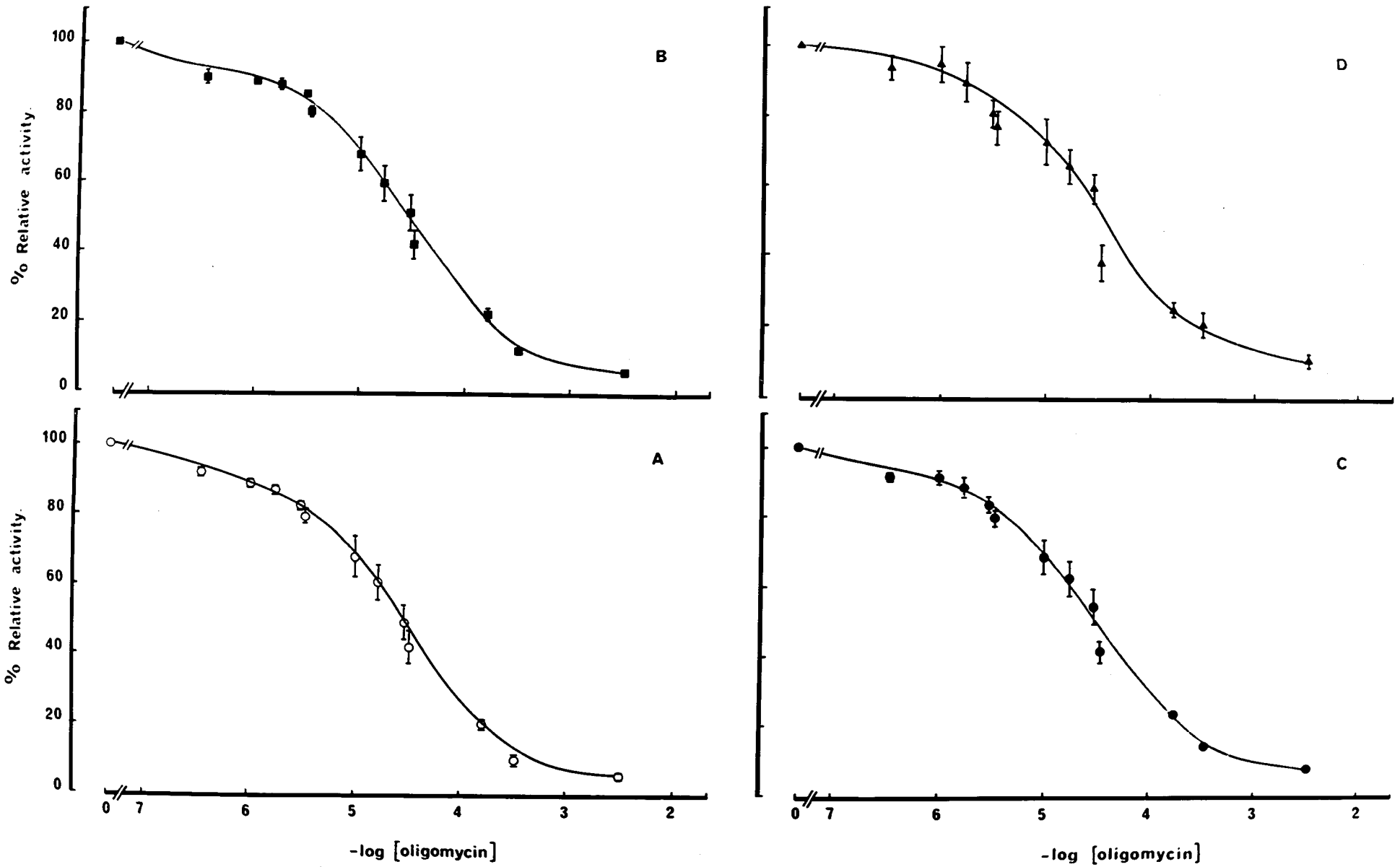
C Mg^{2+}-dependent HCO_3^--stimulated ATPase

D HCO_3^--stimulated ATPase activity.

The results represent the mean $\pm$ S.E.M. percentage activity relative to the activity without adding oligomycin. The data is derived from five experiments in each case.

Ordinate: % Relative ATPase activity

Abscissa: -log [oligomycin] (M).


B
D
A
C
% Relative activity
100
80
60
40
20
0
0
7
6
5
4
3
2
-log [oligomycin]

(Figures 3.16, 3.17, 3.18 and Table 3.15; Appendix 3, Tables 15, 16, 17).

3.18 Intracellular distribution of ATPase activity

In the present study an attempt was made to determine the intracellular localization of the Mg^{2+}-dependent ATPase and Mg^{2+}-dependent HCO_3^--stimulated ATPase in the locust Malpighian tubules. The activities of the enzymes were assayed in three fractions and the results compared with the mitochondrial marker enzyme, succinate dehydrogenase (SDH) (IZUTSU and SIEGEL, 1972; KERSTETTER and KIRSCHNER, 1974; HEGNER and ANIKA, 1975; SOLOMON et al., 1975; and COLE, 1979) in the same three fractions (600g, 20,000g and 100,000g fractions). The assays were carried out on the same homogenates for both enzymes.

Figure 3.19 shows the distribution of Mg^{2+}-dependent ATPase, Mg^{2+}-dependent HCO_3^--stimulated ATPase and SDH. Maximum ATPase and SDH activities were observed in the 20,000g fraction. However, examination of Table 3.16 clearly shows that the SDH/Mg^{2+}-HCO_3^- ATPase ratio is not constant for the three fractions; minimum SDH activity being observed in the 100,000g fraction whilst the lowest Mg^{2+}-HCO_3^- ATPase activity was located in the 600g fraction. The results show that the distribution of Mg^{2+}-dependent HCO_3^--stimulated ATPase is not the same as the distribution of the mitochondrial marker enzyme, succinate dehydrogenase (see Appendix 3, Table 18).

3.19 Carbonic anhydrase activity

The data presented in Table 3.17 clearly demonstrates the presence of carbonic anhydrase activity in the Malpighian tubules

TABLE 3.16

Relative distribution of Mg^{2+}-dependent HCO_3^--stimulated ATPase in relation to SDH in three fractions

Enzyme	600g	20,000g	100,000g	P
SDH	100	161.8 ± 10.8	27.4 ± 4.9	<0.001
Mg^{2+}-dependent HCO_3^--stimulated ATPase	100	286.0 ± 5.9	160.2 ± 11.8	<0.001
SDH/Mg^{2+}-HCO_3^-	1	0.57± 0.05	0.18 ± 0.04	<0.001

100% activity of SDH was 0.067 ± 0.007 µmoles succinate oxidised/mg protein/min.

100% activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase was 259.2 ± 22.8 nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

TABLE 3.17

Carbonic anhydrase activity in the Malpighian tubules of Locusta.

	Carbonic anhydrase unit/mg protein	
- sodium acetazolamide	a	4.17
	b	4.20
+ sodium acetazolamide (1mM)	a	0.019
	b	0.016

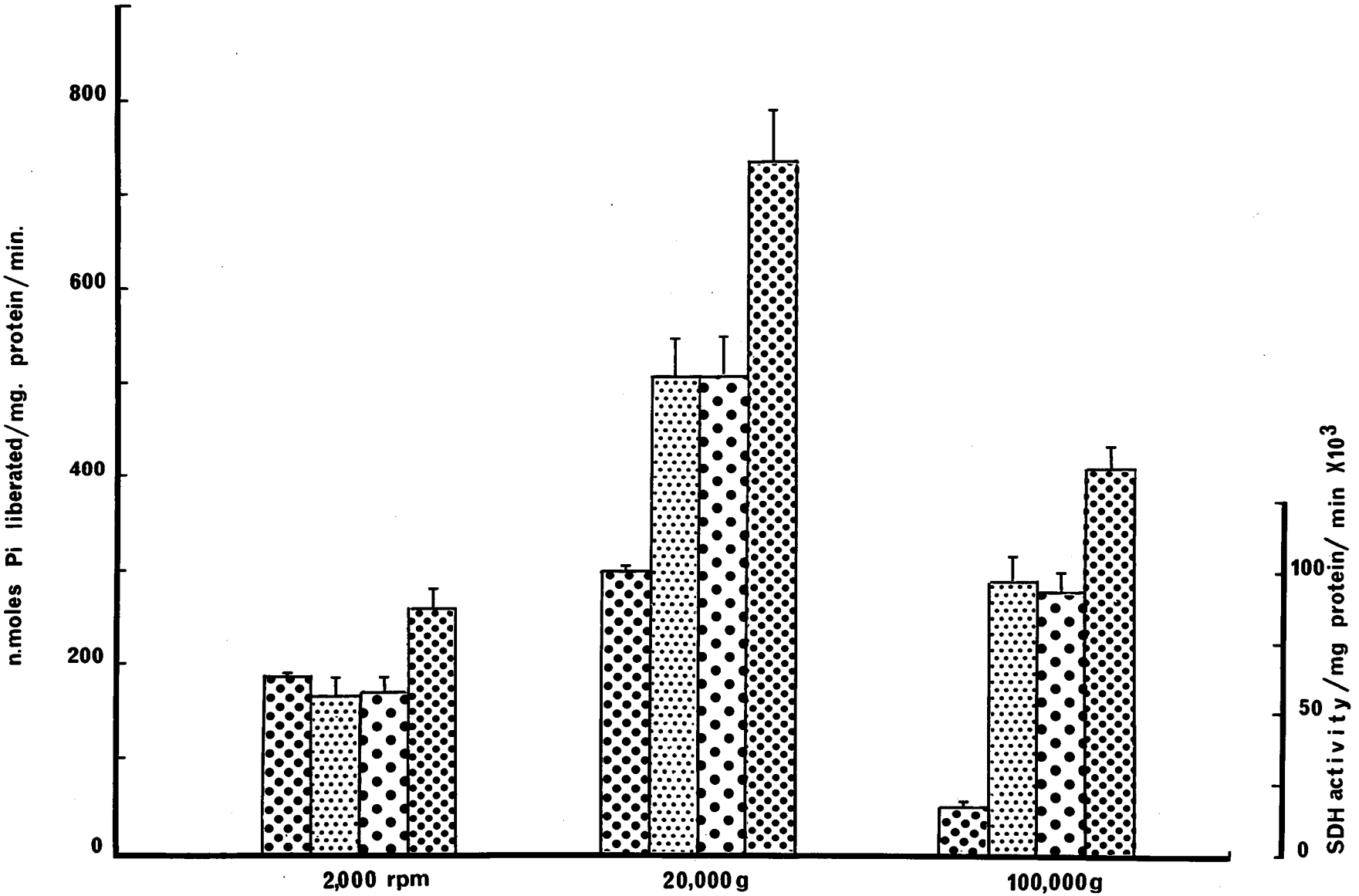
a and b represent the data obtained in two separate experiments.

Figure 3.19

The intracellular distribution of ATPase and SDH in locust Malpighian tubules. ( ) Succinate dehydrogenase; ( ) Mg^{2+}-dependent ATPase (- NaCl); ( ) Mg^{2+}-dependent ATPase (+ NaCl); ( ) Mg^{2+}-dependent HCO_3^--stimulated ATPase.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min. SDH activity as µmoles succinate oxidised/mg protein/min x 10^3.

Abscissa: Different fraction.


n.moles Pi liberated/mg. protein/min.
800
600
400
200
0
2,000 rpm
20,000 g
100,000 g
SDH activity/mg protein/ min X10³
100
50
0

of Locusta migratoria. In common with carbonic anhydrase from other sources (LIANG and SACKTOR, 1976; HOUSTON and Mc CARTY, 1978), that from Locusta tubules is inhibited by sodium acetazolamide. The presence of 1mM sodium acetazolamide reduced the activity of the enzyme from 4.17 units/mg protein to 0.019 units/mg protein (see Table 3.17 'a').

3.20 Effects of HCO_3^- on Na^+-K^+ ATPase activity

The effects of varying concentrations of HCO_3^- were assayed on Na^+-K^+ ATPase activity. The anion concentration was kept constant but 100mM NaCl was reduced by substitution with $NaHCO_3$. As shown in Figure 3.20, replacing NaCl by $NaHCO_3$ decreased the activity of Mg^{2+}-dependent Na^+-K^+ activated ATPase. In the presence of 100mM $NaHCO_3$ and zero NaCl the enzyme activity decreased to 81.3 $\pm$ 0.9%; however, in the presence of 80mM NaCl and 20mM $NaHCO_3$ the relative activity of the Na^+-K^+ ATPase was 98.6 $\pm$ 3.6% of maximal (Table 3.18). The same series of assays were carried out in the presence of 1mM sodium acetazolamide. As shown in Figure 3.20 and Table 3.18 the presence of 1mM sodium acetazolamide did not affect the Na^+ - K^+ ATPase activity (see Appendix 3, Table 19).

3.21 Effect of NaSCN on Na^+ - K^+ ATPase

Na^+-K^+ATPase was assayed in reaction media in which the anion concentration was kept constant but 0-10mM NaSCN was added to the reaction media by substitution for NaCl. SCN^-, which substantially inhibited the Mg^{2+}-dependent HCO_3^--stimulated ATPase (see 3.7), did not markedly inhibit the Na^+-K^+ATPase activity (Table 3.19). The replacement of NaCl with 10mM NaSCN decreased the relative

TABLE 3.18

Effect of $NaHCO_3$ on $Na^+ - K^+$ ATPase

Concentration (mM)		$Na^+ - K^+$ ATPase activity	
NaCl	$NaHCO_3$	- acetazolamide	+ acetazolamide
100	0	100	100
90	10	102.0 $\pm$ 1.7	100.8 $\pm$ 2.2
80	20	98.6 $\pm$ 3.6	96.5 $\pm$ 4.4
50	50	90.6 $\pm$ 3.6	89.4 $\pm$ 5.9
0	100	81.3 $\pm$ 0.9	85.8 $\pm$ 1.8

Activity is expressed as a % of that observed in the presence of 100mM NaCl. The 100% activity in the presence of 100mM NaCl (-acetazolamide) was 230.4 $\pm$ 43.2 and (+acetazolamide) 222.6 $\pm$ 40.7 nmoles Pi liberated/mg protein/min. Each value represents the mean value of three experiments $\pm$ S.E.M.

TABLE 3.19

Effect of NaSCN on $Na^+ - K^+$ ATPase

Concentration (mM)		$Na^+ - K^+$ ATPase activity
NaCl	NaSCN	
100	0	100
99	1	93.9 ± 2.0
95	5	94.9 ± 4.0
90	10	89.8 ± 3.4

Activity is expressed as a % of that observed in the presence of 100mM NaCl.

The 100% activity was 230.4 ± 43.2 nmoles Pi liberated/mg protein/min.

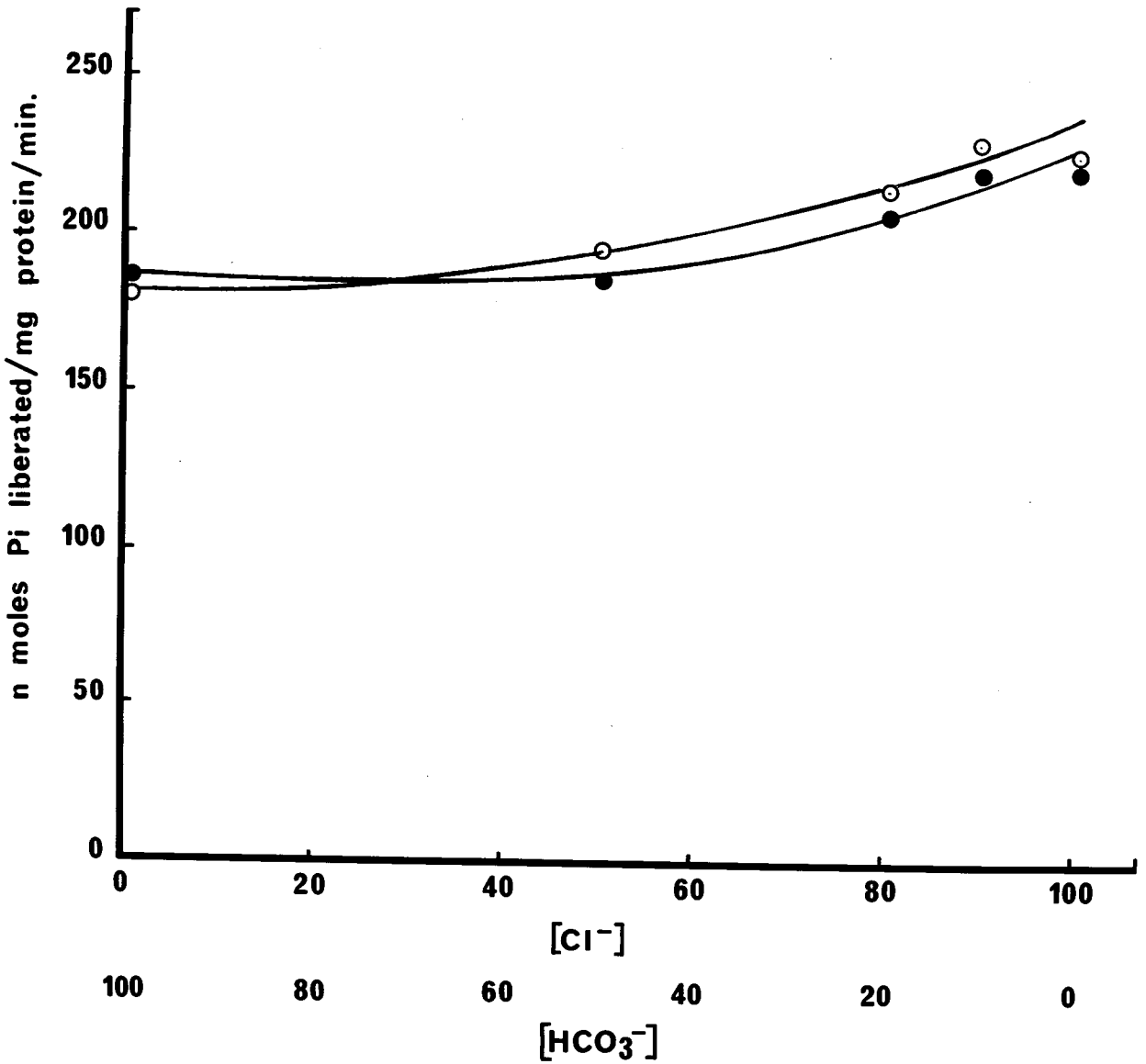
Each value represents the mean value of three experiments ± S.E.M.

Figure 3.20

Effect of HCO_3^- on Na^+-K^+ATPase activity in the absence (O) and presence (●) of 1mM sodium acetazolamide. Typical experiment representative for three experiments.

Ordinate: ATPase activity as nmoles Pi liberated/mg protein/min.

Abscissa: Cl^-/HCO_3^- concentration (mM).


n moles Pi liberated/mg protein/min.
250
200
150
100
50
0
0
20
40
60
80
100
[Cl⁻]
100
80
60
40
20
0
[HCO3⁻]

activity of the Na^+-K^+ ATPase by only 11-12%. (see Appendix 3 Table 20).

3.22 Effect of Br^- and NO_3^- on Na^+ - K^+ ATPase

Na^+ - K^+ ATPase was assayed in reaction media in which NaBr or $NaNO_3$ was substituted for NaCl. Neither Br^- nor NO_3^- alter the activity of Na^+-K^+ ATPase. The activity of Na^+-K^+ ATPase was 167.0 $\pm$ 8.3 in the presence of Cl^-, 166.6 $\pm$ 8.5 in the presence of Br^- and 168.3 $\pm$ 11.6 nmoles Pi liberated/mg protein/min (in the presence of NO_3^-).

3.23 Electron microscopy of subcellular fractions

The 600g, 20,000g and 100,000g pellets were fixed as described before (see Materials and Methods). Figure 3.21 shows the fine structure of the 600g pellet. This fraction consisted largely of unbroken or incompletely broken cells; basement membrane, basal infoldings of the basal cell membrane, large vesicles and mitochondria being recognisable. In contrast, the 20,000g fraction (Figure 3.22 a) consisted mainly of circular and elongated empty vesicles of various sizes cut in different planes. Figure 3.22 b and 3.22 c show higher magnification of the same fraction in which mitochondria and fragments of rough endosplasmic reticulum can be identified. Figure 3.23 a and 3.23 b show the electron microscopic structure of the 100,000g pellet which consisted mainly of small vesicles and a large proportion of free ribosomes. No mitochondria or mitochondrial fragments were recognisable.

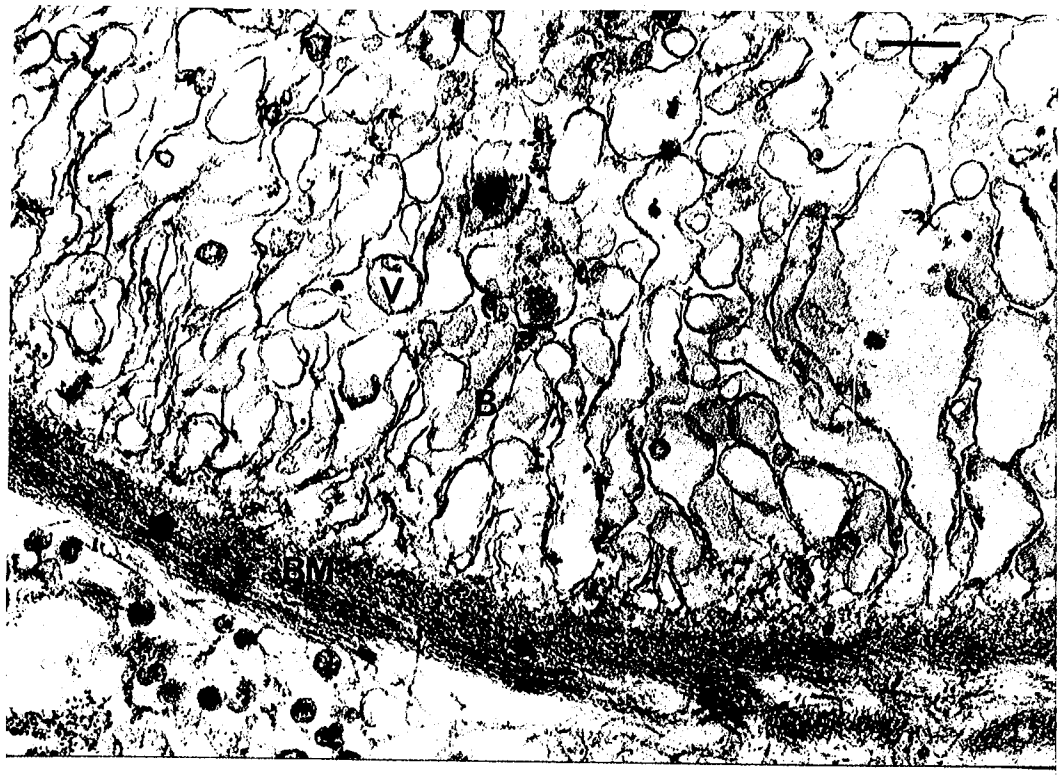
Figure 3.21

(a) Electron micrograph of a section taken through a 600g fraction.

(b) Electron micrograph of the same pellet with higher magnification.

Note: Basement membrane (BM), basal infolding (B), vesicle (V), mitochondria (M).

Scale: 0.5μm


V
B
BM

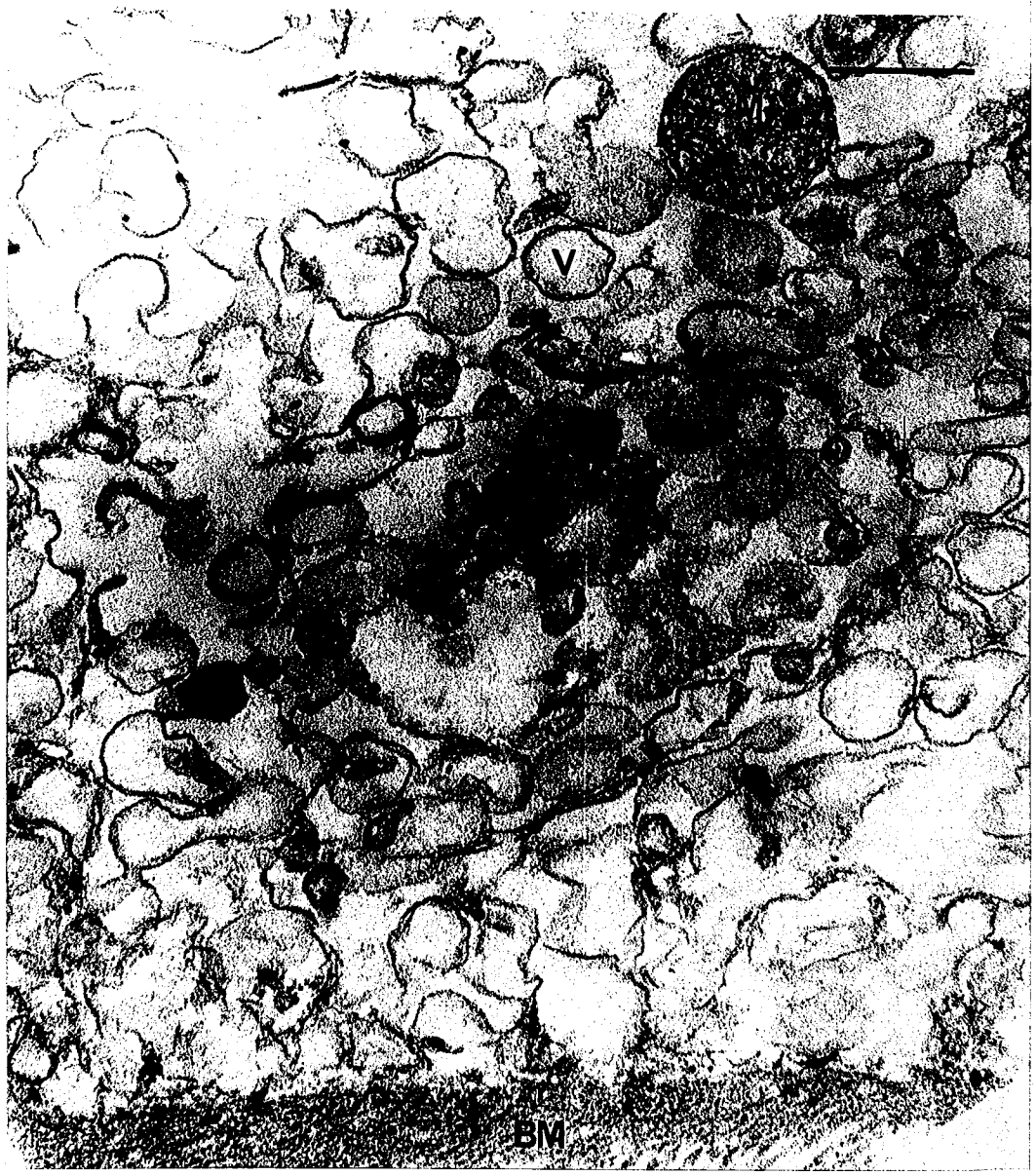

V
BM

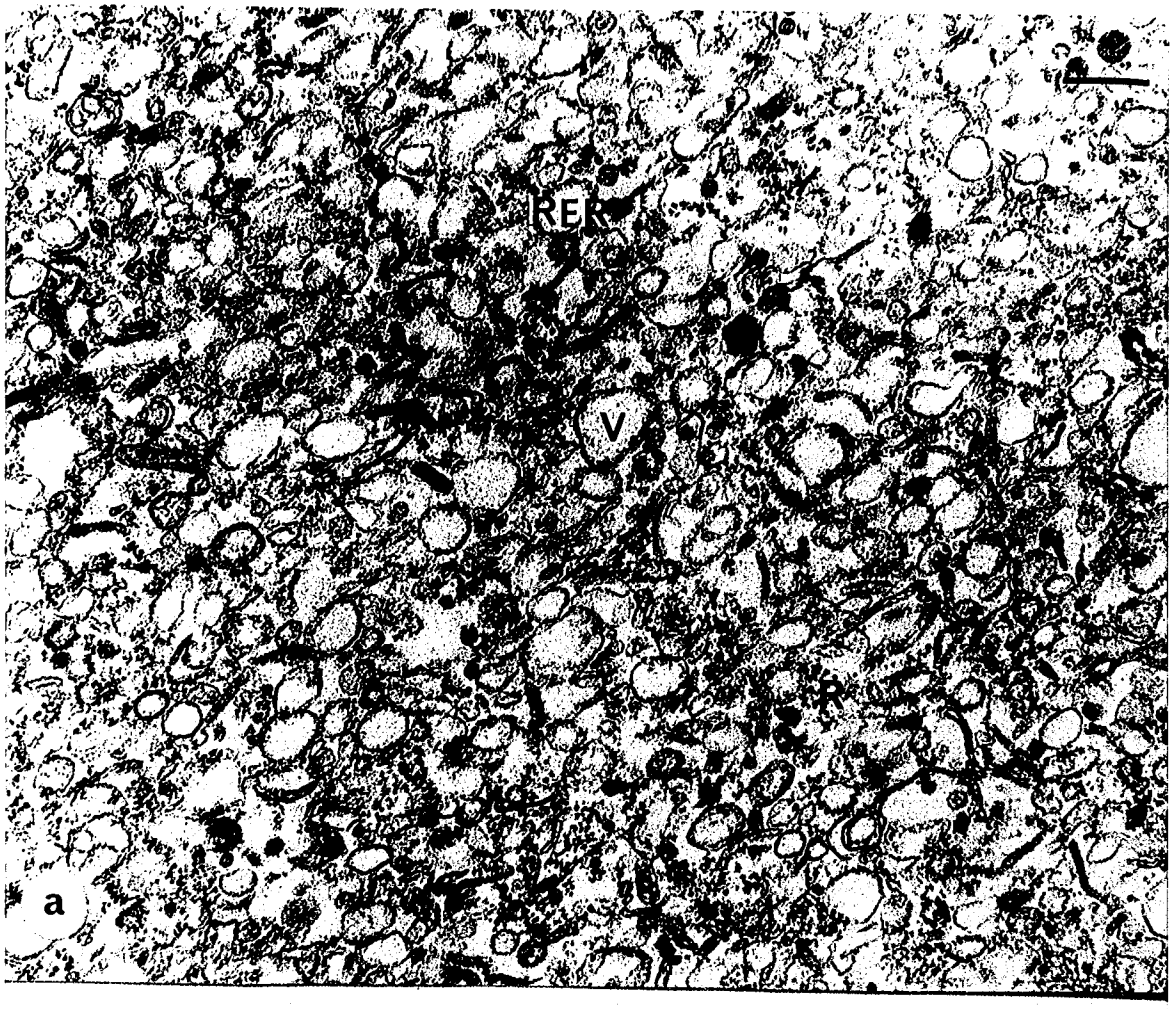
Figure 3.22

(a) Electron micrograph of a section taken through a 20,000g fraction.

(b),(c) Electron micrograph of the same pellet with higher magnification.

Note: Rough endoplasmic reticulum (RER), vesicles (V), mitochondria (M), free ribosomes (R).

Scale= 0.5μm


RER
V
R
a


M
b

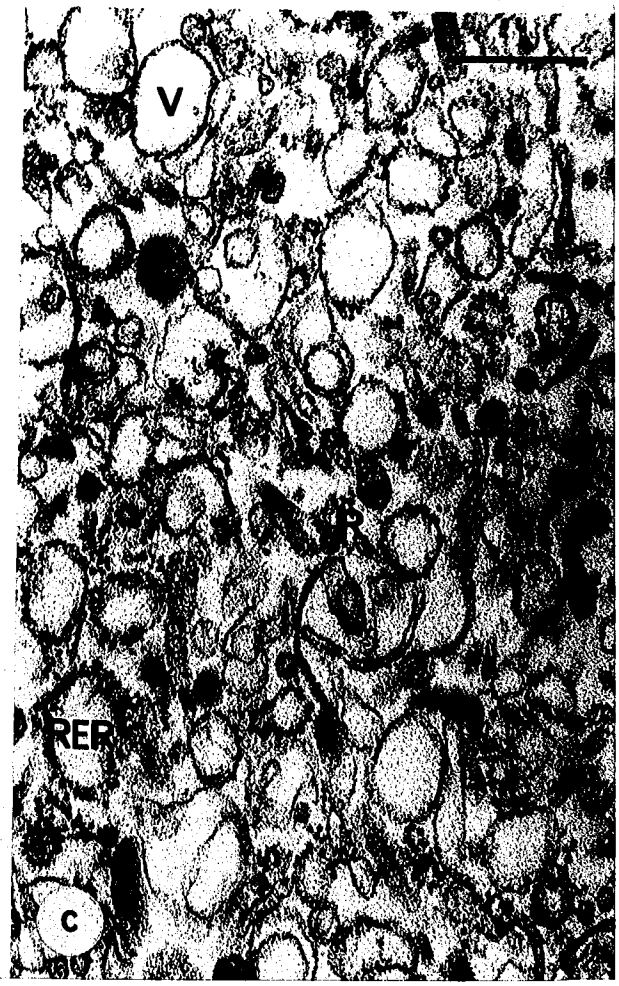

V
R
RER
c

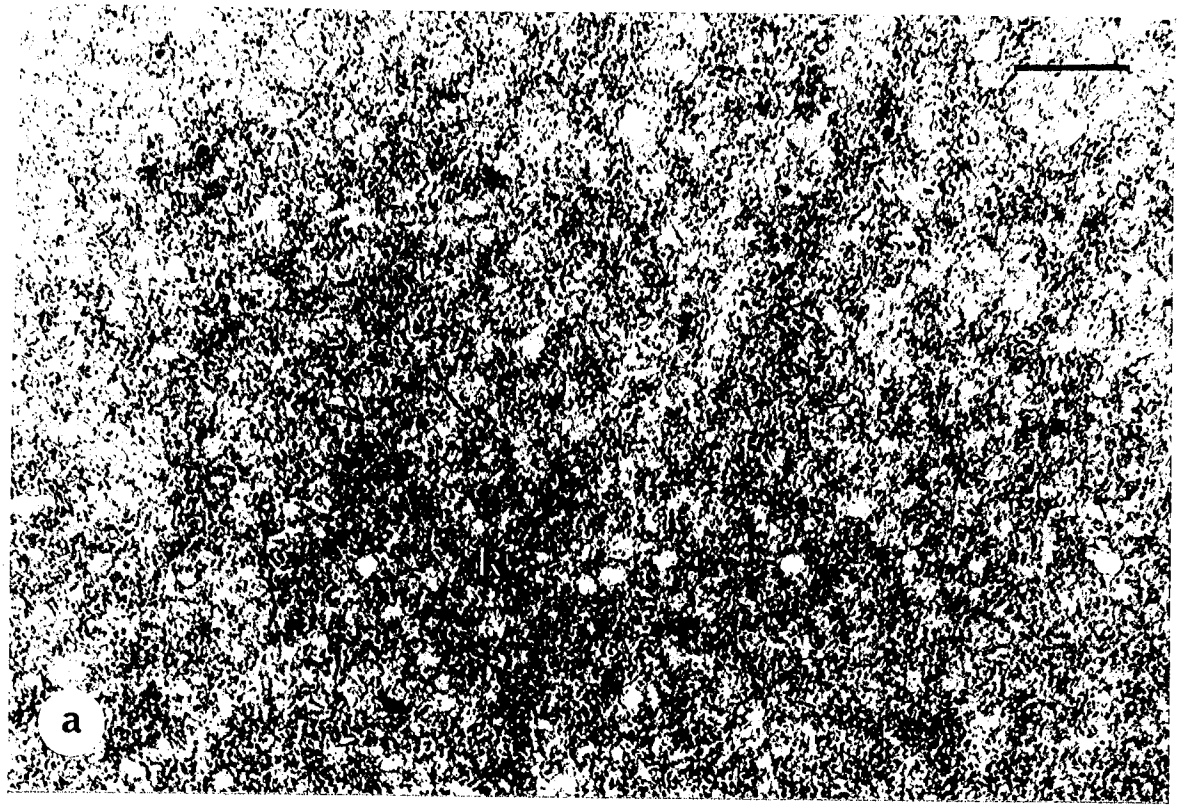
Figure 3.23

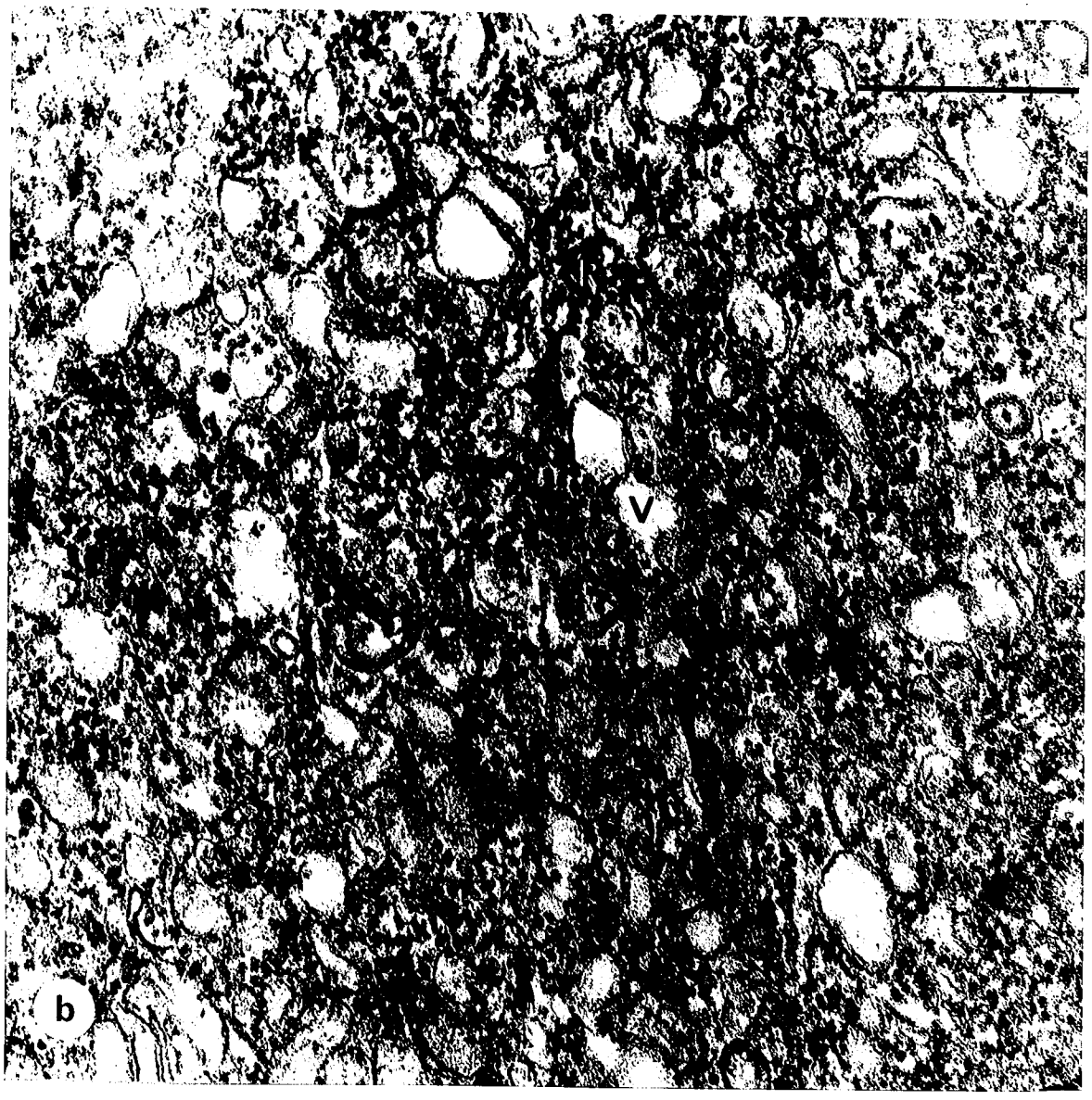
(a) Electron micrograph of a section taken through a 100,000g fraction.

