MALPIGHIAN TUBULES OF A. DORSALIS MOSQUITO LARVAE: GENERAL CHARACTERISTICS AND MECHANISM OF MAGNESIUM TRANSPORT

by

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ABSTRACT

Malpighian tubules of A. dorsalis mosquito larvae, studied in vitro, actively transported magnesium at high rates against concentration gradients as large as 16-fold and transepithelial potential gradients of approximately -15mV. Fluid secretion rates, determined over 90 minute periods, in the presence and absence of cAMP, indicated that A. dorsalis tubules were viable and had secretion rates of the same magnitude as those reported for A. taeniorhynchus tubules. Having characterized the in vitro preparation of Malpighian tubules, the main hypothesis that Mg^{2+} transport is driven predominately by counter transport with Na^{+} was tested. This hypothesis was not supported by kinetic, Na-substitution, or inhibitor studies. Kinetic and Bumetanide studies suggest backflux of K drives J_{Mg}; however, this was not consistently found in other studies.

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LIST OF ABBREVIATIONS

mM	millimoles per liter
uM	micromoles per liter
M	moles per liter
ml	milliliter
nl	nanoliter
mm	millimeter
mV	millivolt
h	hour
mOsm	milliosmole
pmol/min	picomole per minute
pl/min	picoliter per minute
mEquiv/l	milliequivalent per liter
U/P	ratio of urine-to-plasma
Sec/Ext	ratio of secreted fluid to external fluid
J_{ion}	flux of ion
Jv	flux of fluid
Vt	transepithelial potential
Km	Michaelis constant
Vmax	maximum velocity
cAMP	cyclic 3',5'-adenosine monophosphate
PAH	para-amino hippurate
cTAL	cortical thick ascending limb
HEPES	N-2-hydroxyethyl piperazine-N'-2ethanesulfonic acid (a weak acid buffering compound)

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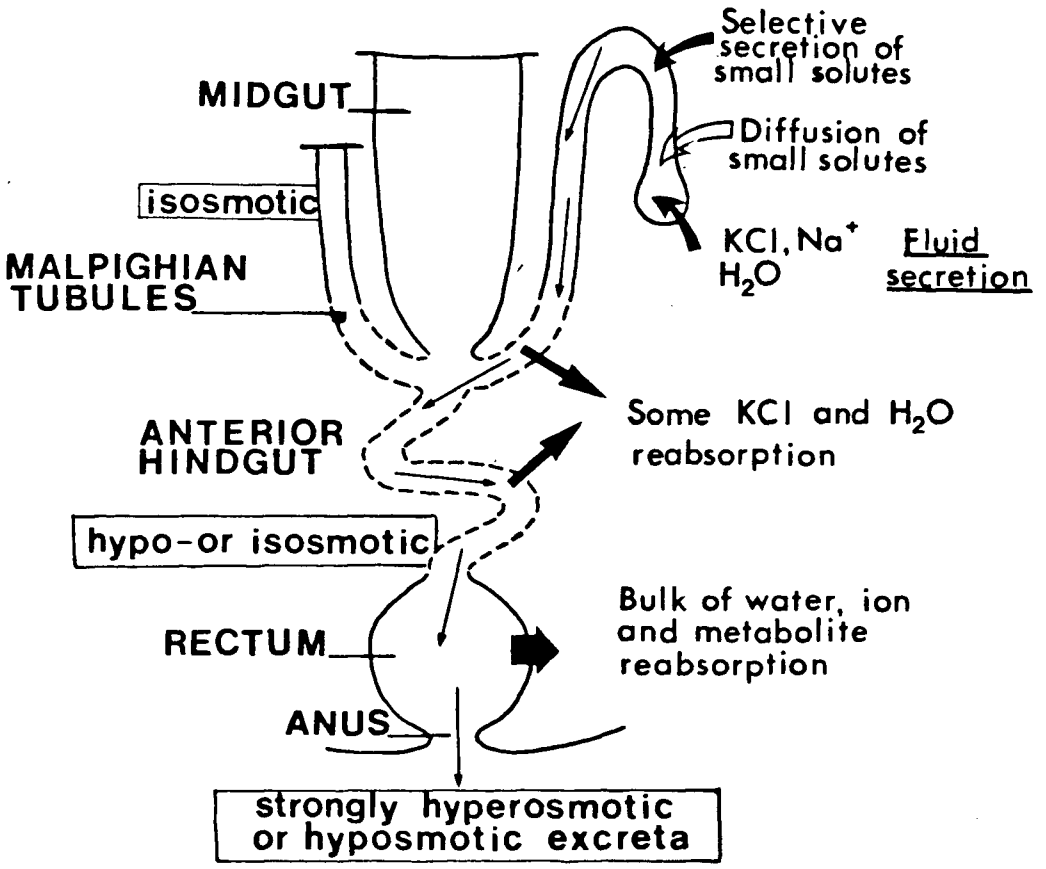
INTRODUCTION

Mosquito larvae, Aedes dorsalis are commonly found in saline ponds of diverse ionic composition in the Great basin of western North America. Fourth-instar stage of Aedes dorsalis larvae have a survival rate near 100% in 200mM magnesium sulphate medium (Bradley and Sheplay, 1982). These larvae drink the external saline media at a rate equal to 130% of their body weight per day (Strange et al, 1982). It follows that in order to survive in such Mg-rich waters, these mosquito larvae must eliminate excess Mg via the excretory system, i.e. Malpighian tubules or rectum, at high rates.

It is unlikely that hemolymph Mg^{2+} concentrations in Aedes dorsalis vary substantially under different external conditions, because hemolymph concentrations of this cation are maintained at low levels (1.5-4mM Mg^{2+}) in a closely related species, Aedes campestris, when exposed to a wide range of external magnesium levels (4-95mM);(Kiceniuk and Phillips, 1975). Strange(1982) measured magnesium levels of 3.8mM in hemolymph of Aedes dorsalis reared in medium containing 0.5mM magnesium. As explained later, the excretory system of A. dorsalis offers an unusual opportunity to investigate the nature of magnesium transport across insect epithelia and indeed epithelia in general.

Insect excretory systems work on the same basic principles as other renal organs (see Fig. 1). Malpighian

Figure 1: Schematic illustration of the insect excretory system.



tubules of insects are commonly found attached to the alimentary canal delineating the separation of the midgut and the hindgut. A. dorsalis larvae have 5 Malpighian tubules which lie free in the hemocoel. They are approximately 2.8mm in length and 0.15mm in diameter, and consist of large principal cells (probably the primary site of salt and water secretion) and smaller stellate cells of unknown function which make up less than 10% of the total tissue mass (Bradley et al, 1983). It has been postulated that principal cells with their abundant mitochondria might secrete a Na-rich fluid while stellate cells reabsorb Na^+ and secrete K^+ (Berridge et al., 1969) but there is no direct evidence for this suggestion.

Histologically, the Malpighian tubules consist of a simple tubular epithelium. The hemolymph side of the epithelium is covered by a basal lamella whose probable function is to filter out particles and cells so as to prevent clogging of infolds of the basal cellular membrane. The high degree of folding of the basal plasma membrane increases surface area and increases the number of transport proteins which can be inserted to maximize solute-water coupling during fluid transport (Berridge and Oschman, 1969). Mitochondria are found in close proximity to the basal membrane infolds (Bradley, 1983). Mitochondria are also found near the apical surface which is extensively covered by microvilli. However, microvilli of the insect

are different from microvilli of other animals in that they commonly contain smooth endoplasmic reticulum, rough endoplasmic reticulum, and mitochondria.

Septate junctions, the major junctions between Malpighian tubule cells, are found on the lateral membranes near the apical surface (Lane and Skaer, 1980). Below the septate junctions are gap junctions, a common structure in insect Malpighian tubules. Intercellular clefts occupy very little surface area of the Malpighian tubule because individual cells of the tubule are large and often extend halfway around each tubule.

Intracellular storage granules, possibly for storage of excretory products, are present in the primary cells of A. taeniorhynchus. These intracellular crystals contain calcium, magnesium, and phosphate (probably PO_4^{2-}; Ryerse, 1979).

In this thesis, the mechanism of magnesium transport across Malpighian tubules of A. dorsalis was investigated. In addition, general characteristics of ionic and fluid secretion in the Malpighian tubules of this species were examined. Before considering Mg transport in this epithelia, a general review of Mg transfer and control in animals is in order.

Considering the important role of Mg^{2+} in many physiological processes, in particular neuromuscular events,

remarkably little is known about the mechanisms of magnesium transport across biological membranes, especially epithelia involved in regulating blood magnesium levels in most animals. Some specific processes that are strongly influenced by magnesium levels include: nucleic acid and protein synthesis (Lindahl et al, 1966), reactions with pyruvate kinase (Sloan et al, 1976; Mildvan et al, 1976), creatine kinase (Cohn, 1963), enolase (Winstead et al, 1965), phosphoglucomutase (Ray et al, 1973), alkaline phosphatase (Vallee et al, 1970), adenylate cyclases (Alvarez et al, 1977), adenosine triphosphatases (Racker, 1977), brain hexokinase (Bachelard, 1970), pyruvate dehydrogenase (Hucho, 1974), creatine phosphokinase (Saks et al, 1975), aspartate transcarbamoylase (Christopherson et al, 1977), second messenger events during insulin stimulation (Lostroh et al, 1974), and muscle contraction (Stephenson et al, 1977; Solaro et al, 1976).

Regulation and transport of magnesium is best understood in single bacteria and invertebrate cells, i.e. for transfer across single plasma membranes rather than epithelia. Lusk and Kennedy (1969) showed that a transport system specific for magnesium exists in E. coli. This transport system is inhibited by azide, dinitrophenol, or potassium cyanide indicating an energy requiring process. However, it is unclear whether it involves a primary or secondary transport process. When temperature is lowered or

when cells are treated with a sulfhydryl poison such as N-ethylmaleimide, magnesium uptake decreases. Cobalt also appears to be transported into E. coli by the same transport system; however, this system has a higher affinity for magnesium. A Na-Mg exchanger found in some invertebrate cells will be reviewed in detail later.

Experiments on mammalian systems have provided little information on magnesium transport across epithelia. However, factors that might influence magnesium excretion in the kidney have been investigated (Quamme et al, 1982). These factors include effects of volume expansion, diuretics, hypercalcemia, and a number of conditions such as acidosis, phosphate depletion, and chronic alcohol ingestion. The possibility of hormonal control of renal magnesium transfer has also been investigated (Quamme, 1982).

Experiments performed on the proximal tubule suggest possible active transport of Mg^{2+} from the lumen which is dependent on sodium and water transport (Wong et al, 1981). The nature of magnesium flux in the deep descending limb is more controversial. This is probably due to species differences (DeRouffignac et al, 1973); diffusional flux of magnesium from interstitium into the lumen may account for all observations. The ascending limb of the loop of Henle appears to be the major site for magnesium reabsorption; 50-60% of filtered magnesium is reabsorbed between the

proximal and early distal tubule (Morel et al, 1973, 1969). There appears to be some interaction between calcium and magnesium transport in this segment (Quamme, 1981; Mussny et al, 1973). Furosemide inhibits transport of sodium, calcium, and magnesium in this portion of the tubule and there is greater inhibition of absolute magnesium and calcium transport as compared to sodium transport (Quamme,1978). In addition, calcitonin, in the presence of low plasma levels of calcium, and parathyroid hormone both increase magnesium reabsorption in the loop of Henle (Quamme, 1980). The distal convoluted tubule and collecting duct both reabsorb approximately 2-5% and 1-3% respectively of filtered magnesium (Quamme, 1980; Brunette, 1978).