(b) Electron micrograph of the same pellet with higher magnification.

Note: Free ribosomes (R), vesicle (V)

Scale: 0.5μm


R
a


V
b

DISCUSSION

The presence of a Mg^{2+}-dependent HCO_3^--stimulated ATPase has been demonstrated in a microsomal preparation from Locusta Malpighian tubules. The concentration of HCO_3^- effecting maximal stimulation of the Mg^{2+}-dependent ATPase was 20mM. This value agrees well with those reported for frog gastric mucosa (KASBEKAR and DURBIN, 1965), rabbit erythrocytes (DUNCAN, 1975), mammalian pancreas (SIMON and THOMAS, 1972), rat cerebral cortex (KIMELBERG and BOURKE, 1973), rat fundus mucosa (SOUMARMON et al., 1974) and gills of Carassius auratus (DE RENZIS and BORNANCIN, 1977). Somewhat higher values (25mM HCO_3^-) have been reported elsewhere; DE PONT et al. (1972) for lizard gastric mucosa, SANTIAGO et al. (1977) for rat liver and heart, HEGNER and ANIKA (1975) for rumen epithelium of Bos primigenius taurus and IZUTSU and SIEGEL (1975) for rat liver. SUZUKI (1978) observed maximal activity with 30mM and KATZ and EPSTEIN (1971) with 40mM HCO_3^- using mouse and rat kidney respectively. Whereas, TURBECK et al. (1968) using midgut epithelium of Hyalophora cecropia larvae and COLE (1979) using rat renal cortex observed maximal activity with 50mM HCO_3^-. Perhaps the highest value so far reported is 70mM HCO_3^- for rainbow trout ATPase (KERSTETTER and KIRSCHNER, 1974). In contrast, IZUTSU and SIEGEL (1972) observed maximal activity with 10mM HCO_3^- using dog submandibular gland ATPase.

In the present study, the percentage stimulation achieved by including 20mM HCO_3^- in the reaction medium was approximately 50%.

This level of stimulation is similar to that reported for mammalian pancreas (SIMON and THOMAS, 1972), rat renal proximal tubule (KINNE-SAFFAREN and KINNE, 1974), rabbit erythrocytes (DUNCAN, 1975), rat pancreatic membranes (LAMBERT and CHRISTOPHE, 1976), brush border membranes of rabbit kidney (LIANG and SACKTOR, 1976), mitochondrial preparation of mouse kidney (SUZUKI, 1978) and rabbit gastric mucosa (VAN AMELSVOORT et al., 1977A). Somewhat different values have been reported elsewhere; SENER et al. (1979) reported 88% stimulation with rat pancreatic islets, KIMELBERG and BOURKE (1973) and GRISOLIA and MANDELSON (1974) reported 100% stimulation with rat cerebral cortex and 124% stimulation with outer membrane of rat liver mitochondria respectively. IVASHCHENKO et al. (1975) reported 15-77% stimulation using different tissues of rat, SUZUKI (1978) 10-15% stimulation using mouse kidney microsomal preparation, and VAN AMELSVOORT et al. (1978A) 27% stimulation using rabbit erythrocytes.

The pH optimum of the Mg^{2+}-dependent and the Mg^{2+}-dependent HCO_3^-- stimulated ATPase preparations which have been studied to date range 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; HEGNER and ANIKA, 1975; IZUTSU and SIEGEL, 1972, 1975; SUZUKI, 1978). In the present study the Mg^{2+}-dependent and the Mg^{2+}-dependent HCO_3^--stimulated ATPase exhibited maximal activity at approximately pH 7.5 with maximal stimulation due to the presence of HCO_3^- lying between pH 7.5 and pH 8.0. Similar results were reported for dog gastric mucosa (BLUM et al., 1971), and rabbit

red cell ghosts (IZUTSU et al., 1977). It would seem, therefore, that the enzyme from insect Malpighian tubules exhibits a pH optimum towards the lower end of the range of recorded pH values.

The concentration of Mg^{2+} giving maximum Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase activity was 2mM; the ratio between Mg^{2+} and ATP (3mM) concentration being 1:1.5. KIMELBERG and BOURKE (1973) used the same concentration of Mg^{2+} and 3.3 mM ATP with rat cerebral cortex, whilst DE PONT et al. (1972) used 2mM Mg^{2+} and 2mM ATP (ratio 1:1) with lizard gastric mucosa and more recently COLE (1979) used the same concentration of Mg^{2+} (2mM) but only 1mM ATP using rat cerebral cortex. KASBEKAR and DURBIN (1965) obtained maximal HCO_3^--stimulated ATPase activity with 1mM Mg^{2+} (Mg^{2+}/ATP ratio being 1:2) for frog gastric mucosa and SUZUKI (1978) recorded maximum activity with mouse kidney ATPase using a Mg^{2+}/ATP ratio 1:2 (1.5mM Mg^{2+}). SIMON et al. (1972A) and WIZEMANN et al. (1974) used 3mM Mg^{2+} and 3mM ATP in their assays, whereas DUNCAN (1975) used 3mM Mg^{2+} and 2mM ATP using rabbit erythrocytes and TURBECK et al. (1968) recorded maximal activity with 5mM Mg^{2+} and 5mM ATP using midgut epithelium of Hyalophora cecropia. In contrast, VAN AMELSVOORT et al. (1977A) using rabbit gastric mucosa, found that maximum activity of the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase was obtained with 0.6mM Mg^{2+} (Mg^{2+}/ATP ratio 0.3). In spite of this, however, they used 2mM Mg^{2+} and 2mM ATP in the reaction medium throughout their experiments.

Unlike the Mg^{2+}-dependent Na^{+}-K^{+}-activated ATPase reported in insect Malpighian tubules and hindgut (ANSTEE and BELL, 1975, 1978; PEACOCK et al., 1972, 1976; ANSTEE and BOWLER, 1979) the Mg^{2+}-dependent HCO_3^--stimulated ATPase of Locusta Malpighian tubules (both the 20,000g and the 100,000g fractions) is not inhibited by the cardiac glycoside, ouabain. This agrees with the results obtained by COLE (1979) in rat renal cortex, SACHS et al. (1965) in frog gastric mucosa, and IZUTSU and SIEGEL (1975) in rat liver. In contrast, IZUTSU and SIEGEL (1972) reported that the Mg^{2+}-dependent HCO_3^--stimulated ATPase from dog submandibular gland is sensitive to ouabain and the presence of 0.1-1mM ouabain in the reaction medium reduced the activity of enzyme by 30%.

Cyclic AMP which is thought to mediate the action of a variety of hormones and stimulates fluid secretion in the Malpighian tubules of Carausius and Rhodnius (MADDRELL et al., 1971), and Locusta (BELL, 1977) did not alter the activity of either the Mg^{2+}-dependent ATPase or the Mg^{2+}-dependent HCO_3^--stimulated ATPase.

The Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase, as previously reported (ANSTEE and FATHPOUR, 1979) is insensitive to sodium acetazolamide in both the 20,000g and the 100,000g fractions. This is in agreement with the findings of 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 submandibular gland is inhibited up to 40% in the presence of 1mM sodium acetazolamide.

In the present study, sodium azide was found to be the most potent inhibitor of the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase. The presence of 1mM sodium azide in the reaction medium almost completely inhibited the activity of these enzymes in both the 20,000g and the 100,000g fractions. Similar results have been found by other investigators; VAN AMELSVOORT et al. (1977A) reported 90% inhibition of rabbit gastric mucosal Mg^{2+}-ATPase and LAMBETH and LARDY (1971) found 0.1mM azide inhibited beef heart mitochondrial ATPase by 65% and rat liver mitochondrial ATPase by 80%. In contrast, VAN AMELSVOORT et al. (1978A) found that sodium azide inhibited erythrocyte Mg^{2+}-ATPase by only 5% and ANADA et al. (1977) reported 85% and 8% inhibition by 1mM azide using mitochondrial and microsomal Mg^{2+}-ATPase from rat heart. SACHS et al. (1972A) reported that sodium azide inhibited both mitochondrial and soluble gastric ATPase from gastric mucosa.

Thiocyanate inhibited both the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase. The presence of 10mM SCN^- in the reaction medium inhibited the relative activity of the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase by 84% and 78% respectively, while stimulation due to HCO_3^- was only inhibited by 67%. Similar results have been reported by

other investigators (see Table 3.20). As shown in Table 3.20 the sensitivity of the enzyme varies in different tissues; between 0-100%inhibition being observed. This raises the question as to whether the SCN^- sensitivity of the mitochondrial and microsomal ATPases is different. The literature is in conflict on this issue. SOUMARMON et al. (1974) reported 90% inhibition of the mitochondrial Mg^{2+}-dependent ATPase and Mg^{2+}-dependent HCO_3^--stimulated ATPase by 50mM SCN^- using rat fundus mucosa, whereas less than 10% inhibition was observed with microsomal ATPase from the same tissue. LIANG and SACKTOR (1976) reported less than 30% inhibition with Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase from rabbit renal proximal tubules. These authors state that their preparation was 85% microsomal fraction. KASBEKAR and DURBIN (1965) and WIEBELHAUS et al. (1971) observed a Mg^{2+}-dependent HCO_3^--stimulated ATPase inhibited by SCN^- which was concentrated in the microsomal fraction of homogenates from both amphibian and mammalian gastric mucosa. SACHS et al. (1965) reported 53% inhibition for mitochondrial ATPase and 46% inhibition for microsomal ATPase from frog gastric mucosa. Whilst, SUZUKI (1978) found no difference in SCN^- inhibition of mitochondrial and microsomal Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase of mouse kidney. In contrast, DUNCAN (1975) reported that the Mg^{2+}-dependent ATPase of rabbit erythrocyte ghosts (where there can be no doubt that mitochondria are absent) is not inhibited by SCN^-. Indeed, 5mM SCN^- produced a small increase in ATPase activity in the presence of Mg^{2+}, Mg^{2+} +

NaCl and Mg^{2+} + $NaHCO_3$. However, VAN AMELSVOORT et al. (1978A) reported 15% inhibition of Mg^{2+}-dependent ATPase activity by 50mM SCN^- using rabbit erythrocyte ghosts, which supports the earlier observation of IZUTSU et al. (1977).

There is also disagreement, in the literature, concerning the mode of action of SCN^- on ATPase activity. DE RENZIS and BORNANCIN (1977) suggest that SCN^- is a competitive inhibitor of HCO_3^- stimulation of Cl^-/HCO_3^- ATPase activity of Carassius auratus gills. Whereas, SOLOMON et al. (1975) have shown that SCN^- acts as a non-competitive inhibitor of the Mg^{2+}-dependent ATPase extracted from homogenates of eel gills. DURBIN (1963) reported that SCN^- acts by competition with Cl^- in a reaction leading to the formation of acid in gastric mucosa. Whilst, KASBEKAR and DURBIN (1965) observed that SCN^- was an uncompetitive inhibitor of gastric mucosal Mg^{2+}-ATPase. Finally, DE PONT et al. (1972) reported that the inhibition of the Mg^{2+}-dependent ATPase by SCN^- in lizard gastric mucosa seems to be due to a high affinity of this anion for a general binding site on the Mg^{2+}-ATPase.

In the present study, HCO_3^- increased both the K_m and V_{max} of the ATPase. Similarly, both K_m and V_{max} were increased by HCO_3^- for ATPase from dog gastric mucosa (BLUM et al., 1971). However, IRITANI and WELLS (1976) reported no change in K_m with microsomal ATPase from rat uterus although an increase in K_m did occur with mitochondrial ATPase from the same tissue.

TABLE 3.20

Summary of SCN^- inhibition of ATPases in different tissues

Species	Tissue and ATPase	Conc. of SCN^- (mM)	% Inhibition	Reference
Frog	Gastric mucosa Mg^{2+}-ATPase	100	90	KASBEKAR and DURBIN (1965)
Frog	Gastric mucosa Mg^{2+}-HCO_3^--ATPase	1	33	"
Hyalophora cecropia larvae	Midgut Mg^{2+}-ATPase	100	83	TURBECK et al. (1968)
Lizard	Gastric mucosa Mg^{2+}-HCO_3^--ATPase	50	83	DE PONT et al. (1972)
Dog	Submandibular gland HCO_3^--stimulated ATPase	1	60	IZUTSU and SIEGEL (1972)
Rainbow trout	Gill HCO_3^--stimulated ATPase	5	100	KERSTETTER and KIRSCHNER (1974)
Rat	Kidney Mg^{2+}-ATPase	120	60	SOLOMON et al. (1975)
Eel	Gill Mg^{2+}-ATPase	120	60	"
Rat	Liver Mg^{2+}-ATPase	10	64	IZUTSU and SIEGEL (1975)
Rat	Liver Mg^{2+}-HCO_3^--ATPase	10	65	"
Carassius auratus	Gill HCO_3^--stimulated ATPase	10	100	DE RENZIS and BORNANCIN (1977)
Necturus	Oxyntic cells Mg^{2+}-HCO_3^--ATPase	10	51	WIEBELHAUS et al. (1971)
Bos primigenius taurus	Rumen HCO_3^--stimulated ATPase	10	90	HEGNER and ANIKA (1975)
Rat	Kidney Mg^{2+}-ATPase	-	48	KATZ and EPSTEIN (1971)
Rabbit	Gastric mucosa Mg^{2+}-ATPase	-	72	VAN AMELSVOORT et al. (1977A)
Mouse	Kidney Mg^{2+} and Mg^{2+}-HCO_3^--ATPase	100	88	SUZUKI (1978)
Locusta	Malpighian tubules Mg^{2+}-ATPase	10	84	ANSTEE and FATHPOUR (1979)
Locusta	Malpighian tubules Mg^{2+}-HCO_3^--ATPase	10	78	"
Rat	Fundus mucosa Mg^{2+} and Mg^{2+}-HCO_3^--ATPase	50	90	SOUMARMON et al. (1974)
Rat	Gastric Mg^{2+}-ATPase	10	30-40	NARUMI and KANNO (1973)
Rat	Gastric Mg^{2+}-HCO_3^--ATPase	10	20-30	"
Rabbit	Erythrocyte Mg^{2+} and Mg^{2+}-HCO_3^--ATPase	5	no effect	DUNCAN (1975)
Rabbit	Erythrocyte Mg^{2+}-ATPase	25	16	IZUTSU et al. (1977)
Rabbit	Erythrocyte Mg^{2+}-ATPase	50	15	VAN AMELSVOORT et al. (1978A)
Rabbit	Kidney Mg^{2+} and Mg^{2+}-HCO_3^--ATPase	10	30	LIANG and SACKTOR (1976)

KIMELBERG and BOURKE (1973) and SUZUKI (1978) observed a decrease in K_m when using mitochondrial ATPase from rat cerebral cortex and both mitochondrial and microsomal ATPase from mouse kidney.

Sodium sulphite was found to be the most potent stimulator of the locust Malpighian tubule Mg^{2+}-ATPase (both in the presence and absence of NaCl). In the presence of 10mM SO_3^{2-} (lowest optimal concentration) the relative activity of the Mg^{2+}-dependent ATPase increased to 200% (i.e. 100% stimulation of activity). Similar levels of stimulation were reported by KIMELBERG and BOURKE (1973) with rat cerebral cortex. Other investigators report even higher values of stimulation; LIANG and SACKTOR (1976) 199% for rabbit brush border membrane, VAN AMELSVOORT et al. (1978A) 159% with rabbit gastric mucosa, BLUM et al. (1971) 381% using dog gastric mucosa (solubilized form of enzyme). IZUTSU and SIEGEL (1975) reported 65% stimulation with rat liver mitochondria, SIMON et al. (1972A) 70% with rabbit glandula submandibularis, whilst, IZUTSU et al. (1977) 16% using rabbit red cell ghosts. In contrast, VAN AMELSVOORT et al. (1978A) observed 21% inhibition of rabbit erythrocyte ATPase by SO_3^{2-}. In the present study when the reaction media contained both SO_3^{2-} and HCO_3^-, the stimulation due to SO_3^{2-} decreased suggesting that HCO_3^- was, in some way, interfering with the SO_3^{2-} stimulation of the Mg^{2+}-dependent ATPase. However, this interference was incomplete; the activity in the presence of both anions being higher than that observed in the presence of HCO_3^- alone.

Sodium borate was also found to stimulate Mg^{2+}-dependent ATPase activity; the level of stimulation (approx. 50%) being of the same order as that observed with HCO_3^-. This finding is in

agreement with the results obtained by BLUM et al. (1971) using solubilized enzyme from dog gastric mucosa (52%). EBEL and LARDY (1975) reported that $B_4O_7^{2-}$ increased the activity of rat liver mitochondrial ATPase by 100%, whilst TURBECK et al. (1968) reported 320% stimulation (relative to activity in the presence of NaCl) with midgut ATPase from Hyalophora cecropia larvae. In contrast, IZUTSU and SIEGEL (1975) reported that $B_4O_7^{2-}$ did not activate the Mg^{2+}-dependent ATPase from rat liver mitochondria, and IZUTSU et al. (1977) found that $B_4O_7^{2-}$ effected a 53% inhibition of the Mg^{2+}-ATPase from rabbit red cells membrane. SACHS et al. (1972A) have suggested that $B_4O_7^{2-}$ only exerts a stimulatory effect on microsomal ATPase and inhibits the mitochondrial enzymes. However, as mentioned above, EBEL and LARDY (1975) report stimulation of rat liver mitochondrial ATPase by $B_4O_7^{2-}$.

The presence of increasing concentrations of $B_4O_7^{2-}$ in the reaction media containing HCO_3^-, inhibited the stimulatory effect of HCO_3^- on the Mg^{2+}-dependent ATPase indicating that the effect of both anions is not cumulative.

Another activator of Mg^{2+}-dependent ATPase activity from locust Malpighian tubules was SeO_3^{2-}. In the presence of 40mM SeO_3^{2-}, 41% and 63% stimulation of activity was observed in the presence and absence of NaCl respectively. Similar results were obtained by IRITANI and WELLS (1976) who reported 44-48% stimulation for rat uterus Mg^{2+}-dependent ATPase. While SIMON et al. (1972B) reported a 4.3-fold, and EBEL and LARDY (1975) a 9.4-fold increase in the activity of the Mg^{2+}-dependent ATPase extracted from dog

pancreatic tissue and rat liver mitochondria respectively. Similarly, TURBECK et al. (1968) found a 10-fold increase (relative to activity in the presence of NaCl) in the activity of midgut Mg^{2+}-dependent ATPase from Hyalophora cecropia larvae. In contrast, IZUTSU et al. (1977) reported that the Mg^{2+}-dependent ATPase prepared from red cell ghosts was inhibited by 42%.

Unlike $B_4O_7^{2-}$, the presence of SeO_3^{2-} in reaction media containing HCO_3^- did not alter the stimulatory effect of HCO_3^- on the Mg^{2+}-dependent ATPase. However, the highest concentration of SeO_3^{2-} (40mM) appeared to decrease slightly the relative activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase. Both Cl^- and HCO_3^- reduced the stimulatory effect of SeO_3^{2-} on the Mg^{2+}-ATPase activity.

In the present study, Br^- could be substituted for Cl^- without substantially effecting the activity of the Mg^{2+}-dependent ATPase. There was, however, a small (11%) increase in enzyme activity when 20mM NaBr replaced NaCl in the reaction medium. In contrast, the presence of the same concentration of Br^- in reaction media containing 20mM $NaHCO_3$ reduced the relative activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase by 10%. These results are in agreement with those of KASBEKAR and DURBIN (1965) for frog gastric mucosa. They found that the optimal concentration of Br^- (20mM) increased the relative activity of the Mg^{2+}-dependent ATPase, whilst the same concentration of Br^- in the reaction media containing optimal concentrations of $NaHCO_3$ did not lead to an

additional stimulation of enzyme activity and larger amounts of Br^- reduced HCO_3^- stimulation. Thus the effect of both ions are not cumulative. IZUTSU et al. (1977) observed 5% inhibition of Mg^{2+}-dependent ATPase with Br^- in preparation of rabbit red cell ghosts, whilst EBEL and LARDY (1975) reported Br^- increased the relative activity of rat liver mitochondrial Mg^{2+}-dependent ATPase by 220%.

Unlike bromide, $NaNO_3$ inhibited the activity of the Mg^{2+}-dependent ATPase; the relative activity of the enzyme being decreased by 29% and 41% (in the presence and absence of 20mM NaCl respectively) when 20mM NO_3^- was present in the reaction media. Similar results were reported by other investigators; VAN AMELSVOORT et al. (1977A) obtained 35% inhibition with rabbit gastric mucosa, TURBECK et al. (1968) observed 36% inhibition with midgut of Hyalophora cecropia larvae, and DE PONT et al. (1972) reported 41% inhibition with lizard gastric mucosa. Similarly, IZUTSU and SIEGEL (1975) observed 48% inhibition with rat liver, whilst KATZ and EPSTEIN (1971) and IZUTSU et al. (1977) reported 18% and 10% inhibition with rat kidney and rabbit red cell ghosts repsectively.

Recently, HERRERA et al. (1978) reported that Mg^{2+}-dependent ATPase extracted from rectum of Schistocerca gregaria was stimulated 4.1-fold in the presence of 60mM Tris-Cl. Addition of 10mM Tris-HCO_3 to the reaction media lead to a further stimulation of the order of 100%. However, if 60mM Tris-Cl was replaced by 60mM Tris-NO_3, the Mg^{2+}-dependent HCO_3^--stimulated ATPase was inhibited by 67%. In the present study, when 20mM $NaNO_3$ was added to reaction media containing 20mM $NaHCO_3$ the activity of the Mg^{2+}-dependent HCO_3^--

stimulated ATPase was inhibited by only 34%. The different levels of response to Cl^-, HCO_3^- and NO_3^- reported by HERRERA et al. (1978), compared to the present study, may be due, in part, to the fact that the basic Mg^{2+}-ATPase activity was determined, by these authors, in the presence of 5mM $Mg(NO_3)_2$ rather than $MgCl_2$. Both the present study and that of HERRERA et al. (1978) indicate that NO_3^- inhibits ATPase activity. However, Cl^-, which stimulates ATPase from rectum of Schistocerca gregaria did not affect the activity of the Malpighian tubules ATPase in the present study. It may be that the Cl^- stimulation, they report, is a feature of Schistocerca gregaria rectal preparation. Alternatively, it is possible such stimulation is to some extent, if not exclusively, due to the removal of NO_3^- inhibition of the Mg^{2+}-ATPase by the addition of 60mM Tris-Cl. Certainly, in the present study the inhibitory effect of NO_3^- was reduced by the inclusion of 20mM Cl^- in the reaction media. Such an effect on the basic Mg^{2+}-ATPase activity of Schistocerca gregaria rectal preparation would appear as a Cl^- stimulation of activity.

The diuretic agent amiloride inhibits fluid secretion by the Malpighian tubules of Glossina morsitans (GEE, 1976B), Locusta (present study), the salivary glands of Calliphora (BERRIDGE et al., 1976) and the movement of Na^+ across the mucosal cell membrane of toad urinary bladder (SUDOU and HOSHI, 1977; CANESSA et al., 1978). However, amiloride did not significantly affect the activity of either the Mg^{2+}-dependent ATPase or the Mg^{2+}-dependent HCO_3^--stimulated ATPase.

TABLE 3.21

Summary of the effects of different anions on Mg^{2+}-dependent ATPase (in the presence and absence of 20mM NaCl) from Locusta Malpighian tubules. Relative activities are presented with S.E.M.; activity in the presence of 2mM $MgCl_2$ and 2mM $MgCl_2$ + 20mM NaCl being taken as 100 arbitrary units respectively.

Major anion	*Relative activity 2mM Mg^{2+}	2mM Mg^{2+} + 20mM NaCl
Cl^-	98.3 ± 0.9	100.0
HCO_3^-	149.0 ± 2.0	152.0 ± 2.0
SO_3^{2-}	200.5 ± 16.0	176.2 ± 15.0
$B_4O_7^{2-}$	151.9 ± 3.5	143.6 ± 1.6
SeO_3^{2-}	163.1 ± 6.4	141.1 ± 4.1
Br^-	111.0 ± 0.3	107.0 ± 1.9
NO_3^-	59.0 ± 0.5	71.7 ± 0.8
SCN^-	16.5 ± 2.7	16.7 ± 4.6
N_3^-	0.0	0.0

* For more details see the Results section

Ethacrynic acid is a diuretic agent which is known to inhibit Na^+-K^+ ATPase from a variety of tissues (DUGGON and NOLL, 1965; CHARNOCK et al. 1970; DAVIS, 1970; PEACOCK et al., 1976). However, the effect of ethacrynic acid appears to be non-specific and it has been shown to effect a variety of cellular processes. These include the inhibition of glycolysis in turtle urinary bladder, renal cortex and medulla of rabbit, rat and human red blood cells (KLAHR et al., 1971), active uptake of various sugar in rabbit kidney cortex cells (KLEINZELLER and EPSTEIN, 1969), anion movement in ox red blood cells (MOTAIS and COUSIN, 1976). Ethacrynic acid has also been reported to inhibit Na^+ transport by interfering with oxidation and phosphorylation in the mitochondria of transporting cells in toad kidney (CASE et al., 1973). It has also been shown to effect oxygen uptake and produce a rapid dimunition of tissue ATP content in rabbit kidney cortex (EPSTEIN, 1972). GEE (1976B) has shown that ethacrynic acid inhibits fluid secretion by Glossina morsitans, whilst SEJERSTED et al. (1978) report that it effects increased tubular K^+ secretion in dog kidney. In the present study, 1mM ethacrynic acid inhibited the activity of the Mg^{2+}-dependent ATPase by 25% and 18% (in the presence and absence of NaCl). Replacing NaCl by $NaHCO_3$ did not change the level of inhibition. Somewhat higher levels of inhibition have been reported by WALD et al. (1979). These authors report 35-80% inhibition of microsomal Mg^{2+}-dependent ATPase by ethacrynic acid by using various tissues of rat, and kidney from a variety of different species. They conclude that the inhibition of membrane Mg^{2+}-dependent ATPase by ethacrynic acid could be either a non-

specific phenomenon or could lead to the identification of a specific energy-coupling mechanism for transport.

The localization of the Mg^{2+}-dependent HCO_3^--stimulated ATPase is in dispute. Three main opinions exist concerning the subcellular localization of this enzyme. Each bases its conclusion on the results of studies involving one or more of the following: mitochondrial marker enzymes, microsomal marker enzymes, electron microscopy, and the differing effects of various inhibitors on mitochondrial and microsomal preparations.

The three schools of thought may be summarised as follows:

(i) Those workers who have concluded that the microsomal Mg^{2+}-dependent HCO_3^--stimulated ATPase activity is derived from mitochondria, mainly due to contamination (TURBECK et al., 1968; KIMELBERG and BOURKE, 1973; SOUMARMON et al., 1974; VAN AMELSVOORT et al.,1977A,B; IZUTSU et al., 1978). IZUTSU and SIEGEL (1975) and VAN AMELSVOORT et al. (1978B) have shown that microsomal Mg^{2+}-ATPase with undetectable mitochondrial contamination, had no detectable HCO_3^--ATPase activity.

(ii) Those who believe that Mg^{2+}-dependent HCO_3^- stimulated ATPase is localized in the microsomal membrane fraction and is not of mitochondrial origin (SIMON et al., 1972A; SIMON and THOMAS, 1972; SACHS et al., 1972B; SPENNEY et al., 1973; KINNE-SAFFAREN and KINNE, 1974; HEGNER and ANIKA, 1975; DE RENZIS and BORNANCIN, 1977). The fact that such an enzyme has been demonstrated in rabbit red cell membranes, where no mitochondrial contamination

could exist, tends to support this contention (DUNCAN, 1975). However, VAN AMELSVOORT et al. (1978A) suggest that the erythrocyte enzyme is different from the anion-sensitive Mg^{2+}-ATPase of other tissues, and is part of the (Ca^{+} + Mg^{2+}) - ATPase system of the erythrocyte membrane.

(iii) Finally, those investigators who have concluded that their homogenates may not be pure microsomal preparation but may contain varying amounts of mitochondrial membranes. Thus, LIANG and SACKTOR (1976) and COLE (1979) reported 10-15% mitochondrial contamination in their preparations from rabbit renal tubule and rat renal tissue. This calculation was based on the activity of the mitochondrial marker enzyme, SDH, present in the microsomal fraction. They concluded that whilst a proportion of their activity is due to mitochondrial contamination, not all was (BLUM et al., 1971; KERSTETTER and KIRSCHNER, 1974; SUZUKI, 1978).

A number of workers have attempted to distinguish between mitochondrial Mg^{2+}-dependent ATPase and the microsomal enzyme on the basis of their response to treatment with 2,4-DNP and oligomycin. Thus SACHS et al. (1972A) and ANADA et al. (1977) report that 2,4-DNP stimulates mitochondrial ATPase activity but not the activity of the microsomal ATPase. In the present study 1mM DNP effected an 22% stimulation of Mg^{2+}-dependent ATPase activity (7% in the presence of 20mM $NaHCO_3$). This represents a very small response to DNP when compared with the data reported by LAMBETH and LARDY (1971) for pure mitochondrial ATPase from

rat liver. These authors observed 100% stimulation of Mg^{2+}-ATPase by DNP, whilst EBEL and LARDY (1975) observed 470% stimulation with rat liver mitochondria. On this basis, one might suppose that the present preparation was relatively insensitive to DNP and this might be considered evidence for its microsomal origin. However, more detailed examination of the literature leadsone to question the original supposition. Thus ANADA et al. (1977) observed 20% stimulation of mitochondrial ATPase from rat heart although the microsomal Mg^{2+}-ATPase from the same tissue was stimulated by only 4%. Similarly, mitochondrial ATPase from rat liver (SANTIAGO et al., 1977) was stimulated by 21%. Both responses of these mitochondrial ATPase are lower than that for frog gastric mucosa (KASBEKAR and DURBIN, 1965) and are similar to the level of stimulation reported for microsomal/mitochondrial ATPase from dog submandibular gland (IZUTSU and SIEGEL, 1972). The latter authors suggest that the stimulation of the microsomal Mg^{2+}-dependent ATPase by DNP is due to contamination of this fraction with mitochondria and is evidence for the mitochondrial origin of the HCO_3^--stimulated ATPase activity. If this is true for the other preparation referred to above, different responses to DNP are clearly poor indications to membrane origin.

Oligomycin is known to inhibit oxidative phosphorylation by mitochondria from a variety of tissues (BONTING, 1970; AZZI and SANTATO, 1970; CATTEDRALL and PEDERSEN, 1974; LEHNINGER, 1976; DROBINSKAYA et al., 1978). It is suggested that this is effected

by inhibition of mitochondrial ATPase activity (CATTEDRALL and PEDERSEN, 1974; LEHNINGER, 1976; DROBINSKAYA et al., 1978; WHITTAKER and DANKS, 1978). However, LAMBETH and LARDY (1971) reported that highly purified rat liver mitochondrial Mg^{2+}-ATPase (F_1ATPase) is insensitive to oligomycin. Subsequent studies have revealed that mitochondrial oligomycin-sensitive ATPase of heart and rat liver consists of three parts, F_1 ATPase, F_o (oligomycin-sensitivity-conferring factor, OSCP) and a membrane section consisting of a minimum of four proteins (LEHNINGER, 1976; WHITTAKER and DANKS, 1978). The F_1ATPase is a soluble coupling factor necessary for the restoration of energy-coupling activity in the mitochondrial membrane. In the presence of Mg^{2+} it catalyses the slow hydrolysis of ATP to ADP and phosphate, this reaction is not inhibited by oligomycin. This ATPase activity is believed to represent reversal of the normal function of F_1 in intact mitochondria, namely, the synthesis of ATP from ADP and phosphate. The F_o factor (OSCP) is another specific protein, which is responsible for the coupling of ATP synthesis to electron transport. This coupling is necessary before the ATPase is inhibitable by oligomycin.

LIANG and SACKTOR (1976) reported that mitochondrial Mg^{2+}-ATPase from rabbit renal cortex was 95% inhibited by $12.5 g/cm^3$ of oligomycin. In contrast, the brush border membrane ATPase from the same tissue was inhibited by only 36% and 23% in the presence and absence of HCO_3^-. If such a difference existed in locust preparation it might well indicate the source of the ATPase activity. However, in the present study, oligomycin was

found to inhibit the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase in all subcellular fractions studied. Indeed, the pI50 for the 100,000g fraction was significantly higher than that of the 20,000g and 5,000g fractions. It would seem, therefore, that the mitochondrial (5,000g fraction) ATPase is less oligomycin-sensitive than the microsomal (100,000g fraction) ATPase. A similar result was reported by VAN AMELSVOORT et al. (1977B); the pI50 being 7.0 for rabbit kidney mitochondria and 7.8 for microsomal preparations from the same tissue. This higher sensitivity of the microsomal fraction to oligomycin, as VAN AMELSVOORT et al. (1977B) pointed out, may be due to the shape of inhibition curve, which possibly reflects the presence of a mixture of Mg^{2+}-ATPase activities with different sensitivities towards oligomycin. The same workers (1978B) reported the pI50 to be 7.75 for mitochondrial ATPase from rat pancreas. It is clear, then, that both the Mg^{2+}-dependent and Mg^{2+}-dependent HCO_3^--stimulated ATPase of Locusta Malpighian tubules are considerably less sensitive to oligomycin than rabbit kidney ATPase. Indeed, the pI50 values obtained with Locusta preparations agree most closely with those reported by VAN AMELSVOORT et al. (1978A) for rabbit erythrocyte ATPase (pI50 = <5.6) where mitochondrial ATPase is clearly absent. Nevertheless, it is impossible to conclude, on the basis of oligomycin-sensitivity, whether the locust ATPase is mitochondrial or non-mitochondrial in origin.

In the present work, the distribution of Mg^{2+}-dependent HCO_3^--stimulated ATPase, between various subcellular fractions, was compared with that of the mitochondrial marker enzyme succinate

dehydrogenase (LIANG and SACKTOR, 1976). The highest SDH activity was measured in the 20,000g fraction which coincided with the highest ATPase activity. However, the activity of SDH in the 100,000g was only 17% of that found in the 20,000g fraction, suggesting that there was mitochondrial contamination of the same order as elsewhere (LIANG and SACKTOR, 1976; COLE, 1979). The fact that, SDH activity is not sharply limited to the mitochondrial fraction is probably explained by mitochondrial disruption during the preparatory processes. The ratio of SDH/Mg^{2+}-dependent HCO_3^--stimulated ATPase activity was 0.57 at 20,000g fraction, compared with 0.16 at 100,000g fraction. These findings might be taken to indicate that Locusta Malpighian tubule Mg^{2+}-dependent HCO_3^--stimulated ATPase is non-mitochondrial in origin. Alternatively, the decrease in SDH/Mg^{2+}-dependent HCO_3^--stimulated ATPase ratio may reflect a relative activation of the ATPase through loss of an ATPase inhibitor protein or an increase in substrate accessibility of the ATPase during fractionation. (VAN AMELSVOORT et al. 1977B).

Further support for the non-mitochondrial source of Mg^{2+}-dependent HCO_3^--stimulated ATPase activity is provided by electron microscopy. Such studies indicated that whilst mitochondria were clearly recognisable in the 20,000g fraction, these organelles were apparently absent from the 100,000g fraction. However, disrupted mitochondria would probably have been unrecognisable. Further studies are clearly necessary before the localization of the enzyme can be finally accepted.

In those tissues (SIMON et al., 1972B; LIANG and SACKTOR, 1976; HEGNER and ANIKA, 1975) where a HCO_3^--stimulated ATPase has been shown to be involved in ion transport, the enzyme carbonic anhydrase is usually implicated. Carbonic anhydrase is thought to play a major role in restoring enough HCO_3^- for stimulating the 'pump'. This being the case, a series of experiments were carried out to ascertain whether this enzyme is present in locust Malpighian tubules. EDWARDS and PATTON (1967) failed to detect carbonic anhydrase activity in the Malpighian tubules of Acheta domesticus. However, the present study indicates that it is clearly present in crude homogenates of the Malpighian tubules of Locusta migratoria; the specific activity of 4.17 units/mg protein observed being somewhat higher than the value reported for rat uterus (IRITANI and WELLS, 1976). These authors reported carbonic anhydrase activity of 0.52 and 0.43 units/mg protein for mitochondrial and microsomal fractions. SUZUKI (1978) reported somewhat higher values for carbonic anhydrase activity with the supernatant of mouse kidney microsomal fraction (62.9 units/mg protein), mouse kidney mitochondria (8.6 units/mg protein), mouse kidney 20,000g fraction (9.5 units/mg protein) and mouse kidney 105,000g fraction (6.5 units/mg protein). The presence of 1mM sodium acetazolamide inhibited the activity of carbonic anhydrase by 99.5%, in the present study, which agrees with the result of other investigators. In fact, HOUSTON and Mc CARTY (1978), HOUSTON and MEAROW (1979), and SMEDA and HOUSTON (1979) determined the activity of carbonic anhydrase in the presence and absence of sodium acetazolamide.

In the present study, it is clear that the $Na^+ - K^+$ ATPase and the Mg^{2+}-dependent HCO_3^--stimulated ATPase are different enzymes. When Cl^- was substituted for HCO_3^- in the reaction media, the anion-stimulated ATPase activity was reduced. In contrast, a similar substitution effected a stimulation of $Na^+ - K^+$ ATPase activity. In addition, 10mM NaSCN inhibited the level of enzyme activity of the Mg^{2+}-dependent HCO_3^--stimulated ATPase by 78%, whereas, the same concentration of SCN^- inhibited the activity of Na^+-K^+ ATPase by only 10%. The latter results are supported by the findings of SOLOMON et al. (1975) who reported that 12mM SCN^- had no effect on $Na^+ - K^+$ ATPase activity of eel gill and rat kidney, and DE PONT et al. (1972) who observed that 5mM SCN^- did not alter the $Na^+ - K^+$ ATPase activity of lizard gastric mucosa.

Further support for this argument is the effect of Br^- and NO_3^- on $Na^+ - K^+$ ATPase. Br^- which decreased the relative activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase (10%) did not alter the activity of $Na^+ - K^+$ ATPase. Similarly, NO_3^- which markedly decreased the relative activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase (34%) did not effect the activity of $Na^+ - K^+$ ATPase.

Conclusion. A Mg^{2+}-dependent ATPase which is stimulated by HCO_3^- and inhibited by SCN^- is present in the Malpighian tubules of Locusta migratoria. The exact localization of the enzyme is uncertain. The evidence available suggests that the enzyme is both microsomal and mitochondrial in origin. In the following chapters, the role of this enzyme in fluid and ion secretion will be investigated.

CHAPTER 4

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

INTRODUCTION

Urine formation by the Malpighian tubules of a number of insect species depends on an active transport of K^+ and it is thought that the active pumping of these ions from the haemolymph to the lumen creates the osmotic gradient necessary for water to flow passively (RAMSAY, 1953, 1955; BERRIDGE, 1967, 1968; BELL, 1977; ANSTEE *et al.*, 1979). However, this is not the case for all insects which have been investigated. For example, *Rhodnius* (MADDRELL, 1969) can produce 'urine' in the presence of Na^+ or K^+ whereas *Glossina austeni* (GEE, 1975) and *G. morsitans* (GEE, 1976A) use Na^+.

Despite this role of K^+ (or Na^+) as the 'prime mover' in fluid secretion, a number of workers have shown that these cations alone will not support water transport across the tubule wall, unless accompanied by an appropriate anion. The mechanism of transporting anions to accompany the active secretion of K^+ (or Na^+) is possibly different in different species. For example, in *Calliphora*, Cl^- transport occurs by passive diffusion down an electrochemical gradient created by active K^+ transport (BERRIDGE, 1969). In contrast, Cl^- is thought to be actively transported across the basal cell membrane of *Rhodnius* Malpighian tubules cells (MADDRELL, 1977). Active Cl^- transport has been reported in other insect epithelia. PHILLIPS (1964, 1977); HERRERA *et al.*, (1977, 1978) and WILLIAMS *et al.*, (1978) have shown that a Cl^- 'pump' is involved in the rectum of *Schistocerca gregaria*. Similarly, KAUFMAN and PHILLIPS (1973)

intracellular Na^+ concentrations in Locusta Malpighian tubules, and BERRIDGE and SCHLUE (1978) working on salivary glands of Calliphora noticed that the intracellular level of K^+ was lowered in the presence of ouabain and the absence of stimulator (5-HT). ANSTEE et al. (1979) reported that ouabain increased the ratio of Na^+/K^+ in secreted fluid by Locusta. Furthermore, they observed that ouabain affects the rate of oxygen consumption by the locust tubules. In contrast, GEE (1976B) reported that ouabain did not effect the Na^+ concentration of secreted fluid by the 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.

It has been reported that amiloride inhibits fluid secretion by the Malpighian tubules of Calliphora (BERRIDGE et al., 1976) and Glossina morsitans (GEE, 1976B). Similarly, amiloride interferes with Na^+ transport in toad bladder (BENTLEY, 1968; SUDOU and HOSHI, 1977) and frog skin (NAGEL and DORGE, 1970). In general, it is considered that amiloride acts by blocking passive Na^+ entry into the cell (BENTLEY, 1968; NAGEL and DORGE, 1970; BERRIDGE et al., 1976; SUDOU and HOSHI, 1977).

In the previous chapter, the presence of a Mg^{2+}-dependent HCO_3^--stimulated ATPase in the 100,000g fraction of Locusta Malpighian tubules was reported and its relation with carbonic anhydrase was discussed. As was mentioned, a close-link is reported to exist between the two enzymes in those cells in which the Mg^{2+}-dependent HCO_3^--stimulated ATPase has been implicated in ion transport (e.g. in mammalian pancreatic tissue, SIMON et al., 1972B; in rumen

epithelium, HEGNER and ANIKA, 1975; in rabbit renal proximal tubule, LIANG and SACKTOR, 1976; and in mouse kidney, SUZUKI, 1978). Sodium acetazolamide is known as an inhibitor of carbonic anhydrase. This enzyme is implicated in fluid secretion in a variety of different tissue including the Malpighian tubules of Glossina morsitans (GOODING, 1975), rabbit proximal tubules (BURG and GREEN, 1977; Mc KINNEY and BURG, 1977), and rat seminiferous tubules (CHEUNG et al., 1977). In contrast, MADDRELL (1969) and BERRIDGE (1968) failed to show acetazolamide inhibition of fluid secretion by the Malpighian tubules of Rhodnius or Calliphora. Acetazolamide is also reported to effect Na^+ flux in rat and dog proximal tubules (GREEN and GIBISCH, 1975; MATHISEN et al., 1978) and Cl^- flux in turtle and toad urinary bladder (LESLIE et al., 1973; SQBOSLAI et al., 1977).

Sodium thiocyanate is known to inhibit Mg^{2+}-dependent HCO_3^--stimulated ATPase in a variety of tissues (see Chapter 3). In addition, it also inhibits acid secretion in gastric mucosa (DAVENPORT, 1940; HARRIS and EDELMAN, 1959; FORTE and DAVIES, 1964; SACHS et al., 1972A) and Na^+ flux in red cells (WIETH, 1969); a fact which has been taken in many instances to indicate a role of Mg^{2+}-dependent HCO_3^--stimulated ATPase in the transport mechanisms (KASBEKAR and DURBIN, 1965; BLUM et al., 1971; SIMON and THOMAS, 1972; DE RENZIS and BORNANCIN, 1977).

The present study has been carried out to provide further information on the nature of the fluid and ion secretory mechanisms of Locusta tubules and to determine the extent to which the Mg^{2+}-dependent HCO_3^--stimulated ATPase, carbonic anhydrase and Na^+-K^+ ATPase enzymes may be involved.

MATERIALS AND METHODS

Adult locusts, of both sexes, were used throughout all the following experiments.

An alternative Ringer solution (MADDRELL, 1969) was used in certain studies on the Na^+ and K^+ composition of the urine. The composition of this Ringer solution was (mM): NaCl 129; KCl 8.6; $MgCl_2$ 8.5; $CaCl_2$ 2; $NaHCO_3$ 10.2; NaH_2PO_4 4.3; glucose 34. The pH was adjusted to 7.2.

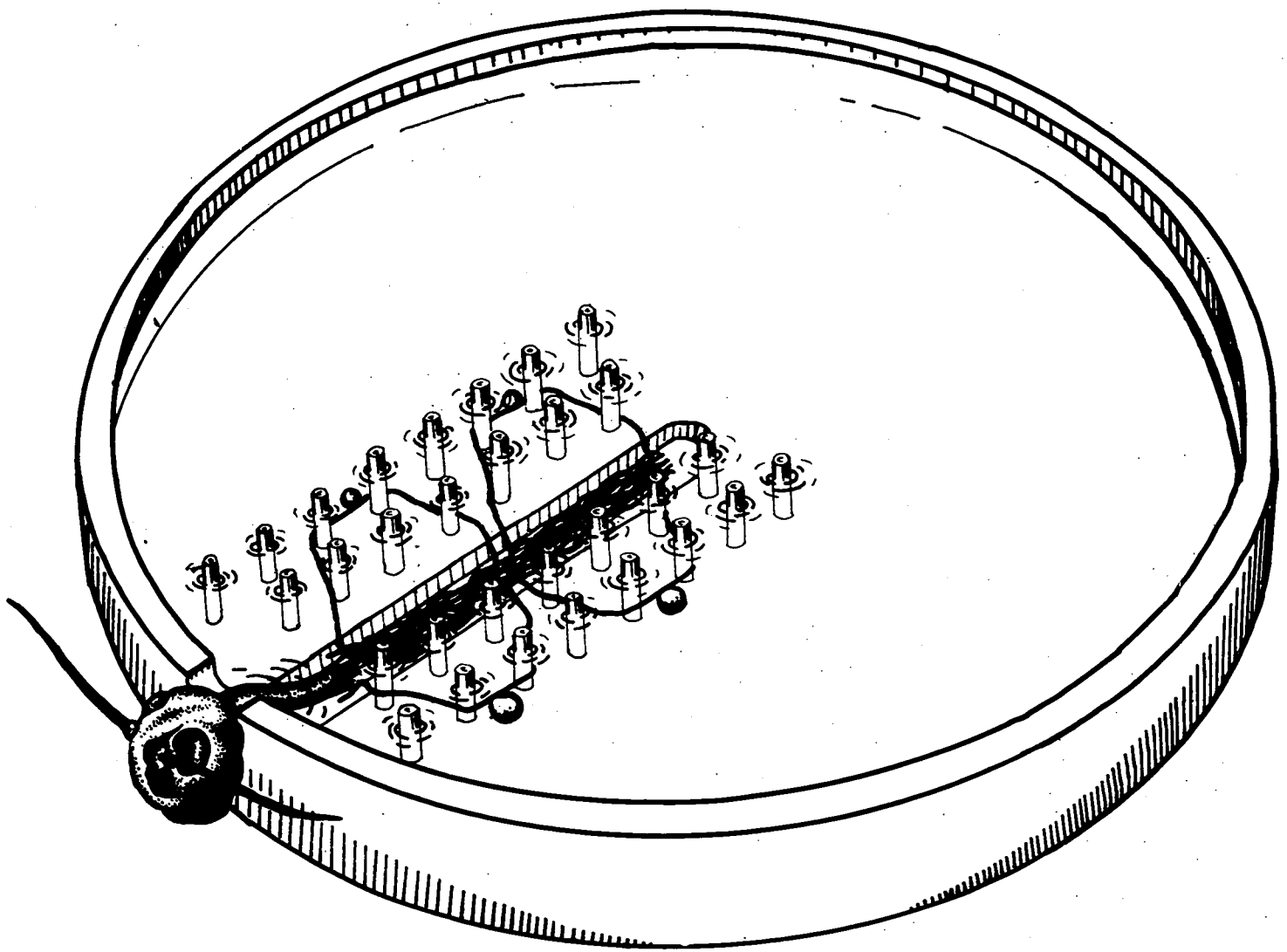
1. The determination of the rate of fluid secretion in vitro.

In vitro measurement of fluid secretion by the Malpighian tubules were carried out using essentially the same method as that described by MADDRELL and KLUNSUWAN (1973) with minor modifications. The gut of an experimental animal with the head attached was dissected free from the thorax and abdomen, and immersed in a small volume of Ringer solution in the Ringer-well of an experimental dish (see Figure 4.1). The head remained outside the dish and care was taken to prevent contamination of the bathing media by regurgitated digestive fluid. The entire preparation was covered with liquid paraffin. The major modification introduced was that the tubules were not completely cut at the point where they feed into the alimentary canal. Instead, individual Malpighian tubules were drawn out of the Ringer trough into the liquid paraffin and looped around small stainless steel pegs (Figure 4.1). The tubule was then partially severed along its length between two pegs using a fine tungsten needle. At the end of a 15 min equilibration period,

Figure 4.1

Experimental arrangement involved in setting up *in vitro* preparations of Malpighian tubules.

Note: the hollow in the perspex dish containing Ringer in which the gut is placed. Individual Malpighian tubules are looped around the pegs which are out of the Ringer solution. The entire preparation is covered with liquid paraffin.



any secreted 'urine' droplet was removed and discarded. The secretion rate for each tubule was then determined by measuring the diameter of the secreted 'urine' droplet at 5 min intervals for 30 min. At the end of this time the Ringer solution was replaced by a fresh solution which had either a different (experimental) or the same composition (control). Following an equilibration period in the fresh solution (which varied between 5 min and 30 min in different experiments) the secreted fluid was removed and the rate of secretion redetermined for a further 30 min. The volume of the secreted droplet was calculated in nls, by assuming the droplet to be a sphere, and the effect of the treatment was estimated by comparing the rates of secretion over the two periods on the basis of a paired 't' test. In all experiments controls were used to indicate the extent to which any changes in the rate of secretion over the two periods of measurement, were the result of ageing of the preparation.

The temperature throughout was maintained at $30\pm0.5^{o}C$ by placing the perspex dish inside a brass water-heated chamber.

2. To determine the Na^{+} and K^{+} concentration of the bathing medium and 'urine'.

In vitro preparations were set up as described above. The 'urine' secreted during the 15 min equilibration period was removed and discarded. This was to ensure that the secreted fluid which was to be analysed was formed from the Ringer solution and not from the haemolymph. The 'urine' secreted by the individual tubules over the next 30 min period was collected and pooled under liquid paraffin.

0.5 or 1µl aliquots were then transferred by micropipette to a small test-tube containing 1.5 or 3cm^3 of deionized water (the volume of deionized water depended on the volume of 'urine').

The Na^+ and K^+ concentration of the 'urine' samples were determined by atomic emission spectroscopy using a Pye Unicam SP90 spectrophotometer. Emission readings were compared with standard calibration curves constructed with known concentrations of Na^+ and K^+ (see Appendix 4, Figures A.4.1, A.4.2).

3. <u>To determine the effects of various inhibitors and HCO_3^--free Ringer on Na^+ and K^+ concentration in the 'urine'</u>.

The gut preparations were set up in Hepes Ringer solution as outlined above. 'Urine' samples were collected at the end of the initial 30 min period and analysed in the manner described in (2) above. The Ringer solution around the gut was then replaced with fresh Hepes Ringer solution containing one of the following inhibitors; 1mM sodium acetazolamide; 10mM NaSCN; 1mM ouabain. In other experiments, HCO_3^--free Hepes Ringer or HCO_3^--free Hepes Ringer + 1mM acetazolamide was used. Following an equilibration period of 30 min, in the presence of the modified Hepes Ringer solution, any secreted fluid was discarded. The 'urine' collected over the next 30 min was analysed to determine its Na^+ and K^+ concentration. Control experiments were carried out in which Hepes Ringer solution was used throughout.

RESULTS

4.1 The effect of sodium acetazolamide on fluid secretion by the Malpighian tubules.

The effect of sodium acetazolamide on fluid secretion was studied by determining the rate of fluid secretion over an initial period of 30 min in 'normal' Hepes Ringer solution. Then, the tubules were surrounded by the same Hepes Ringer solution containing 3×10^{-6} - 10^{-2}M sodium acetazolamide. Following a 20 min equilibration period, the rate of fluid secretion was redetermined over a second 30 min. Control tubules were run in 'normal' Hepes Ringer solution throughout. The results are shown in Table 4.1 and Figure 4.2 which clearly show that sodium acetazolamide inhibits fluid secretion over the concentration range 1×10^{-4} -10^{-2}M. The presence of 1mM inhibitor, significantly decreased the mean rate of fluid secretion by approx. 40% when control and experimental are compared ($P<0.001$). No such reduction was observed with the control tubules.

4.2 The effect of HCO_3^--free Ringer solution on fluid secretion

Experiments were carried out to compare the rate of fluid secretion in 'normal' Hepes Ringer solution with that observed in HCO_3^--free Ringer solution. The rate of fluid secretion was determined over an initial period of 30 min, then the tubules were surrounded by HCO_3^--free Ringer solution. Following a 20 min equilibration period, the rate of fluid secretion was redetermined over a second 30 min period. Table 4.2 shows the effect of HCO_3^--

TABLE 4.1

The effect of different concentrations of acetazolamide on fluid secretion by the Malpighian tubules.

Concentration of acetazolamide (M)	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min		expressed as % original rate	P
		Rate 1	Rate 2		
0 (Control)	29	3.41 ± 0.65	3.18 ± 0.55	97.7 ± 2.4	>0.1
3×10^{-6}	30	4.32 ± 0.56	4.23 ± 0.60	97.5 ± 6.5	>0.1
3×10^{-5}	25	3.25 ± 0.34	2.69 ± 0.33	87.3 ± 5.9	<0.02
1×10^{-4}	31	1.89 ± 0.25	1.40 ± 0.19	81.8 ± 8.3	<0.002
3×10^{-4}	27	3.33 ± 0.35	2.19 ± 0.36	71.4 ± 8.8	<0.001
1×10^{-3}	33	3.64 ± 0.45	2.21 ± 0.33	61.4 ± 5.6	<0.001
3×10^{-3}	26	4.01 ± 0.55	1.99 ± 0.37	53.4 ± 7.5	<0.001
1×10^{-2}	48	3.90 ± 0.31	1.91 ± 0.23	46.8 ± 4.8	<0.001

P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

TABLE 4.2

The effect of HCO_3^--free Ringer solution on the secretion of fluid by the Malpighian tubules

Treatment	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Rate 2	expressed as % original rate	P
Control	12	3.47 ± 0.74	3.53 ± 0.70	104.6 ± 6.3	>0.1
Experimental (HCO_3^--free Ringer present for rate 2)	63	3.86 ± 0.30	3.17 ± 0.29	81.4 ± 4.3	<0.001

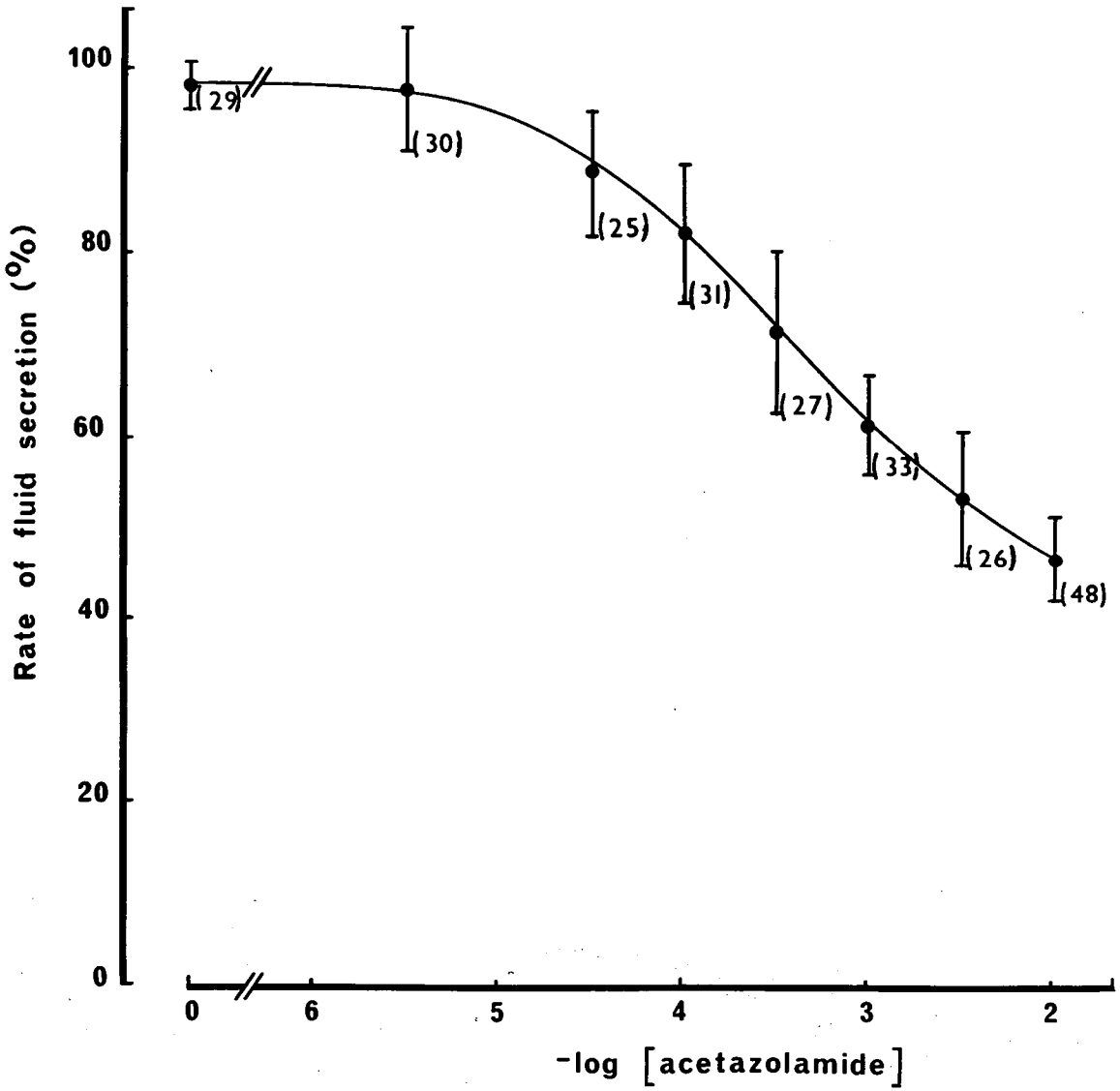
P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

Figure 4.2

The effect of various concentrations of sodium acetazolamide on fluid production. The vertical lines represent $\pm$ S.E.M. and the figures in parentheses indicate the number of determinations.

Ordinate: Rate of fluid secretion (% original rate)

Abscissa: -log [acetazolamide] (M).


Rate of fluid secretion (%)
100
80
60
40
20
0
(29)
(30)
(25)
(31)
(27)
(33)
(26)
(48)
0
6
5
4
3
2
-log [acetazolamide]

free Ringer solution on fluid secretion. It can be seen that, in the absence of HCO_3^-, the mean rate of fluid secretion was reduced by approx. 24%, when control and experimental are compared. The composition of HCO_3^--free Ringer solution used was (mM): NaCl 104; KCl 8.6; $CaCl_2$ 2; $MgCl_2$ 8.5; NaH_2PO_4 4; glucose 34; Hepes 25; NaOH 11 (i.e. the $NaHCO_3$ of Hepes Ringer solution was replaced by NaCl).

4.3 The effect of HCO_3^--free Ringer containing sodium acetazolamide on fluid secretion by the Malpighian tubules.

It has already been shown that both sodium acetazolamide and HCO_3^--free Ringer solution inhibit fluid secretion. To determine, whether the effect of both treatment is cumulative, experiments were set up in which the initial rate of secretion was determined over a 30 min period in Hepes Ringer solution. The tubules were then surrounded by either the HCO_3^--free Ringer containing 1mM sodium acetazolamide (experimental) or the same Ringer solution (control). Following an equilibration period of 20 min, the rate of secretion was redetermined for 30 min. The results are shown in Table 4.3. It can be seen that the presence of 1mM acetazolamide in the absence of HCO_3^- effected approximately 55% inhibition of fluid secretion, when control and experimental are compared. This value is a greater inhibition than that observed in the presence of either 1mM sodium acetazolamide or HCO_3^--free Ringer solution alone.

4.4 The effect of sodium thiocyanate on fluid secretion by the Malpighian tubules.

As was demonstrated above (Chapter 3), sodium thiocyanate inhibits

TABLE 4.3

The effect of 1mM acetazolamide in HCO_3^--free Ringer solution on fluid secretion by the Malpighian tubules.

Treatment	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Rate 2	expressed as % original rate	P
Control	11	3.69 ± 0.74	3.64 ± 0.67	102.7 ± 11.5	>0.1
Experimental (1mM acetazolamide present in HCO_3^--free Ringer for rate 2)	32	3.73 ± 0.46	1.72 ± 0.23	46.8 ± 4.1	<0.001

P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

the Mg^{2+}- dependent HCO_3^-- stimulated ATPase. Since it has been proposed that this enzyme is involved in ion transport across various tissues, in a number of different species (LIANG and SACKTOR, 1976; HEGNER and ANIKA, 1975), it was important to establish whether fluid secretion by Locusta Malpighian tubules was sensitive to SCN^-. To this end, the experimental procedure was carried out as described above (see 4.1). SCN^- (1-10mM) was included in the Hepes Ringer solution in determining rate 2. This was achieved by substitution for NaCl thus ensuring that the cation concentration remained unchanged. Control tubules were run in Hepes Ringer solution throughout. The results are shown in Table 4.4. It is clear that SCN^- inhibits fluid secretion by Malpighian tubules of Locusta. Comparison between the rates observed in the first and second 30 min periods, by means of a paired 't' test confirm these results.

4.5 The effect of Cl^- concentrations on fluid secretion by the Malpighian tubules.

In vitro preparations were set up in 'normal' Hepes Ringer solution as outlined above (see Materials and Methods). The rate of fluid secretion over initial period of 30 min in 'normal' Hepes Ringer solution was determined. Then, the Ringer solution was replaced with modified Ringer in which varying concentrations of NO_3^- were substituted for Cl^-. Following a 15 min equilibration period, the rate of fluid secretion was redetermined over a second 30 min period, Control tubules were run in Ringer solution throughout. The results are shown in Table 4.5 and Figure 4.3. It can be seen that in the absence of Cl^-, fluid secretion was inhibited by approx.

TABLE 4.4

The effect of different concentrations of thiocyanate on fluid secretion by the Malpighian tubules.

Concentration of NaSCN (mM)	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Rate 2	expressed as % original rate	P
0 (Control	23	2.90 ± 0.36	2.72 ± 0.34	100.5 ± 6.9	>0.1
1	21	6.01 ± 0.64	3.88 ± 0.49	66.8 ± 5.2	<0.001
5	55	4.30 ± 0.36	2.77 ± 0.29	64.8 ± 4.6	<0.001
10	21	2.95 ± 0.38	1.48 ± 0.19	55.7 ± 6.5	<0.001

P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

TABLE 4.5

The effect of Cl^- concentration on fluid secretion by the Malpighian tubules

Concentration (mM) Cl^-	Concentration (mM) NO_3^-	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 2	Mean rate of fluid secretion ± S.E.M. expressed as % original rate	P
120	0	29	3.41 ± 0.65	3.18 ± 0.55	97.7 ± 2.4	>0.1
90	30	23	2.74 ± 0.45	2.63 ± 0.45	99.4 ± 8.3	>0.1
75	45	43	2.11 ± 0.21	1.38 ± 0.17	69.2 ± 4.8	<0.001
60	60	42	2.28 ± 0.17	1.12 ± 0.14	49.6 ± 4.9	<0.001
30	90	42	2.84 ± 0.26	1.40 ± 0.20	46.4 ± 4.9	<0.001
0	120	49	2.00 ± 0.19	0.74 ± 0.10	37.6 ± 0.1	<0.001

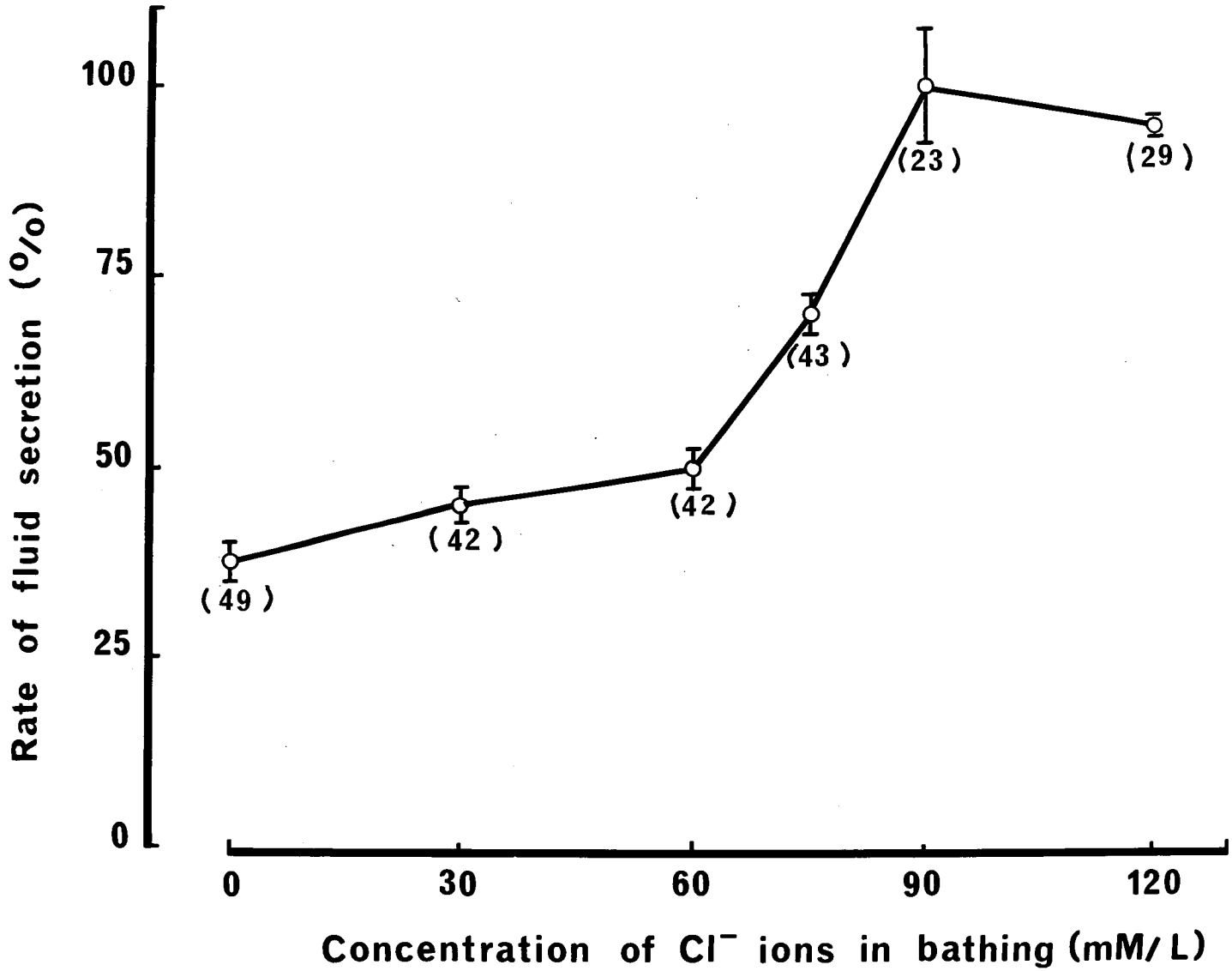
P values were obtained by comparing Rate 1 and Rate 2 by paired 't' test.

Figure 4.3

The effect of various concentrations of Cl^- on fluid secretion. The vertical lines represent $\pm$ S.E.M. and the figures in parentheses indicate the number of determinations.

Ordinate: Rate of fluid secretion (% original rate)

Abscissa: Cl^- concentration (mM).


100
75
50
25
0
Rate of fluid secretion (%)
(49)
(42)
(42)
(43)
(23)
(29)
0
30
60
90
120
Concentration of Cl⁻ ions in bathing (mM/L)

60%, when control and experimental are compared. Furthermore, the results show that fluid secretion is sensitive to the concentration of Cl^- in the bathing medium; 75-90mM Cl^- being necessary for normal tubule secretion rates to be maintained.

4.6 The effects of Br^- on fluid secretion by the Malpighian tubules

Experiments were carried out in which the initial rate of fluid secretion was determined over a period of 30 min in the presence of 'normal' Hepes Ringer solution. Then, the tubules were surrounded by a Cl^--free Ringer solution in which all the Cl^- was replaced by the equivalent Br^- salt. Once again, the rate of fluid secretion was redetermined over a second 30 min period, following an equilibration period of 15 min. Examination of the results (Table 4.6) shows that the replacement of Cl^- with Br^- did not significantly reduce the rate of fluid secretion by the Malpighian tubules ($P>0.05$).

4.7 The effect of ouabain on fluid secretion by the Malpighian tubules

The rate of fluid secretion was determined over an initial period of 30 min in the presence of 'normal' Hepes Ringer solution. The tubules were then surrounded by the same Ringer solution containing 1mM ouabain. Following an equilibration period which varied from 5-30 min, the rate of secretion was redetermined over a second 30 min period. Controls were run in which Hepes Ringer (minus ouabain) was used throughout. The results are shown in Figure 4.4 and Table 4.7. It can be seen that the effect of ouabain on fluid secretion was dependent on the time allowed for equilibration. Thus, following equilibration periods of 5 or 10 min there was no significant reduction in the rate of fluid secretion ($P>0.1$). In contrast, the

TABLE 4.6

The effect of Br^- on fluid secretion by the Malpighian tubules

Treatment	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Rate 2	expressed as % original rate	P
Control	14	1.80 ± 0.28	1.71 ± 0.36	101.2 ± 10.6	>0.1
Experimental (120mM Br^- was substituted for Cl^-)	29	3.60 ± 0.44	3.13 ± 0.38	92.6 ± 7.4	>0.05

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

TABLE 4.7

The effect of ouabain on fluid secretion by the Malpighian tubules

Treatment	Equilibration time (min) prior to determination rate 2	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Rate 2	expressed as % original rate	P
Control	5	19	2.62 ± 0.42	2.85 ± 0.49	107.1 ± 8.0	>0.1
Control	10	20	1.87 ± 0.35	1.67 ± 0.39	97.0 ± 17.2	>0.1
Control	15	19	2.51 ± 0.30	2.42 ± 0.30	102.7 ± 8.1	>0.1
Control	20	20	2.45 ± 0.26	2.13 ± 0.20	93.1 ± 7.8	>0.1
Control	30	20	2.54 ± 0.42	2.51 ± 0.36	103.5 ± 9.0	>0.1
Experimental (1mM ouabain present for rate 2)	5	20	3.39 ± 0.47	3.01 ± 0.44	95.2 ± 8.5	>0.1
	10	20	2.20 ± 0.28	1.83 ± 0.33	84.5 ± 11.1	>0.1
	15	20	2.55 ± 0.30	1.89 ± 0.30	68.4 ± 7.5	<0.01
	20	20	1.98 ± 0.24	0.73 ± 0.16	38.9 ± 7.4	<0.001
	30	20	2.70 ± 0.34	1.02 ± 0.23	36.9 ± 7.4	<0.001

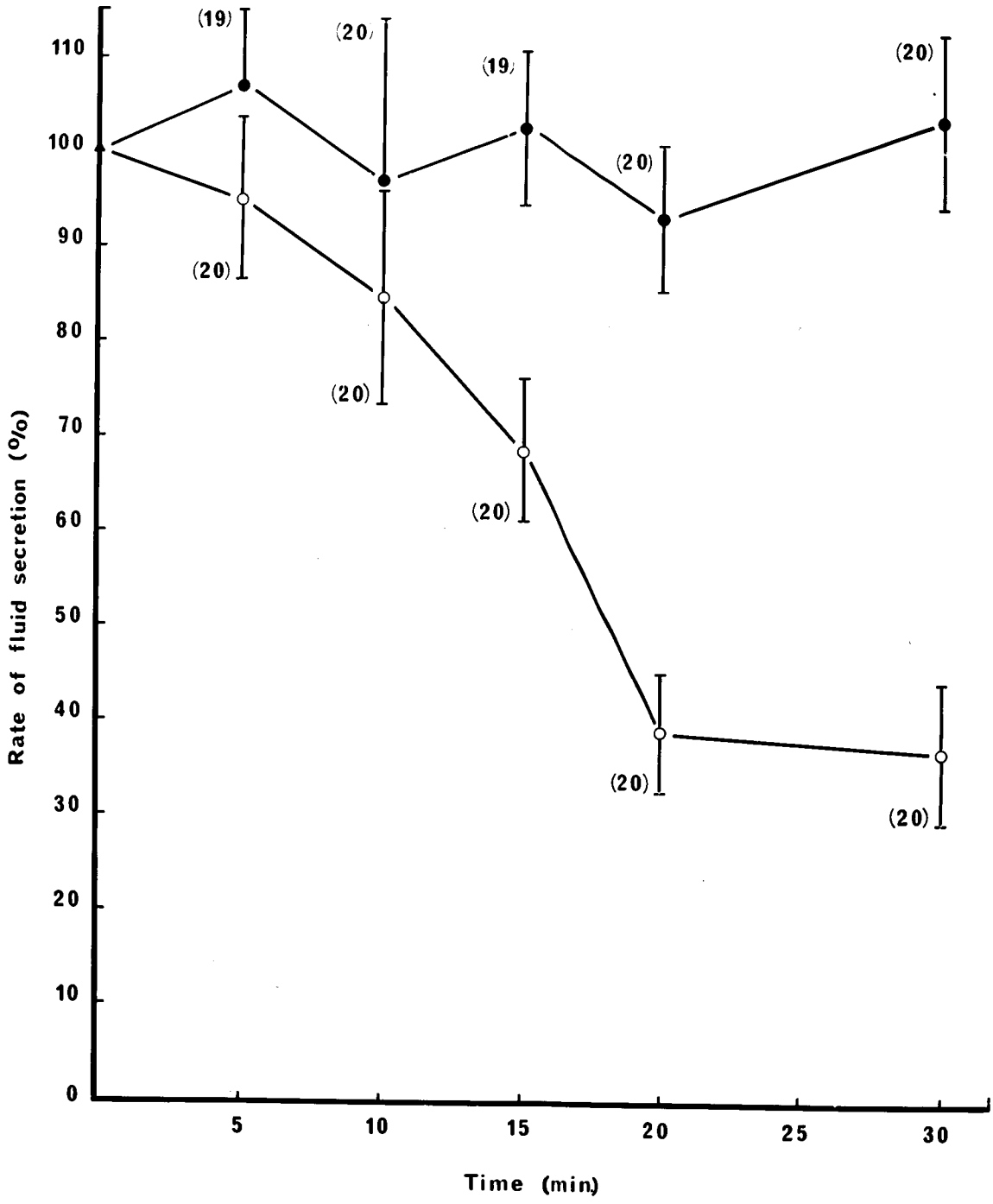
P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

Figure 4.4

The effect of preincubation time in the presence (o) and absence (●) of ouabain on fluid secretion. The vertical lines represent ± S.E.M. and the figures in parentheses indicate the number of determinations.