Since it is difficult to elucidate mechanisms of magnesium transport across renal tubules, it may be more rewarding to study epithelia known to transport magnesium at high rates and against large electrochemical differences. The Malpighian tubules and recta of mosquito larvae which inhabit magnesium and sodium sulphate lakes appear ideal for this purpose. These epithelia have cells of unusually large size and simple structure which can probably be impaled with microelectrodes. Studies of Malpighian tubules in nine species of insects indicate lumen positive potentials with respect to the hemocoel; the range of potential readings is between 10 and 30 mV. The cell interior of the 4 species studied is electrically negative to both lumen and hemocoel

(Maddrell,1971; Ramsey, 1953; see Figure 2). Concentrations of K^+ in the secretion are normally 3-30 times higher than those in the hemolymph and do not change much when bathing Na^+/K^+ is varied in vitro over a wide range (Maddrell, 1971). Clearly, K^+ transport at the apical membrane is the predominate ion transport process in most insect tubules. Of particular interest, Aedes larvae tubules continue to secrete fluid containing high concentrations of potassium for some time when larvae are bathed in a pure NaCl solution (Ramsay, 1953). In all cases to date, Malpighian tubule fluid is either isosmotic or slightly hyperosmotic (Phillips, 1981).

Unlike plant feeders, the blood-sucking species Rhodnius prolixus must eliminate excess NaCl rather than K^+ after feeding, and fluid secretion is driven by both Na^+ and K^+ transport with recovery of K^+ in a lower tubule segment (Maddrell,1969,1977). Experiments on Malpighian tubules of this species suggest transport of Na^+ and K^+ by a common pump situated on the apical plasma membrane (Maddrell, 1977,1978). Entry of K^+ (from the external solution to cell interior) may possibly involve exchange for Na^+ by a Na^+/K^+-ATPase which is ouabain-insensitive (Anstee et al, 1979; Berridge,1968). Therefore, it is possible that some Na^+ may be recycled from the tubule lumen to the cell interior because both the electrical and concentration gradients across both the apical and basal membrane favour

passive entry of Na^+ into the cell. The concentration of Na^+ in the cell is probably maintained at low levels by a Na^+/K^+-ATPase on the basolateral membrane.

There is evidence for control of fluid secretion in Malpighian tubules in 20 species of insects (Maddrell,1981) by a family of diuretic hormones (reviewed by Phillips, 1981). Diuretic hormone is thought to increase tubular secretion by raising intracellular levels of cAMP. In support of this view, externally applied cAMP mimics the action of diuretic hormone in 8 species. Furthermore, diuretic hormone stimulation of tubules in Rhodnius increases tissue levels of cAMP (Anstee and Bowler, 1979) and causes a transient reversal of the transmural electropotential difference (Maddrell, 1980). As a result of accelerated salt and hence fluid secretion, other substances in the secreted fluid are diluted in the lumen of the tubule. This increases the favourable concentration gradient for substances that enter passively and reduces opposing concentration differences created by active transport (thus reducing back diffusion of transported substances from the lumen). In both cases, substances are thereby removed from the hemolymph at a higher rate after stimulation of KCl transport by cAMP.

Phillips et al (1974;1975) demonstrated that active transport of magnesium can occur against both large electrical and concentration gradients in Malpighian tubules

of A. campestris. Larvae capable of surviving in medium of 100mM magnesium actively transported magnesium against a tenfold concentration gradient and an electrical potential difference of +15mV. Magnesium transport approaches saturation when the bathing medium contains 5-6mM Mg^{2+} and half maximal rate is reached at 2.5mM. The rate of transport of magnesium is not affected by changes in the rate of fluid secretion which suggests that backflux of this cation is negligible. When Malpighian tubules were exposed to artificial hemolymph containing 2mM Mg^{2+} and either 4mM or 76mM K^{+}, tubules secreted Mg^{2+} at average rates of 6.72 pmol/min and 6.95 pmol/min respectively. The tubules in K^{+}-rich medium secreted fluid containing 8.2mM Mg^{2+} at an average rate of 852 pl/min, whereas tubules in K^{+}-poor medium secreted fluid containing 25.5mM Mg^{2+} at an average rate of 260 pl/min. Therefore, at high fluid secretion rates, the Mg^{2+} concentration in the tubular fluid is lower (Phillips and Maddrell, 1974).

The major hypothesis tested in this thesis rests on evidence for a Na^{+}-Mg^{2+} exchange mechanism in giant squid axon (Baker et al, 1972) and single crustacean muscle fibers (Ashley et al, 1972), which extrudes Mg^{2+} actively from these cells. Cyanide and low temperatures both reduce this magnesium efflux, indicating the presence of a metabolically dependent pump. Ouabain does not inhibit Mg^{2+} transport in either squid axon or crustacean fibers, suggesting that

Na^+/K^+-ATPase is not directly and immediately involved. Palaty (1974) has demonstrated that an outwardly directed Mg^{2+} flux in rat vascular smooth muscle utilizes the energy available in the spontaneous influx of sodium. In addition, many other cellular transport mechanisms are known to involve cotransport with, or exchange for, external sodium. In all these cases low intracellular levels of sodium are maintained by Na^+/K^+-ATPase in the plasma membrane (Harrison et al, 1980). The inward directed backflux of Na^+ is used to drive secondary transport of many substances: eg. absorption of D-hexose (Busse et al,1972) (Crane, 1977), di and tricarboxylic acids (Kippen et al, 1978), bile acids (Wilson et al, 1980), (Lucke et al 1978), L-ascorbate (Silliprandi et al, 1979), L-lactate (Barac et al, 1980), (Hildmann et al, 1980), dipeptides (Sigrist-Nelson, 1975), L-aminoacids (Burckhardt et al, 1980),(Fromter, 1979), (Hopfer,1977a), inorganic sulfate (Lucke et al, 1979), inorganic phosphate (Murer et al, 1980), (Berner et al, 1976), chloride (Eveloff et al, 1980), calcium (Gmaj et al, 1979), (Hildmann et al, 1980), secretion of chloride (Eveloff et al, 1978), and H^+ secretion (Murer et al, 1977).

Based on this widespread occurrence of sodium-coupled transport processes, including that of magnesium transport in some single cells, it seemed possible that magnesium secretion by mosquito Malpighian tubules is also driven by a sodium exchange process located at the

mucosal border. It is unlikely that magnesium could cross the apical membrane by diffusion since the cell interior would have to contain greater than 200mM Mg^{2+} to explain secretion of this cation (20mM in lumen) against an opposing apical potential, as low as 29mV (see Fig. 2).

If Malpighian tubules of A. dorsalis larvae have transepithelial potential profiles similar to those of other insects described above, then downhill movement of Na^+ across the apical membrane into the cell could drive active extrusion of Mg^{2+} from cell to lumen. However, given that Na^+ levels are often low in the secretions of insect tubules, including A. dorsalis (Phillips, 1981), alternate mechanisms may have evolved in these epithelia, e.g. exchange of cellular magnesium for luminal K^+, H^+, or Ca^{2+}. Alternately, magnesium may be co-transported with sulphate or bicarbonate. Phillips and Maddrell found that tubules of A. taeniorhynchus (1978) and A. campestris (Maddrell,S.H.P. et al, 1975) actively secrete sulphate but they did not consider the possibility of co-transport with magnesium. Induction of sulphate transport was shown by Maddrell and Phillips (1978) in Malpighian tubules of A. taeniorhynchus. They found that fourth stage larvae were able to regulate hemolymph sulphate levels at low values (<10mM) even when external levels were as high as 89mM. Larvae exposed to 33.3mM sulphate were able to secrete this anion at a rate nearly four times faster than insects reared in

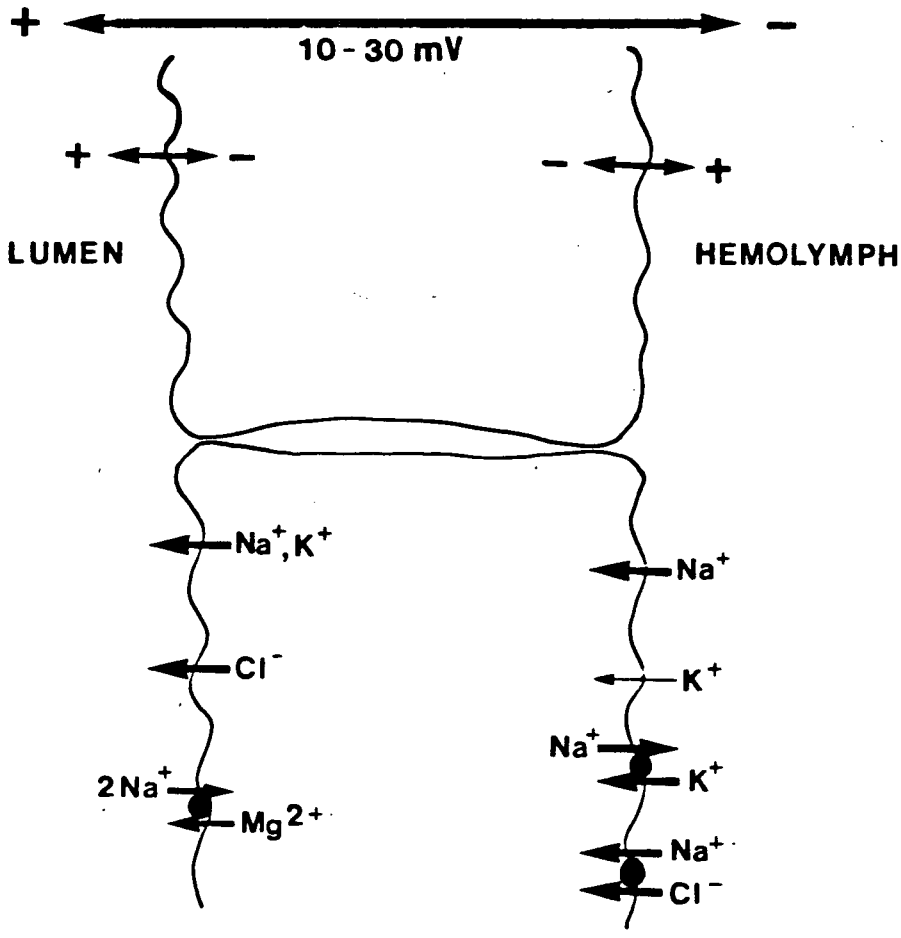


Figure 2: Electric potential across an insect epithelium.

sulphate-free water. Conceivably the Mg^{2+} transport system in A. dorsalis Malpighian tubules is also inducible. This possibility was investigated in the present study. A remaining possibility is that Mg-ATPase exists in A. dorsalis Malpighian tubules; however, such a transport ATPase has not been reported to date in any epithelia or single cell.

To determine whether magnesium secretion in A. dorsalis Malpighian tubules occurs by a Na^{+}-Mg^{2+} exchange mechanism, the following studies were performed. Malpighian tubules were bathed in varying concentrations of magnesium (0-4mM) and secreted droplets were analyzed to determine the relationship between Mg^{2+} and Na^{+} (as well as with HPO_4^{-}, SO_4^{2-}, Cl^{-}, and K^{+}). Second, secreted droplets from Malpighian tubules bathed in sodium-free salines were analyzed for any correlation between residual Na^{+} and Mg^{2+} content. These studies also yielded information concerning the relationship of other secretory processes (e.g. HPO_4^{-}, SO_4^{2-}, Cl^{-}, K^{+}) with Mg^{2+}. Third, the effects of inhibitors of transepithelial sodium transport (e.g. amiloride, bumetanide) on Mg^{2+} secretion were studied.

MATERIALS AND METHODS

Aedes dorsalis eggs were kindly provided by Dr. Laura Kramer, Department of Biomedical and Environmental Health Sciences, School of Public Health, Berkeley, California. Eggs were hatched as previously described (Bradley, 1976) and larvae were reared at 28°C in 50% seawater. Composition of seawater is shown in Table 1.