Ordinate: Rate of fluid secretion (% original rate)

Abscissa: Time (min) allowed for equilibration prior to determination of rate 2.


(19)
(20)
(19)
(20)
(20)
(20)
(20)
(20)
(20)
(20)
110
100
90
80
70
60
50
40
30
20
10
0
Rate of fluid secretion (%o)
5
10
15
20
25
30
Time (min)

same concentration of ouabain (1mM) effected 67% inhibition of fluid secretion following a 30 min equilibration period, when control and experimental are compared ($P<0.001$). No such reduction was observed with the control tubules. Indeed, as is shown (Table 4.7) a number of the control tubules showed a slight increase in their mean rates of secretion over the second period of measurement.

4.8 The effect of amiloride on fluid secretion by the Malpighian tubules

Experiments were carried out, in which 1mM or 0.1mM amiloride was present in Hepes Ringer solution for the second period of rate determination. The initial rate was determined over a 30 min in 'normal' Hepes Ringer solution, then the tubules surrounded with Hepes Ringer solution containing amiloride. Following a 5 min equilibration period, the rate of fluid secretion was redetermined over a second 30 min period. The results show that 1mM amiloride almost totally inhibited secretion of 'urine' by the Malpighian tubules of Locusta (Table 4.8). Indeed, out of 22 tubules which were examined, 18 tubules failed to secrete any fluid over the second 30 min period. Similarly, substantial inhibition of fluid secretion (ca. 77%) was observed in the presence of 0.1mM amiloride.

4.9 The relationship between concentration of Na^+ and K^+ in the bathing medium and that of the 'urine'

'Urine' was collected from the tubules of preparations bathed in Ringer solutions (based on MADDRELL, 1969) of varying Na^+ and K^+ concentrations. The solutions were adjusted to maintain the total concentration of Na^+ and K^+ at 152mM, and only the relative concentrations

TABLE 4.8

The effect of amiloride on fluid secretion by the Malpighian tubules

Treatment	n	Mean rate of fluid secretion ± S.E.M. expressed in nl/min Rate 1	Rate 2	expressed as % original rate	P
Control	14	1.80 ± 0.28	1.71 ± 0.36	101.2 ± 10.6	>0.1
Experimental (1mM amiloride) present for rate 2)	22	3.62 ± 0.40	0.13 ± 0.06	6.04 ± 2.9	<0.001
(0.1mM amiloride present for rate 2)	25	3.71 ± 0.49	0.98 ± 0.22	23.2 ± 3.9	<0.001

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

of the two cations were altered. The results obtained are shown in Figure 4.5 (also see Appendix 4, Table 1). It can be seen that K^+ ions were secreted preferentially even against considerable gradients. At K^+ concentrations greater than 20mM there was more than three times as much K^+ secreted as Na^+. However, at very low concentrations of K^+, the amount of Na^+ present in the 'urine' increased considerably until it comprised approx. 80% of the total Na^+ plus K^+ secreted. The Na^+ plus K^+ concentration remained fairly constant with a mean value of 180.2mM thoughout.

4.10 The effect of sodium acetazolamide on the Na^+ and K^+ concentration of the 'urine'.

A study was carried out to determine the effect of 1mM sodium acetazolamide on the $Na^+$and K^+ levels in the fluid secreted by the Malpighian tubules. Samples of 'urine' were collected over the two 30 min periods as described in the Materials and Methods section of this chapter. Due to inhibition of secretion by acetazolamide, it was sometimes difficult to obtain exactly 1µl samples of 'urine' for analysis. To overcome this problem and the difficulties encountered in accurately determining the exact volume collected it was necessary to compare Na^+/K^+ ratios in the two samples, rather than the exact concentration of each ion present. The results are shown in Table 4.9. It can be seen that acetazolamide did not significantly alter the ratio of Na^+/K^+ in the secreted fluid.

4.11 The effect of HCO_3^--free Ringer solution on the Na^+ and K^+ concentration in the 'urine'.

Experiments were carried out to investigate the effects of HCO_3^--free Ringer on the Na^+ and K^+ levels of secreted fluid.

TABLE 4.9

The effects of HCO_3^--free Ringer and acetazolamide on the Na^+ and K^+ ratio in 'urine'.

Treatment	n	Na^+/K^+ Ratio ± S.E.M. Ratio 1	Na^+/K^+ Ratio ± S.E.M. Ratio 2	2 as a % of 1	P
Control	10	0.268 ± 0.037	0.305 ± 0.031	121.4 ± 10.5	>0.1
Experimental (1mM acetazolamide present for rate 2)	9	0.282 ± 0.036	0.370 ± 0.96	144.2 ± 20.5	>0.1
(HCO_3^--free Ringer was substituted for rate 2)	9	0.198 ± 0.017	0.271 ± 0.036	135.6 ± 12.4	<0.05
(HCO_3^--free Ringer throughout and in the presence of 1mM acetazolamide also for rate 2)	10	0.286 ± 0.026	0.556 ± 0.100	230.5 ± 60.5	<0.05

P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

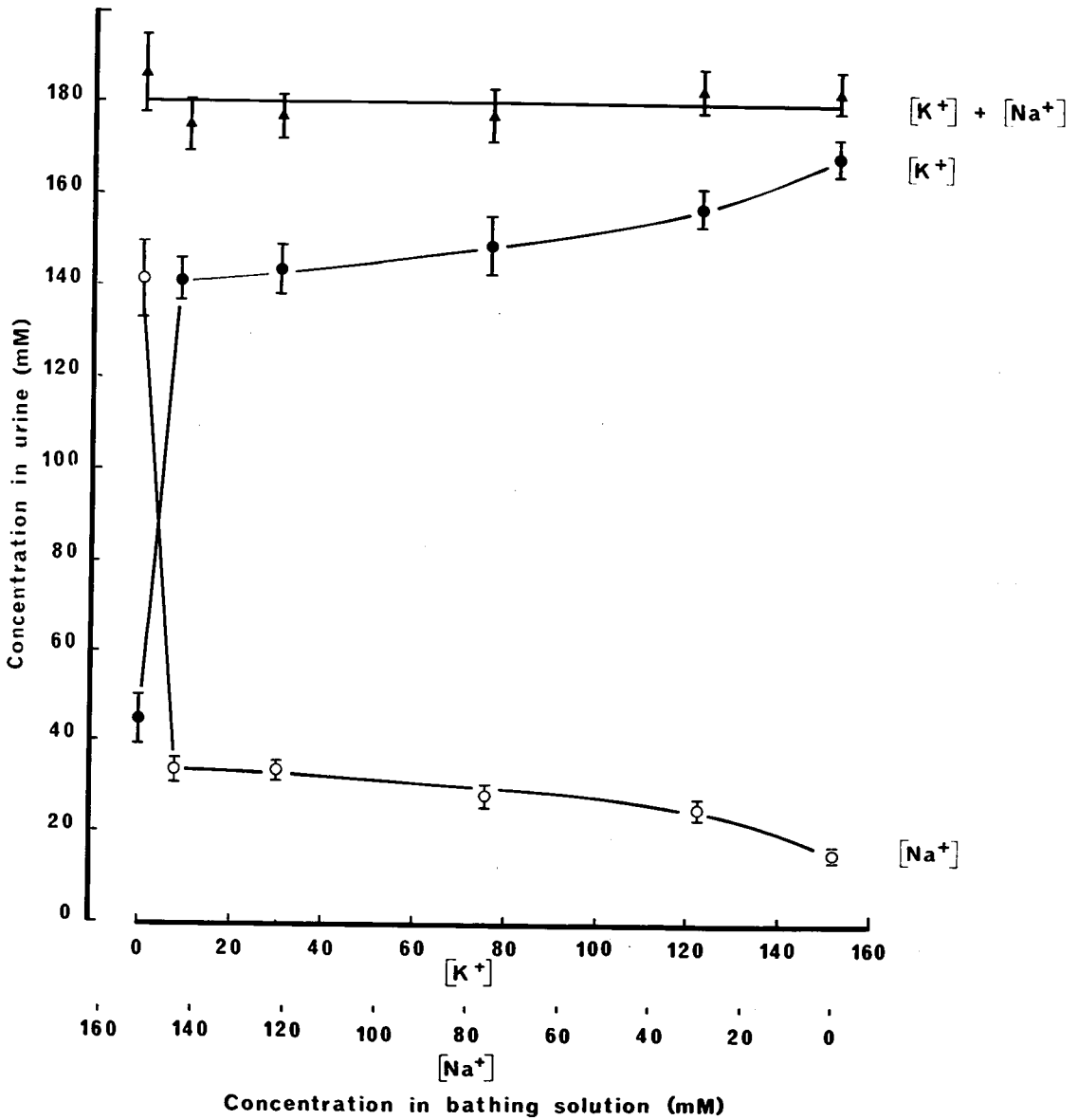
Figure 4.5

The relationship between the concentration of Na^+ and K^+ in the secreted fluid and the bathing medium. The vertical lines represent $\pm$ S.E.M.

Ordinate: Concentration in the urine (mM)

Abscissa: Concentration in the bathing medium (mM)

The Ringer solution was based on that of MADDRELL (1969, see Materials and Methods). As stated in the text, the Na^+ and K^+ concentrations were varied by mutual substitution; the Na^+ plus K^+ concentrations remaining unchanged throughout.


180
160
140
120
100
80
60
40
20
0
Concentration in urine (mM)
[K+] + [Na+]
[K+]
[Na+]
0 20 40 60 80 100 120 140 160
[K+]
160 140 120 100 80 60 40 20 0
[Na+]
Concentration in bathing solution (mM)

Once again, Na^+/K^+ ratios in 'urine' collected over the initial 30 min period were compared to those obtained over the second 30 min period (in which HCO_3^--free Ringer was present). The effects of HCO_3^--free Ringer on the Na^+/K^+ ratios in the 'urine' are shown in Table 4.9 where they can be compared with the results of similar measurements made with control tubules. It can be seen that HCO_3^--free Ringer effected a significant increase in the Na^+/K^+ ratio of 'urine' ($P<0.05$).

4.12 The effects of HCO_3^--free Ringer solution on the Na^+ and K^+ concentration in the 'urine' in the presence of acetazolamide.

The Na^+/K^+ ratios were determined for 'urine' samples collected from tubules bathed in HCO_3^--free Ringer solution for 30 min. These were then compared with the Na^+/K^+ ratios observed after tubules had been soaked for a second 30 min in HCO_3^--free Ringer solution containing 1mM sodium acetazolamide. In contrast, to the effect of acetazolamide in 'normal' Hepes Ringer, which did not significantly alter the Na^+/K^+ ratio in the 'urine', the presence of this inhibitor in HCO_3^--free Ringer solution effected a significant increase in the ratio of these two cations (Table 4.9); the increase in Na^+/K^+ ratio being even greater than that observed in HCO_3^--free Ringer alone.

4.13 The effect of sodium thiocyanate on the Na^+ and K^+ concentration in the 'urine'.

A study was carried out to determine the effect of NaSCN on the Na^+ and K^+ levels in the fluid secreted by the Malpighian tubules.

10mM NaSCN was included in the Hepes Ringer solution in determining ratio 2. This was achieved by substitution for NaCl. The Na^+/K^+ ratios in 'urine' collected over the initial 30 min period were compared to those obtained over the second 30 min period (in which NaSCN was present), following an equilibration period of 30 min. It can be seen (Table 4.10) that NaSCN did not significantly alter the Na^+/K^+ ratio of the secreted fluid.

4.14 The effect of ouabain on the Na^+ and K^+ concentration in the 'urine'.

The Na^+ and K^+ concentration in samples of 'urine' obtained from tubules immersed in 'normal' Hepes Ringer solution for 30 min was compared with the concentrations of these ions after the tubules had been immersed for a second 30 min in the same Ringer solution containing 1mM ouabain. The results are shown in Table 4.10 where the effects of ouabain on the Na^+/K^+ ratio are shown as are the results of similar measurements made with control tubules. It can be seen that ouabain effected a significant increase in the Na^+/K^+ ratio ($P<0.001$).

TABLE 4.10

The effect of thiocyanate on the Na^+ and K^+ concentrations in the 'urine'.

Treatment	n	Na^+/K^+ Ratio ± S.E.M. Ratio 1	Ratio 2	2 as a % of 1	P
Control	10	0.268 ± 0.037	0.305 ± 0.031	121.4 ± 10.5	>0.1
Experimental (10mM NaSCN was substituted for NaCl for rate 2)	9	0.446 ± 0.036	0.538 ± 0.076	122.2 ± 16.7	>0.1
(1mM ouabain present for rate 2)	11	0.335 ± 0.026	0.912 ± 0.095	289.8 ± 55.0	<0.001

P values were obtained by comparing rate 1 and rate 2 by paired 't' test.

DISCUSSION

The effects of different concentrations of Na^+ and K^+ in the bathing medium on the concentrations of these cations in the 'urine' show that the Malpighian tubules of Locusta, in common with a number of other species which have been studied (e.g. Calliphora, BERRIDGE, 1968; Tipula paludosa, COAST, 1969; Schistocerca gregaria, MADDRELL and KLUNSUWAN, 1973), are able to transport K^+ against a chemical gradient over a wide range of external K^+ concentrations. In addition, K^+ is transported in preference to Na^+ even when present as much as lower concentrations in the bathing medium. However, both Na^+ and K^+ are necessary for maximal fluid secretion (ANSTEE and BELL, 1975). Measurements of the potential difference across the Malpighian tubule wall indicates that the lumen is positive with respect to the external medium (BELL, 1977, present study). It is clear, therefore, that the transport of K^+, which takes place against chemical and electrical gradients, must be an active process.

The results presented here demonstrate that the fluid secretion by the Malpighian tubules of Locusta was inhibited by sodium acetazolamide over the concentration range $3 \times 10^{-5} - 10^{-2}$M. This is in contrast with the results reported by BERRIDGE (1968) and MADDRELL (1969) who reported that acetazolamide did not inhibit fluid secretion by the Malpighian tubules of Calliphora or Rhodnius. However, GOODING (1975) found that acetazolamide inhibited diuresis in Glossina morsitans which agrees with the results of the present study. In Schistocerca gregaria, acetazolamide inhibits the

transepithelial potential across the rectum (HERRERA et al., 1977, 1978; WILLIAMS et al., 1978). Similarly, acetazolamide inhibited the transepithelial potential across the Malpighian tubules of Locusta in the present study (Chapter 6). Numerous other workers have reported inhibitory effects of acetazolamide on transport processes. Mc KINNEY and BURG (1977), BURG and GREEN (1977) have shown that fluid absorption by rabbit renal proximal tubules is inhibited 22-40% by acetazolamide and CHEUNG et al. (1977) found that acetazolamide caused a rapid and dramatic fall in the secretory rate, to about 10% of the control value in isolated seminiferous tubules of the rat. Acetazolamide inhibits 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 (Mc KINNEY and BURG, 1978A, B), and rat renal tubules (HOPPE et al., 1976; LUCCI et al., 1979). Other workers report acetazolamide inhibition of secretion with 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).

The rate of fluid secretion by the Malpighian tubules of Locusta decreased by approx. 24% in HCO_3^--free Ringer, in the present study. This agrees well with the 22% inhibition in fluid absorption with rabbit proximal tubule reported by Mc KINNEY and BURG (1977) when Cl^- replaced HCO_3^- in the perfused fluid and bath. The importance of HCO_3^- to fluid secretion by the Malpighian tubules was more pronounced when acetazolamide was included in HCO_3^--free

Ringer. Application of 1mM acetazolamide in HCO_3^--free Ringer caused 55% inhibition of the rate of fluid secretion which was more or less equal to the sum of inhibitions observed with each individual of treatment (i.e. acetazolamide in 'normal' Hepes Ringer, and HCO_3^--free Ringer). Similarly, Mc KINNEY and BURG (1977) observed increased inhibition (30%) in fluid absorption by rabbit proximal tubules when 10^{-4}M acetazolamide was included in HCO_3^--free Ringer.

As already mentioned, the inhibitory activity of acetazolamide, reported by GOODING (1975) and also observed in the present study, contrasts markedly with the lack of effect reported by BERRIDGE (1968) and MADDRELL (1969) with Calliphora and Rhodnius respectively. One possible explanation for these different results is related to the time allowed for preincubation in the presence of the inhibitor. POWELL et al., (1962) and BERKOWITZ and JANOWITZ (1967) working on dog gastric mucosa, observed that acetazolamide exhibited its maximum effect on water secretion and ion flux approx. 30 min after initial application (except for H^+ and Na^+ where 'steady state' secretion was not reached until 90-120 min after acetazolamide administration, POWELL et al., 1962). Similarly, GONZALES (1969) working on turtle bladder, reported that acetazolamide affected Cl^- transport after 10-15 min but took about 1 hour to reach a new steady state. In the present study, tubules were equilibrated for 20 min before determining the new rate of secretion. However, no indication is shown by BERRIDGE (1968) or MADDRELL (1969) whether such an equilibration period was allowed in their studies. Alternatively, the form in which acetazolamide is applied may be important. Thus, in the present

study sodium acetazolamide was used as it is known to go into solution much more readily than the sparingly soluble acetazolamide itself (WINDHOLZ et al., 1976). A further possible explanation concerns the temperature of experimentation. The present investigation was carried out at 30°C whilst MADDRELL (1969) made his determinations at room temperature (ca. 24°C) and BERRIDGE (1968) did not mention it.

The involvement of HCO_3^- and carbonic anhydrase in 'urine' production is further supported by the effects of HCO_3^--free Ringer and 1mM acetazolamide in HCO_3^--free Ringer on the Na^+/K^+ ratio of the secreted 'urine'. In both cases, the Na^+/K^+ ratio of the 'urine' was significantly increased. Several workers have reported changes in cation secretion as a result of treatment with acetazolamide. GREEN and GIBISCH (1975) reported that replacement of HCO_3^- by acetate resulted in a decrease of Na^+ flux in rat proximal tubule to 45% of that when HCO_3^--Ringer was used. They also found that the net Na^+ flux was inhibited by 36% with the addition of acetazolamide to HCO_3^--Ringer solution. MAUDE (1974) observed that in the absence of $NaHCO_3$, or in the presence of acetazolamide, the rate of reabsorption of NaCl in rat proximal tubule decreased. Similarly, MATHISEN et al. (1978) reported that acetazolamide inhibits Na^+ reabsorption in dog proximal tubule and TURNBERG et al. (1970B) observed acetazolamide markedly inhibits Na^+ movement in human ileum. POWELL et al. (1962) and BERKOWITZ and JANOWITZ (1967) reported that application of acetazolamide to dog gastric

mucosa, reduced both H^+, K^+ and water secretion, whereas the concentration of Na^+ secreted was increased. In addition, acetazolamide inhibits Cl^- flux across the turtle and toad urinary bladder (GONZALES, 1969; LESLIE et al., 1973; SOBOSLAI et al., 1977) and human ileum (TURNBERG et al., 1970B). Similar results have been reported across the frog skin (GARCIA-ROMEU and EHRENFELD, 1975; EHRENFELD and GARCIA-ROMEU, 1978). In contrast, SLEGERS and MOONS (1968) working on human sweat glands, observed that acetazolamide markedly increase Cl^- concentration in the sweat. In view of the importance of Cl^- to the secretion of 'urine' by Locusta tubules (to be discussed below) it would have been of interest to determine the effect of acetazolamide on the Cl^- concentration of the secreted fluid. Unfortunately, time prevented this study from being carried out.

The fact that there is no specific inhibitor of Mg^{2+}-dependent HCO_3^--stimulated ATPase currently available means that, of necessity, the evidence for its role in secretory processes must be of a circumstantial nature. Thus, the fact that SCN^- inhibits both the Mg^{2+}-dependent HCO_3^--stimulated ATPase and a variety of transport processes (KASBEKAR and DURBIN, 1965; BLUM et al., 1971; SACHS et al., 1972A; SIMON and THOMAS, 1972; DE RENZIS and BORNANCIN, 1977) has been taken by a number of researchers as possible evidence for the involvement of this ATPase in these processes. In the present investigation, the effect of SCN^- was studied on 'urine' production by the Locusta Malpighian tubules. 1mM NaSCN decreased the rate of fluid secretion by 33%, whereas, 10mM SCN^- increased

the level of inhibition to 45% compared with the control. BERRIDGE (1969) working with Calliphora Malpighian tubules, observed that the replacement of Cl^- by SCN^- inhibited the rate of fluid secretion by 25%. Inhibitory effects of SCN^- have been reported elsewhere; SACHS et al., (1972A) reported that gastric mucosal acid secretion was completely inhibited by 10mM SCN^-. Similarly, HARRIS and EDELMAN (1959) reported that frog gastric acid production was almost completely inhibited by 12mM SCN^-, and FORTE and DAVIES (1964) observed that acid secretion by bullfrog gastric mucosa was inhibited 70-90% by 1-10mM SCN^-.

Despite its effects on fluid secretion and ATPase activity (Chapter 3), sodium thiocyanate did not significantly alter the Na^+/K^+ ratio of the 'urine'. This agrees with the findings of DE RENZIS (1975) working on gills of Carassius auratus. He found that whilst SCN^- did not produce any significant variations in the Na^+ flux, although it strongly inhibited Cl^- influx when placed in the external medium. In contrast, WIETH (1969), working on human red cells, observed that the net flux of Na^+ and K^+ was reduced by some 30% in the presence of relatively high concentrations of SCN^- (120mM).

ANSTEE and BELL (1975) and ANSTEE et al. (1979) have shown ouabain inhibits secretion by the Malpighian tubules of Locusta migratoria. However, as mentioned earlier the effect of ouabain on insect epithelia is in dispute (see review by ANSTEE and BOWLER, 1979). Some investigators have failed to demonstrate an effect

of ouabain on Malpighian tubule function (BERRIDGE, 1968; MADDRELL, 1969; PILCHER, 1970; RAFAELI-BERNSTEIN and MORDUE, 1978) and on this basis have questioned the role of Na^+-K^+ ATPase in the fluid secretory mechanism (see review by MADDRELL, 1971). Other workers have demonstrated ouabain sensitivity of Malpighian tubules function. These include ATZBACHER et al. (1974) who showed that the rate of excretion of the two dyes, azocarmine and indigocarmine is significantly reduced in presence of ouabain. FARQUHARSON (1974) has shown that fluid secretion by the pill millipede, Glomeris marginata was sensitive to ouabain. These differing responses to ouabain cannot be explained on the basis of species differences because conflicting results have been reported on the same species. For example, GOODING (1975) observed that 'urine' production in the tsetse fly, Glossina morsitans is inhibited by ouabain. Whereas, GEE (1976A,B) reported that ouabain had no effect on Malpighian tubules function in the same species. Similarly, in contrast to the reports of ANSTEE and BELL (1975) and ANSTEE et al. (1979), RAFAELI-BERNSTEIN and MORDUE (1978) failed to show ouabain inhibition of fluid secretion by Malpighian tubules of Locusta migratoria L.. Recently, SACHS et al. (1978) have pointed out a defect in the use of ouabain to determine the role of Na^+-K^+ ATPase in various secretory processes. This concerns the time course of inhibition which may require 60 min or more. This delay, it is suggested, may be due to restricted access of ouabain to the basal surface of the cell in intact tissue. Further support for this argument is provided by the present study in which the period of preincubation with ouabain substantially affected the level of inhibition observed. Thus, the rate of fluid secretion was unaffected after 10 min but was 67% at 30 min. Similarly, GOH and PHILLIPS (1978) observed that 10^{-3}M ouabain placed

on the lumen side of locust rectum (Schistocerca gregaria) caused 50% inhibition of water absorption within 30 min. More recently, REUSS et al. (1979) studying the time course of ouabain on Necturus gall bladder observed that 10^{-4}M ouabain inhibited fluid transport by about 18% in the initial 5 min period, 70% in 15 min and completely in 30 min. The same authors reported that the transepithelial and cell membrane potentials depolarized progressively, for at least 3 hours, after exposure to ouabain. A further possible explanation for the different responses to ouabain concerns temperature at which the various studies were carried out. It has been shown that ouabain binding to Na^+-K^+ATPase is temperature sensitive; PEACOCK et al. (1976) working on Malpighian tubules and hindgut of Homorocoryphus nitidulus vicinus and CHARNOCK et al. (1975) working on rabbit cortex demonstrated that increasing the incubation temperature produces an increase in the ouabain-inhibition of Na^+-K^+ATPase. More recently, DONKIN and ANSTEE (1980) reported that the inhibitory effect of ouabain on both Na^+-K^+ATPase and fluid secretion by Locusta Malpighian tubules is dependent on temperature.

In addition to inhibiting fluid secretion by Locusta Malpighian tubules, 1mM ouabain effected a significant increase in the Na^+/K^+ ratio of 'urine'. Similar effects have been reported elsewhere. For example, ARCHIBALD and WHITE (1974) showed that ouabain (1mM) produced a complete reversal of the Na^+ and K^+ content of synaptosomes of rat brain. Unfortunately, studies on insect tissue are somewhat limited. However, O'RIORDAN (1969), working with cockroach midgut, reported a 55% inhibition of Na^+ efflux and an approx. 40% inhibition of K^+ influx with concentrations of ouabain between 10^{-6} and 10^{-3}M.

TREHERNE (1966), using insect nerve, found that approx. 50% of the Na^+ efflux was blocked by ouabain at concentration levels of 10^{-5}M for Periplaneta and 10^{-4}M for Carausius. WEBER-VON GROTTHUS et al. (1974) demonstrated that the injection of 2 x 10^{-4}M ouabain into the body cavity of Drosophila hydei produced significant changes in the Na^+ and K^+ concentrations in the haemolymph. More recently, MORDUE and RAFAELI-BERNSTEIN (1978) have shown the level of Na^+, in the fluid secreted by the Malpighian tubules of Locusta increased as a result of treatment with 1mM ouabain. This agrees well with the results obtained in the present study. However, FARQUHARSON (1974) found no change in the Na^+ and K^+ levels in the 'urine' of the pill millipede, Glomeris marginata in response to ouabain despite the fact that secretion was inhibited.

The rate of fluid secretion by the Malpighian tubules of Locusta depends markedly on the concentration of Cl^- in the bathing solution. In Ringer solutions in which NO_3^- replaced Cl^- the secretory rate was reduced to 38% of that observed in 'normal' Ringer solution; the rate of fluid secretion increasing as the concentration of Cl^- increased from zero to approx. 90mM. Similar results were reported by KAUFMAN and PHILLIPS (1973) with the salivary glands of the tick, Dermacentor andersoni, in which the rate of fluid secretion observed in NO_3^--Ringer solution was reduced to 5% of that observed in Cl^--Ringer solution. The rate of fluid secretion increasing as the concentration of Cl^- increased. Similarly, MADDRELL (1969) working on Rhodnius Malpighian tubules reported that the rate of fluid secretion was reduced to 45% of the rate of the controls in

NO_3^--Ringer solution. In contrast, BERRIDGE (1969) reported that NO_3^- had no inhibitory effect on the rate of 'urine' production by the Malpighian tubules of Calliphora. Indeed, the rate of secretion increased by approx. 25-30% in the presence of NO_3^-. However, the replacement of Cl^- with SO_4^{2-} resulted in a progressive decrease in the rate of 'urine' production such that when the Cl^- concentration was reduced below 30mM,'urine' flow ceased. Similarly, MADDRELL (1969, 1972) showed that fluid secretion by Rhodnius Malpighian tubules ceased when SO_4^{2-} replaced Cl^- in the bathing medium; maximum secretion being observed at about 30mM Cl^-.

In contrast to the results obtained when NO_3^- replaced Cl^- in the bathing medium, substitution of Br^- for Cl^- had no significant effect on the rate of fluid secretion by the Malpighian tubules of Locusta migratoria L.. Similar results have been reported by BERRIDGE (1969) with the Malpighian tubules of Calliphora; he observed that Br^- was able to support fluid secretion equally as well as Cl^-. KAUFMAN and PHILLIPS (1973) reported the same effect with the salivary glands of the tick, Dermacentor andersoni.

Amiloride which is thought to inhibit Na^+ transport by blocking the entry of Na^+ into the transporting cells (GEE, 1976B), markedly inhibited the 'urine' production by the Malpighian tubules of Locusta. In the presence of 1mM amiloride the secretory rate was reduced to 5% of the rate observed in its absence. Somewhat similar results have been reported with the Malpighian tubules of Glossina morsitans (GEE, 1976B) and the salivary glands of Calliphora (BERRIDGE et al., 1976). The sensitivity of the Malpighian tubules to amiloride

indicates that they require the basal cell membrane to be permeable to Na^+ for fluid secretion (GEE, 1976B). This idea is supported by the results of BERRIDGE et al. (1976) which showed that amiloride did not effect fluid secretion by Calliphora salivary glands in high K^+ Ringer solution (120mMK^+, 55mM Na^+), whereas in 'normal' Ringer solution (20mMK^+, 155mM Na^+) the saliva production fell dramatically. They concluded that "the effect of amiloride is apparently specific for Na^+ transport". Similarly in toad bladder (BENTLEY, 1968; SUDOU and HOSHI, 1977; CANESSA et al., 1978), it has been shown that amiloride restricts the entry of Na^+ into the cell across the mucosal membrane and thereby reduces the intracellular pool of Na^+ available to the Na^+ 'pump' on the opposite cell membrane. In the case of Locusta Malpighian tubules, where K^+ is the 'prime mover' the action of amiloride might be explained on the basis of inhibition of the entry of Na^+ into the cell across the basal cell membrane. The resulting reduction in the intracellular Na^+ level would be expected to affect normal functioning of the proposed Na^+-K^+ exchange 'pump'. This would ultimately reduce the supply of K^+ to the proposed electrogenic K^+ 'pump' (BERRIDGE, 1967; BERRIDGE and OSCHMAN, 1969) on the apical cell membrane.

Conclusion. The results presented here, clearly implicate the Na^+-K^+ATPase, the Mg^{2+}- dependent HCO_3^--stimulated ATPase and carbonic anhydrase enzymes in fluid secretion by the Malpighian tubules of Locusta migratoria L. . Chloride is clearly important in maintaining 'normal' secretion rate; although Br^- can support secretion equally as well as Cl^-, fluid secretion is inhibited by NO_3^-.

CHAPTER 5

Studies of the effect of K^+, HCO_3^-, SCN^- and various pharmacological agents on oxygen consumption by Malpighian tubules of Locusta migratoria L.

INTRODUCTION

A characteristic of all living organisms is the presence of solute gradients across their membranes. The gradients are developed and maintained, directly or indirectly, by active transport, which is in turn sustained by energy derived from cellular metabolism. Early studies with frog skin (LUND, 1928) demonstrated that the potential difference produced by active Na^+ transport across this epithelium is decreased when oxygen availability is reduced. Similarly, MUDGE (1951A,B) and WHITTAM and DAVIES (1953) working with kidney tissue, showed that energy from respiration was essential for the maintenance of Na^+ and K^+ transport. The main evidence for this was the fact that active transport decreased if respiration was inhibited with substances such as cyanide. These studies did not, however, reveal what fraction of the total energy produced by respiration was expended on ion movements, or whether the latter, in turn, influenced the rate of respiration. This information was provided by ELSHOVE and VAN ROSSUM (1963) and VAN ROSSUM (1963) who investigated the relationship between metabolism and cation transport by rat liver slices. These investigators found that 90% of the calculated cation transport could be inhibited by addition of cyanide and therefore appeared

to be dependent on respiration. Inhibition of transport, by omitting Na^+ or K^+ from the incubation medium or by adding the glycoside, strophantin-K, inhibited tissue respiration by 30-40%. Therefore, approximately one-third of tissue respiration appeared to be directly associated with cation transport. Similar studies with kidney cortex (WHITTAM and WILLIS, 1962, 1963) and other tissues (WHITTAM, 1975) have also shown that 30-40% of oxygen consumption was inhibited by ouabain or the absence of Na^+. In 1971, VAN ROSSUM et al., found that complete inhibition of the net, active movements of K^+ and water by ouabain caused a 30% inhibition of respiration of rat liver slices, whereas concentrations of cyanide which produced up to 50% inhibition of respiration did not effect the cation transport. WEINER and MAFFLY (1978) suggest that this implies that,(i) the rate of synthesis of ATP by cells is considerably in excess of that required to maintain cation transport, and (ii) if metabolism is inhibited, the remaining energy is preferentially utilized to maintain ion transport.

The diuretic, ethacrynic acid, inhibits oxygen consumption of rat and Necturus renal cortical slices (WHITTEMBURY, 1968; MACKNIGHT, 1969; YOSHIDA et al., 1971; EPSTEIN, 1972), isolated rat, rabbit and dog kidney mitochondria (YOSHIDA et al., 1971; CASE et al., 1973; SUKI et al., 1973; EKNOYAN,et al., 1975), and ATP content of transporting cells (DANIEL et al., 1971; EPSTEIN, 1972). In addition, ethacrynic acid has been shown to inhibit Na^+ transport in kidney by interfering with oxidative phosphorylation

in the mitochondria of transporting cells (CASE et al., 1973). Similarly, amiloride, inhibits renal oxygen consumption by kidney slices (CASE et al., 1973) and interferes with passive Na^+ entry across the membrane (BENTLEY, 1968; SUDOU and HOSHI, 1977; LAU et al., 1979). In contrast, YOSHIDA et al., (1971) reported that amiloride did not significantly effect the rate of oxygen consumption by rat kidney slices or mitochondria. The cellular mechanisms of diuretic action, however, remain obscure. Evidence has been advanced that many diuretics may exert their effects by interfering with various aspects of cellular metabolism, including glycolysis (CANNON et al., 1968; LANDON and FITZPATRICK, 1970; KLAHR et al., 1971; DANIEL et al., 1971), Na^+-K^+ATPase activity (PROVERBIO et al., 1970; CHARNOCK et al., 1970; SUKI et al., 1973; PEACOCK et al., 1976) and Na^+ transport (CASE et al., 1973; BERRIDGE et al., 1976; GEE, 1976B, SUDOU and HOSHI, 1977; LAU et al., 1979).

As discussed earlier (Chapter 3,4) a close-link has been proposed between carbonic anhydrase and Mg^{2+}-dependent HCO_3^--stimulated ATPase in those cells in which the latter enzyme has been implicated in active transport processes. In addition, the carbonic anhydrase inhibitor, is known to affect ion transport (LESLIE et al., 1973; GREEN and GIBISCH, 1975; SOBOSLAI et al., 1977; MATHISEN et al., 1978) and fluid secretion in a variety of tissues (GODDING, 1975; BURG and GREEN, 1977; McKINNEY and BURG, 1977). In view of the relationship between Na^+-K^+ATPase activity and metabolism, discussed above, if carbonic anhydrase and Mg^{2+}-dependent HCO_3^--stimulated ATPase are also involved in active ion transport across

the cell membranes of Locusta Malpighian tubules, one might expect that inhibition of these latter enzymes would, similarly, result in a reduced rate of oxygen consumption. To date, little information is available concerning the effects of acetazolamide on oxygen consumption. YOSHIDA et al. (1971) reported that the carbonic anhydrase inhibitor, acetazolamide, did not effect any significant inhibition in the oxygen uptake by slices of rat renal cortex and medulla or by kidney mitochondria. In contrast, EKNOYAN et al. (1975) observed that acetazolamide reduced the rate of mitochondrial respiration in dog kidney. More recently, SENER et al. (1979) and MALAISSE (personal communication) reported that the range of concentrations ($\leqslant$3mM) in which HCO_3^- exerts its maximum stimulation on HCO_3^--ATPase activity, affects oxygen consumption and insulin release (HENQUIN and LAMBERT, 1975) by the pancreatic islets of rat. They concluded that a membrane - associated HCO_3^--activated ATPase might participate in the regulation of ATP utilization by pancreatic islet cell.

As Na^+-K^+ATPase, Mg^{2+}-dependent HCO_3^--stimulated ATPase and carbonic anhydrase have been demonstrated in Locusta Malpighian tubules (ANSTEE and BELL, 1975; 1978; ANSTEE and FATHPOUR, 1979; present study), and since these enzymes have been implicated in a variety of transport processes, the present study was carried out to establish the extent to which conditions which affect the activation of these enzymes, and tubule function, also influenced Malpighian tubules respiration. In addition, such a study should indicate the proportion of the total energy consumption which may be attributed to the activity of the HCO_3^--ATPase and the Na^+-K^+ ATPase 'pumps'.

MATERIALS AND METHODS

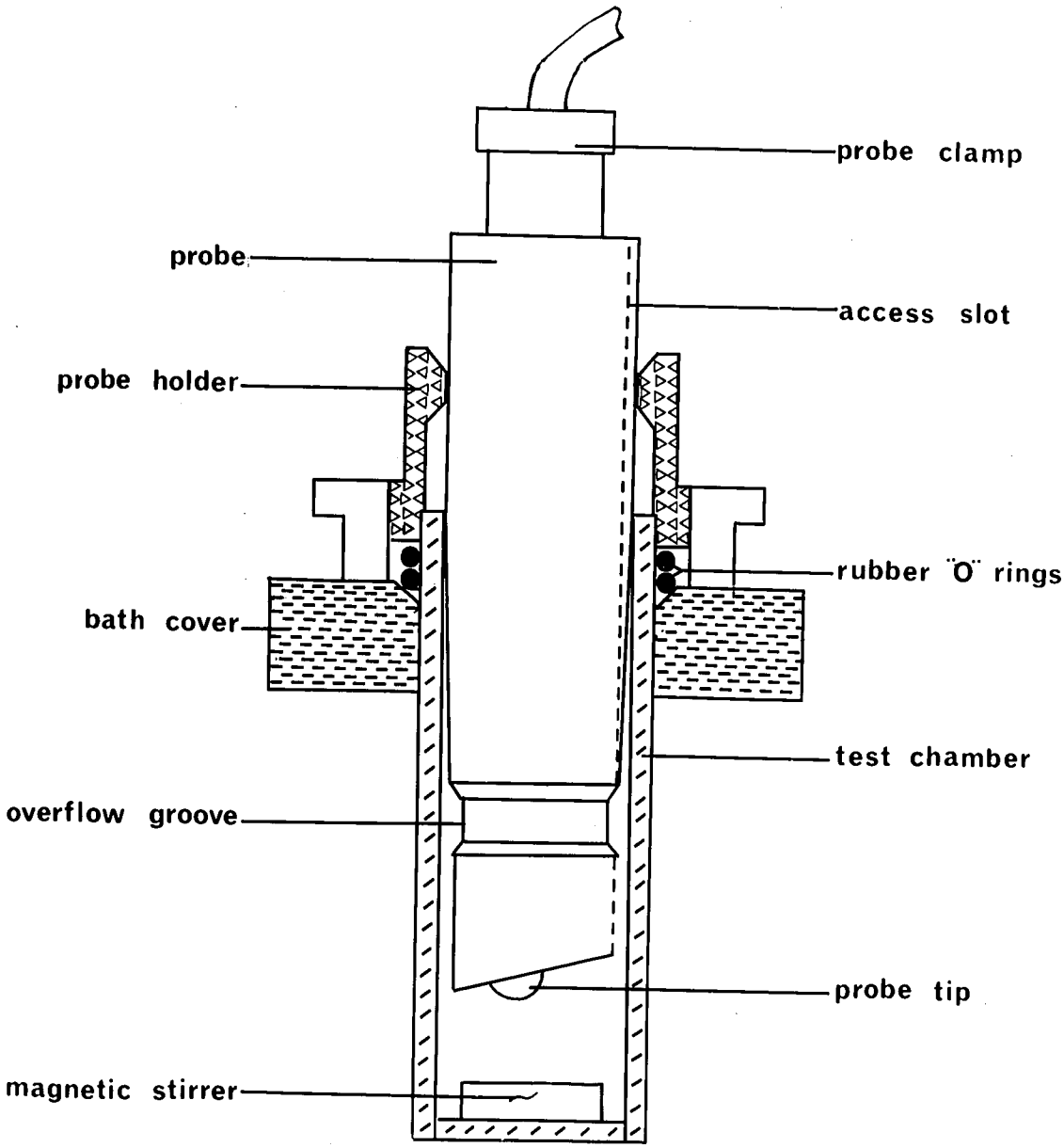
Oxygen consumption was measured with a Clark-type polarographic electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A., YSI MODEL 53). In this model the oxygen probe consists of a 0.025" diameter platinum cathode and a silver anode cased in an epoxy block. Prior to starting the experiment, KCl solution was placed on the electrode, which was then covered with a thin, tightly fitting, teflon membrane which was held in position by a rubber "O" ring. The membrane was examined for physical damage and tests were carried out to ensure that the electrode was working correctly (performed once a day as routine).

The apparatus included a constant temperature bath surrounding 4 incubation chambers and the temperature was maintained at $30 \pm 0.02^{\circ}C$ by continuous circulation from a constant temperature water bath (LAUDA-THERMOSTAT, TYPE K2, W. GERMANY). The oxygen content of the Ringer solution in the incubation chamber was monitored on a Servoscribe pen recorder (Goerz Electro) which was calibrated to give maximum output at 100% air saturation, at the beginning of each experiment. The Ringer solution in the chamber was stirred continuously during the experiments using a small magnetic stirrer (Figure 5.1). Results were expressed as µmoles O_2/g wet wt/min (for the method of calculation see Appendix 5).

Mature adult locusts, Locusta migratoria L. of both sexes were used. Animals were killed by decapitation and the Malpighian

Figure 5.1

Diagram of a Y.S.I. oxygen electrode sample chamber with the probe in position.



PROBE, PLUNGER & SAMPLE CHAMBER.

tubules were quickly dissected out under ice-cold Ringer solution. The tubules together with a narrow 'collar' of gut, to which they were attached, were then subjected to one of two different experimental procedures:

(i) The tubules were placed in an incubation chamber containing $3cm^3$ of air saturated 'normal' Ringer solution. Following a 10 min equilibration period, the rate of oxygen consumption was determined. The tubules were then removed from the incubation chamber, rinsed in ice-cold K^+-free Ringer solution and then soaked in fresh ice-cold (Ca.4^oC) K^+-free Ringer solution for 5 min. This was to partially deplete the tissues of K^+. At the end of this time, the tubules were returned to the incubation chamber which now contained $3cm^3$ of air saturated K^+-free Ringer solution at 30^oC. Following a 10 min equilibration period, the rate of oxygen consumption was redetermined. A series of control experiments were carried out where the procedure was as described above except that 'normal' Ringer solution was used throughout.

(ii) The tubules were placed in an incubation chamber containing $3cm^3$ of 'normal' Ringer solution and allowed to equilibrate for 10 min at 30^oC. Thereafter, the rate of oxygen consumption was determined, $1cm^3$ of 'normal' Ringer was then removed using a Finnpipette.

This was replaced with $1cm^3$ of 'normal' Ringer solution containing 3mM inhibitor (ouabain, acetazolamide, or ethacrynic acid; final concentration 1mM). The Malpighian tubules were left in the incubation chamber and the rate of oxygen consumption measured after 30 min. Control tubules were treated in exactly the same manner except that inhibitor was omitted from the replacement Ringer solution.

Experimental solutions. The Hepes buffered Ringer solution was used in most experiments (the composition of Hepes Ringer solution is described in the General Materials and Methods, Chapter 2). However, an alternative Ringer solution was used in some earlier studies on the effect of K^+-free Ringer solution and ouabain on oxygen consumption. The composition of this alternative 'normal' Ringer solution was (mM): NaCl 129; KCl 8.6; $MgCl_2$ 8.5; $CaCl_2$ 2; $NaHCO_3$ 10.2; NaH_2PO_4 4.3; glucose 34. The pH was adjusted to 7.2. K^+-free Ringer solution was prepared by substitution with the appropriate Na^+ salt.

P values referred to in the text were obtained by applying student's 't' test. Whereas P values shown in the tables were obtained by applying paired 't' tests.

RESULTS

5.1 The effect of K^+ on oxygen consumption by the Malpighian tubules.

Soaking the Malpighian tubules in K^+-free Ringer solution effected a significant decrease in the rate of oxygen consumption when compared to the original rate in 'normal' Ringer solution ($P<0.001$) (Table 5.1, Figure 5.2). In control tubules, no comparable decrease was observed and rate 2 was not significantly different from rate 1 (Table 5.1, Figure 5.3). When the tubules from the K^+-free Ringer were washed and resoaked in 'normal' Ringer for 5 min, the rate of oxygen consumption by the tubules showed a slight recovery (rate 3). It is difficult, however, to determine accurately the extent of the recovery as a decrease in the rate of oxygen consumption was recorded in the control tubules at this time (Table 5.1). When the results are expressed as a percentage of the original rate in 'normal' Ringer solution there is an approx. 25% decrease in the rate of oxygen consumption, in the absence of K^+, compared with the control ($P<0.001$) (Table 5.1).

5.2 The effect of ouabain on oxygen consumption by the Malpighian tubules.

A series of experiments were carried out which involved comparing the rates of oxygen consumption by Malpighian tubules which had been soaked in 'normal' Ringer solution, with their rates of consumption at the end of a 30 min period in the presence of

TABLE 5.1

The effects of K^+ on oxygen consumption by the Malpighian tubules

Treatment	n	Rate of O_2 consumption ± S.E.M. (µmoles O_2/g wet wt/min)			Rate 2 expressed as % Rate 1	Rate 3 expressed as % Rate 1	P
		Rate 1	Rate 2	Rate 3			
Control	10	2.69 ± 0.22	2.30 ± 0.20	1.96 ± 0.16	85.9 ± 2.5	73.1 ± 1.7	<0.001
Experimental (K^+-free for Rate 2)	11	3.63 ± 0.27	2.17 ± 0.15	2.30 ± 0.16	60.9 ± 3.5	62.3 ± 3.6	<0.001

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

Figure 5.2

Typical example of the effect of the absence of K^+ on the rate of oxygen consumption by Malpighian tubules.

Rate 1 (●) Immediately after dissection

Rate 2 (o) After 15 min in K^+-free Ringer solution (the first 5 min being at 4°C, thereafter at 30°C).

Rate 3 (▲) After a further 15 min in 'normal' Ringer solution.

Ordinate: Rate of oxygen consumption in µmoles O_2/g wet wt.

Abscissa: Time in seconds.

Figure 5.3

Typical example of the rate of oxygen consumption in control experiments where the tubules were soaked in 'normal' Ringer solution.

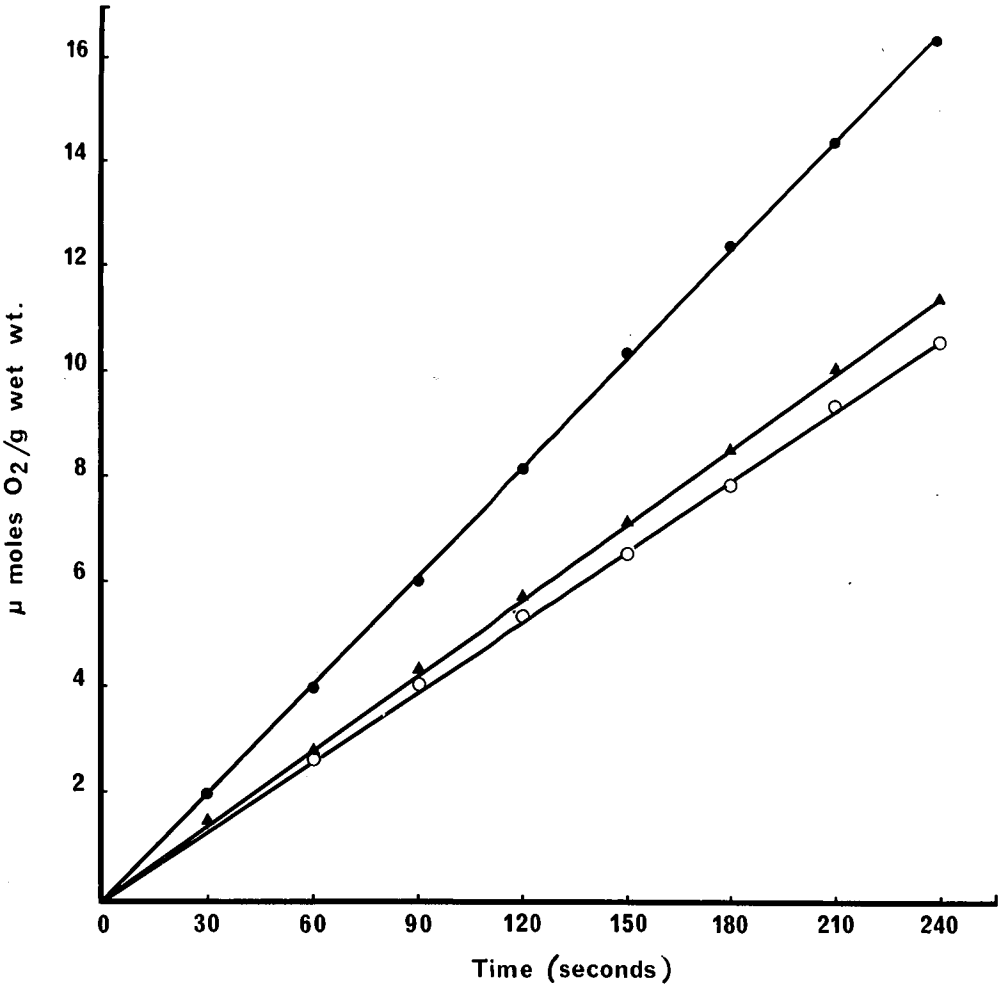
Rate 1 (●) Immediately after dissection

Rate 2 (o) After 15 min in 'normal' Ringer solution (the first 5 min being at 4°C, thereafter at 30°C).

Rate 3 (▲) After a further 15 min in 'normal' Ringer solution.

Ordinate: Rate of oxygen consumption in µmoles O_2/g wet wt.

Abscissa: Time in seconds.


16
14
12
10
8
6
4
2
μ moles O2/g wet wt.
0
30
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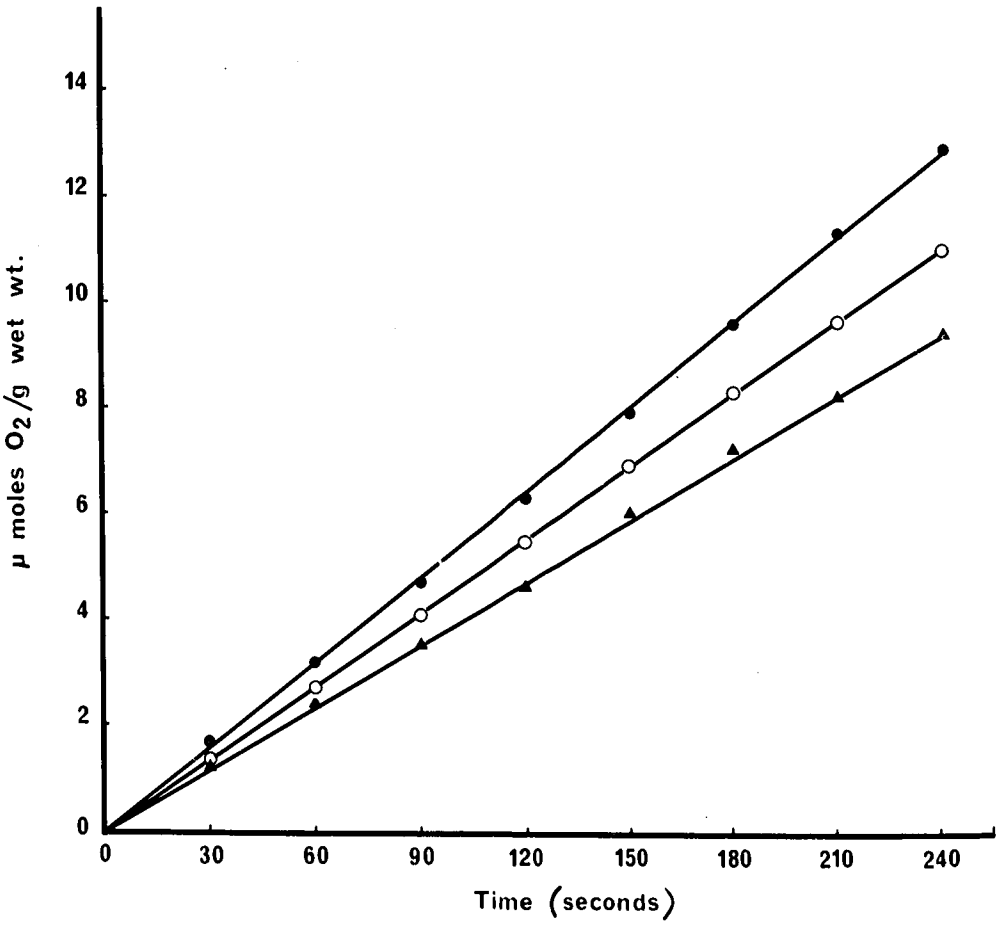
Time (seconds)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μ moles O2/g wet wt.
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Time (seconds)

1mM ouabain. The results are shown in Table 5.2. It can be seen that there has been an 18% reduction in the rate of oxygen consumption when experimental and control are compared ($P<0.001$).

5.3 The effect of acetazolamide on oxygen consumption by the Malpighian tubules.

Experiments were carried out in which the rates of oxygen consumption by the Malpighian tubules were determined in 'normal' Hepes Ringer solution. Following a 30 min period of soaking in 'normal' Hapes Ringer solution containing 1mM acetazolamide, the rates were redetermined. Controls were run in 'normal' Hepes Ringer solution throughout. The results are shown in Table 5.3. It can be seen that the presence of acetazolamide effected a slight, but significant, reduction in rate of oxygen consumption when experimental and control are compared ($P<0.02$).

5.4 The effect of HCO_3^--free Ringer containing acetazolamide on oxygen consumption by the Malpighian tubules.

A series of experiments was carried out in which the rates of oxygen consumption by the Malpighian tubules in 'normal' Hepes Ringer solution were compared with their rates of oxygen consumption at the end of a 30 min period in the presence of 1mM acetazolamide and the absence of HCO_3^- in the bathing medium. The results are similar to those obtained in 5.3 above; 1mM acetazolamide in HCO_3^--free Ringer effects a small but significant reduction in the rate of oxygen consumption ($P<0.03$) (Table 5.3).

TABLE 5.2

The effect of 1mM ouabain on oxygen consumption by the Malpighian tubules.

Treatment	n	Rate of O_2 consumption ± S.E.M. (µmoles O_2/g wet wt/min) Rate 1	Rate 2	Rate 2 expressed as % Rate 1	P
Control	20	2.83 ± 0.25	2.51 ± 0.21	89.3 ± 2.3	<0.001
Experimental (1mM ouabain present for Rate 2)	30	2.72 ± 0.21	1.91 ± 0.17	71.5 ± 2.6	<0.001

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

TABLE 5.3

The effect of 1mM acetazolamide in the presence and absence of HCO_3^-, and 1mM ethacrynic acid on oxygen consumption by the Malpighian tubules.

Treatment	n	Rate of O_2 consumption ± S.E.M. (µmoles O_2/g wet wt/min)		Rate 2 expressed as % Rate 1	P
		Rate 1	Rate 2		
Control	7	2.70 ± 0.12	2.74 ± 0.11	101.8 ± 2.5	>0.1
Experimental (1mM acetazolamide present for Rate 2)	9	3.07 ± 0.21	2.73 ± 0.17	89.6 ± 4.3	<0.02
(1mM acetazolamide present in HCO_3^--free Ringer for Rate 2)	8	2.82 ± 0.18	2.56 ± 0.17	91.1 ± 3.2	<0.02
(1mM ethacrynic present for Rate 2)	10	3.26 ± 0.11	2.22 ± 0.07	68.5 ± 2.5	<0.001

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

5.5 The effect of thiocyanate on oxygen consumption by the Malpighian tubules.

As was shown earlier (Chapter 3,4), sodium thiocyanate inhibits the Mg^{2+}-dependent HCO_3^--stimulated ATPase and fluid secretion by the Malpighian tubules of Locusta. It was of interest, therefore, to establish whether SCN^- affected the rate of oxygen consumption. The experimental procedure was as described in the Materials and Methods. 'Normal' Ringer solution was used in the determination of rate 1 oxygen consumption. 10mM SCN^- was included in the 'normal' Hepes Ringer solution in determining rate 2. $NaSCN^-$ was added by substitution for NaCl. Control tubules were run in 'normal' Hepes Ringer solution throughout. It is difficult to assess the effect of SCN^- on oxygen consumption. The results show that whilst 10mM SCN^- effected a slight reduction in the rate of oxygen consumption (by 7%) the effect was not significantly different when experimental and control are compared ($P>0.1$). However, application of a paired 't' test revealed that the difference between two rates are highly significant from experimentals (Table 5.4), whereas for the controls they were not.

To find out whether such a reduction in the rate of oxygen consumption is related to the inhibitory effect of SCN^- or simply because of reducing the amount of Cl^- in the Ringer solution, a series of experiments were carried out in which 90mM NaCl was present for rate 2 (instead of 100mM). The results show that the rate of oxygen consumption was reduced by 5% when the experimental and control are compared. But the application of a paired 't' test, yielded results similar to those obtained for SCN^-, i.e. the

TABLE 5.4

The effect of 10mM SCN^- on oxygen consumption by the Malpighian tubules

Treatment	n	Rate of O_2 consumption $\pm$ S.E.M. (μmoles O_2/g wet wt/min)		Rate 2 expressed as % Rate 1	P
		Rate 1	Rate 2		
Control	8	2.18 $\pm$ 0.11	2.05 $\pm$ 0.07	94.6 $\pm$ 2.7	>0.05
Experimental (10mM SCN^- present for Rate 2)	10	2.14 $\pm$ 0.13	1.86 $\pm$ 0.10	87.7 $\pm$ 2.7	<0.001
(90mM Cl^- present for Rate 2 instead of 100mM)	8	2.28 $\pm$ 0.09	2.04 $\pm$ 0.08	89.7 $\pm$ 1.8	<0.001

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

differences between the two rates are again highly significant (Table 5.4). It would seem, therefore, that the apparent inhibition of oxygen consumption by SCN^- may be due to a reduction of the Cl^- concentration in the Ringer solution, and not to SCN^- inhibition of the HCO_3^--ATPase enzyme.

5.6 The effect of ethacrynic acid on oxygen consumption by the Malpighian tubules

Experiments were carried out in which tubules were soaked in 'normal' Hepes Ringer solution and the rates of oxygen consumption were determined (rate 1). Following a 30 min soaking in 'normal' Hepes Ringer solution containing 1mM ethacrynic acid, the rates of oxygen consumption were redetermined (rate 2). Results are shown in Table 5.3. It can be seen that there has been a 33% decrease in the rate of oxygen consumption when experimental and control are compared ($P<0.001$).

5.7 The effect of amiloride on oxygen consumption by the Malpighian tubules.

The procedure for determining the rate of oxygen consumption in the presence of amiloride (rate 2) was somewhat different from that referred to above. The following modification of procedure was necessary, because of the relative insolubility of amiloride in Ringer solution. In the above experiments, $1cm^3$ of 'normal' Hepes Ringer solution was replaced by $1cm^3$ of Ringer solution containing 3mM inhibitor (see Materials and Methods). Whereas, in the case of amiloride, all $3cm^3$ of Hepes Ringer solution were replaced by $3cm^3$ of Hepes Ringer solution containing 1mM

amiloride. Control tubules were run in 'normal' Hepes Ringer solution throughout. The results are shown in Table 5.5. It can be seen that there has been an approx. 22% reduction in the rate of oxygen consumption when experimental and control are compared ($P<0.001$).

TABLE 5.5

The effect of 1mM amiloride on oxygen consumption by the Malpighian tubules

Treatment	n	Rate of O_2 consumption ± S.E.M. (µmoles O_2/g wet wt/min)		Rate 2 expressed as %	P
		Rate 1	Rate 2	Rate 1	
Control	8	2.42 ± 0.13	2.29 ± 0.11	94.9 ± 1.7	>0.02
Experimental (1mM amiloride present for Rate 2)	11	2.62 ± 0.12	1.91 ± 0.11	73.2 ± 3.0	<0.001

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

DISCUSSION

In the present study, the presence of 1mM ouabain in the bathing medium caused a fall of about 18% in the rate of oxygen consumption by the Malpighian tubules. Similarly, when tubules were soaked in K^+-free Ringer, the respiratory rate decreased by 25% when experimental and control are compared. It would appear, therefore, that approx. 18-25% of the oxygen consumed by the Malpighian tubules of Locusta migratoria is involved in active ion transport. Since ouabain is a specific inhibitor of Na^+-K^+ATPase, and since this enzyme has been shown to be present in the Malpighian tubules of Locusta (ANSTEE and BELL, 1975, 1978), the results presented here further support the proposals (BERRIDGE and OSCHMAN 1969; ANSTEE and BELL, 1975) that it is involved in active cation transport across the tubules. Somewhat similar results have been reported elsewhere. WHITTAM (1961), using rabbit brain and kidney slices, reported that ouabain inhibited oxygen consumption by 32-48% whilst the omission of Na^+ from the medium caused a fall of 33-42% in the rate of oxygen consumption. ELSHOVE and VAN ROSSUM (1963) reported omission of Na^+ or K^+ from the incubation medium or addition of glycoside, strophantin-K, inhibited the respiration of rat liver slices by 30-40%. WILLIS (1968A,B) working on renal cortex of squirrel and hamster reported that addition of ouabain or the omission of K^+ from the incubation medium decreased the rate of oxygen consumption by 22-35%. Somewhat higher values of inhibition have been reported (50-55%) with rabbit brain and chick embryo brain due to addition of ouabain or with the omission of Na^+ from the reaction

medium (WHITTAM, 1962; SEDLACEK, 1973). Whereas, NOE and CRABBE (1975) observed that treatment of renal cortex slices from guinea pig with ouabain resulted in a lesser inhibition of oxygen consumption (27%) than when Na^+ was withdrawn from the bathing medium (50%). WHITTAM and WILLIS (1962, 1963) working on rabbit kidney reported 35-45% reduction in oxygen consumption following the addition of ouabain or omission of Na^+ from medium. They also found that when kidney slices were incubated in Na^+ free medium with ouabain, no further reduction in oxygen consumption occurred. They concluded from this, that both the lack of sodium and the presence of ouabain in normal media act upon a single factor, cation transport, which sets the pace of part of the cell's metabolism. In contrast, to the studies referred to above, HARVEY et al. (1967) reported that, although the K^+ transport in the midgut of Hyalophora cecropia is 5-10 times larger than the Na^+ transport in the frog skin for a similar mass of tissue, the K^+ transport has no effect on the oxygen consumption. They concluded that the ion transport mechanism of the midgut is complex, with unidentified links between oxygen consumption and K^+ transport. Whereas, HOULIHAN (1977) reported a 200% increase in the respiration rate during the uptake of water by the eversible abdominal sacs of Petrobius brevistylis. He proposed that the uptake of water could be due to the linkage between ion secretion and water uptake.

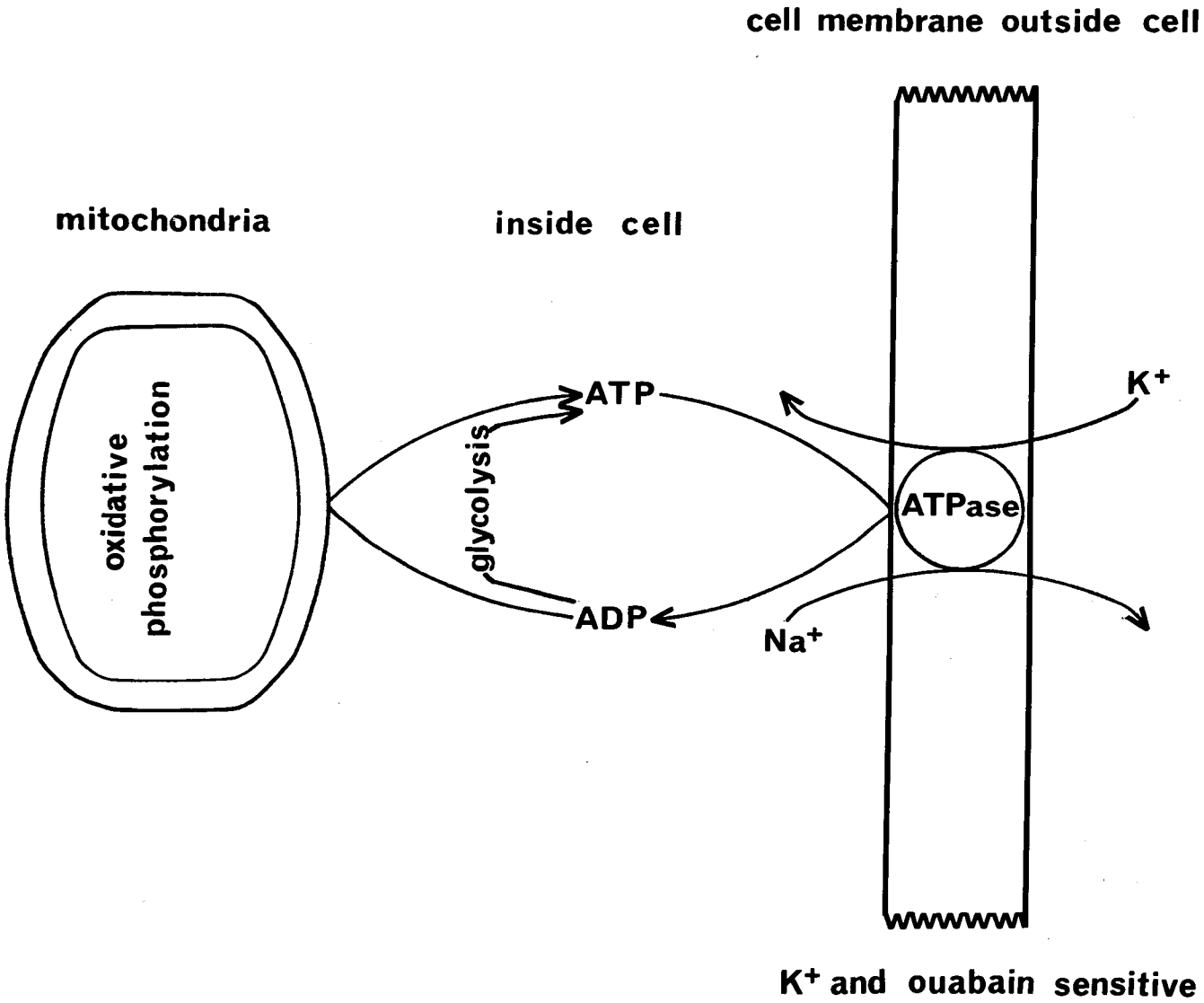
The above results are comparable with those obtained in the present study; since Na^+ is the major cation transport in kidney tissue, whereas K^+ is the 'prime mover' in Locusta Malpighian

tubules. The proposed interrelationship between ATPase activity and oxidative phosphorylation is as follows (Figure 5.4). The Na^+-K^+ ATPase activity transport K^+ from the haemolymph into the cytoplasm across the basal cell membrane and Na^+ is transported in the opposite direction. The energy for this process is obtained from splitting ATP to ADP and phosphate. As ADP and phosphate accumulate, these, in turn, stimulate oxidative phosphorylation (BLOND and WHITTAM, 1964; WHITTAM and WILLIS, 1963; LANDON and FITZPATRICK, 1970; WEINER and MAFFLY, 1978) and glycolysis (PARKER and HOFFMAN, 1967; LANDON and FITZPATRICK, 1970; WHITTAM, 1975; WEINER and MAFFLY, 1978) which results in the replenishment of the supply of ATP and enhancement of the level of oxygen consumption. Na^+-K^+ATPase activity can therefore continue as there is always sufficient ATP available to provide the energy for active transport. It would seem clear, therefore, that active transport, respiration and Na^+-K^+ATPase activity are very closely coupled in Locusta Malpighian tubules.

In earlier chapters, the presence of Mg^{2+}-dependent HCO_3^--stimulated ATPase and carbonic anhydrase were demonstrated. In addition, the response of tubule fluid secretion to SCN^- and acetazolamide was taken as circumstantial evidence for the involvement of these enzymes in 'urine' production by Locusta Malpighian tubules. If these enzymes are involved in active ion transport across the cell membranes, it would be expected that oxygen consumption would be reduced by the addition of known

Figure 5.4

Scheme for the coupling between active transport and respiration in a Malpighian tubule. Oxidative phosphorylation in mitochondria and glycolysis provide ATP required to support active movement of K^+ and other energy requiring processes in the cell.


cell membrane outside cell
mitochondria
inside cell
oxidative phosphorylation
glycolysis
ATP
ADP
ATPase
K+
Na+
K+ and ouabain sensitive

enzyme inhibitors. This was in fact the case ; the presence of 1mM acetazolamide in the bathing medium, inhibited the rate of oxygen consumption by ca. 12%. No further reduction was observed by excluding HCO_3^- from Ringer solution containing 1mM acetazolamide. These results suggest that the depletion of intracellular HCO_3^- reduces the metabolic activity of the Malpighian tubule cell, possibly by depriving the Mg^{2+}-ATPase of HCO_3^- which stimulates its activity. If this is so, the energy demands of the HCO_3^--ATPase would appear to count for ca. 12% of the total metabolic activity of the cells. Similarly, EKNOYAN et al. (1975) reported that acetazolamide inhibited the respiration of cortical and outer medullary mitochondria from dog kidney. They observed that the 50% inhibitory dose for both cortical and medullary mitochondria was the same 10.8×10^{-3}M (complete inhibition being observed at 13.2×10^{-3}M). More recently, SENER et al. (1979) and MALAISSE (personal communication) reported that the range of concentrations ($\leqslant$3mM) in which HCO_3^- exerts its most pronounced stimulation action on ATPase activity coincides with the range of concentrations in which extracellular HCO_3^- affects oxygen consumption and insulin release by the islets of rat pancreas (HENQUIN and LAMBERT, 1975). In contrast, YOSHIDA et al. (1971) reported that 3×10^{-4}M acetazolamide did not significantly effect the oxygen consumed by rat renal slices or renal mitochondrial respiration (in the presence of 10^{-4}M inhibitor in the case of the latter).

In view of the slight, but significant inhibition of oxygen consumption by acetazolamide it was, perhaps, surprising that 10mM SCN^-, which inhibits HCO_3^--ATPase, did not, also,

convincingly reduce the rate of oxygen consumption by the Malpighian tubules. Although, application of paired 't' test showed that rate 1 and rate 2 are significantly different ($P<0.001$), it was concluded that this difference is largely due to reduced $[Cl^-]$ rather than SCN^- inhibition. Similarly, FORTE and DAVIES (1964) working with bullfrog gastric mucosa reported that 15mM SCN^- did not significantly reduce the rate of oxygen consumption after 1.0-1.5 hour (2% reduction). However, they found that the same concentration of SCN^- did reduce the rate of oxygen consumption (by approx. 47%) after 3.0-3.5 hours and that it took at least 1 hour for SCN^- to effect the rate of oxygen consumption. This might be an explanation for the results obtained in the present study, in which tubules were soaked only 30 min in Ringer solution containing 10mM SCN^- prior to experiment. However, such an explanation is inconsistent with the observation that fluid secretion was inhibited by the same concentration of SCN^- after similar (30 min) period of exposure (Chapter 4). It would seem, therefore, that the links between HCO_3^--ATPase activity, carbonic anhydrase activity, and O_2 consumption are complex.

The diuretic agent, ethacrynic acid, which exerts its inhibitory effects on several levels of cell metabolism (see Chapter 3), reduced the rate of oxygen consumption in the locust tubules by about 33%. This agrees well with the results of JONES and LANDON (1967), who reported that ethacrynic acid inhibited oxygen uptake by 28% in rat kidney. Somewhat higher value of inhibition has been reported for kidney tissue elsewhere.

MACKNIGHT (1969) working on rat recorded 54%, WHITTEMBURY (1968) working on Necturus reported 55%, EPSTEIN (1972) and CASE et al. (1973) in rabbit and Bufo observed 90% and 70% reduction in oxygen consumption by addition of ethacrynic acid. EKNOYAN et al. (1975) working on dog kidney observed that the 50% inhibitory dose of inhibitor for mitochondrial respiration was 4.5×10^{-4} (with succinate as the substrate) and 7.1×10^{-4}M drug completely inhibited the mitochondrial respiration. Whereas, CASE et al. (1973) reported that 1mM ethacrynic acid inhibits 57% of Bufo kidney mitochondrial respiration. Similarly, SUKI et al. (1973) reported that 0.1-1.0mM ethacrynic acid depressed respiration and decreased intracellular K^+ concentration of kidney slices from rat, rabbit and dog. In addition, they showed that ethacrynic acid inhibited the respiration of isolated kidney mitochondria. In contrast, FULGRAFF (1969) reported that ethacrynic acid did not significantly change the rate of oxygen consumption of dog kidney.