Table 1: Composition of 50% Seawater

Ion	Concentration(mM)
Na^+	229.43
K^+	4.89
Mg^{2+}	26.30
Ca^{2+}	7.54
Cl^-	272.57
HCO_3^-	1.25
Br^-	0.38
SO_4^{2-}	13.90

Fourth instar larvae were used in all experiments because they survive in higher magnesium waters compared to earlier stages (Bradley and Sheplay, 1982). Larvae were fed daily with dry fish food (Tetramin Staple Food; D4520 Melle 1,W.Germany) and the rearing media was aerated gently and changed periodically to prevent stagnation.

Survival studies were performed by transferring fourth instar larvae, five to six days after being hatched in 50% seawater, to solutions containing 50% seawater plus

one of the following combination of salts: (a) 0mM $MgSO_4$+500mM NaCl (abbreviated 0/500), (b)50/450, (c)100/400. After 24 hours, the number of dead and surviving animals was determined, as described by Bradley(1982). Based on these survival studies, in all subsequent experiments larvae were transferred to 100mM $MgSO_4$/400mM NaCl medium five to six days after being hatched; experiments were performed on larvae before and after transfer to 100mM $MgSO_4$/400mM NaCl medium.

Physiological salines used to bathe isolated Malpighian tubules were similar in composition to saline solutions used by Strange (1982) and were based on measured ionic, osmotic, and organic solute concentrations of natural hemolymph (Table 2). To study kinetics of Mg^{2+} transport, $MgCl_2$ concentrations of this physiological saline were adjusted between 0.5 and 4 mM. In sodium-free salines, Na^+ was replaced by K^+ or choline and all other components were the same. The final concentration of K^+ was 15.0mM in control saline as compared to 75.4mM in the K^+-substituted sodium-free saline. The "sodium-free" saline contained approximately 1mM Na, as measured by electron microprobe analysis (Cameca MBX). This concentration of Na found in "sodium-free" saline may be due to sodium from reagents or may be due to background interference of other ions as measured by the electron microprobe. As the source of Na was not determined, this value of Na (1mM) found in "Na-

Table 2: Composition of Experimental Salines

Saline#	Na^+	K^+	Mg^{2+}	Ca^{2+}	HCO_3^-	Cl^-	SO_4^{2-}	Cycl.	Succ.	Citrate	Hepes	Amil.	Bumet.	cAMP	pH	osmol
i)	60.4	15.0	0	4.0	7.9	41.3	5.0	10.3	7.4	2.4	3.9				7.2	195
ii)	"	"	0.5	"	"	"	"	"	"	"	"				"	200
iii)	"	"	1.0	"	"	"	"	"	"	"	"				"	200
iv)	"	"	2.0	"	"	"	"	"	"	"	"				"	200
v)	"	"	4.0	"	"	"	"	"	"	"	"				"	200
vi)	"	13.4	2.0	"	"	"	"	"	"	"	2.0			1.0	"	200
vii)	"	15.0	2.0	"	"	"	"	"	"	"	1.0			2.0	"	210
viii)	"	"	"	"	"	62.4	"			"	2.0			1.0	"	220
ix)		75.4	2.0	4.0	"	62.4	5.0			"	2.0			1.0	"	240
x)		15.0	"	1.0	"	20.6	8.1			"	3.9				7.3	300
xi)	60.4	"	"	4.0	"	73.5					2.0			1.0	7.2	220
xii)	"	"	"	"	"	"					2.0	1.0		1.0	"	210
xiii)	"	"	"	"	"	"					2.0			1.0	"	220
xiv)	"	"	"	"	"	"					2.0		0.05	1.0	"	220

Cycl. = cyclamate
Amil. = amiloride
Bumet. = bumetanide

(All experimental salines contain 10mM glucose, 4mM glutamine, 20mM proline, 5mM alanine, and 3mM glycine).

free" saline was not subtracted from all Na values. Because of solubility problems, sulphate, succinate, citrate, and cyclamate were omitted from the salines containing the inhibitor amiloride (1mM). The control saline in this case was the same as the experimental saline, but without amiloride. Amiloride was a gift from W.D. Dorian, Merck Merck Frosst Laboratories (Dorval, Quebec). Bumetanide was a gift from Hoffman-La Roche Ltd. (Etobicoke, Ontario).

The in vitro preparation of Malpighian tubules from A. dorsalis follows the procedure described by Ramsey (1953) and Phillips and Maddrell (1974). A pair of forceps was used to hold the head of a larva on a glass dish. The cuticle of the posterior region (from midgut to anal papillae) was cut and freed from the rest of the larva by grasping the trachea (siphon) with another pair of forceps and pulling. The 5 Malpighian tubules came free and were separated from each other and from the mid-gut with glass needles made from capillary tubes(Fisher,Pittsburgh,Pa.) which were pulled to obtain fine tips using a vertical glass electrode puller(Model 700C, David Kopf instruments, Tujunga, California) and the tips sealed. Approximately 8 tubules were placed in 0.1 ml droplet of physiological saline immersed under hydrated light paraffin oil(specific gravity=0.845-0.875 at 25°C, MCB reagents) in a glass dish half-filled with Sylgard (Midland, Michigan). The cut ends of tubules were pulled out of the saline drop into the oil and wrapped around fine glass pegs stuck into the Sylgard

floor of the dish. Pegs were placed 0.8mm from the bathing drop to prevent backflux of secreted fluid into the bathing drop. Secretion rates of fluid and electrolytes were determined over a period of time as described by Phillips and Maddrell(1974).

Fluid secretion rates were determined by measuring the diameter of the secreted droplets at 30,60, and 90 minutes using an eye-piece micrometer and a Wild dissecting microscope, (Heerbrugg, Switzerland) and then determining the volume of fluid secreted by the equation, $V=4/3\ \pi r^3$. The accuracy of this method has been repeatedly confirmed for drops of less than 0.4mm diameter(Phillips and Maddrell, 1974).

After the volumes were determined, secreted droplets were drawn up in capillary tubes(Fisher, Pittsburgh,Pa.) previously rinsed with nitric acid and filled with hydrated paraffin oil containing Sudan Black B (MCB Reagents). Each fluid sample was analyzed for concentrations of Mg, Ca, K, Na, Cl, total S and total P, using a Cameca MBX electron microprobe. Methods are described by Morel and Roinel (1969) and Roinel (1975). This method of element analysis is suitable for determining concentrations of elements in nanoliter samples to an accuracy of 2% for a concentration of 1mM (Roinel et al., 1982). Samples of external saline

bathing tubules were also analyzed to confirm the accuracy of the pipetting procedure and of probe analysis. In addition, droplets of distilled water were analyzed to determine background. At 30, 60, and 90 minutes, the rate of secretion of various electrolytes was estimated from the volume and the respective concentration of the secreted droplets.

Osmotic concentrations of nanoliter fluid samples were measured with a 'Clifton Technical Physics' nanoliter osmometer (Hartford, N.Y.) and larger samples with an osmometer from Advanced Instruments Inc. (Newton Highlands, Mass.). NaCl solutions (100, 200, 300mOsm) were used as standards for the Clifton Technical Physics osmometer.

Studies with ^{14}C-inulin were performed initially to confirm the integrity of dissected tubules and to indicate whether any leakage occurred along the tubule surface in the oil between bathing and secreted droplets. This tracer was added at high specific activities to the bathing saline. Droplets (approximately 24.0 nl) of secreted fluid were collected with time and placed in plastic vials containing 1.0ul non-radioactive saline solution and 3.33 ml of ACS II aqueous counting scintillate fluid (Amersham Corporation, Oakville, Ontario). Samples of the labelled bathing saline were similarly prepared. The ^{14}C-activity of these samples were measured by liquid scintillation counting(Beckmann 9000 scintillation counter) with appropriate corrections for

background radiation and quenching. These studies on A. dorsalis tubules yielded a U/P ratio of 0.0181±0.0041 for ^{14}C-inulin (n=9). This low value proves that tubules were not significantly damaged during dissection and that no mixing occurs externally between bathing and secreted drops. U/P ratios for inulin (of 0.007-0.05) are commonly reported for other insect tubules (Maddrell, 1974). If there is a major leak in the tubules U/P could approach 1; if a compound is actively net secreted, U/P could be greater than 1.

The transepithelial potential difference for the tubules bathed in saline iv was recorded using fine glass microelectrodes filled with 3M KCl, as described by R.C. Thomas (1978). Capillary tubing (Frederick Haer and Co., Brunswick, Maine, U.S.A.) was used to make these glass electrodes. These were inserted in microelectrode holders (type EH-3FS) from WPI (New Haven, CT.) which contained internal Ag-AgCl half cells. The two microelectrodes, were inserted with the aid of micromanipulators (Kanetsu, MB-W, Tokyo, Japan) into the bathing and secreted droplets respectively, and were connected to a high impedance voltmeter (Keithley Model 602, Cleveland, Ohio). The transepithelial potential difference (P.D.) was recorded as described by Phillips and Maddrell (1974). The asymmetry potential was determined with both salt bridges in the bathing droplet and was subtracted from transepithelial readings.

Phenol red (MCB) was used as pH indicator to determine pH of the bathing saline with time as well as the secreted droplets from tubules. Approximately 4.10×10^{-6} ml of phenol red was mixed with an equal volume of either standard buffer (pH=7.0-7.5) or test solution(bathing drop or secreted droplet). These drops were immersed in a dish containing hydrated paraffin oil. The pH of these test solutions were determined by visually comparing the colour of phenol red in unknown solutions with standard buffers of known pH.

RESULTS

Survival of Aedes dorsalis in Waters of High Mg^{2+} Content

Experiments similar to those conducted by Bradley et al.(1982) on A. dorsalis larvae were repeated to determine the upper survival limit to external Mg^{2+} and to see if a Mg^{2+} tolerance strain could be selected by exposing successive generations to high Mg^{2+} waters. Such larvae would be expected to have well developed Mg transport mechanisms in their excretory systems. Eggs were hatched and larvae were reared for 2 days in 50% seawater containing 26mM $MgSO_4$ and % survival was then determined 1 and 2 days (i.e. 3rd instar) after transfer to test solutions of varying Na^+ and Mg^{2+} solution (Table 3, Exp. 1).

Closely related species of saline-water mosquito larvae reach new steady levels of hemolymph ions within 1-2 days of transfer to new salines (Kiceniuk and Phillips, 1974); therefore, this time period was judged appropriate in the current study. Third instar larvae survived well the first day in 126mM Mg^{2+} but mortality exceeded 50% after 1 day and was nearly 100% after 1 day at 226mM Mg^{2+} (Table 3, Exp. 1). On this basis, 126mM Mg^{2+} was subsequently used for routine rearing of larvae. In contrast, Bradley reports survival rates of 85% and 15% for third instar larvae of this species in 226mM and 326mM Mg^{2+} respectively. He also reports good survival (80-100%) for larvae hatched in 26 to

Table 3: Percentage survival of larvae hatched in distilled water and reared in 50% seawater after they were transferred at day 2 to test solutions of different Mg^{2+} and Na^{+} content. Two separate experiments were performed. (n=original number of larvae in each experiment)

CONCENTRATION OF TEST SOLUTION (mM)		%SURVIVAL AFTER TRANSFER 1 Day		2 Days		n	
Na	Mg	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2
729	26	95	98	90	98	132	112
629	126	91	92	27	79	215	100
579	170	-	48	-	0	-	92
529	226	0.01	-	0	-	-	156
429	326	0	-	0	-	-	154
329	426	0	-	0	-	-	142
229	526	0	-	0	-	-	149

Table 4: Percentage survival of larvae hatched in distilled water and reared in 50% seawater after they were transferred at day 5 to solutions containing different amounts of Mg^{2+}. (n=original number of larvae in each experiment).