In kidney, energy metabolism is utilized primarily for sodium reabsorption. Sodium reabsorption accounts for approx. 57% of total renal transport and a linear relationship exists between sodium reabsorption and oxygen consumption by the kidney (KIIL et al., 1961). Mitochondria are a major source of cellular energy production (phosphate utilization and ATP production) and oxygen consumption, they may, therefore, have an important role in active transport of sodium in the renal tubules (EKNOYAN et al., 1975). Further support to this argument is provided by the results of

EPSTEIN (1972) who observed that ethacrynic acid brings about a rapid fall in rabbit kidney cortex ATP content. He postulated that the fall in oxygen consumption brought about by ethacrynic acid is secondary to inhibition of mitochondrial ATP production. Furthermore, ethacrynic acid inhibits Na^+-K^+ATPase of a variety of species (PROVERBIO et al., 1970; CHARNOCK et al., 1970; INAGAKI et al., 1973; PEACOCK et al., 1976). The drug block phosphorylation of the enzyme, prevent the ADP-ATP exchange reaction, and leads to estabilization of the spontaneous disappearance of the phosphorylated intermediate (SUKI et al., 1973). In addition, ethacrynic acid, inhibits glycolysis in rat and rabbit kidney (JONES and LANDON, 1967), dog kidney (CANNON et al.,1968; EKNOYAN et al., 1975), and rat uterus (DANIEL et al., 1971). In the case of the Malpighian tubules, as discussed in the previous Chapter, K^+ is the 'prime mover'; it may be suggested therefore that the action of ethacrynic acid is complex and that it exerts its inhibitory effect on K^+ transport in two ways; (i) inhibition of ATP production (both mitochondrial and glycolytic). This has implications for all energy requiring processes including the activity of ATPase enzymes; (ii) direct inhibition of Na^+-K^+ATPase itself.

Amiloride which inhibits 'urine' production markedly (Chapter 4), caused a fall of 22% in oxygen consumed by the Malpighian tubules of Locusta. This agrees well with the level of inhibition (19%) reported for rabbit renal cortical slices following administration of 1mM amiloride (CASE et al., 1973).

These workers observed that addition of amiloride caused the intracellular Na^+ and K^+ concentration to fall significantly, without a change in cell water content. More recently, LAU et al. (1979) working with frog skin and toad bladder observed that more than 30% of oxygen consumption is inhibited in the presence of 10^{-5}M amiloride. These workers referred to the fact that the action of amiloride is specific, resulting in the inhibition of movement of Na^+ across the external plasma membrane into the 'active transport' pool, without affecting the flux of other ions via cellular pathway. They suggested that it is superior to ouabain for the evaluation of basal metabolism, for although the glycoside is considered to act specifically to inhibit the Na^+-K^+ATPase associated with the Na^+ 'pump', it might be expected to interfere with not only transepithelial active Na^+ transport but also with the pumping of Na^+ out of muscle, connective tissue and/or other cells in the contiguous serosal adventitial layer. In contrast, YOSHIDA et al. (1971) reported that amiloride did not influence oxygen consumption by rabbit kidney slices and isolated mitochondria. As was discussed above, amiloride interferes with passive Na^+ entry across the cell membrane (BENTLEY, 1968; SUDOU and HOSHI, 1977; LAU et al., 1979). However, this may not be the sole action of this inhibitor. Studies on kidney tissue suggest that amiloride may also inhibit one of the enzymes of Krebs cycle, viz, α-ketoglutarate dehydrogenase (LOSERT et al., 1969). In addition, amiloride also decreases K^+ secretion in the rat distal tubules but has no perceptible effect on Na^+ absorption in this segment

(DUARTE et al., 1971). As was discussed earlier, amiloride may interfere with fluid secretion by Locusta Malpighian tubules, indirectly, by depriving the Na^+-K^+ exchange 'pump' of the intracellular Na^+ (Chapter 4). This would agree with the observation that in the presence of ouabain, a specific inhibitor of Na^+-K^+ATPase, the 'pump' can not operate normally, and the rate of oxygen consumption by the tubules is reduced (see above). In addition, this is consistent with the observation that whenever Na^+ extrusion is reduced, respiration is diminished in proportion (WILLIS, 1968B).

Conclusion. Results presented here, clearly demonstrate that active transport, respiration and Na^+-K^+ATPase activity are very closely coupled in Locusta Malpighian tubules. The reduction of the rate of oxygen consumption by amiloride might be further support for this claim. Whether the same statement is valid for Mg^{2+}-dependent HCO_3^--stimulated ATPase is uncertain.

CHAPTER 6

The effects of various ions and pharmacological agents, on the transepithelial potential across the Malpighian tubules of Locusta migratoria L.

INTRODUCTION

One of the characteristic features of living cells is the fact that the ionic concentrations of the intracellular fluid are generally quite different from those of extracellular fluid. For example, K^+ is commonly found at a concentration higher inside cells than outside, whereas the concentration gradients of Na^+ and Cl^- are usually opposite to that of K^+. Therefore, there are concentration gradients tending to move ions through plasma membranes in one direction or another. For any given ionic species, the concentration gradient across a membrane, selectively permeable to that ion, will result in a transmembrane potential difference across the membrane which can be calculated by applying the NERNST equation. This is normally expressed as:

$$E = \frac{RT}{ZF} \log_e \frac{\gamma_1 \cdot \text{concentration side A}}{\gamma_2 \cdot \text{concentration side B}}$$

where E = P.D. in volts, R = the gas constant (8.314 J/Mole), T = temperature in °A, Z = valency (the charge number of the ion), F = the Faraday constant (96,500 international coulombs), γ_1 and γ_2 = the activity coefficients which are usually taken to equal 1 at physiological concentrations. Any deviation of potential from that predicted by the NERNST equation may be taken as indicative of either

membrane permeability to more than one ion, or of electrogenic active ion transport across the membrane.

RAMSAY (1953) demonstrated that in the eight different species of insects which he studied the transepithelial P.D. across the Malpighian tubules did not obey the NERNST equation for K^+. On this basis, he concluded that in these insects K^+ is actively secreted into the tubule lumen, whereas transport of Na^+ is apparently brought about by passive diffusion. In contrast, PILCHER (1970) and MADDRELL (1971) working with Carausius and Rhodnius, respectively, report that a 10-fold increase in K^+ concentration in the bathing medium increased the transepithelial potential in a manner similar to that predicted by the NERNST equation.

It is widely agreed that active K^+ transport occurs in the majority of insect species which have been studied; notably Carausius, Calliphora, Rhodnius, Locusta, Schistocerca (RAMSAY, 1953, 1955; BERRIDGE, 1967, 1968; BERRIDGE and OSCHMAN, 1969; PILCHER, 1970; MADDRELL, 1971, 1972, 1977; MADDRELL and KLUNSUWAN, 1973; BELL, 1977; ANSTEE et al., 1979). On the basis of such studies, MADDRELL (1971) has proposed a hypothetical scheme to explain the mechanism of Malpighian tubules function in Carausius and Calliphora. The main features of this model are that active ion transport occurs both at the basal and apical cell membranes. It is proposed that K^+ is actively transported into the cell by an electrogenic 'pump' which is stimulated by Na^+ and 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, once again, considered to be passive. In the case of Rhodnius a slightly different model is suggested (MADDRELL, 1971, 1972). Here

it is proposed that an electrogenic K^+ 'pump' which is stimulated by Na^+ and an electrogenic Na^+ 'pump' which is stimulated by K^+ are present in the basal cell membrane. Cl^- enters the cell passively. It is proposed that in this way, Na^+, K^+ and Cl^- are made available to three electrogenic 'pumps' (for Na^+, K^+ and Cl^-) situated in the apical cell membrane and transporting these ions into the lumen.

Active K^+ transport, active Na^+ transport, or both have been reported across various other insect epithelia. For example, active K^+ transport across salivary glands of Calliphora (OSCHMAN and BERRIDGE, 1970; PRINCE and BERRIDGE, 1973; BERRIDGE et al., 1975, 1976; BERRIDGE and SCHLUE, 1978), active K^+ and Na^+ transport across the midgut of larval silkworm, Hyalophora cecropia (HARVEY et al., 1967; ZERAHN, 1971; HARVEY and ZERAHN, 1971; HARVEY et al., 1975; WOOD and HARVEY, 1975; ZERAHN and KOEFOED, 1979). Similarly, active Na^+ transport has been reported across the midgut of larval Sarcophaga bullata (PRUSCH, 1978).

Transepithelial potentials have been measured across the epithelia of many tissues of a variety of arthropods. These include the Malpighian tubules of different insects (RAMSAY, 1953; COAST, 1969; PILCHER, 1970; MADDRELL, 1971; MADDRELL and KLUNSUWAN, 1973; BELL, 1977) and certain other arthropods (FARQUHARSON, 1974; KAUFMAN and PHILLIPS, 1973), the salivary glands of Calliphora (BERRIDGE and PRINCE, 1972; PRINCE and BERRIDGE, 1973; GUPTA et al., 1978), the midgut of larval Hyalophora cecropia and Sarcophaga bullata (HARVEY et al., 1967; PRUSCH, 1978), and the hindgut of Schistocerca gregaria (HERRERA et al., 1976; 1977, 1978; WILLIAMS et al., 1978).

Numerous investigators have studied the effects of a variety of different inhibitors on the P.D. across these various epithelia, in an attempt to establish the mechanism of ion and fluid transport. For example, the cardiac glycoside, ouabain, a specific inhibitor of Na^+-K^+ATPase (SKOU, 1969) has been shown to decrease the trans-epithelial potential across cockroach intestine (O'RIORDAN, 1969; DATTA, 1971) and the midgut of larval Sarcophaga bullata (PRUSCH, 1978). Similarly, ouabain affects the membrane potential of Calliphora salivary glands in the absence of 5-hydroxytryptamine (5-HT) (BERRIDGE and SCHLUE, 1978). Ouabain has also been shown to affect the P.D. in a variety of other epithelia, e.g. toad urinary bladder (CIVAN, 1970), rabbit salivary glands (AUGUSTUS, 1976), rabbit collecting tubules (McKINNEY and BURG, 1978A,B), rabbit gall bladder (FREDERIKSEN, 1978), and Necturus gall bladder (REUSS et al., 1979). In contrast, PILCHER (1970) failed to demonstrate an effect of ouabain on the potential difference across the Malpighian tubules of Carausius.

The carbonic anhydrase inhibitor, acetazolamide, has also been reported to affect the transepithelial potential across a variety of epithelia; the rectal wall of Schistocerca gregaria (HERRERA et al., 1977, 1978), turtle bladder (GONZALES, 1969; GONZALES and SCHILB, 1969) and toad bladder (CHEN and WALSER, 1977). In contrast, McKINNEY and BURG (1978A, B) observed that acetazolamide did not change the transepithelial potential across the rabbit cortical collecting tubules. Recently, WILLIAMS et al., (1978) reported that carbonic anhydrase has been detected in rectal tissue of the desert locust (S. gregaria) and acetazolamide inhibited the short-circuit current (Isc) across

the rectal wall. Similarly, HASKELL et al., (1965) reported that the carbonic anhydrase inhibitor, ethoxzolamide, inhibits the short-circuit current of Hyalophora cecropia midgut. Absence of HCO_3^- in the bathing medium has also been reported to effect the trans-epithelial P.D. in turtle bladder (GONZALES and SCHILB, 1969), Necturus kidney (ANAGNOSTOPOULOS and EDELMAN, 1977), rabbit corneal endothelium (FISCHBARG and LIM, 1974; HODSON and MILLER, 1976) and rat proximal tubules (FROMTER, 1979).

Sodium thiocyanate is reported to inhibit Mg^{2+}- dependent HCO_3^-- stimulated ATPase in a variety of different tissues (Chapter 3) and fluid secretion (Chapter 4), however, there is little information in the literature concerning the effect of SCN^- on transepithelial potential. HARRIS and EDELMAN (1959) observed that in the presence of normal K^+ concentration (4.1mM) in the submucosal solution, introduction of SCN^- caused an increase in P.D. across the frog gastric mucosa,whereas increasing the concentration of K^+ to 9.0mM (in the presence of SCN^-),decreased the transepithelial potential. FORTE and DAVIES (1964) reported that SCN^- increased the transmucosal potential difference across the bullfrog gastric mucosa.

Ethacrynic acid, which interferes with different aspects of cell metabolism and the transport of ions (Chapter 3,5) has been shown to reduce the transepithelial potential across the salivary glands of Calliphora (BERRIDGE et al., 1976), the cortical collecting limb of Henle's loop in the rabbit (BURG and GREEN, 1973) and isolated toad bladder (CASE et al., 1973). In contrast, FREDERIKSEN (1978)

reported that ethacrynic acid when applied to the mucosal side caused a small and gradual increase in the potential difference across rabbit gall bladder epithelium, in spite of a marked inhibitory effect on fluid transport rate.

Amiloride, the inhibitor of Na^+ transport (NAGEL and DORGE, 1970; SUDOU and HOSHI, 1977; see Chapter 4), also affects the transepithelial potential across the salivary glands of Calliphora (BERRIDGE et al., 1976), rabbit cortical collecting tubules (Mc KINNEY and BURG, 1978A,B), toad bladder (BENTLEY, 1968; SUDOU and HOSHI, 1977), and frog skin (NAGEL and DORGE, 1970).

In the present study, the effect of varying the Na^+ and K^+ composition of the bathing medium on the transepithelial potential across the Malpighian tubules of Locusta has been examined. In addition, the effects of various pharmacological agents, HCO_3^-, SCN^-, SO_3^{2-} and substitution of Cl^- by NO_3^- or Br^- were investigated to provide further information concerning the likely nature of the ion 'pumps' and their contributions to the transepithelial potential difference observed.

MATERIALS AND METHODS

Mature adult locusts, of both sexes were used throughout. The experimental dish described earlier (Chapter 4) was used with some modifications (Figure 6.1). Individual tubules were drawn out of the Ringer trough into the liquid paraffin, looped around the small stainless steel pegs and partially severed as described previously (Chapter 4). Only actively secreting tubules were used. Once it had been established that a given tubule was actively secreting 'urine', Ringer solution was added to cavity Y such that there was continuity between it and the lumen of the tubule. The lumen of the tubule was now electrically continuous with the Ringer solution in cavity Y. Thus the transepithelial potential difference (P.D.) could be measured across its wall, by placing one electrode in cavity Y and the other in the Ringer trough (cavity X, Figure 6.1).

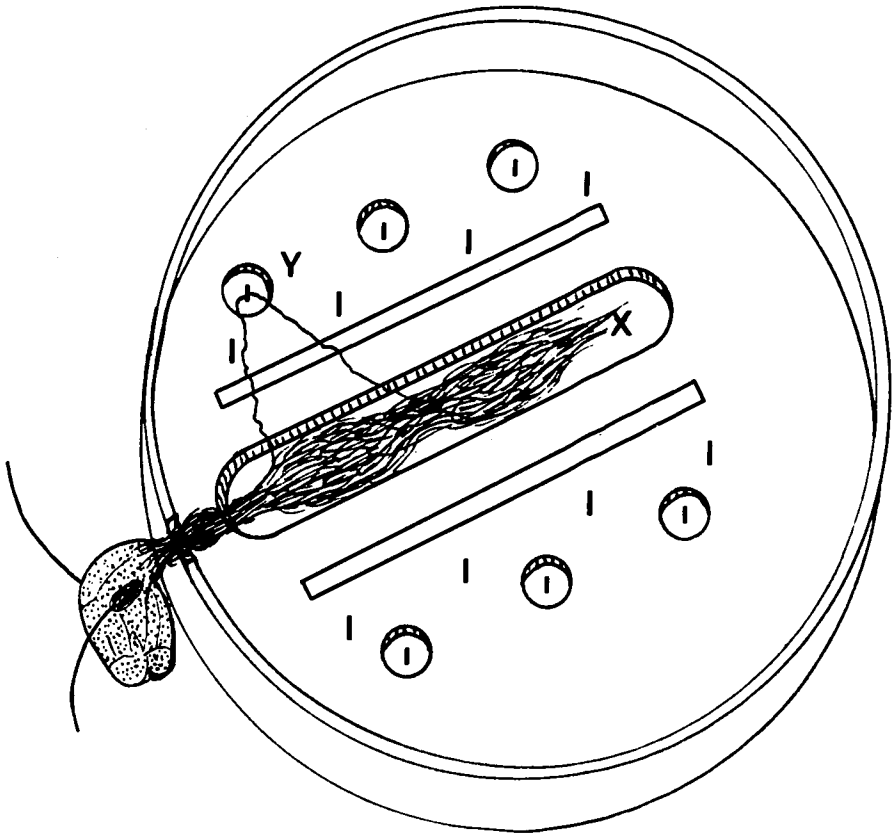
The potential was measured using calomel electrodes connected to 3mM KCl via KCl-agar bridges. A high imput impedance differential amplifier with a gain of 10X was used. This incorporated a zero back-off facility and a voltage calibrator was used in the imput circuit of the amplifier. Transepithelial potential was recorded by placing the reference electrode in cavity X (outside the tubule) and the recording electrode in cavity Y (i.e. in contact with the lumen, Figure 6.1). Prior to experiments the initial potential was adjusted to zero by placing the electrodes in the same Ringer pool (the circuit diagram is shown in Figure 6.2). This being done, the initial potential across a tubule was measured with the gut bathed in 'normal' Ringer solution, and continuous recordings were taken for

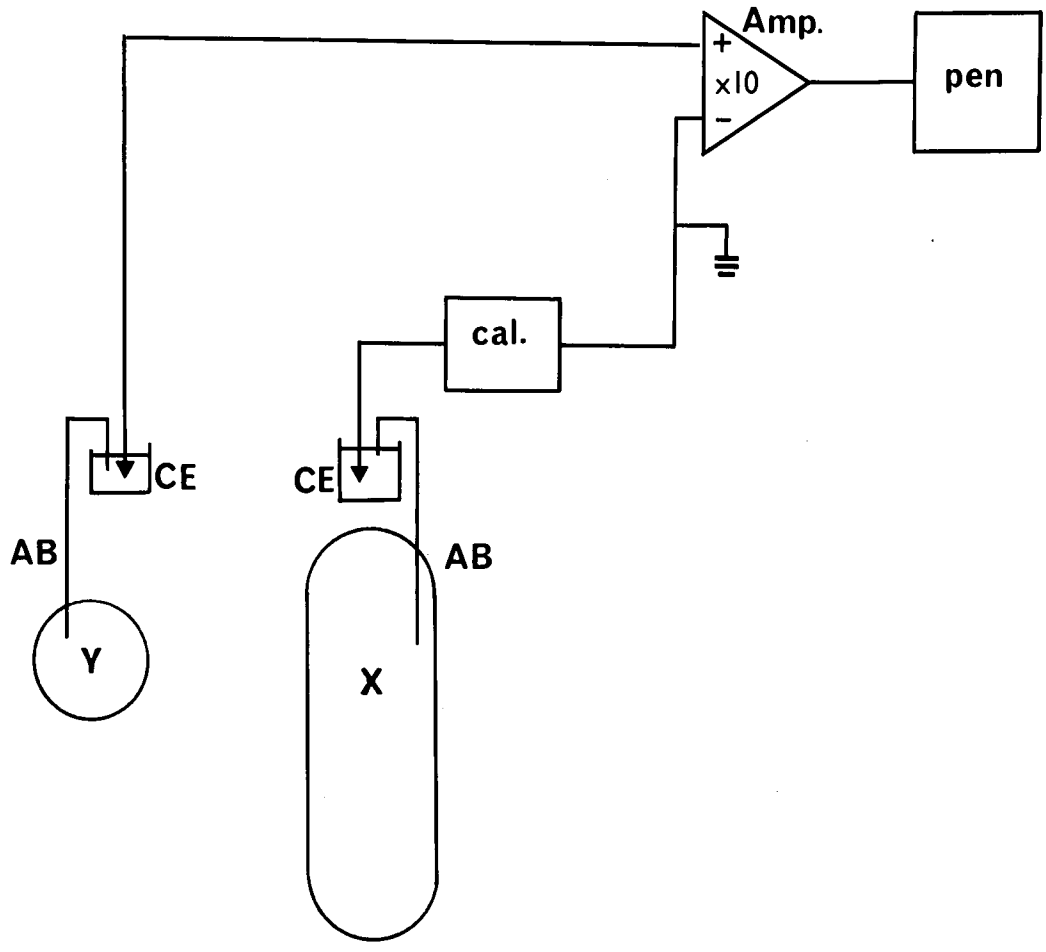
Figure 6.1

Modified chamber used to measure the transepithelial potential across individual Malpighian tubules. The method is described in the text.

Figure 6.2

Diagram of circuit used to measure the transepithelial potential across the Malpighian tubules. AB = agar bridge; CE = calomel electrode; cal = calibrator; Amp = amplifier; pen = pen recorder. X and Y represent the two chambers containing the gut, and an individual severed tubule respectively.


Y
X


Amp.
+
x10
−
pen
cal.
CE
CE
AB
AB
Y
X

15 min to ensure that the potential was stable. The 'normal' Ringer solution around the gut was then replaced with an appropriate experimental solution. The transepithelial potential was continuously recorded for the next 45 minutes. Control experiments were carried out in which the same procedure was followed except the 'normal' Ringer solution was used throughout.

Experimental solutions. The Hepes Ringer solution was used in most experiments. However, an alternative Ringer solution (MADDRELL, 1969) was used in studying the effect of different concentration of Na^+ and K^+ on the transepithelial potential. The composition of the Ringer solutions used has been described previously.

The P value was obtained by the application of paired 't' test to the data for the initial and final potentials.

RESULTS

The Malpighian tubules were set up in 'normal' Hepes Ringer solution as outlined above, and the transepithelial potential was measured across the wall of the tubules. The potential was found to be 8.74 ± 0.29 mV (n = 85) with the lumen being positive with respect to the bathing medium. The P.D. remained stable for at least 1 hour provided the Ringer solution surrounding the gut was changed regularly (approx. every 15 min).

6.1 The effect of HCO_3^- and acetazolamide on the transepithelial potential.

Following the measurement of the transepithelial potential in 'normal' Hepes Ringer solution, the bathing medium was changed for Ringer solution containing various concentrations (10^{-5} - 10^{-3}M) of sodium acetazolamide. All these inhibitor concentrations effected a gradual decrease in P.D., the final potential being significantly different from the initial value (Table 6.1). No such effect was observed with control tubules. The most marked effect on transepithelial P.D. was observed with 10^{-3}M acetazolamide; the potential dropped from an initial value of $+9.7 \pm 1.0$ to $+0.9 \pm 0,9$mV. The mean time for a new stable potential to be reached was 29.8 ± 1.1 min.

When 'normal' Hepes Ringer solution was replaced by HCO_3^- free Ringer solution the potential was not significantly changed ($P>0.1$). The inclusion of 10^{-3}M acetazolamide in the HCO_3^- free Ringer solution resulted in a marked decrease in potential and eventually lumen became negative with respect to the bathing medium, possibly suggesting a somewhat more marked effect compared to that observed with acetazolamide in 'normal' Hepes Ringer (Table 6.1).

TABLE 6.1

Effect of acetazolamide, SCN^-, SO_3^{2-}, and HCO_3^--free Ringer on the transepithelial potential of the Malpighian tubules.

Treatment	n	Initial potential ± S.E.M. (mV)	Final potential ± S.E.M. (mV)	Mean difference ± S.E.M.	Mean time taken to establish new stable P.D.	P
Control	11	+7.6 ± 0.7	+7.4 ± 0.8	-0.5 ± 0.4		>0.1
10^{-5}M acetazomalide	8	+7.5 ± 0.8	+6.1 ± 0.9	-1.4 ± 0.5	33.8 ± 1.5	<0.02
10^{-4}M acetazomalide	15	+7.6 ± 0.8	+4.6 ± 1.3	-2.9 ± 1.0	30.8 ± 0.8	<0.01
10^{-3}M acetazomalide	15	+9.7 ± 1.0	+0.9 ± 0.9	-8.2 ± 0.7	29.8 ± 1.1	<0.001
HCO_3^-- free Ringer	7	+10.1± 1.1	+9.3 ± 1.2	-0.7 ± 0.4	44.1 ± 3.2	>0.1
10^{-3}M acetazomalide in HCO_3^--free Ringer	15	+9.5 ± 0.7	-1.6 ± 0.8	-10.2± 0.8	30.1 ± 1.2	<0.001
10^{-2}M NaSCN	10	+10.1± 1.6	+1.6 ± 2.0	- 7.7± 2.1	22.9 ± 2.6	<0.001
10^{-2}M Na_2SO_3	11	+10.1± 1.2	-1.5 ± 2.4	-13.4± 2.5	31.6 ± 2.3	<0.001

P were obtained by comparing rate 1 and rate 2 values by paired 't' test.

6.2 The effect of thiocyanate on the transepithelial potential

The initial potential was measured in 'normal' Hepes Ringer, then the Ringer solution surrounding the gut was replaced by Hepes Ringer solution containing 10mM NaSCN. Thiocyanate was added by substitution for NaCl. The presence of SCN^- in Ringer solution around the gut decreased the potential (Table 6.1). The mean time for a new stable potential to be established was 22.9 ± 2.6 min, which is a significantly shorter time than the average time observed for acetazolamide in 'normal' Hepes Ringer ($P<0.05$).

6.3 The effect of SO_3^{2-} on the transepithelial potential

As was shown earlier (Chapter 3), sodium sulphite stimulates the Mg^{2+}-dependent ATPase of the locust Malpighian tubules. It was of interest, therefore, to establish the effect of SO_3^{2-} on the transepithelial potential. 10mM SO_3^{2-} was included in the 'normal' Hepes Ringer solution by substitution for NaCl. The transepithelial potential, however, decreased when Ringer solution containing SO_3^{2-} was substituted for 'normal' Hepes Ringer, and eventually the lumen became negative with respect to the bathing medium (Table 6.1).

6.4 The effect of ouabain on the transepithelial potential

When 'normal' Hepes Ringer solution surrounding the gut was replaced by Hepes Ringer solution containing 10^{-3}M ouabain, the potential decreased; the initial value of $+9.1 \pm 0.7$ mV reaching a new stable potential of $+1.8 \pm 0.6$ mV in 27.6 ± 1.0 minutes. The results of these experiments are shown in Table 6.2. Application of a paired 't' test showed that ouabain significantly reduced the

transepithelial potential ($P<0.01$). Whereas, in the case of control, potential had not significantly altered.

6.5 The effect of amiloride on the transepithelial potential

The presence of 1mM amiloride in Hepes Ringer solution rapidly reduced the P.D. The initial potential of ca. + 9.7mV dropped to a new stable value of ca. -4.5mV in less than 10 min (Table 6.2).

Another series of experiments was carried out in which the initial potential was determined in 'normal' Hepes Ringer solution, the Hepes Ringer solution containing 10^{-3}M ouabain was introduced and the new stable potential established (+0.5 $\pm$ 1.3mV). The ouabain-Ringer solution was then replaced with another Ringer solution containing 10^{-3}M amiloride instead of 10^{-3}M ouabain. The presence of 10^{-3}M amiloride in the Ringer solution caused a further reduction in P.D. which reached a new steady state level (of ca. -4.5mV) after 10 min (Table 6.2). These experiments clearly show that the inhibitory effect of amiloride on P.D. is greater than that of ouabain.

6.6 The effect of ethacrynic acid on the transepithelial potential

When 10^{-3}M ethacrynic acid in Hepes Ringer solution was substituted for 'normal' Hepes Ringer solution surrounding the gut, the transepithelial potential dramatically decreased and the lumen became negative (-8.5mV) with respect to the bathing medium. The difference between the initial and final potentials was 17.6 $\pm$ 1.8 mV ($n = 12$), indicating a greater effect than that obtained with the other drugs examined. The time for the establishment of a new stable P.D. was longer than for amiloride, but about the same as for ouabain (Table 6.2).

TABLE 6.2

The effect of ouabain, amiloride, and ethacrynic acid on the transepithelial potential of the Malpighian tubules

Treatment	n	Initial potential ± S.E.M. (mV)	Final potential ± S.E.M. (mV)	Mean difference ± S.E.M.	Mean time taken to establish new stable P.D.	P
Control	11	+7.6 ± 0.7	+7.4 ± 0.8	-0.5 ± 0.4		>0.1
10^{-3}M ouabain	21	+9.1 ± 0.7	+1.8 ± 0.6	-7.3 ± 0.8	27.6 ± 1.1	<0.001
10^{-3}M amiloride	16	+9.7 ± 0.9	-4.5 ± 1.6	-13.8± 1.4	8.8 ± 0.4	<0.001
10^{-3}M ouabain*	12	+9.6 ± 1.1	+0.5 ± 1.3	-9.1 ± 3.9	20.3 ± 1.7	<0.001
10^{-3}M amiloride*	12	+0.5 ± 1.3	-4.6 ± 1.2	-5.1 ± 1.0	9.9 ± 1.1	<0.05
10^{-3}M ethacrynic acid	12	+9.2 ± 0.7	-8.4 ± 1.7	-17.6± 1.8	26.3 ± 1.6	<0.001

P were obtained by comparing rate 1 and rate 2 values by paired 't' test.

* After treatment with ouabain, the Ringer solution containing ouabain was replaced by Ringer solution containing amiloride (for more details see text).

6.7 The effect of Br^- on the transepithelial potential

In chapter 4, it was shown that Br^- can replace Cl^- in Hepes Ringer solution without significantly affecting the rate of fluid secretion. It was of interest, therefore, to establish whether the substitution of Br^- for Cl^- affected the transepithelial potential. The initial potential was determined in 'normal' Hepes Ringer solution, then it was replaced by Cl^- free Ringer (i.e. Br^--Ringer solution). As is shown in Table 6.3, the P.D. across the locust Malpighian tubules did not significantly change.

6.8 The effect of NO_3^- on the transepithelial potential

As was shown earlier (Chapter 4), $NaNO_3$ reduced the rate of fluid secretion by the Malpighian tubules of Locusta and inhibited the Mg^{2+}- dependent ATPase both in the presence of NaCl and $NaHCO_3$ (Chapter 3). To determine whether the transepithelial potential was affected by NO_3^-, experiments were carried out in which all Cl^- in Hepes Ringer solution was replaced by NO_3^- (NO_3^--Ringer solution). When 'normal' Hepes Ringer solution surrounding the gut was replaced by NO_3^-- Ringer solution the P.D. was dramatically reduced; the initial potential of +10.5 $\pm$ 1.1mV settling to a new steady potential of -9.4 $\pm$ 3.4mV in less than 12 minutes (Table 6.3).

6.9 The effect of Na^+ and K^+ on the transepithelial potential

A series of experiments was carried out in which the transepithelial potentials across Malpighian tubules bathed in 'normal' Ringer solution (MADDRELL, 1969), were compared to those obtained with Ringer solution containing different concentrations of Na^+ and K^+. The solutions were adjusted to maintain the total concentration

TABLE 6.3

The effect of Br^- and NO_3^- on transepithelial potential of the Malpighian tubules

Treatment	n	Initial potential ± S.E.M. (mV)	Final potential ± S.E.M. (mV)	Mean difference ± S.E.M.	Mean time taken to establish new stable P.D.	P
Control	13	+8.1 ± 1.1	+8.2 ± 1.3	+0.1 ± 0.7		>0.1
Br^--Ringer solution	14	+9.2 ± 1.0	+8.1 ± 1.1	-1.1 ± 0.7	26.3 ± 1.4	>0.1
NO_3^--Ringer solution	16	+10.5 ± 1.1	-9.4 ± 3.4	-19.9 ± 3.3	11.1 ± 1.9	<0.001

P value were obtained by comparing rate 1 and rate 2 values by paired 't' test

of Na^+ and K^+ at 152mM, and only relative concentrations of the two cations were altered. The results are shown in Table 6.4. In the absence of K^+, the transepithelial potential of the tubules was rapidly reduced, and lumen became negative with respect to the bathing medium in less than 4 min. Increasing the concentration of K^+ in the bathing solution, increased the P.D. such that in the presence of Na^+- free Ringer solution (i.e. in presence of 152mM K^+) the potential increased from +11.1 $\pm$ 0.9mV in 'normal' Ringer to a new stable potential of +35.6 $\pm$ 2.4mV. The relationship between the external K^+ concentration and the increase in lumen positivity, shown in Figure 6.3, is non-linear.

TABLE 6.4

The effect of varying Na^+ and K^+ concentrations on the transepithelial potential of the Malpighian tubules.

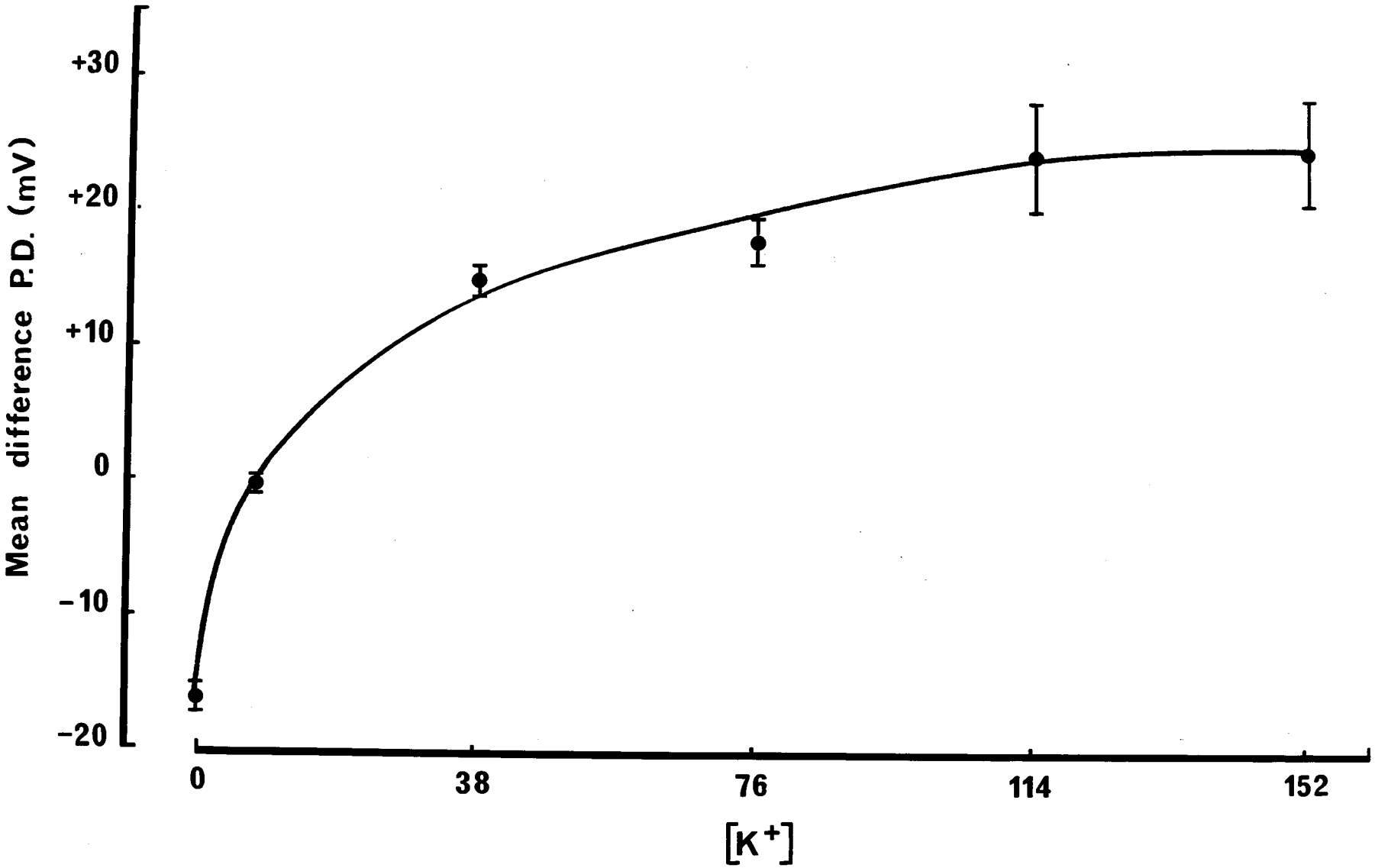
Concentration (mM)		n	Initial potential ± S.E.M. (mV)	Final potential ± S.E.M. (mV)	Mean difference ± S.E.M.	Mean time taken to establish new stable P.D.
K^+	Na^+					
0	152	13	+10.6 ± 0.8	-5.3 ± 1.1	-16.0 ± 1.2	3.4 ± 0.4
8.6	143.4	22	+10.1 ± 1.2	+9.6 ± 1.3	- 0.2 ± 0.5	4.2 ± 0.2
38	114	14	+ 8.4 ± 0.8	+23.1 ± 1.4	+14.7 ± 1.3	2.1 ± 1.6
76	76	15	+11.3 ± 1.0	+29.0 ± 2.5	+17.7 ± 1.9	1.4 ± 0.2
114	38	17	+10.8 ± 1.1	+35.1 ± 2.7	+24.3 ± 2.3	2.1 ± 0.3
152	0	15	+11.1 ± 0.9	+35.6 ± 2.4	+24.5 ± 1.9	1.8 ± 0.3

Figure 6.3

The relationship between K^+ concentration in bathing medium and P.D. across the Malpighian tubules of Locusta.

Ordinate: Mean P.D. across the tubules (mV)

Abscissa: K^+ concentration in the bathing medium (mM).


Mean difference P.D. (mV)
+30
+20
+10
0
−10
−20
0
38
76
114
152
[K+]

DISCUSSION

The transepithelial potential across the Malpighian tubules of Locusta reported in the present study was approximately 9mV with the lumen being positive with respect to the bathing medium. This agrees with the previous value of +10.8mV reported by BELL (1977), whilst RAMSAY (1953) recorded a transepithelial potential of -16mV across the Malpighian tubules of the same species. However, RAMSAY'S data were obtained with in vivo preparations and therefore are difficult to compare with those obtained in the present study and that of BELL (1977). In Table 6.5 the transepithelial potential observed in the present study is compared with data from other studies involving a variety of different species.

The theoretical transepithelial potential for the Malpighian tubules of Locusta bathed in 'normal' Ringer solution can be calculated for both Na^+ and K^+ by applying the NERNST equation. At 30^oC, this may be expressed as:

$$E = -60.1 \log_{10} \frac{\text{concentration in the bathing medium}}{\text{concentration in the lumen}}$$

the potential being given in millivolts (mV).

The concentration of Na^+ and K^+ secreted were determined previously (Chapter 4) by atomic emission spectroscopy and are shown in Table 6.6 in relation to their levels in the bathing medium.

The results obtained by the application of the NERNST equation to these data for Na^+ and K^+ are shown in Table 6.7, where they are

TABLE 6.5

Transepithelial potential values reported for Malpighian tubules of different species.

Species	Potential with respect to the haemolymph (mV)	Reference
Rhodnius	-35	RAMSAY, 1953
Rhodnius	-30	MADDRELL, 1971
Dixippus (*Carausius*)	+21	RAMSAY, 1953
Aedes	+21	RAMSAY, 1953
Dytiscus	+22	RAMSAY, 1953
Pieris	+28	RAMSAY, 1953
Tenebrio	+45	RAMSAY, 1953
Locusta	-16	RAMSAY, 1953
Locusta	+10.8	BELL, 1977
Locusta	+8.7	present study
Tipula	+32	COAST, 1969
Calpodes	+25	IRVINE, 1969
Glomeris (the pill millipede)	-0.75	FARQUHARSON, 1974

compared to those obtained by RAMSAY (1953) and BELL (1977) for the same species. It can be seen that for K^+, in the present study, and that of RAMSAY (1953) and BELL (1977), the P.D. was such that the lumen was considerably more positive than would be predicted by the NERNST equation. One might conclude from this, that locust Malpighian tubules do not obey the NERNST equation for K^+ transport, and that the potential is not the result of passive K^+ permeability. It may be that the potential results from active ion pumping, or alternatively, that the membranes are permeable to more than one ion. In the case of Na^+, the P.D. is less than that predicted by the NERNST equation, in both the present study and that of RAMSAY (1953). This deviation of the predicted NERNST value, again, indicates that Na^+ transport across the tubules is not passive, or as mentioned above, that the potential results from membrane permeability to a variety of other ions. RAMSAY (1955) and TAYLOR (1971) suggested that active Na^+ transport occurs across the Malpighian tubules of Carausius (Dixippus). Whereas, the value recorded for Na^+ by BELL (1977) is close to that calculated by NERNST. She concluded that the transepithelial potential is due to the active pumping of some other ion, probably K^+, which results in a redistribution of Na^+ ions such that they appear to agree with the potential predicted by NERNST.

TABLE 6.6

Concentration of Na^+ and K^+ in the bathing medium and 'urine' (mM).

Ion	Bathing medium	Urine
Na^+	143.4	33.4
K^+	8.6	141.7

TABLE 6.7

Comparison of the observed transepithelial potential across the Locusta Malpighian tubules with the values calculated by NERNST equation for Na^+ and K^+(mV).

Recorded P.D.	Calculated P.D. Na^+	Calculated P.D. K^+	Calculated P.D.-recorded P.D. Na^+	Calculated P.D.-recorded P.D. K^+	Reference
+8.74 ± 0.29 (n = 85)	+38.0	-73.1	+29.3	-81.8	Present study
+10.8 ± 2.1 (n = 74)	+ 4.8	-53.4	+6.0	-64.2	BELL (1977)
-16.0	+ 5.0	-46.0	+21.0	-30.0	RAMSAY (1953)

In all cases the sign indicated is that of the Malpighian tubules lumen with respect to the bathing medium.

The lumen positivity with respect to the bathing medium increased when the concentration of K^+ was increased in the bathing medium. However, NERNST equation does not account for the distribution of this cation across the epithelium, and one might suggest that an active K^+ transport is responsible for the potential. At higher K^+ concentration the transepithelial P.D. reached a plateau. It may be, therefore, that at the higher $[K^+]$ the 'pump' became saturated or alternatively that Na^+ concentration may have become rate limiting (Figure 6.3, Table 6.4). Furthermore, reference to Figure 6.4, shows that for a 10-fold increase in K^+ concentration in the bathing medium the P.D. increased only to the value of 19.25mV not the 60.1mV predicted by the NERNST equation. Whilst this is in agreement with findings of BELL (1977) it is in contrast to the situation reported for Carausius (PILCHER, 1970) and Rhodnius (MADDRELL, 1971) where the P.D. across the Malpighian tubules responded to a 10-fold increase in K^+ concentration in the bathing medium in a manner similar to that predicted by the NERNST equation. These researchers proposed that the basal cell membrane is more permeable to K^+ than Na^+. BERRIDGE et al. (1976) studied the effect of varying the external K^+ concentration on the potential across the basal membrane of Calliphora salivary glands both at rest and during stimulation with 5-HT. These workers observed that the NERNST plot in the presence of 5-HT (53mV) is closer to the theoretical value of 58mV than that obtained in the absence of 5-HT (44mV) and suggested that 5-HT increases K^+ permeability.

In the present study, addition of 10^{-3}M ouabain to the bathing medium significantly reduced the transepithelial potential across the

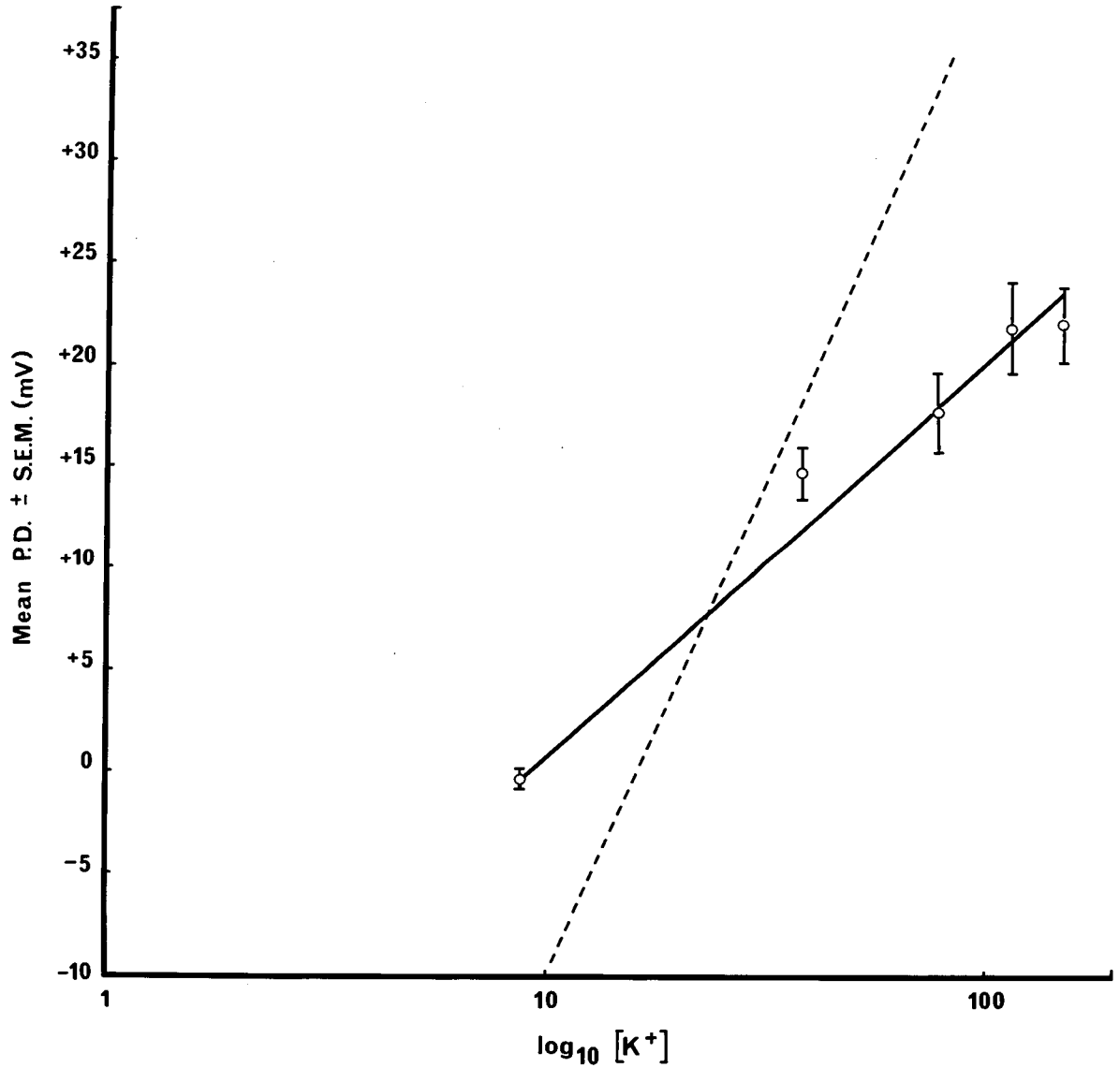
Figure 6.4

Effect of K^+ concentration on the transepithelial potential of Malpighian tubules. The vertical lines represent $\pm$ S.E.M.

Ordinate: Mean difference in the P.D. (mV)

Abscissa: $\log_{10}$ K^+ concentration (mM).

Note: The dashed line is that of a 60.1mV change in P.D. for a 10-fold change in external K^+ concentration predicted by NERNST.


+35
+30
+25
+20
+15
+10
+5
0
−5
−10
Mean P.D. ± S.E.M. (mV)
1
10
100
log10 [K+]

Malpighian tubules. This result together with the above discussion, indicates that active ion transport is responsible for the maintenance of the P.D. and that a Na^+-K^+ATPase is involved in cation secretion. This interpretation is supported by the following observations; (i) fluid secretion by the Malpighian tubules of Locusta is inhibited by ouabain (ANSTEE and BELL, 1975; ANSTEE et al., 1979; DONKIN (personal communication); present study), (ii) Na^+/K^+ ratio in the secreted fluid is affected by ouabain (ANSTEE et al., 1979; present study), and (iii) a Na^+-K^+ATPase enzyme is present in the Malpighian tubules of Locusta (ANSTEE and BELL, 1975, 1978; BELL, 1977). Ouabain has been shown to decrease the transepithelial potential in other tissues. O'RIORDAN (1969) and DATTA (1971) working on cockroach intestine observed that a 50-75% decreased in transepithelial potential when 10^{-4}M ouabain was applied on the haemolymph side. BERRIDGE and SCHLUE (1978) have shown that 10^{-4}M ouabain inhibits the basal membrane potential and decreased the intracellular K^+ level in unstimulated salivary gland (i.e. in absence of 5-HT) of Calliphora. However, on addition of $1x10^{-8}$M 5-HT there was a rapid restoration of both potential and K^+ level to value exceeding those seen in the resting glands. They suggested that there was some indication that in unstimulated glands ouabain could prevent the reaccumulation of K^+ by depleted glands and that a Na^+-K^+ exchange 'pump' is responsible for maintaining high K^+ levels in resting Calliphora salivary glands whereas the potential-dependent mechanism for K^+ entry takes over when large amounts of K^+ are required to generate the rapid flow of saliva during the action of 5-HT. Similarly, PRUSCH (1978) reported that 10^{-5}M ouabain in the external medium of midgut of larval

Sarcophaga bullata brought about a rapid and irreversible drop in the transepithelial potential to zero. Similar results have been reported in vertebrates tissues; toad urinary bladder (CIVAN, 1970); rabbit salivary glands (AUGUSTUS, 1976); rabbit renal cortical collecting tubules (Mc KINNEY and BURG, 1978A,B); rabbit gall bladder (FREDERIKSEN, 1978); Necturus gall bladder (REUSS et al., 1979). In contrast, PILCHER (1970) found that 10^{-4}M ouabain had no effect on the transepithelial potential of Carausius Malpighian tubules.

Sodium acetazolamide, even at low concentrations (10^{-5}M) slightly but significantly reduced the transepithelial potential of locust Malpighian tubules ($P<0.02$). Whilst 10^{-3}M acetazolamide reduced the P.D. almost to zero. Similarly, the inclusion of 10^{-3}M acetazolamide in HCO_3^-- free Ringer caused a marked reduction in P.D. across the tubules. Indeed, under these conditions, the potential became slightly negative. In contrast, HCO_3^--free Ringer alone did not affect the P.D. It may be that sufficient HCO_3^- was generated from respiration to make up for its exclusion from the bathing medium. Sodium acetazolamide is a specific inhibitor of carbonic anhydrase (FISCHBARG and LIM, 1974; LIANG and SACKTOR, 1976; HERRERA et al., 1978; FROMTER, 1979), an enzyme which is known to be present in Locusta Malpighian tubules (Chapter 3). The inhibition of this enzyme by acetazolamide might be expected to decrease the intracellular HCO_3^- concentration and thereby affect the activity of Mg^{2+}-dependent HCO_3^--stimulated ATPase. The observation, therefore, that sodium acetazolamide affects fluid secretion and the transepithelial potential suggest that carbonic anhydrase and perhaps HCO_3^-- stimulated ATPase are involved in fluid and ion transport across the tubule wall. This interpretation is supported by the fact that; (i) the HCO_3^--

stimulated ATPase is present in Locusta Malpighian tubules (ANSTEE and FATHPOUR, 1979; present study) and (ii) fluid secretion by the Locusta Malpighian tubules is inhibited by acetazolamide and SCN^-, the latter being considered a specific inhibitor of Mg^{2+}-dependent HCO_3^--stimulated ATPase (BLUM et al., 1971; SIMON and THOMAS, 1972; DE RENZIS and BORNANCIN, 1977).

Depletion of HCO_3^- from the bathing medium or addition of acetazolamide to Ringer solution have been reported to effect the P.D. in other tissues. HERRERA et al., (1977, 1978) observed that acetazolamide inhibited the transepithelial potential across the rectal wall of Schistocerca gregaria in the absence of HCO_3^- in the bathing medium, however, no effect was observed when HCO_3^- was present in the medium. These workers also reported that P.D. across the rectum decreased in the absence of HCO_3^- in the bathing medium. Similarly, WILLIAMS et al., (1978) reported that acetazolamide (5×10^{-4}M) caused a 25-40% inhibition of short-circuit current (Isc) across the rectum of S. gregaria. GONZALES (1969) and GONZALES and SCHILB (1969) reported that addition of acetazolamide to the serosal bathing medium of turtle bladder or decreasing the concentration of HCO_3^- in mucosal fluid from 20mM to 0.2mM decreased the potential difference. These workers suggested that acetazolamide inhibits the active transport of Cl^- and HCO_3^-. Other studies also demonstrate the importance of HCO_3^- in the maintenance of trans-epithelial potentials. ANAGNOSTOPOULOS and EDELMAN (1977) working on Necturus kidney reported that depletion of HCO_3^- from the bathing medium decreased the P.D. by 50%. Similarly, HODSON and MILLER (1976) working on rabbit corneal endothelium reported that when 'normal' Ringer

solution was replaced by HCO_3^--free Ringer, the transepithelial potential decreased. Addition of carbonic anhydrase inhibitor, ethoxzolamide, has also been reported to reduce the P.D. across rabbit corneal endothelium (HODSON and MILLER, 1976). They concluded that the endothelial P.D. is generated by electrogenic HCO_3^- transport. CHEN and WALSER (1977) observed that addition of acetazolamide or replacement of HCO_3^- by phosphate in the bathing medium led to a significant reduction in the transepithelial potential across toad bladder. They concluded that HCO_3^- facilitate active Na^+ transport and also may be actively transported from serosa to mucosa in this species. In contrast, FISCHBARG and LIM (1974) and Mc KINNEY and BURG (1978A,B) reported that acetazolamide had no significant effect on transepithelial potential across the corneal endothelium and cortical collecting tubules of rabbit respectively.

In the present study, the presence of 10mM SCN^- in the bathing medium decreased the P.D. across locust Malpighian tubules. As discussed above, SCN^- inhibited fluid secretion (Chapter 4) and Mg^{2+}-dependent HCO_3^--stimulated ATPase (Chapter 3) of Locusta Malpighian tubules. This effect of SCN^- on P.D. may be taken as further support for HCO_3^--stimulated ATPase involvement in fluid and ion secretion. HARRIS and EDELMAN (1959) studied the effect of SCN^- on P.D. across the frog gastric mucosa in the presence of different concentration of K^+ in the submucosal solution. These workers concluded that the progressive decline in P.D. during exposure to low K^+-SCN^- solutions, may reflect inhibition of the active anion transport mechanism when faced simultaneously with abnormally low K^+ and an abnormal anion.

FORTE and DAVIES (1964) observed that SCN^- caused a rapid increase in the transmucosal potential difference across the bullfrog gastric mucosa.

Replacement of Cl^- in the Hepes Ringer solution by Br^-, did not change the transepithelial potential across the tubules. This result is consistent with the earlier finding that replacement of Cl^- with Br^- did not affect the rate of fluid secretion by locust Malpighian tubules. In contrast, substitution of Cl^- in the Hepes Ringer solution by NO_3^- caused a rapid and dramatic fall in transepithelial potential across the tubule, the initial potential of +10.5mV fell to -9.4mV. Once again, this result is consistent with the earlier observation that NO_3^- inhibited fluid secretion (Chapter 4). It is perhaps significant that whilst Br^- did not affect Mg^{2+}-dependent HCO_3^--stimulated ATPase, NO_3^- did inhibit this enzyme. These observations, together with the effects of SCN^- reported earlier suggest that a HCO_3^--stimulated ATPase is involved in ion and fluid secretion by Malpighian tubules of Locusta. Recently, HERRERA et al., (1976) working on rectal wall of Schistocerca gregaria observed that by substituting a non-penetrating anion such as SO_4^{2-} for Cl^- in the bathing medium, the P.D. fell and disappeared in a few minutes. These workers suggested that transepithelial potential across the rectal wall of S. gregaria are mainly dependent on Cl^- fluxes, and HCO_3^- has an important role in active Cl^- transport, either, because it directly stimulates Cl^- pumping, or, because it aids Cl^- entrance to the cells through an exchange with the outflux of HCO_3^- (HERRERA et al., 1976, 1977). HERRERA et al., (1978) on the basis of their studies on the effects of HCO_3^- on P.D. concluded that effect of HCO_3^- on Cl^- transport is

a direct action on the mechanism of this transport. Their results are supported by those of WILLIAMS et al. (1978).

10mM sodium sulphite which stimulated the Mg^{2+}- dependent HCO_3^-- stimulated ATPase (Chapter 3), reduced the transepithelial potential across the locust Malpighian tubules. The interpretation of this effect is unexplained.

Presence of 1mM ethacrynic acid in the bathing medium markedly decreased the transepithelial potential across the tubules; thus the initial potential of +9.2mV fell to -8.4mV. Similar results have been reported elsewhere. BERRIDGE et al. (1976) working with Calliphora salivary glands have shown that ethacrynic acid abolished the positive phase of the transepithelial P.D. which has been attributed to 'pump' activity. They suggest that an active cation 'pump' which may be electrogenic, and which is inhibited by ethacrynic acid, is situated in the apical cell membrane. CASE et al. (1973) reported that ethacrynic acid significantly reduced the P.D. across isolated toad bladder. BURG and GREEN (1973) observed that when ethacrynic acid was present in the perfusate solution or in the lumen of rabbit cortical thick ascending limbs of Henle's loop, it decreased the P.D. They suggested that although ethacrynic acid has a number of different biological actions (as was discussed in Chapter 5), the drug was, in this case, most likely inhibiting active transport of Cl^- across the epithelium of the cortical thick ascending limbs of Henle's loop. In contrast, FREDERIKSEN (1978) reported that high concentrations of ethacrynic acid (10^{-3}M) when applied to the mucosal side of the rabbit gall bladder epithelium caused a small and gradual increase in the mucosa positive P.D., in spite of the marked inhibitory effect on

fluid transport rate. However, the same concentration of inhibitor when applied to the serosal side reduced the P.D. by 40% after 1.5 hour. As was discussed earlier (Chapters 3,5 and above), ethacrynic acid has a variety of effects on cell-metabolism including interference with the transport of different ions across the cell membranes of various cells. It may be, therefore, that the effect of ethacrynic acid on P.D. across Locusta Malpighian tubules is due to the inhibition of Na^+-K^+ exchange 'pump' or perhaps the transport of Cl^-; or both the Na^+-K^+ exchange 'pump' and Cl^- transport. However, whether active Cl^- transport occurs in Locusta tubule remains to be established.

In the present study, the presence of 1mM amiloride in the bathing medium caused a fall in transepithelial potential across the Malpighian tubules of Locusta. It was observed that amiloride effected a greater change of P.D. than ouabain; replacing Ringer solution containing ouabain with the same Ringer solution including 1mM amiloride caused a further reduction (about 5mV) in P.D. across the tubules. Mc KINNEY and BURG (1978A,B) observed that addition of amiloride to the perfusate fluid of rabbit cortical collecting tubules caused the P.D. immediately to reverse from negative to positive (on the lumen side). KNAUF (1973), working on rat salivary duct, reported that during perfusion with HCO_3^- - Ringer solution containing amiloride, the P.D. was abolished. Similarly, BENTLEY (1968) and SUDOU and HOSHI (1977) working on toad bladder reported that when amiloride was placed at the mucosal surface, the P.D. decreased rapidly. They concluded that amiloride inhibits Na^+ permeation across the mucosal

cell membrane. Similar results have been reported for frog skin epithelia where it is suggested that amiloride inhibits Na^+ influx at the apical cell membrane (NAGEL and DORGE, 1970). Recently, EHRENSPECK et al., (1978) studied the effect of amiloride on turtle bladder in the presence and absence of HCO_3^- and Cl^- in the bathing medium. They observed that 10^{-4}M amiloride in HCO_3^--rich bathing medium increased the P.D. whereas in HCO_3^--free bathing medium the P.D. did not alter. These researchers concluded that amiloride stimulates the active reabsorption of HCO_3^- and this effect is independent of its action on Na^+ transport. BERRIDGE et al., (1976) working on salivary glands of Calliphora reported different results from that observed in the present study. This is almost certainly related to the different conditions under which their experiments were carried out. They showed that the introduction of 5-HT into the bathing medium effected a rapid drop in the potential difference across the tubules. The addition of amiloride, in the presence of 5-HT, caused the P.D. return to the resting value (i.e. the value observed before the addition of 5-HT). These workers suggest that, although, amiloride may inhibit fluid secretion by interfering with the movements of cations across the basal membrane, in the case of Calliphora salivary glands, there are some indications that it may inhibit the influx of Ca^{2+}. In addition, it is suggested that by blocking Ca^{2+} influx, amiloride will reduce the permeability of the basal and apical membranes to Cl^- thus inhibiting the normal secretory and potential responses. In the case of locust Malpighian tubules as was discussed earlier (Chapters 4,5), the effect of amiloride maybe explained in terms of Na^+-K^+ exchange which would be consistent with the involvement of

Na^+-K^+ ATPase in fluid and cation secretion. However, an effect of amiloride on HCO_3^- transport and possibly Cl^- transport similar to that reported above cannot be excluded at this stage.

Conclusion. The results of electrophysiological work on Malpighian tubules of Locusta suggest that the P.D. across the tubule epithelium does not obey the NERNST equation for K^+. This observation, together with the effect of various inhibitors (e.g. ouabain), suggests that the potential difference results from active K^+ pumping. The evidence available suggests the possibility that HCO_3^--ATPase may also be involved in ion transport across the Malpighian tubule epithelium.

CHAPTER 7

Conclusion

The present study has confirmed the presence of a Na^+-K^+ ATPase in microsomal preparations of the Malpighian tubules of Locusta migratoria L. and that this enzyme is involved in cation and fluid transport across the tubules (ANSTEE and BELL, 1975, 1978; PEACOCK, 1975; BELL, 1977). Na^+-K^+ ATPase activity has also been demonstrated in the Malpighian tubules of Schistocerca gregaria and Jamaicana flava (PEACOCK et al., 1972), and Homorocoryphus nitidulus (PEACOCK et al., 1976). However, as described previously the involvement of this enzyme in fluid and cation secretion across the Malpighian tubules of insects is in controversy. The main disagreement seems to arise from the failure of some researchers to demonstrate that tubule secretion is sensitive to the specific Na^+-K^+ ATPase inhibitor, ouabain. This controversy has been reviewed by ANSTEE and BOWLER (1979) and has also been described earlier (Chapters 4 and 6). In the present study ouabain clearly inhibited fluid and cation secretion although the period of pre-incubation in the presence of ouabain was clearly important. Further support for the involvement of Na^+-K^+ ATPase in the locust Malpighian tubule secretion was provided from electrophysiological studies; ouabain caused a substantial reduction of the trans-epithelial potential, indicating decreased ion transport. Similarly, Malpighian tubule oxygen consumption was inhibited by the inclusion of ouabain in the bathing medium. This effect was ascribed to decreased demand for ATP as a result of reduced ion pumping

(see Chapter 5).

In common with a number of other species which have been studied (e.g. Rhodnius, RAMSAY, 1953; Carausius, MADDRELL, 1971, 1972; Schistocerca, MADDRELL and KLUNSUWAN, 1973), K^+ was found to be the major cation transported in preference to Na^+ even when present at much lower concentrations in the bathing medium. However, as ANSTEE and BELL (1975) reported both K^+ and Na^+ are necessary for maximal fluid secretion. This has also been shown to be the case in Calliphora (BERRIDGE, 1968) and Carausius (PILCHER, 1970). The necessity for the presence of both Na^+ and K^+ in the bathing medium might be taken as further evidence to support the involvement of a Na^+-K^+ exchange 'pump' in fluid secretion (BERRIDGE, 1968; ANSTEE and BELL, 1975).

In the present investigation the transepithelial P.D. was shown to increase in lumen positivity with increasing the concentration of K^+ in the bathing medium. However, there was not a significant increase in potential associated with increasing K^+ concentration above 114mM. Application of the NERNST equation by plotting $\log [K^+]_o/[K^+]_i$ ($[K^+]_o = [K^+]$ in the bathing medium and $[K^+]_i = [K^+]$ in the urine) against mean difference in P.D. gave a line with a slope of 21.20mV for a 10-fold change in K^+ concentration gradient (Figure 7.1). This value is clearly different from the value of 60.1mV predicted by the NERNST equation and suggests, once again, that either the membrane is permeable to more than one ion or that K^+ transport is active. It would seem, therefore, that the evidence available is consistent with the suggestion that in Locusta Malpighian tubules, the deviation

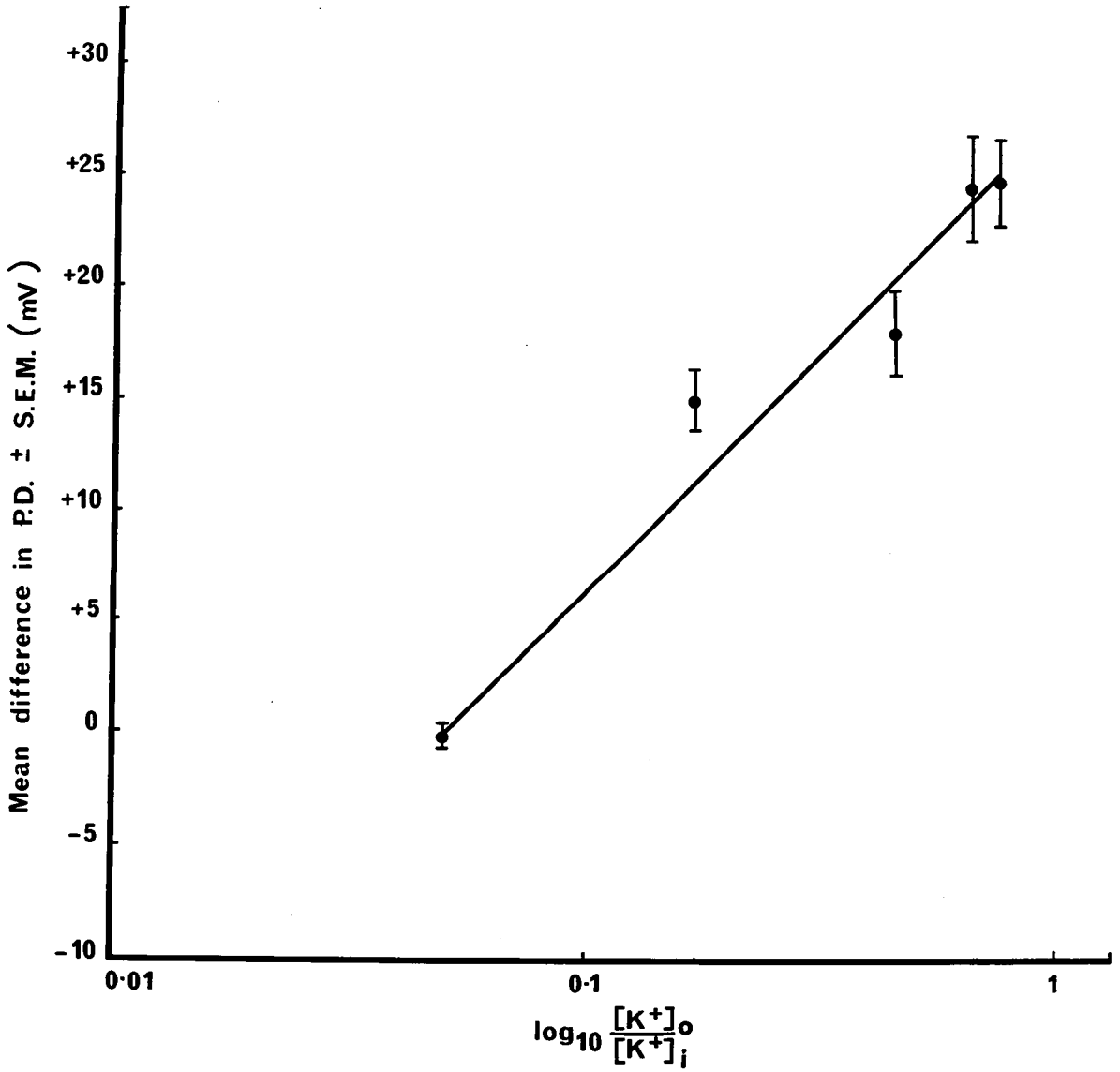
Figure 7.1

The relationship between the transepithelial potential across the Malpighian tubules and the ratio of external to internal K^+ concentration. The vertical lines represent $\pm$ S.E.M.

Ordinate: Mean difference in potential difference across the tubules when compared to that recorded in 'normal' Ringer solution.

Abscissa:

$$\log_{10} \frac{\text{external } K^+ \text{ concentration (Ringer solution)}}{\text{internal } K^+ \text{ concentration (urine)}}$$


+30
+25
+20
+15
+10
+5
0
-5
-10
Mean difference in P.D. ± S.E.M. (mV)
0·01
0·1
1
log10 [K+]o / [K+]i

from the NERNST equation is due to active K^+ pumping by a Na^+-K^+ ATPase 'pump'.

More recently, MADDRELL (1977) proposed an alternative and rather different model to explain the secretion of fluid by the cells tubules. This model attempts to explain how one basic mechanism can account for the secretion of Na^+ rather than K^+ in different species of insect. He suggests that insect Malpighian tubules possess an electrogenic cation 'pump' on the membrane facing the lumen and that this 'pump' has a higher affinity for Na^+ than K^+. The 'pump' would act to maintain the intracellular level of Na^+ lower than that of K^+. The actual rate at which cations are pumped across the tubules from the bathing solution into the lumen by this 'pump' depends not only on the affinity of the 'pump' for the two cations but also how fast these cations enter the cell. This depends partly on the electrochemical gradients across the basal cell membrane facing the bathing solution and also on the permeability of this membrane to these ions. MADDRELL (1977) has confirmed the earlier suggestions by PILCHER (1970) and MADDRELL (1971) that the basal cell membranes of the Malpighian tubules of Carausius and Rhodnius are more permeable to K^+ than Na^+. This has been achieved by studying the effect of changes in K^+ concentration on the P.D. across the basal cell membranes of Rhodnius Malpighian tubules. Whilst, the electrochemical gradient favouring Na^+ movements into Malpighian tubule cells may be steeper than those favouring K^+ entry, K^+ movements may occur at comparable rates because of the higher K^+ permeability. Possibly then, comparatively small changes in the relative permeability of the

basal membrane to Na^+ and K^+ may cause large changes in the ionic composition of the fluid secreted by Malpighian tubules. The ability of tubules from Glossina to secrete a Na^+ rich fluid at a high rate, for example, might be simply explained by their having a higher permeability to Na^+ than have other tubules; as a result Na^+ enter faster than do K^+ and as the 'pump' has a higher affinity for Na^+ in any case, it is these ions which are transported. In contrast, in K^+-secreting tubules, K^+ enters the cell faster than Na^+, Na^+ being virtually excluded, and so it is K^+ which is transported. On this view the very slow secretion by tubules in K^+-free media follows from the very slow entry of Na^+ into the cells. MADDRELL (1977) has presented evidence to suggest that the apical cation 'pump' does indeed show a higher affinity for Na^+ than K^+. Using radioactive Na^+ and K^+ tracers, he measured the intracellular concentrations of these ions and at the same time determined the Na^+ and K^+ levels in the secreted fluid and the bathing medium. In this manner he showed that Na^+ transport is much faster than K^+ transport at low intracellular levels. The extent to which such a model for cation transport might be applicable to all insect Malpighian tubules remains to be established. However, comparison between this model and previous ones proposed by the same author (MADDRELL, 1971) reveal one or two inconsistencies. For example, in earlier models, he proposed a Na^+-stimulated electrogenic K^+ 'pump' situated on the basal cell membrane which was not a Na^+-K^+ ATPase. This was suggested to account for the lack of ouabain sensitivity of the Malpighian tubules of Calliphora

and Carausius. In the more recent model, MADDRELL (1977) suggests that Na^+ and K^+ enter the cells passively across the basal membrane. However, it is difficult to see how this form of cation entry is consistent with certain observed facts. It has been shown that Na^+ can markedly stimulate the rate of K^+ secretion and fluid secretion (BERRIDGE, 1968; MADDRELL, 1971). This fact has been discussed by BERRIDGE (1968) and has been taken as strong support for the presence of a Na^+-K^+ exchange 'pump' on the basal membrane of tubule cells. A second objection to passive cation entry is one raised by MADDRELL himself (1971; see Chapter 1), viz. that due to the higher concentration of K^+ in the cytoplasm compared with that of the bathing medium, it is difficult to see how K^+ entry into the cell can be passive.

In the present work, the importance of HCO_3^- in 'urine' production was indicated by the fact that both the absence of this anion from the bathing medium or the presence of the carbonic anhydrase inhibitor, acetazolamide, inhibited 'urine' production by 24 and 40% respectively. GOODING (1975) observed a similar effect with acetazolamide on Glossina Malpighian tubules, whereas, BERRIDGE (1968) and MADDRELL (1969) found that this inhibitor did not affect fluid secretion in Calliphora or Rhodnius tubules. As discussed earlier, this might be related to the time allowed for pre-incubation in the presence of inhibitor. Alternatively, it may be that the mechanism of tubule function varies from one insect species to another. Since acetazolamide inhibits fluid secretion and since carbonic anhydrase has been demonstrated in

locust Malpighian tubules (Chapter 3), one might conclude that this enzyme has a role in 'urine' production. Further support for this proposal is provided from studies in which sodium acetazolamide inhibited P.D. and oxygen consumption.

The present investigation has identified the presence of a Mg^{2+}-dependent HCO_3^--stimulated ATPase enzyme in the Locusta Malpighian tubules which appears to have a role in fluid production across the tubules. This enzyme is present in both mitochondrial and microsomal fractions of this tissue. However, despite the application of different inhibitors, the determination of mitochondrial marker enzyme (SDH) activity in the different subcellular fractions, and electron microscopy it is not possible to state with absolute confidence the source of the membranes exhibiting HCO_3^--stimulated ATPase activity. Nevertheless, the evidence available suggests that HCO_3^--stimulated ATPase activity is not exclusively associated with the mitochondrial membranes. The fact that there is no specific inhibitor reported for this enzyme, makes it difficult to assess its role in secretory processes. However, some investigators suggest that SCN^- may be taken as a specific inhibitor of the Mg^{2+}-dependent HCO_3^--stimulated ATPase enzyme (KASBEKAR and DURBIN, 1965; BLUM et al., 1971; SIMON and THOMAS, 1972; DE RENZIS and BORNANCIN, 1977). If this is true, the inhibition of fluid by SCN^- strongly implicates this enzyme in 'urine' production. The fact that SCN^- reduces the transepithelial P.D. is further support for this suggestion. More recently, 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 electro-neutrality 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.