CONCENTRATION of TEST SOLUTION (mM)		%SURVIVAL AFTER TRANSFER		n
Na	Mg	1 Day	2Days	
729	26	96	92	50
679	76	92	88	50
629	126	92	80	50

226mM Mg^{2+} solution whereas negligible survival for larvae hatched in 126mM Mg^{2+} was observed in present study (data not shown). Accordingly, larvae were hatched in distilled water and transferred to solution containing 26mM Mg^{2+} and Mg^{2+} was increased to 126mM at the third instar. After approximately 6 generations (3 months), the tolerance of larvae to external Mg^{2+} was retested. In this case larvae were transferred to high Mg^{2+} test solutions after either 2 days (i.e. Table 3, Exp. 2) or 5 days (4th instar, Table 4) because of the suggestion (Bradley et al., 1982) that tolerance increases with developmental stage. As shown in Tables 3 and 4, 20% mortality of 3rd and 4th instar larvae occurred after 2 days in 26 to 126mM Mg^{2+} waters and 100% mortality at 170mM Mg^{2+}, suggesting that there was a small but not a dramatic increase in Mg^{2+} tolerance as a result of repeated selection. As compared to larvae reared at low Mg^{2+} concentration, those exposed to 126mM $MgSO_4$ medium were smaller in size. The lesser tolerance of larvae to Mg^{2+} in our experiments compared to those of Bradley et al. (1982) may reflect the fact that our colony had previously been reared in high HCO_3^-/low Mg^{2+} waters by Strange (1982) for several years.

Viability and Characterization of In Vitro Malpighian Tubule Preparation

The viability of *in vitro* Malpighian tubules was assessed by following the metabolically dependent fluid secretion rate (J_v) with time and its response to the common stimulant of insect tubules, cAMP. The control bathing saline (iv,Table 2) resembled larval hemolymph and is known to sustain ion transport at steady rates for many hours (Strange, 1982).

Unstimulated tubules secreted at an average rate of 0.30±0.06 nl/min-mm over the first 1.5h. (Fig. 3) and J_v declined from 0.54 to 0.20 nl/min-mm during this time, which compares favourably with results for other insect Malpighian tubule preparations;eg., rates of secretion by tubules of fed *A. taeniorhynchus* larvae were found to be 1.95±0.16 nl/min (n=10) during the first 5 minutes after isolation, falling to 0.98±0.06 nl/min (n=27) after 15 min. and to 0.41±0.05 nl/min (n=10) during the subsequent 15 min. (Maddrell et al., 1978). In two experiments at different times of the year (Fig. 3), cAMP stimulated the average J_v by about 6-fold to 1.96±0.23 (1mM cAMP) and 1.6±0.19 (2mM cAMP) nl/min-mm respectively over the first 1.5h. Stimulated J_v declined by about 30% over this period which is a small decrease compared to preparations of other insect tubules. These results demonstrate the capacity of mosquito tubule cells to respond to the metabolic demands of the

greatly increased ion transport rates which drive stimulated J_v. An indication of the range and time course of J_v for individual cAMP-stimulated (2mM) tubules is shown in Fig. 4. While the range in J_v is quite large, the decline with time is reasonably consistent.

The trans-epithelial potential difference of 8 Malpighian tubules isolated in a bathing solution containing 2mM Mg^{2+} (15mM K^+, 60mM Na^+, 4mM Ca^{2+}, 41mM Cl^-, 5mM SO_4^{2-}, 8mM HCO_3^-) varied widely but averaged -15mV at 90 minutes (lumen negative with respect to bathing solution). Variations with time for individual tubules were less than differences between tubules (Table 5).

Table 5: Potential readings across A. dorsalis Malpighian tubules. (mV)

Time(min.)	30	60	90
	-15	-19	-17
	-14.5	-11	-20
	-28	-12	-24
	+5	+8	+4
	+8	+2	+4

Figure 3. The effect of cAMP on secretion rates of A. dorsalis Malpighian tubules when added to the bathing drop. Larvae were reared in seawater containing 126mM $MgSO_4$. Vertical lines attached to the bars represent ±S.E. of the mean.

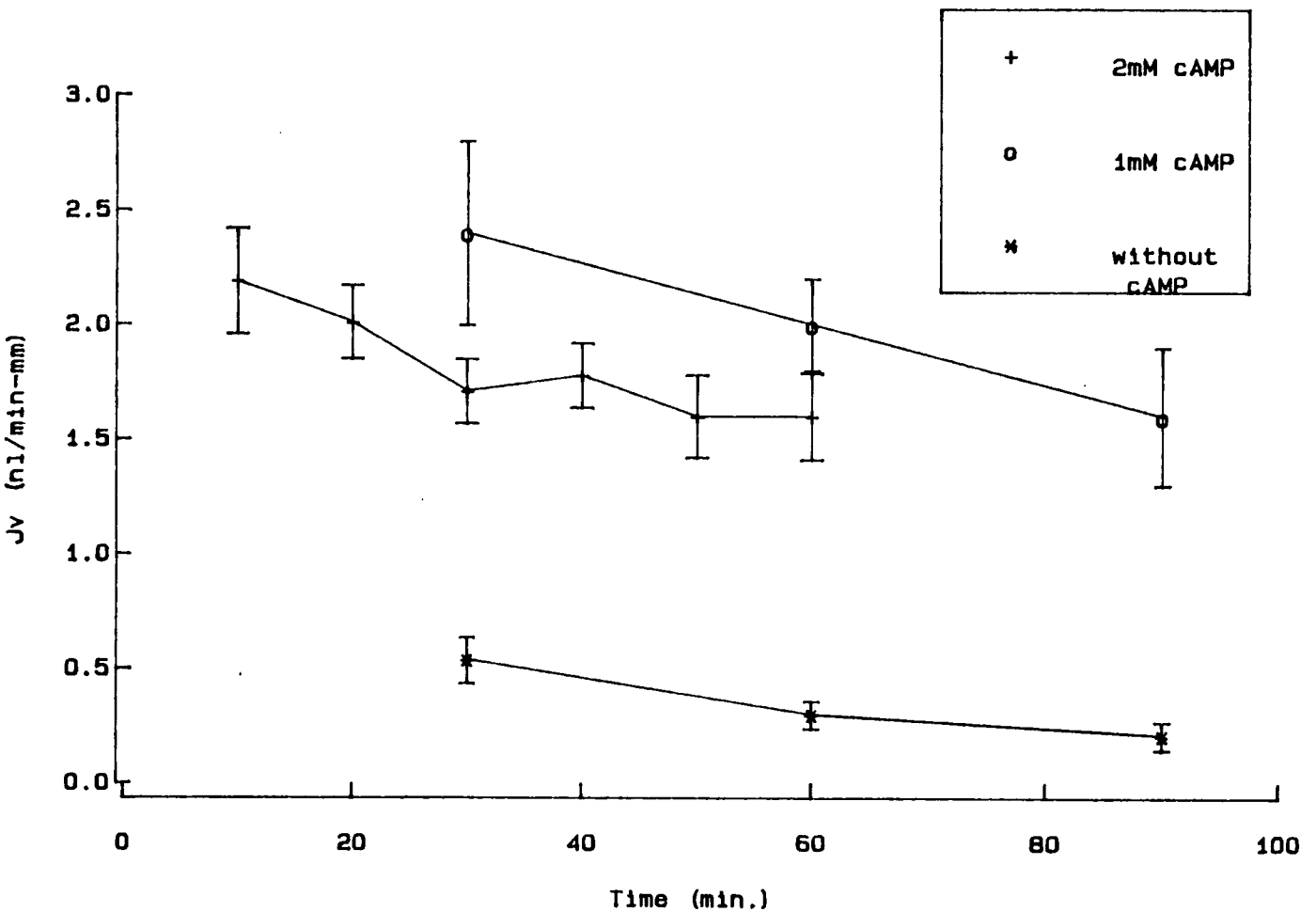

+ 2mM cAMP
o 1mM cAMP
* without cAMP
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Jv (nl/min-mm)
0
20
40
60
80
100
Time (min.)

Figure 4. Secretion rates of individual Malpighian tubules of A. dorsalis reared in 126mM $MgSO_4$ seawater and stimulated with 2mM cAMP (n=9).

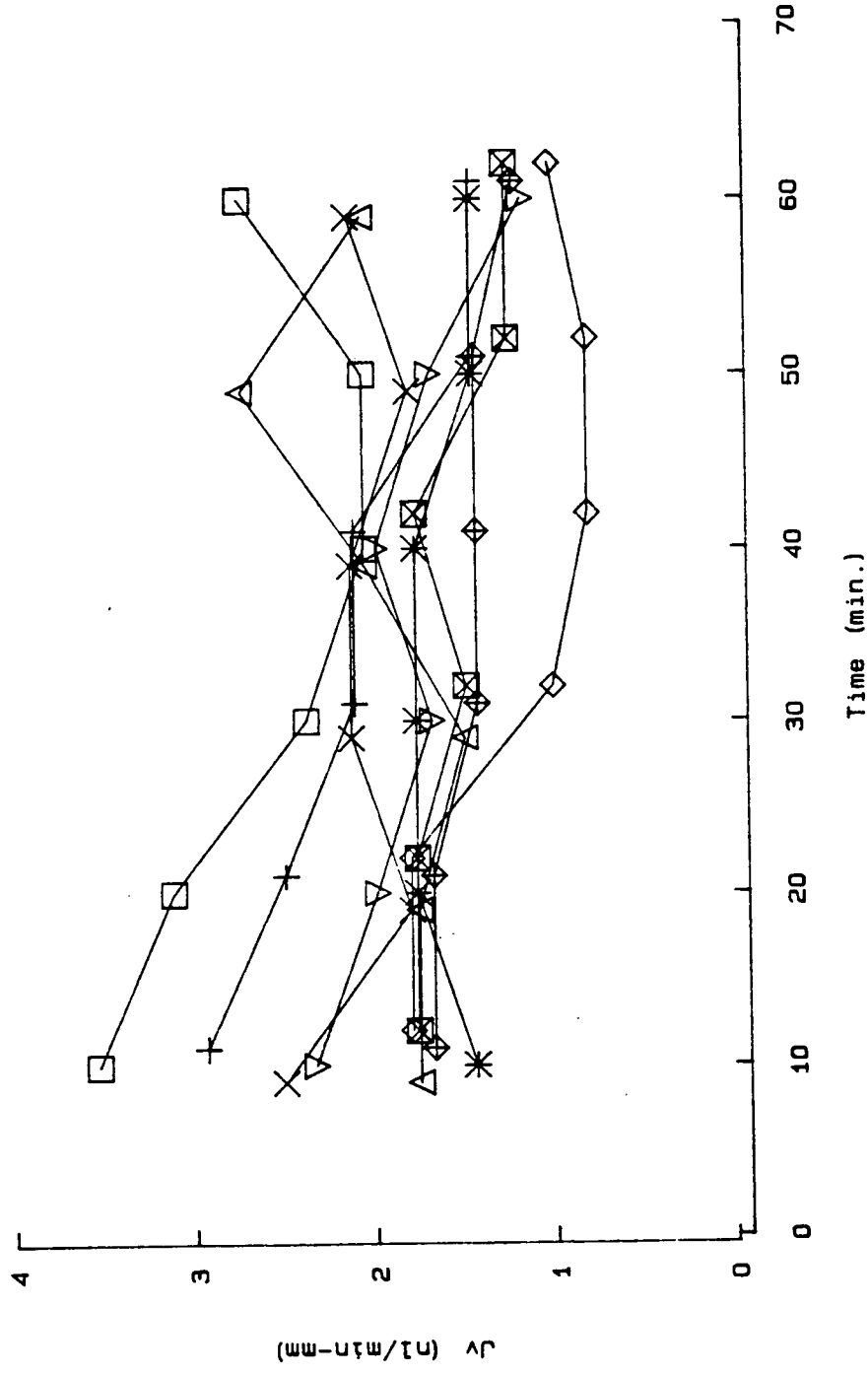
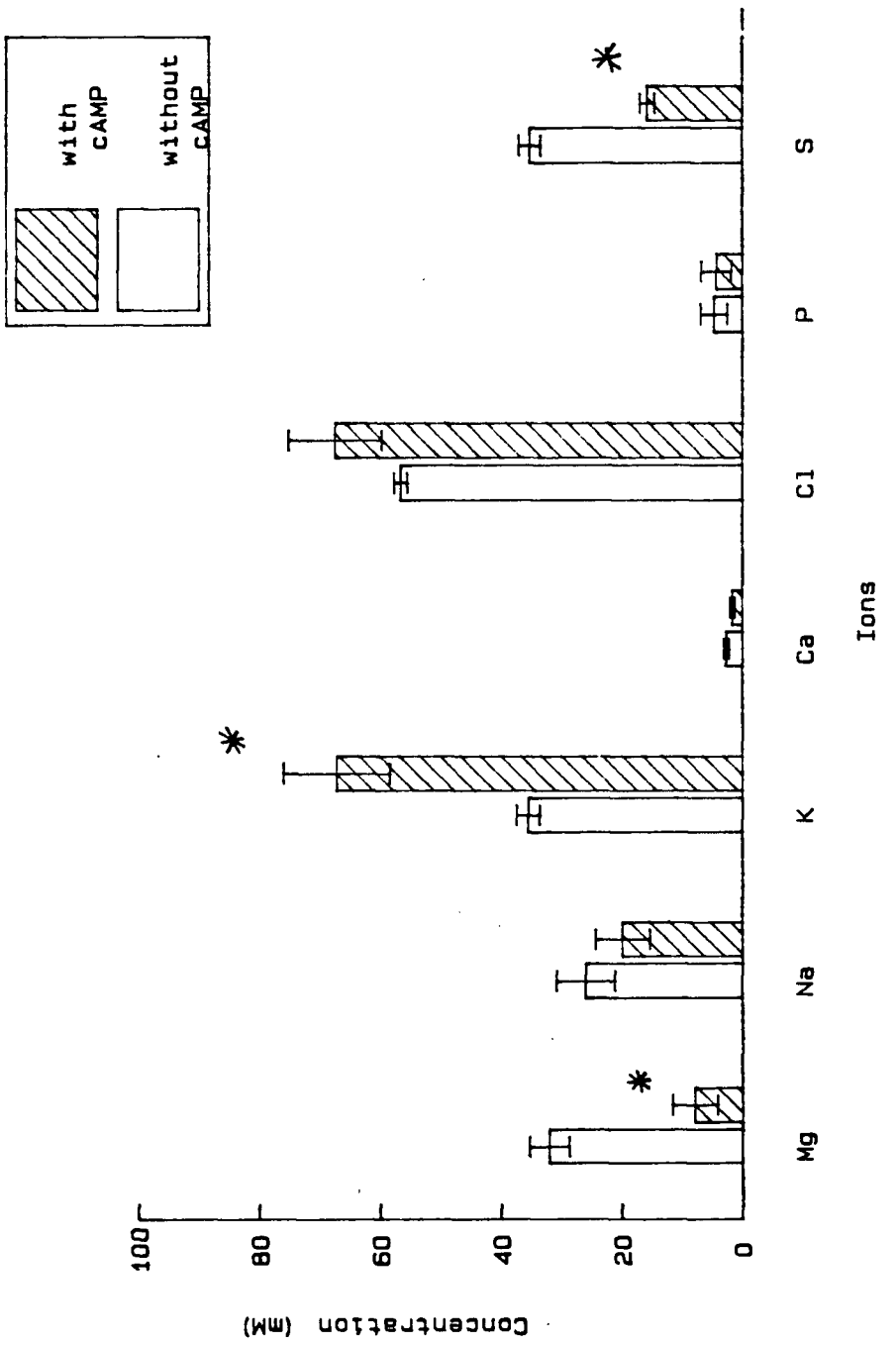

Time (min.)
0
10
20
30
40
50
60
70
0
1
2
3
4
ΔV (nl/min-mm)

Figure 5 shows the average electrolyte composition of the secretion from unstimulated and cAMP-stimulated tubules over the first 1.5h in initial experiments. Like many other insect cAMP-stimulated tubules including A. campestris and A. taeniorhynchus (Phillips and Maddrell, 1974);(Maddrell and Phillips, 1978) those of A. dorsalis larvae secrete a KCl(68mM)-rich fluid (U) as compared to the bathing saline (P) which contained 15mM K^+ and 40mM Cl^-. Clearly, secretion of these ions occurs against concentration differences (U/P ratio of 4.5 for K^+ and 1.7 for Cl^-). Since the transepithelial potential (Vt) ranges between -20 to +8mV (mean =-15mV,sign refers to lumen); (Table 5) for different tubules, clearly K^+ is actively secreted against an average net electrochemical difference of -38mV. Cl^- may also be secreted by active transport against a net electrochemical difference of -8.54 mV, as previously reported for tubules of a closely related larva,(A. campestris, Phillips and Maddrell,1974). Cyclic AMP stimulation significantly increased K^+ but not Cl^- concentration in the secretion (P=0.05).

As for many insect tubules, Na^+ levels in the secretion (16 to 25mM) are low relative to the bathing saline (60mM, U/P ratio of 0.3) and are not changed significantly by cAMP stimulation. Thus Na^+ probably diffuses passively into the tubular fluid due to active KCl secretion. The same may be true for Ca^{2+} since its U/P ratio is less than one.

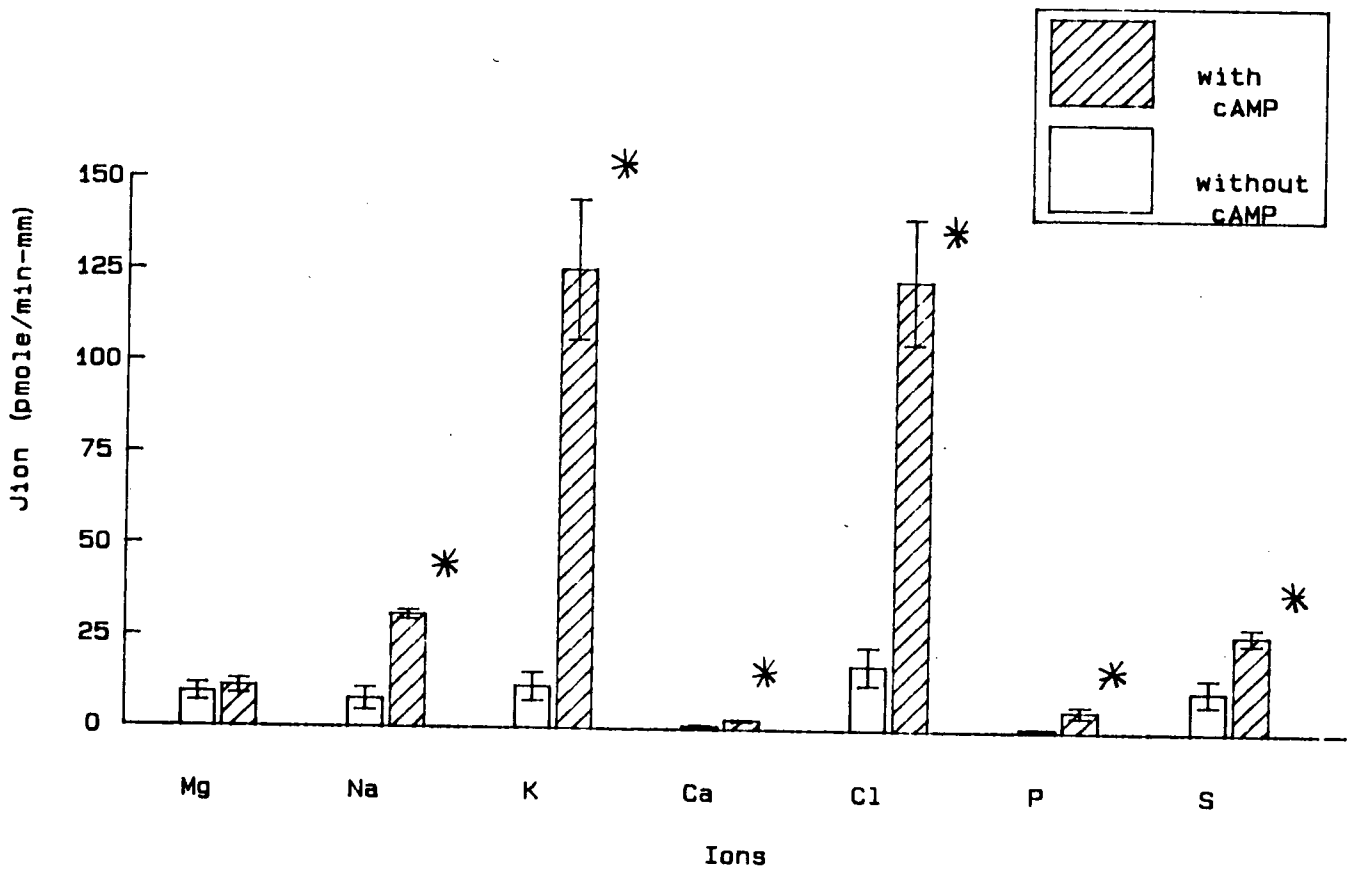
Figure 5. The effect (of adding 1mM cAMP to the bathing medium) on concentrations of ions secreted by A. dorsalis Malpighian tubules. Mean is average of values at 60' and 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=10). * indicates significant difference.


with cAMP
without cAMP
Concentration (mM)
100
80
60
40
20
0
Mg
Na
K
Ca
Cl
P
S
Ions
*
*
*

As previously reported for A. campestris larvae from (Na+Mg)SO_4 lakes, A. dorsalis tubules in saline containing 2mM Mg secrete Mg^{2+} (32mM) against a U/P of 16-fold. Levels of Mg^{2+} in the tubular secretion fall to 8mM after stimulation, not because Mg^{2+} secretion is reduced (see Fig. 6), but because this process is independent of J_v and enhanced KCl-driven J_v dilutes Mg in the tubule lumen. This was previously observed for Mg^{2+} secretion by tubules of A. campestris larvae by Phillips and Maddrell (1974). Given the low transepithelial potential across A. dorsalis tubules (varying from +8mV to -20mV for different tubules), Mg^{2+} secretion must involve active transport assuming that, as for A. campestris, this cation does not exist as a precipitate in the secretion (see Phillips and Maddrell, 1974).

As generally observed for other insect tubules (Phillips, 1981) A. dorsalis tubules secrete phosphate against a large concentration difference (5-fold) and this is not significantly changed by cAMP stimulation. Likewise, total sulphur, presumably as SO_4^{2-}, is secreted against a difference of nearly 2-fold by unstimulated tubules, in agreement with previous observations for A. campestris (Phillips and Maddrell, 1975) and A. taeniorhynchus tubules (Maddrell and Phillips,1978). Cyclic AMP causes levels of SO_4^{2-} to fall in the secreted fluid because of dilution but

Figure 6. The effect (of adding 1mM cAMP to the bathing medium) on secretion rates of ions by A. dorsalis Malpighian tubules. Mean is average of values at 60' and 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=10). * indicates significant difference.


with cAMP
without cAMP
Jion (pmole/min-mm)
150
125
100
75
50
25
0
Mg
Na
K
Ca
Cl
P
S
Ions

stimulation actually increases net SO_4^{2-} secretion rates (see Fig. 6).

Rates of ion secretion (Jion) are a function of both J_v (Fig. 3) and ion levels in the secretion (Fig. 5). The pooled values for tubules determined at 1.0 and 1.5h. are shown in Fig. 6. As expected, if KCl transport largely drives fluid secretion, cAMP causes 11-fold and 7-fold increase respectively ($P<0.05$) in J_K and J_{Cl}, compared to an increase in J_v of 6-fold (Fig. 3). Stimulation also causes smaller but significant increases in Na^+ (4-fold; $P<0.05$), Ca^{2+} (3-fold; $P<0.05$), sulphur (2.5-fold; $P<0.05$), and P (6.5-fold; $P<0.05$) secretion rates. No significant change in Mg^{2+} secretion rate is apparent after addition of cAMP.