Chloride ions play an important role in fluid secretion by Malpighian tubules of Locusta. The total replacement of Cl^- by NO_3^- in the bathing medium reduced the rate of Malpighian tubules 'urine' production by 60%. Similar substitution reduced the P.D. across the tubules from +10.5 mV to -9.4 mV. This suggests that either Cl^- transport is active or absence of Cl^- and presence of NO_3^- in the bathing medium indirectly affected active transport of other ions. The importance of Cl^- transport has been studied in the Malpighian tubules of Rhodnius (MADDRELL, 1971, 1972). This investigator proposed that these tubules have a Cl^- 'pump' which might be situated on the apical side of the cells. However, in the light of his recent studies based on membrane potentials across the basal and apical side of the cells, MADDRELL (1977) suggests that the basal side of the cell now seems a more likely site for Cl^- 'pump'. He stated that typically the cellular interior is at a potential about 50 mV negative to the bathing solution while the lumen is some 20 mV positive to the

cell interior so that Cl^- may well follow cation transport passively from inside the cell into the lumen but may have to be pumped into the cell against the electrical gradient. As MADDRELL points out, this interpretation rests on the assumption that the intracellular Cl^- level is lower than in the bathing and luminal fluids. This assumption is supported by the studies of GUPTA et al. (1976). These workers revealed that the intracellular Cl^- concentration of Rhodnius Malpighian tubules bathed in dextran-Ringer solution ($[Cl^-]$ = 160 mM) is 31 $\pm$ 4 mM Kg^{-1} wet wt, in the cytoplasm and 119 $\pm$ 14 mM Kg^{-1} wet wt in the lumen. Somewhat similar results have been reported for Calliphora Malpighian tubules (GUPTA et al., 1977). In addition, MADDRELL (1977) reported that when the concentration of Cl^- in the bathing solution decreased from 155 mM to 9 mM ($SO^{2}_{4}{}^-$ replacing Cl^-) the P.D. across the basal side of the cell decreases by 3-4 mV, confirming his suggestion that a Cl^- 'pump' is located on the basal cell membrane. Such a Cl^- 'pump' has also been shown to be present in the apical cell membrane of Schistocerca gregaria rectum (HERRERA et al., 1976, 1977; WILLIAMS et al., 1978). This 'pump' is stimulated by HCO_3^- and a HCO_3^-/Cl^--ATPase enzyme has been demonstrated in this tissue (HERRERA et al., 1978). These workers suggested that the 'pump' either is a Cl^-/HCO_3^- exchange or HCO_3^- is directly affecting the Cl^- transport.

Although the importance of Cl^- in fluid secretion has been demonstrated in the present work, it is not possible, at this stage, to suggest that a Cl^- 'pump' is present in this tissue. At present it is only possible to speculate on the relationship

between Cl^- transport and HCO_3^--stimulated ATPase in tubule secretion. The present study demonstrates the presence of two enzymes, carbonic anhydrase and Mg^{2+}-dependent HCO_3^--stimulated ATPase in the Malpighian tubules of Locusta. It has been shown that in those tissue in which HCO_3^--stimulated ATPase is involved in ion transport (SIMON et al., 1972B; HEGNER and ANIKA, 1975; LIANG and SACKTOR, 1976; FROMTER, 1979), carbonic anhydrase is usually implicated. This enzyme is thought to play a major role in providing enough HCO_3^- for stimulation of the HCO_3^--stimulated ATPase. Taking this into account, together with the fact that substitution of Cl^- by NO_3^- decreased HCO_3^--ATPase activity, and also, fluid secretion and the transepithelial P.D., it is tempting to suggest that the HCO_3^--ATPase may be acting as a Cl^-/HCO_3^- exchange 'pump'. Clearly more information is needed before the exact roles of carbonic anhydrase, Mg^{2+}-dependent HCO_3^--stimulated ATPase and Na^+-K^+ ATPase in ion and fluid secretion are established. At this stage, it is possible to conclude that there is evidence to suggest that these enzymes are involved in the Malpighian tubule secretory mechanism(s).

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APPENDIX 3.1

Standard calibration curve for inorganic phosphate. (The method for preparing the calibration curve was described in Materials and Methods, Chapter 3).

Figure A.3.1

Standard curve for inorganic phosphate concentration against absorbancy.

Ordinate: Optical density 390nm.

Abscissa: Phosphate concentration as nmoles.

(line drawn by eye).

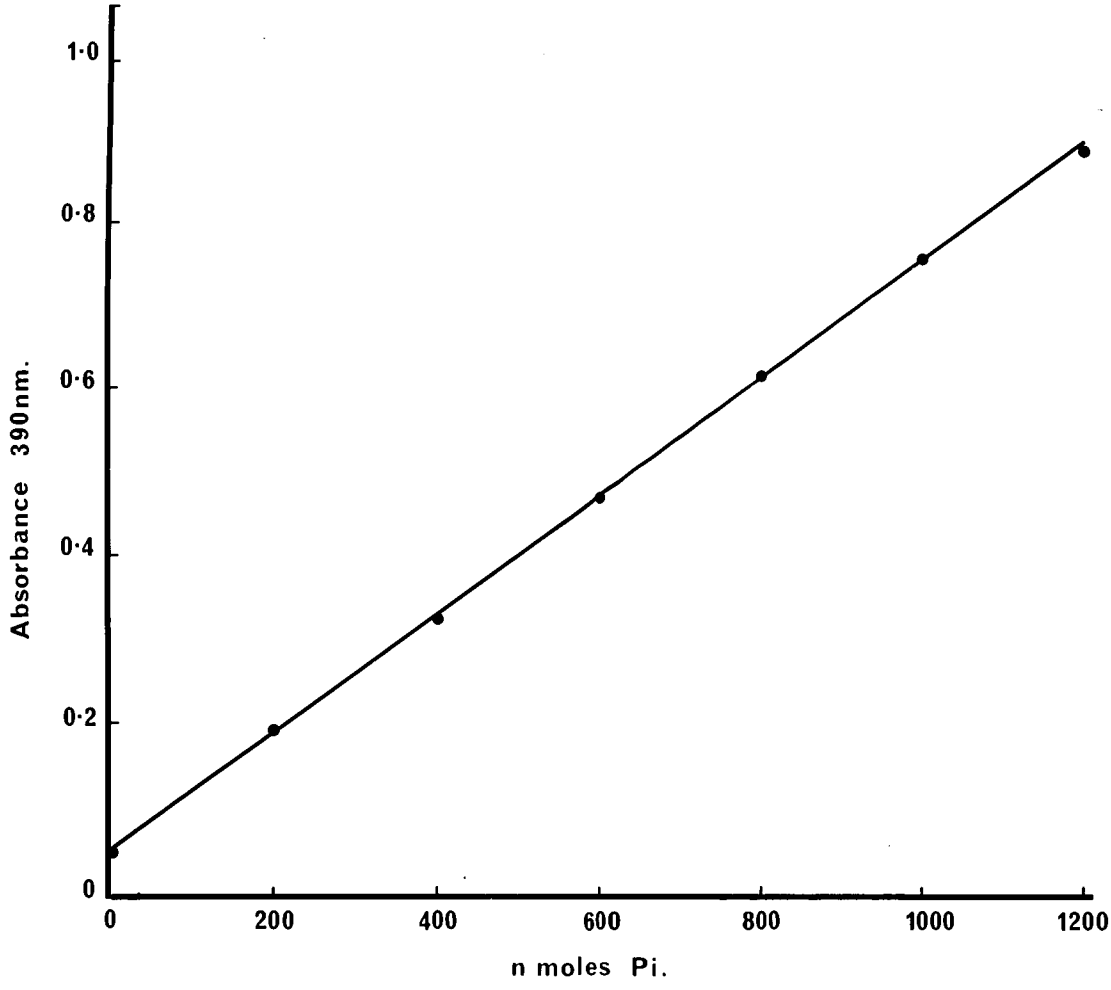
Figure A.3.2

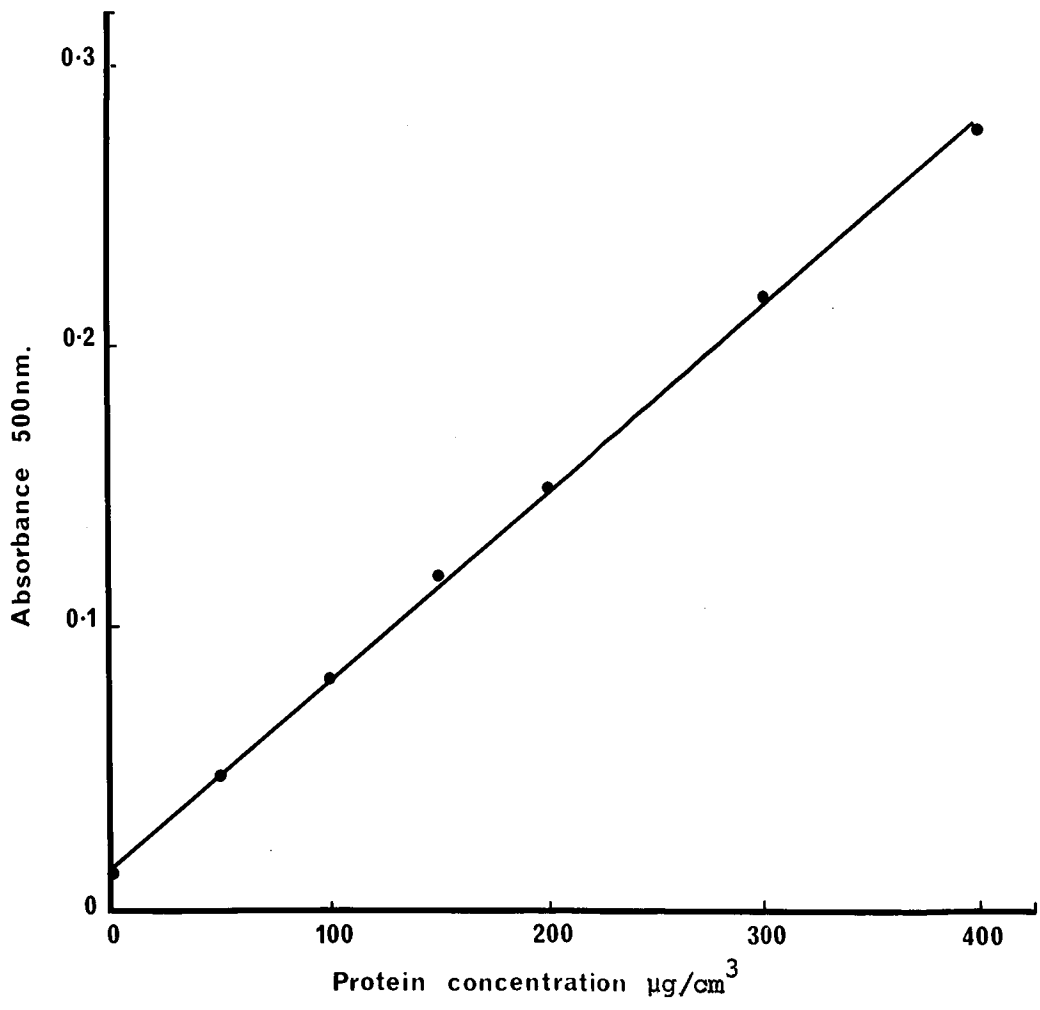
Standard calibration curve for protein assay against optical density.

Ordinate: Optical density 500nm.

Abscissa: Protein concentration as $\mu g/cm^3$.

(line drawn by regression analysis)


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


0·3
0·2
0·1
0
Absorbance 500nm.
0
100
200
300
400
Protein concentration µg/cm3

Appendix 3.2

Folin's method for protein assay (Lowry, 1951)

Bovine serum albumin (BSA), Fraction V was serially diluted to give a concentration of 0, 50, 100, 150, 200, 300, 400 mg/cm^3 protein for standard calibration curve.

1. Reagents

(i) 2% wt/vol. sodium carbonate in 0.1N sodium hydroxide

(ii) 0.5g% cupric sulphate

(iii) 1% potassium, sodium tartrate.

2. Folin A mixture

Equal volumes of (ii) and (iii) were mixed and to 1cm^3 of this mixture, 100cm^3 of solution (i) was added.

3. Folin B mixture

4 volumes of Folin and Ciocalteu's phenol reagent diluted with 6 volumes of deionized water.

4. Experimental procedure

To 0.2cm^3 of protein solution (homogenate or BSA), 3cm^3 of Folin A was added in a test tube and it was left at room temperature for 30 min. Then 0.3cm^3 of Folin B was added and the mixture was allowed to stand at room temperature for another 60 min. The mixture was poured into a glass cuvette and the absorbancy measured at 500nm on a Pye Unicam Spectrophotometer. The protein concentration of the homogenates were determined by reference to a standard calibration curve relating absorbancy to protein concentration (Lowry, 1951). A new calibration curve was made each time a protein assay was carried out. A typical standard curve is shown in Figure A.3.2.

APPENDIX 3.3

Preparation of the Tris salt of adenosine triphosphate (ATP)

Tris ATP was prepared from the sodium salt of the nucleotide using an ion exchange Dowex resin. The Dowex resin was well rinsed with distilled water in a Buchner funnel and the wet weight was noted. The resin was then washed in 3N HCl (AnalaR), using 150cm^3 acid per 25g wet weight of resin. This was followed by washing 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 the resin was in H^+ form. It was then resuspended in distilled water and stored at 0-4°C until required.

A known quantity of Na_2-ATP was dissolved in approximately 10cm^3 of deionized water and this was added to a small quantity of resin (H^+ form) and both were thoroughly mixed using a Whirlmixer (Fisons Scientific apparatus). The resin was allowed to sediment and the supernatant was removed and retained. Then the resin was washed three times with a little deionized water, mixing each time to ensure the removal of all the ATP from the Dowex. The supernatant was pooled each time. The ATP was now in the H^+ form. It was converted to the 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 in boiling tubes at -20°C.

APPENDIX 3

TABLE 1

Effect of pH on ATPase activity

pH	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
7.0	290.0 ± 56.2	276.1 ± 24.8	398.6 ± 20.9
7.5	333.0 ± 61.1	306.5 ± 41.0	441.6 ± 21.7
8.0	296.6 ± 47.1	293.6 ± 26.6	437.8 ± 32.4
8.5	213.0 ± 24.6	268.6 ± 23.0	381.6 ± 41.8
9.0	154.6 ± 18.8	189.6 ± 41.0	311.8 ± 38.5

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of six experiments $\pm$ S.E.M.

APPENDIX 3

TABLE 2

Effect of sodium acetazolamide on ATPase activity (100,000g fraction)

Concentration of acetazolamide (M)	Mg^{2+}-dependent ATPase activity			Stimulation due to the presence of HCO_3^- instead of Cl^-
	-NaCl	+NaCl	+$NaHCO_3$	
0	231 ± 12	223 ± 18	297 ± 15	73.3 ± 15.2
10^{-7}	223 ± 12	222 ± 19	289 ± 17	75.0 ± 10.4
10^{-6}	225 ± 13	219 ± 20	296 ± 21	76.6 ± 16.4
10^{-5}	227 ± 13	219 ± 18	293 ± 17	73.6 ± 11.0
10^{-4}	232 ± 12	220 ± 15	295 ± 15	75.0 ± 8.9
10^{-3}	242 ± 18	217 ± 18	295 ± 15	78.0 ± 6.9

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

APPENDIX 3

TABLE 3

Effect of sodium acetazolamide on ATPase activity (20,000g fraction)

Concentration of acetazolamide (M)	Mg^{2+}-dependent ATPase activity			Stimulation due to the presence of HCO_3^- instead of Cl^-
	-NaCl	+NaCl	+$NaHCO_3$	
0	453.0 ± 42.8	476.6 ± 47.7	607.0 ± 97.2	138.6 ± 47.8
10^{-7}	456.6 ± 32.5	474.0 ± 44.8	621.3 ± 104.8	150.3 ± 63.1
10^{-6}	453.0 ± 30.1	478.6 ± 40.0	611.0 ± 95.8	132.3 ± 56.9
10^{-5}	455.0 ± 34.8	475.6 ± 46.0	617.0 ± 100.0	141.3 ± 54.3
10^{-4}	456.6 ± 34.3	481.6 ± 47.4	623.3 ± 101.2	141.3 ± 53.8
10^{-3}	471.6 ± 42.1	470.3 ± 40.9	616.3 ± 87.4	146.0 ± 49.5

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

APPENDIX 3

TABLE 4

Effedt of sodium thiocyanate on ATPase activity

Concentration of NaSCN (mM)	Mg^{2+}-dependent ATPase activity			Stimulation due to the presence of $NaHCO_3$ instead of NaCl
	-NaCl	+NaCl	$+NaHCO_3$	
0	209.6 ± 61.7	212.6 ± 62.8	318.3 ± 92.3	105.7 ± 30.5
0.5	129.0 ± 40.1	147.6 ± 46.6	251.3 ± 73.6	103.6 ± 26.4
1	99.0 ± 31.1	115.6 ± 37.7	206.0 ± 63.0	90.0 ± 26.0
5	44.6 ± 13.8	58.6 ± 17.4	119.3 ± 36.3	60.6 ± 19.8
10	37.6 ± 14.2	40.6 ± 17.7	74.3 ± 26.1	33.6 ± 8.3

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

APPENDIX 3

TABLE 5

Effect of ATP concentration on ATPase activity

Concentration of ATP (mM)	Mg^{2+}-dependent ATPase activity		Stimulation of activity due to the presence of $NaHCO_3$ instead of NaCl
	+NaCl	$+NaHCO_3$	
3.0	249.2 ± 39.9	376.0 ± 43.8	132.4 ± 6.5
1.0	195.0 ± 27.0	288.6 ± 34.1	97.4 ± 8.8
0.5	165.0 ± 20.0	248.6 ± 22.8	83.4 ± 7.9
0.3	153.0 ± 19.4	205.4 ± 21.3	56.2 ± 11.3
0.2	130.6 ± 14.0	167.8 ± 16.8	39.8 ± 7.8
0.15	114.6 ± 12.7	144.2 ± 16.0	31.2 ± 5.2
0.12	101.8 ± 10.7	124.4 ± 12.7	24.2 ± 4.1
0.10	91.0 ± 9.4	108.2 ± 11.5	18.4 ± 3.1

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

APPENDIX 3

TABLE 6

Effect of SO_3^{2-} concentration on ATPase activity

Concentration of SO_3^{2-} (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+ NaCl	+$NaHCO_3$
0	232.2 ± 33.1	238.2 ± 29.7	348.0 ± 52.4
0.2	304.2 ± 62.0	304.7 ± 53.1	362.2 ± 54.9
0.5	372.5 ± 70.5	351.5 ± 59.5	375.2 ± 55.2
1.0	398.2 ± 74.5	389.2 ± 65.8	384.2 ± 58.9
5.0	461.5 ± 77.0	415.0 ± 68.5	415.7 ± 61.8
10	472.7 ± 82.4	431.0 ± 82.8	416.0 ± 68.4
20	384.7 ± 35.2	385.7 ± 34.9	391.5 ± 38.6
30	403.2 ± 40.5	382.0 ± 31.0	368.2 ± 38.4
40	452.0 ± 64.5	407.7 ± 43.4	427.7 ± 69.4

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

APPENDIX 3

TABLE 7

Effect of $B_4O_7^{2-}$ on ATPase activity

Concentration of $B_4O_7^{2-}$ (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	+$NaHCO_3$
0	166.7 ± 34.0	170.2 ± 34.2	274.7 ± 63.9
1	193.5 ± 29.3	190.7 ± 32.9	267.5 ± 61.3
5	230.0 ± 43.7	231.0 ± 47.0	261.7 ± 63.0
10	251.5 ± 49.3	247.0 ± 46.4	252.0 ± 58.3
20	243.7 ± 47.5	229.2 ± 48.6	222.5 ± 51.8
30	214.5 ± 43.7	202.5 ± 41.2	193.0 ± 43.6
40	189.2 ± 34.6	181.0 ± 37.4	168.5 ± 38.4

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the Mean ± S.E.M. of four experiments

APPENDIX 3

TABLE 8

Effect of SeO_3^{2-} concentration on ATPase activity

Concentration of SeO_3^{2-} (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	214.0 ± 27.4	213.0 ± 24.8	336.6 ± 32.2
1	216.3 ± 29.2	209.6 ± 32.6	303.6 ± 36.7
5	241.0 ± 34.3	228.6 ± 28.5	317.0 ± 38.9
10	269.0 ± 33.7	239.0 ± 25.9	316.6 ± 46.3
20	310.0 ± 43.6	263.0 ± 40.5	320.3 ± 45.2
30	338.6 ± 39.3	291.6 ± 38.3	311.0 ± 51.0
40	349.0 ± 48.1	300.3 ± 36.3	303.0 ± 46.7

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments.

APPENDIX 3

TABLE 9

Effect of Br^- and NO_3^- on ATPase activity in the presence of 20mM Cl^-/HCO_3^-

Concentration of Br^-/NO_3^- (mM)	NaCl + Br^-	NaCl+NO_3^-	$NaHCO_3$+Br^-	$NaHCO_3$+NO_3^-
0	398.6 ± 7.8	398.6 ± 7.8	648.0 ± 5.0	647.3 ± 4.7
1	399.6 ± 8.4	387.6 ± 7.9	630.6 ±12.7	618.3 ±13.7
5	408.6 ± 9.7	353.6 ± 7.9	625.0 ± 11.0	574.0 ±11.5
10	414.0 ±11.2	325.0 ± 8.6	613.3 ± 13.8	520.0 ± 8.3
15	418.0 ±10.6	296.3 ± 5.9	601.6 ± 13.6	473.0 ±10.0
20	428.0 ±13.8	286.0 ± 2.5	587.3 ± 11.5	426.6 ± 7.7

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

APPENDIX 3

TABLE 10

Effect of Br^- and NO_3^- on ATPase activity in the presence of different concentration of Cl^-

Concentration (mM)		Mg^{2+}-dependent ATPase activity	
Cl^-	Br^-/NO_3^-	NaCl + Br^-	NaCl + NO_3^-
20	0	357.2 ± 19.0	357.2 ± 19.0
19	1	360.2 ± 18.2	341.3 ± 16.9
15	5	365.4 ± 19.2	287.5 ± 14.3
10	10	376.6 ± 19.9	254.0 ± 11.5
5	15	388.8 ± 19.2	230.5 ± 11.6
0	20	396.4 ± 20.2	213.8 ± 11.0

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

APPENDIX 3

TABLE 11

Effect of amiloride on ATPase activity

Concentration of amiloride (M)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	342.2 ± 11.5	348.2 ± 15.4	503.2 ± 12.5
5×10^{-8}	338.7 ± 14.3	351.2 ± 16.2	496.7 ± 5.7
5×10^{-7}	339.2 ± 5.4	350.0 ± 17.8	490.2 ± 4.5
5×10^{-6}	343.2 ± 11.5	344.5 ± 15.4	491.2 ± 3.2
5×10^{-5}	334.0 ± 14.7	338.0 ± 18.2	473.0 ± 4.1
5×10^{-4}	319.7 ± 14.9	307.2 ± 13.5	429.5 ± 8.7

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

APPENDIX 3

TABLE 12

Effect of ethacrynic acid on ATPase activity

Concentration of ethacrynic acid (M)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	319.7 ± 20.8	321.7 ± 25.4	470.0 ± 32.1
10^{-7}	316.2 ± 22.8	316.2 ± 22.5	474.0 ± 31.6
10^{-6}	312.5 ± 22.5	317.5 ± 20.1	474.7 ± 30.6
10^{-5}	310.5 ± 22.9	315.5 ± 18.7	478.0 ± 32.3
10^{-4}	298.2 ± 19.4	297.0 ± 16.2	436.7 ± 25.0
10^{-3}	264.0 ± 20.5	238.7 ± 12.9	351.2 ± 20.5

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

APPENDIX 3

TABLE 13

Effect of DNP on ATPase activity

Concentration of DNP (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	234.0 ± 46.3	237.0 ± 48.4	372.7 ± 70.9
1	282.0 ± 65.4	284.7 ± 67.0	405.0 ± 88.4
5	325.5 ± 69.0	361.7 ± 62.4	417.5 ± 82.1

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

APPENDIX 3

TABLE 14

Effect of c.AMP on ATPase activity

Concentration of c.AMP (mM)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	332.1 ± 12.3	338.7 ± 15.1	506.5 ± 21.8
1	341.2 ± 15.4	337.7 ± 14.7	503.5 ± 19.4

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of four experiments ± S.E.M.

APPENDIX 3

TABLE 15

Effect of oligomycin on ATPase activity (5,000g fraction)

Concentration of oligomycin (M)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	290.8 ± 11.6	308.0 ± 9.8	512.6 ± 8.7
3.5×10^{-7}	273.4 ± 12.6	291.8 ± 12.2	496.0 ± 13.7
1×10^{-6}	271.8 ± 13.1	289.8 ± 12.2	490.2 ± 15.6
1.7×10^{-6}	266.8 ± 12.3	285.8 ± 11.5	484.2 ± 14.9
2.6×10^{-6}	265.6 ± 10.7	281.6 ± 11.0	482.0 ± 14.9
3.5×10^{-6}	262.0 ± 9.0	278.0 ± 7.6	470.6 ± 10.6
1.0×10^{-5}	252.8 ± 11.4	261.8 ± 11.8	438.4 ± 9.5
1.7×10^{-5}	231.2 ± 10.7	249.8 ± 16.5	397.0 ± 18.3
2.6×10^{-5}	206.4 ± 11.8	222.2 ± 19.3	372.6 ± 17.5
3.5×10^{-5}	192.8 ± 12.7	204.8 ± 17.7	333.2 ± 18.5
1.7×10^{-4}	101.4 ± 4.5	123.0 ± 7.9	179.6 ± 9.1
3.5×10^{-4}	68.4 ± 3.6	86.0 ± 6.8	131.8 ± 5.6
3.5×10^{-3}	34.4 ± 3.5	43.8 ± 2.1	63.0 ± 3.5

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

APPENDIX 3

TABLE 16

Effect of oligomycin on ATPase activity (20,000g fraction)

Concentration of oligomycin (M)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	497.4 ± 23.7	534.0 ± 22.0	878.8 ± 28.5
3.5×10^{-7}	481.0 ± 26.3	503.6 ± 21.8	845.0 ± 37.4
1×10^{-6}	477.8 ± 27.7	501.2 ± 16.8	830.4 ± 31.2
1.7×10^{-6}	467.0 ± 29.2	490.6 ± 32.3	829.8 ± 34.1
2.6×10^{-6}	447.6 ± 21.3	485.0 ± 18.3	806.2 ± 32.1
3.5×10^{-6}	447.0 ± 22.5	473.4 ± 21.8	784.8 ± 36.8
1×10^{-5}	418.0 ± 25.9	426.0 ± 24.7	714.6 ± 40.6
1.7×10^{-5}	381.4 ± 30.7	394.0 ± 25.4	681.8 ± 37.1
2.6×10^{-5}	319.8 ± 20.1	336.4 ± 17.4	579.6 ± 24.4
3.5×10^{-5}	219.4 ± 32.5	283.6 ± 13.3	461.6 ± 30.1
1.7×10^{-4}	128.2 ± 9.7	145.0 ± 7.5	235.4 ± 12.1
3.5×10^{-4}	84.0 ± 10.6	89.8 ± 4.1	162.6 ± 11.6
3.5×10^{-3}	32.4 ± 3.2	38.4 ± 4.1	78.0 ± 6.7

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

APPENDIX 3

TABLE 17

Effect of oligomycin on ATPase activity (100,00g fraction)

Concentration of oligomycin (M)	Mg^{2+}-dependent ATPase activity		
	-NaCl	+NaCl	$+NaHCO_3$
0	328.2 ± 16.2	328.2 ± 13.9	524.4 ± 21.8
3.5×10^{-7}	299.8 ± 14.9	296.8 ± 15.7	481.6 ± 23.2
1×10^{-6}	290.8 ± 16.2	289.4 ± 14.8	475.4 ± 22.6
1.7×10^{-6}	280.2 ± 17.3	284.4 ± 14.5	457.8 ± 25.8
2.6×10^{-6}	258.8 ± 17.4	271.6 ± 12.7	430.4 ± 22.3
3.5×10^{-6}	248.2 ± 18.0	257.6 ± 13.1	409.2 ± 23.8
1×10^{-5}	211.0 ± 23.4	216.0 ± 23.6	360.0 ± 36.0
1.7×10^{-5}	190.8 ± 24.4	189.0 ± 22.3	318.4 ± 29.9
2.6×10^{-5}	152.4 ± 21.1	161.8 ± 21.2	279.8 ± 27.8
3.5×10^{-5}	132.6 ± 18.2	134.0 ± 12.1	206.4 ± 15.8
1.7×10^{-4}	63.4 ± 6.1	69.4 ± 6.9	118.8 ± 7.9
3.5×10^{-4}	35.6 ± 2.5	36.6 ± 3.5	79.2 ± 9.2
3.5×10^{-3}	24.8 ± 3.5	24.8 ± 4.6	47.2 ± 6.2

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

APPENDIX 3

TABLE 18

Distribution of Mg^{2+}-dependent HCO_3^--stimulated ATPase activity in comparison with SDH activity

Subcellular fraction (g)	Mg^{2+}-dependent HCO_3^--stimulated ATPase	SDH activity (µmoles succinate oxidized/ mg protein/min).
600	259.2 ± 22.8	0.067 ± 0.007
20,000	739.8 ± 61.4	0.107 ± 0.011
100,000	412.0 ± 41.6	0.019 ± 0.004

Activity for ATPase is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of five experiments ± S.E.M.

APPENDIX 3

TABLE 19

Effect of $NaHCO_3$ on Na^+-K^+ ATPase

Concentration (mM)		$Na^+ - K^+$ ATPase activity	
NaCl	$NaHCO_3$	-acetazolamide	+ acetazolamide
100	0	230.4 ± 43.2	222.6 ± 40.7
90	10	233.0 ± 31.1	223.0 ± 36.7
80	20	225.0 ± 36.8	211.0 ± 30.7
50	50	206.3 ± 33.4	195.0 ± 26.4
0	100	180.2 ± 34.0	192.6 ± 39.0

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

APPENDIX 3

TABLE 20

Effect of NaSCN on Na^+-K^+ ATPase

Concentration (mM)		$Na^+ - K^+$ ATPase activity
NaCl	NaSCN	
100	0	230.4 ± 43.2
99	1	214.0 ± 39.0
95	5	220.6 ± 42.2
90	10	206.6 ± 37.3

Activity is expressed as nmoles Pi liberated/mg protein/min. Each value represents the average of three experiments ± S.E.M.

APPENDIX 4

Figure A.4.1

Calibration curve for KOH concentration against % emission (determined by SP 90 spectrophotometer, wavelength 766).

Ordinate: % emission

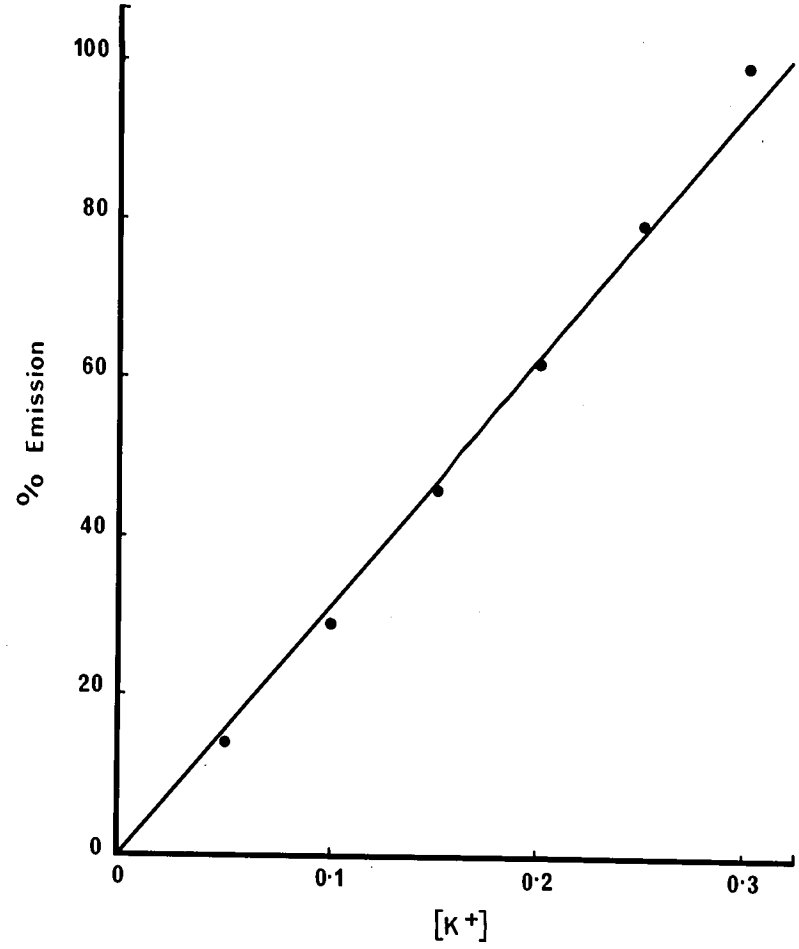
Abscissa: K^+ concentration (mM)

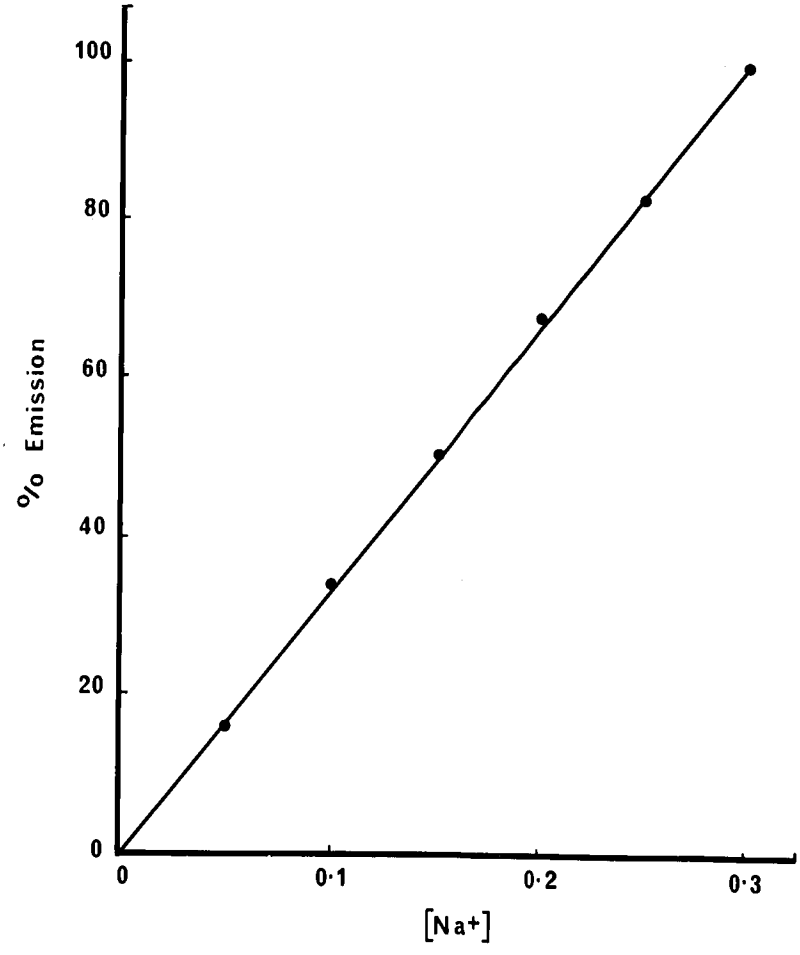
Figure A.4.2

Calibration curve for NaOH concentration against % emission (determined by SP 90 spectrophotometer, wavelength 589nm).

Ordinate: % emission

Abscissa: Na^+ concentration (mM)


100
80
60
40
20
0
% Emission
0
0·1
0·2
0·3
[K+]


100
80
60
40
20
0
% Emission
0
0·1
0·2
0·3
[Na+]

APPENDIX 4

(1) Na^+ and K^+ concentrations of fluid secreted by Malpighian tubules bathed in media of varying Na^+ and K^+ concentrations.

Ringer	Urine			
$[Na^+]$ and $[K^+]$ mM	$[Na^+]$ mM	$[K^+]$ mM	total $[Na^+]$ and $[K^+]$	n
152.0 Na^+ : 0.0 K^+	141.2 ± 8.3	44.9 ± 5.3	186.1 ± 8.8	14
143.4 Na^+ : 8.6 K^+	33.4 ± 2.4	141.7 ± 4.9	175.1 ± 5.8	16
122.0 Na^+ : 30.0 K^+	33.1 ± 1.8	143.9 ± 5.2	177.0 ± 4.6	29
76.0 Na^+ : 76.0 K^+	28.0 ± 2.2	149.1 ± 6.1	177.1 ± 5.8	26
30.0 Na^+ :122.0 K^+	25.1 ± 1.7	157.9 ± 4.4	182.9 ± 4.6	28
0.0 Na^+ :152.0 K^+	15.0 ± 1.1	168.3 ± 4.3	182.9 ± 4.2	35

APPENDIX 5

Calculation of oxygen content of $3cm^3$ of Ringer in oxygen electrode apparatus (Chapter 5).

Oxygen content of $1cm^3$ of reaction medium saturated with air at 30^oC = 0.445µg atoms oxygen (CHAPPELL, 1964). $3cm^3$ of reaction medium was used in the experiments; therefore oxygen content of $3cm^3$ was 1.335µg atoms oxygen = 0.668µmoles oxygen. The Ringer solution was saturated with air prior to each experiment, therefore 100% saturation contains 0.668 µmoles oxygen.