A comparison of total equivalents of cations (Mg^{2+}, Ca^{2+}, Na^+, K^+) and anions (Cl^-, S as SO_4^{2-}, P as $H_2PO_4^-$) indicates no anion deficit in the secretion. In all experiments in Tables 6 and 7, the average difference between total anions and total cations ranged between 0 and 10 mEquiv.L^{-1}. This suggest that HCO_3^- and organic anions are not major components in the secretion. Moreover, significant alkalinization of secretion due to HCO_3^- or OH^- secretion was not observed.

The fluid secreted by A. dorsalis Malpighian tubules (204±1.38 mosm, (n=19)) is not significantly different ($P=0.05$) than the bathing solution (198.8±1.83 mosm, (n=6)). Like most other insect Malpighian tubules, the composition

Table 6: Concentration of Ions in Secreted Fluid (mM) and J_v (nl/min-mm)

Concentration of Mg in Artificial Hemolymph(mM)		Mg^{2+}		Ca^{2+}		K^+		Na^+		Cl^-		P		S		J_v	
		c	$s\sqrt{n}$	c	$s\sqrt{n}$	c	$s\sqrt{n}$	c	$s\sqrt{n}$	c	$s\sqrt{n}$	c	$s\sqrt{n}$	c	$s\sqrt{n}$	J	$s\sqrt{n}$
0.0	a.																
	b.	9.1	0.4	3.3	0.9	52.8	4.1	43.1	11.6	47.5	3.0	4.2	1.8	36.4	7.4	0.28	0.22
0.5	a.	6.5	0.6	3.3	0.7	50.7	5.8	36.2	8.2	55.2	1.4	3.9	0.7	27.5	2.7	0.40	0.11
	b.	8.2	1.0	2.3	0.2	67.7	3.8	25.9	3.4	63.0	2.5	4.9	0.7	18.6	0.5	0.54	0.11
1.0	a.	8.4	0.7	3.1	0.8	44.5	3.7	45.1	2.7	64.5	2.9	4.3	1.7	25.5	5.3	0.37	0.07
	b.	15.0	1.7	3.0	0.5	52.8	3.6	36.6	4.4	62.2	3.6	5.2	0.9	27.9	2.7	0.41	0.06
2.0	a.	22.0	2.9	5.7	1.2	34.7	3.0	40.6	5.9	63.9	1.2	5.4	1.0	39.3	2.9	0.43	0.09
	b.	32.0	3.3	2.7	0.4	35.5	1.9	26.0	4.8	56.6	1.1	4.6	2.2	35.2	1.8	0.35	0.07
4.0	a.	27.9	3.0	6.7	1.2	43.4	3.6	28.1	4.8	87.9	3.3	3.7	0.9	30.3	3.1	0.47	0.06
	b.	30.3	4.1	5.7	1.0	37.3	6.3	33.7	4.5	75.9	1.4	3.6	1.2	35.2	3.5	0.44	0.06

a=larvae reared in 26mM $MgSO_4$ solution.
b=larvae reared in 126mM $MgSO_4$ solution.

Table 7: Flux of Ions from A. dorsalis Malpighian tubules (pmole/min-mm) and J_V (nl/min-mm)

Concentration of Mg in Artificial Hemolymph(mM)		Mg^{2+}		Ca^{2+}		K^+		Na^+		Cl^-		P		S		J_V	
		J	$s\sqrt{n}$	J	$s\sqrt{n}$	J	$s\sqrt{n}$	J	$s\sqrt{n}$	J	$s\sqrt{n}$	J	$s\sqrt{n}$	J	$s\sqrt{n}$	J	$s\sqrt{n}$
0.0	a.																
	b.	3.62	0.9	1.1	0.2	21.8	6.3	16.0	4.2	19.5	5.2	1.4	0.3	13.8	3.8	0.28	0.22
0.5	a.	2.8	0.9	1.5	0.6	21.2	6.2	15.5	4.7	23.6	6.9	1.6	0.6	11.5	3.2	0.40	0.11
	b.	3.7	0.6	1.0	0.1	33.6	7.3	11.6	2.1	30.4	5.7	2.2	0.3	9.0	1.7	0.54	0.11
1.0	a.	2.5	0.4	0.8	0.1	14.5	3.4	13.9	2.2	20.3	3.8	1.5	0.6	7.4	1.1	0.37	0.07
	b.	5.4	1.0	1.1	0.2	18.5	2.7	10.9	2.2	21.9	3.3	1.9	0.6	9.9	1.7	0.41	0.06
2.0	a.	6.8	1.7	1.8	0.6	10.1	1.7	11.6	2.5	19.1	3.6	1.5	0.4	12.1	2.8	0.43	0.09
	b.	9.3	2.4	0.9	0.3	11.4	3.8	7.7	3.0	17.4	5.2	0.9	0.2	11.2	3.6	0.35	0.07
4.0	a.	10.1	1.5	2.6	0.6	16.2	2.6	9.5	2.3	33.0	4.7	1.3	0.4	10.6	1.3	0.47	0.06
	b.	11.5	3.8	2.2	0.6	15.3	5.0	12.1	2.0	29.1	5.5	1.1	0.2	13.7	2.7	0.44	0.06

a=larvae reared in 26mM $MgSO_4$ solution.
b=larvae reared in 126mM $MgSO_4$ solution.

of secreted fluid is isosmotic to the bathing solution. In addition, no significant differences were found between osmolarity of fluid secreted by tubules bathed in either 0.5, 1, 2, or 4mM Mg saline solution and between larvae reared in either 26mM or 126mM Mg^{2+} medium (P=0.05).

A comparison of pH of bathing droplets (saline iv, Table 2) before and after the 90 minute experimental period indicated a change in pH of 0.2 units (n=4) from 7.2 to 7.4 respectively. Secreted droplets had an average pH of 7.24±0.07 (n=5) at the end of the 90 minute experimental period. Therefore, pH of bathing and secreted droplets were not significantly different (P=0.05).

Another indicator of tubule viability is the constancy in the composition of the secretion with time, since a deterioration in cellular metabolism should reduce active secretion of ions as opposed to passive diffusion and thereby change ionic ratios. Significant changes in ion concentration in the secretion did not occur between 0.5, 1.0, and 1.5h. after the start of experiments (data in Appendix A, Fig.5 for unstimulated and stimulated tubules).

The 30% decline in stimulated J_v over 1.5h., which clearly reflects KCl secretion rate, may indicate either some deterioration in tissue viability or alternately a regulatory decrease in cAMP stimulation of transport processes with time, or both.

Having characterized secretion by *in vitro* tubules of *A. dorsalis* and having confirmed Mg^{2+} secretion against large concentration differences in this species, the kinetics of Mg^{2+} secretion was next investigated to decide on a suitable concentration of this cation to be used in a later studies of Na-dependency.

Since the Mg^{2+} gradients developed across the tubule wall are much larger in unstimulated tubules (Fig. 5) and since J_{Mg} is not changed by cAMP stimulation (Fig. 6), subsequent experiments were conducted on unstimulated tubules.

Influence of External Mg^{2+} Levels on Fluid and Ion Secretion Rates

Unstimulated tubules were bathed in salines containing 0, 0.5, 1.0, 2.0, or 4.0 mM Mg^{2+} but otherwise identical in composition, and secretion was measured. Since SO_4^{2-} secretion in A. campestris is increased by exposure of larvae to higher external levels of this anion for a day (Phillips and Maddrell,1975), a similar stimulation of tubular Mg^{2+} secretion might be anticipated. Accordingly, tubules were compared from A. dorsalis larvae reared totally in 26mM Mg^{2+} and others exposed for 1 to 2 days to 126mM Mg^{2+} just before use. As shown in Fig. 7, J_v is not significantly affected by Mg^{2+} concentrations (0-4mM) in the saline bathing tubules or in the waters (26 and 126mM) in which larvae were reared.

The ionic composition and secretion rates at 1.0 and 1.5h (pooled) are given in Tables 6 and 7. As shown in Fig. 8, tubules in nominally Mg-free saline (measured concentration of 0.07mM Mg) continue to secrete fluid containing 9mM Mg, presumably from the cellular contents. Mg^{2+} levels in the secretion reach a maximum of about 30mM when the bathing saline contains 2-4mM Mg^{2+}. As shown in Fig. 9, the Mg^{2+} ratio developed across the tubule wall declines, as expected for a saturable transport process, as the external level of this cation increases but the trend is not marked over the limited range of concentrations tested.

Figure 7. Rates of fluid secretion of secreted droplets from A. dorsalis Malpighian tubules versus concentration of Mg in external saline solution. Mean is average of values at 60' and at 90'. Vertical lines attached to points indicate ±S.E. of the mean (n=52).

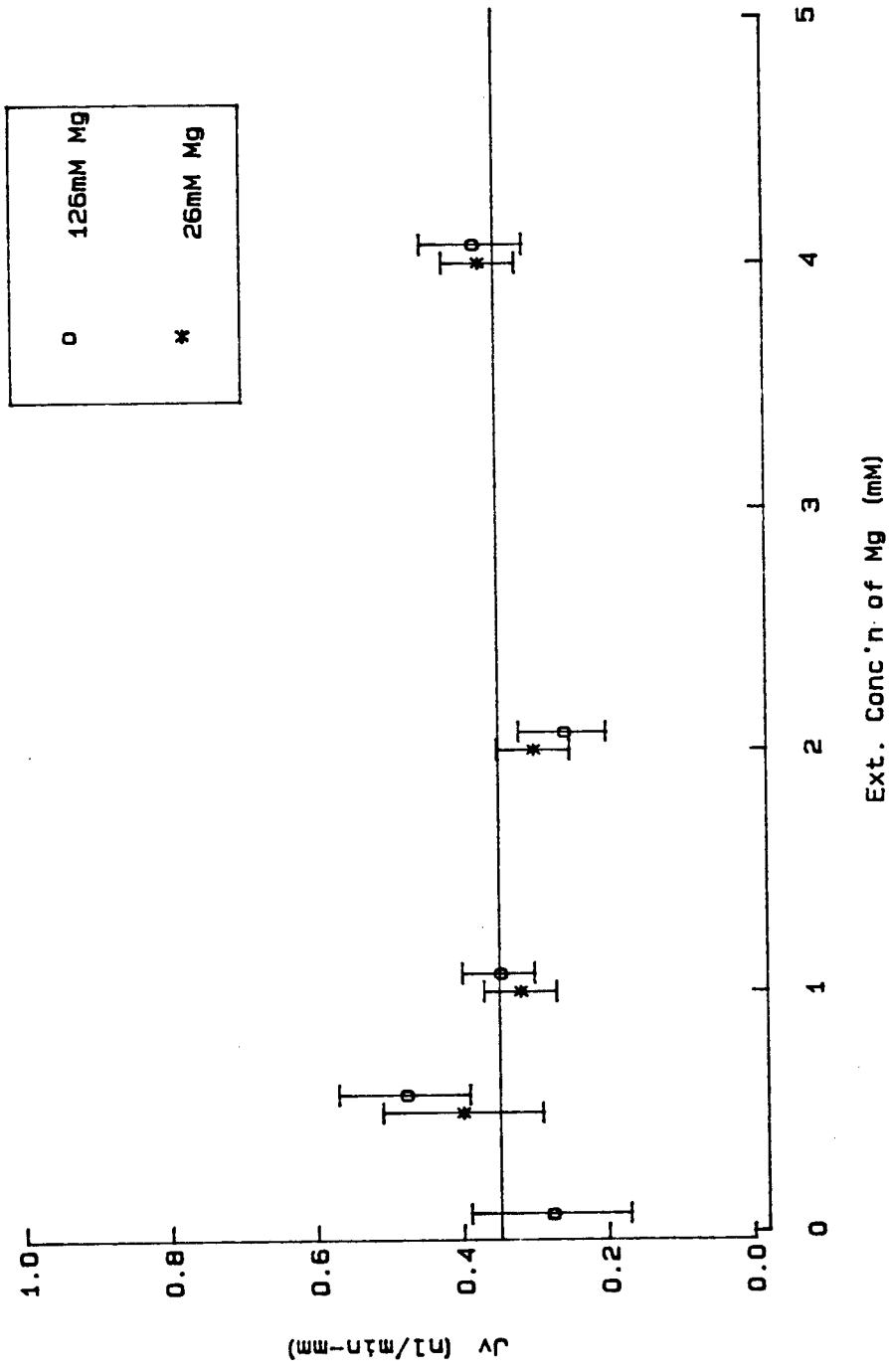

126mM Mg
26mM Mg
o
*
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Ext. Conc'n of Mg (mM)
Jv (nl/min-mm)

Figure 8. Concentrations of Mg secreted versus external concentrations of Mg bathing A. dorsalis Malpighian tubules. Larvae were reared in 126mM $MgSO_4$ medium. Mean is average of values at 60' and at 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).

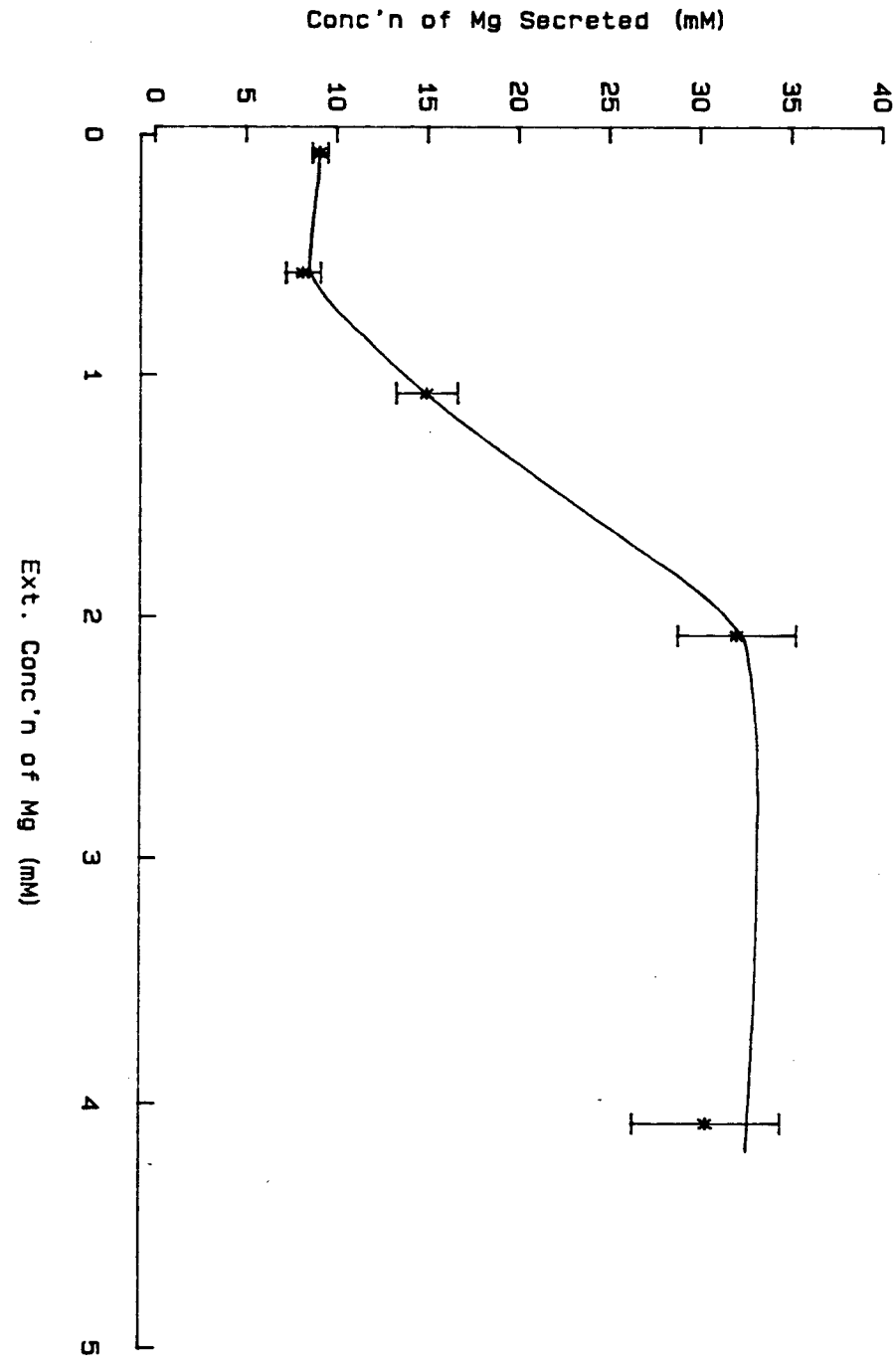
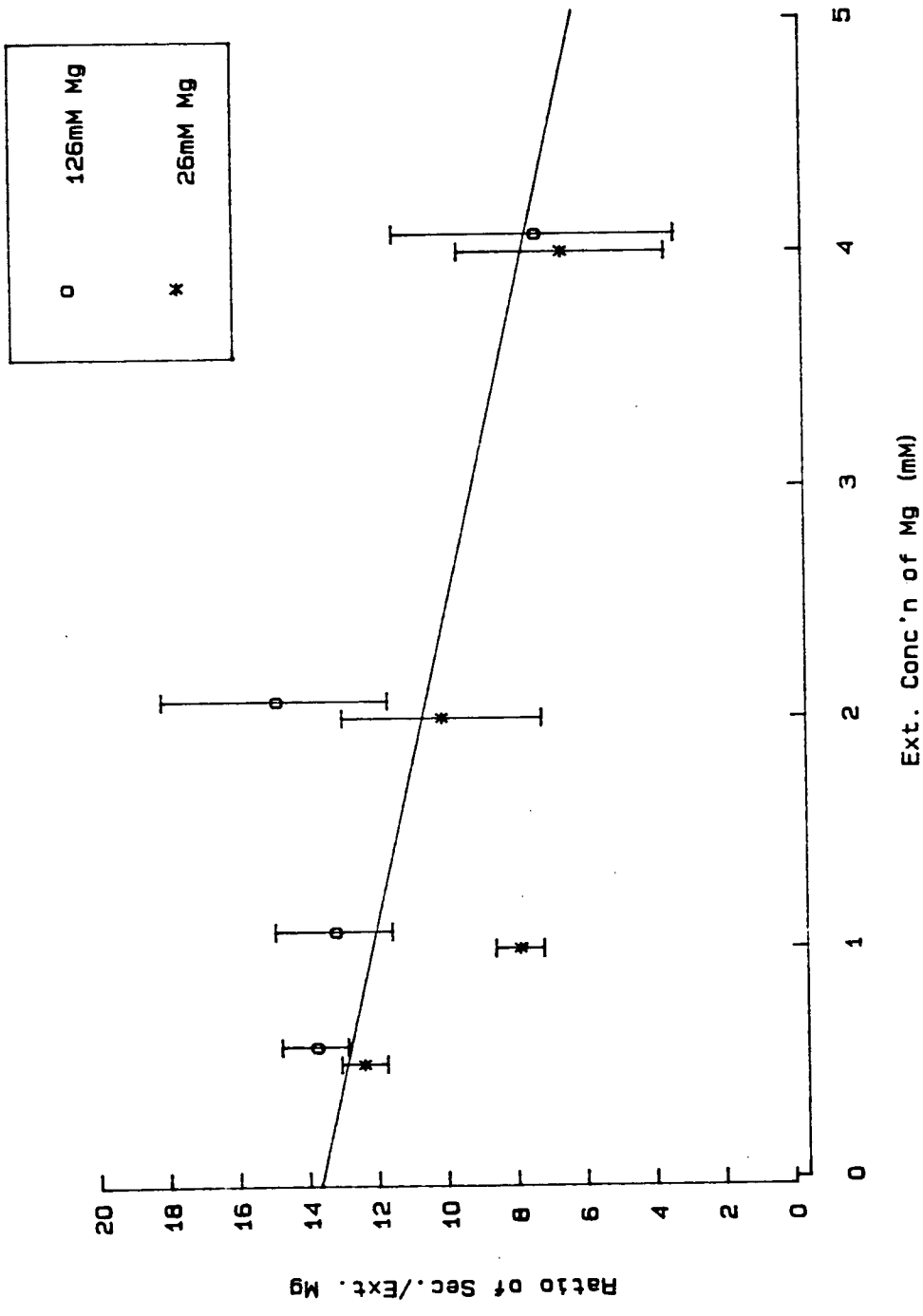

Conc'n of Mg Secreted (mM)
0
5
10
15
20
25
30
35
40
Ext. Conc'n of Mg (mM)
0
1
2
3
4
5

Figure 9. Ratio of Sec/Ext concentrations of Mg versus external (ext.) concentrations of Mg. Mean is average of values at 60' and at 90'. Vertical lines attached to points represent ±S.E. of the mean (n=52).


126mM Mg
26mM Mg
Ratio of Sec./Ext. Mg
Ext. Conc'n of Mg (mM)
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5

Two mM Mg is the lowest concentration at which J_{Mg} is markedly increased over the baseline level (0mM external Mg) and at which the U/P ratio is still very large (10-15). Therefore, 2mM Mg^{2+} in the saline was selected for later experiments on Na-dependency. Rearing larvae in 126 as opposed to 26 mM Mg had a slight but not dramatic effect on J_{Mg} (Fig. 10) and tubular Mg concentration (Fig. 8). Only the difference at 1mM Mg is significant ($P<0.05$). Perhaps if larvae were reared in Mg-free waters, a more significant effect on J_{Mg} might be demonstrated as was the case for $J_{SO_4^{2-}}$ in A. taeniorhynchus tubules (Phillips and Maddrell,1978).

Expressed as mEquiv.L^{-1}, the levels of secreted Mg^{2+}(60) from tubules bathed in 2 and 4 mM Mg^{2+} actually exceed those of both K^+ (36) and Na^+ (26-34) and indeed almost equals these two monovalent ions (Tables 6 and 7). The data suggests that as levels of Mg^{2+} in the secretion rise, those of K^+ decline whereas Na^+ does not change (Figs. 12.1, 13.1). Total Equivalents of all ions (assuming monovalent P, divalent S as SO_4^{2-}) are higher at 4mM Mg (293 mEquiv. L^{-1}) than at 0.5mM Mg (220 mEquiv. L^{-1}) suggesting an increase in osmolarity (Table 6). Increased Mg levels seem to be associated with increased Cl^- levels (compare 0 and 0.5mM Mg vs. 4mM Mg in Table 6 and 7) rather than other anions (eg. SO_4^{2-}). In addition, Figure 14 indicates general increases in Cl with Mg at 4mM Mg (compared to

0mM Mg) although values fluctuate in between these concentrations. However, there is enough variability between different experiments (Table 6 and 7) to exclude any rigorous statement at this time that increased Mg secretion significantly alters levels of other ions in the secretion (Tables 6 and 7).

If the increase in J_{Mg} above baseline in Fig.10 is considered, then J_{Mg} secretion appears to be a saturable process as expected for carrier mediated active transport with a V_{max} of 14.94±2.89 pmoles/min-mm and a K_m of 1.57±0.16 mM Mg^{2+} (for larvae reared in 126mM $MgSO_4$). These values are similar to those reported by Phillips and Maddrell (1974) for J_{Mg} in tubules of A. campestris. For larvae reared in 26mM Mg medium, V_{max} was 9.17±8.16 pmole/min-mm while K_m was 1.40±0.77 (Fig. 11).

Figure 10. J_{Mg} of A. dorsalis Malpighian tubules with increasing external Mg concentrations. Vertical bars attached to points represent ±S.E. of the mean (n=52). * indicates significant difference.

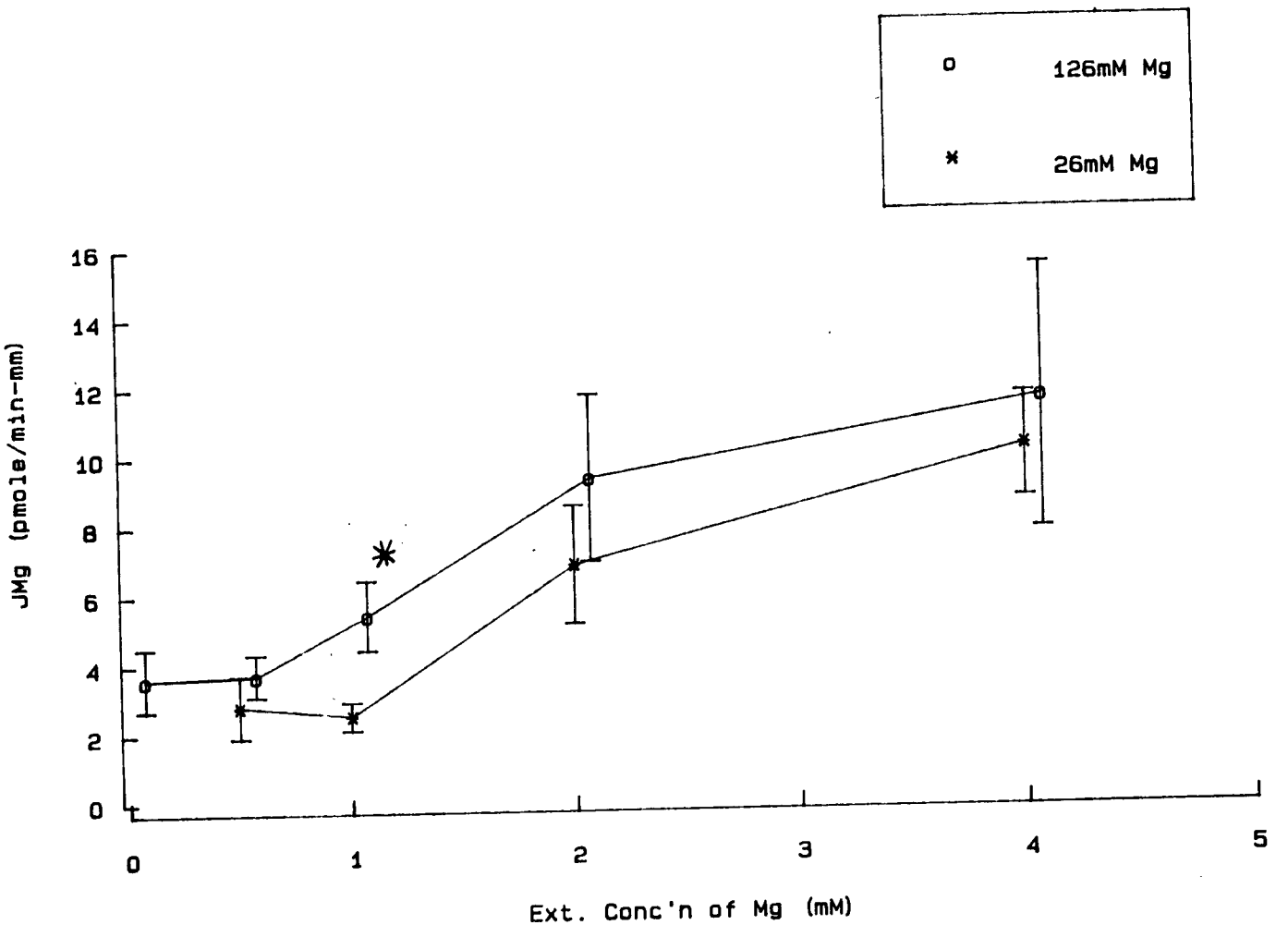

o 126mM Mg
* 26mM Mg
JMg (pmole/min-mm)
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
Ext. Conc'n of Mg (mM)

Figure 11. A double-reciprocal (Lineweaver-Burk) plot of J_{Mg} versus external concentration of Mg (n=52).

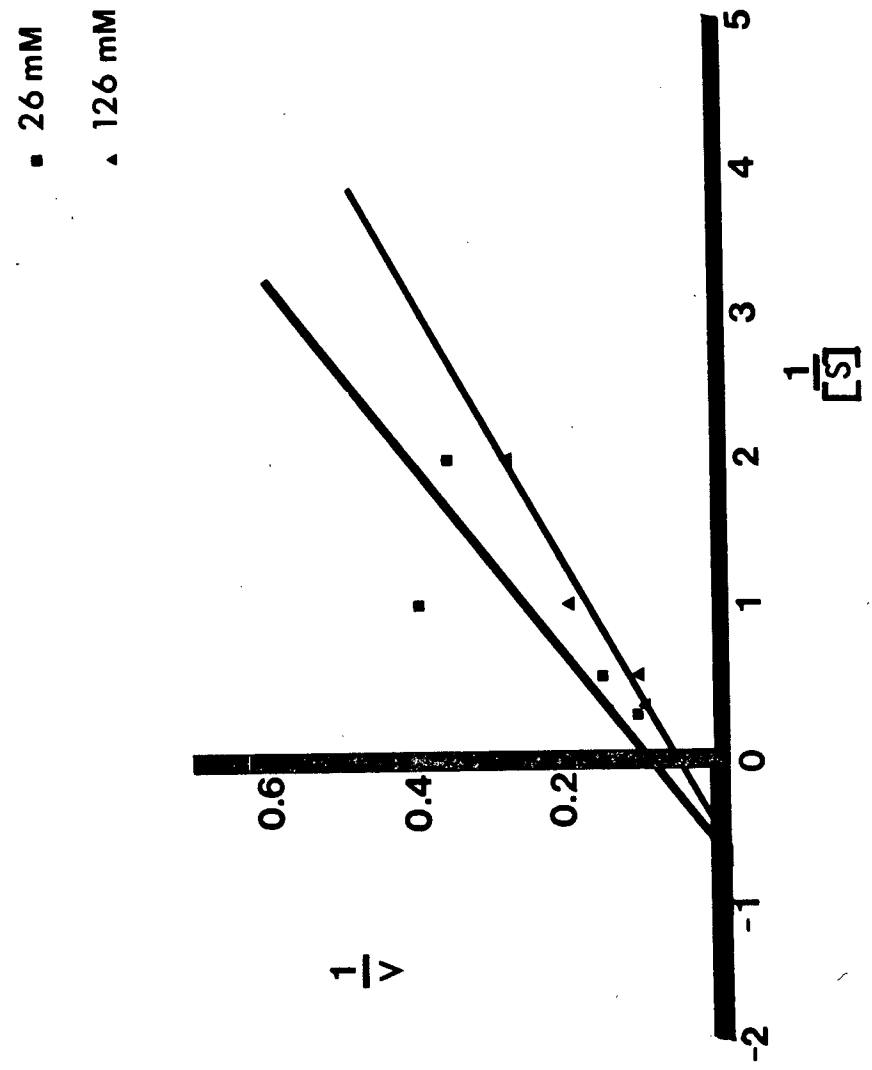

▪ 26 mM
▲ 126 mM
0.6
0.4
0.2
0
-1
-2
1
2
3
4
5
1/v
1/[S]

Figure 12.1. Concentrations of Mg and K in the secreted fluid versus external concentration of Mg. Mean is average of values at 60' and 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).

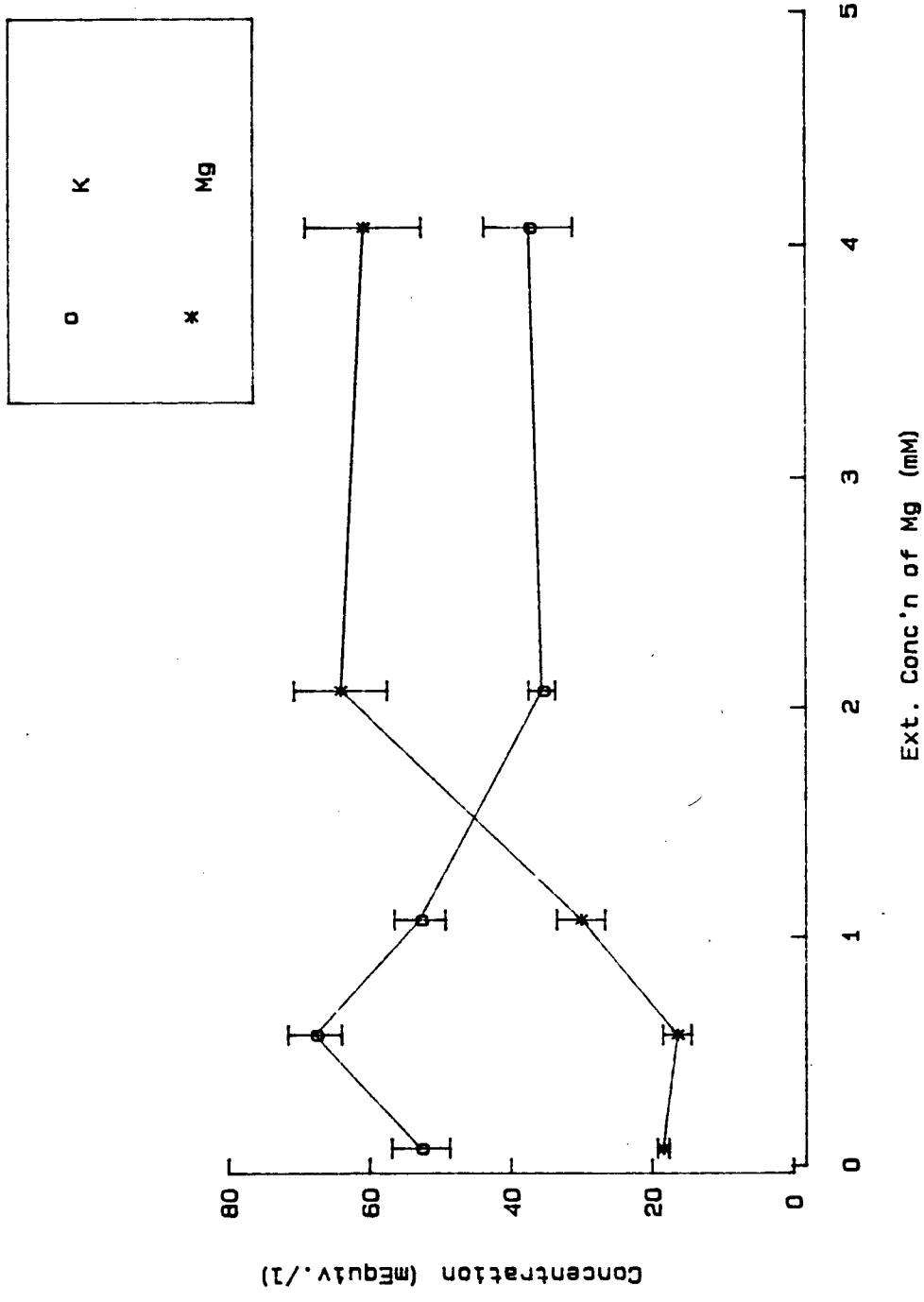

K
Mg
o
*
80
60
40
20
0
0
1
2
3
4
5
Concentration (mEquiv./l)
Ext. Conc'n of Mg (mM)

Figure 12.2. Secretion rates of Mg and K versus external concentration of Mg. Mean is average of values at 60' and 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).

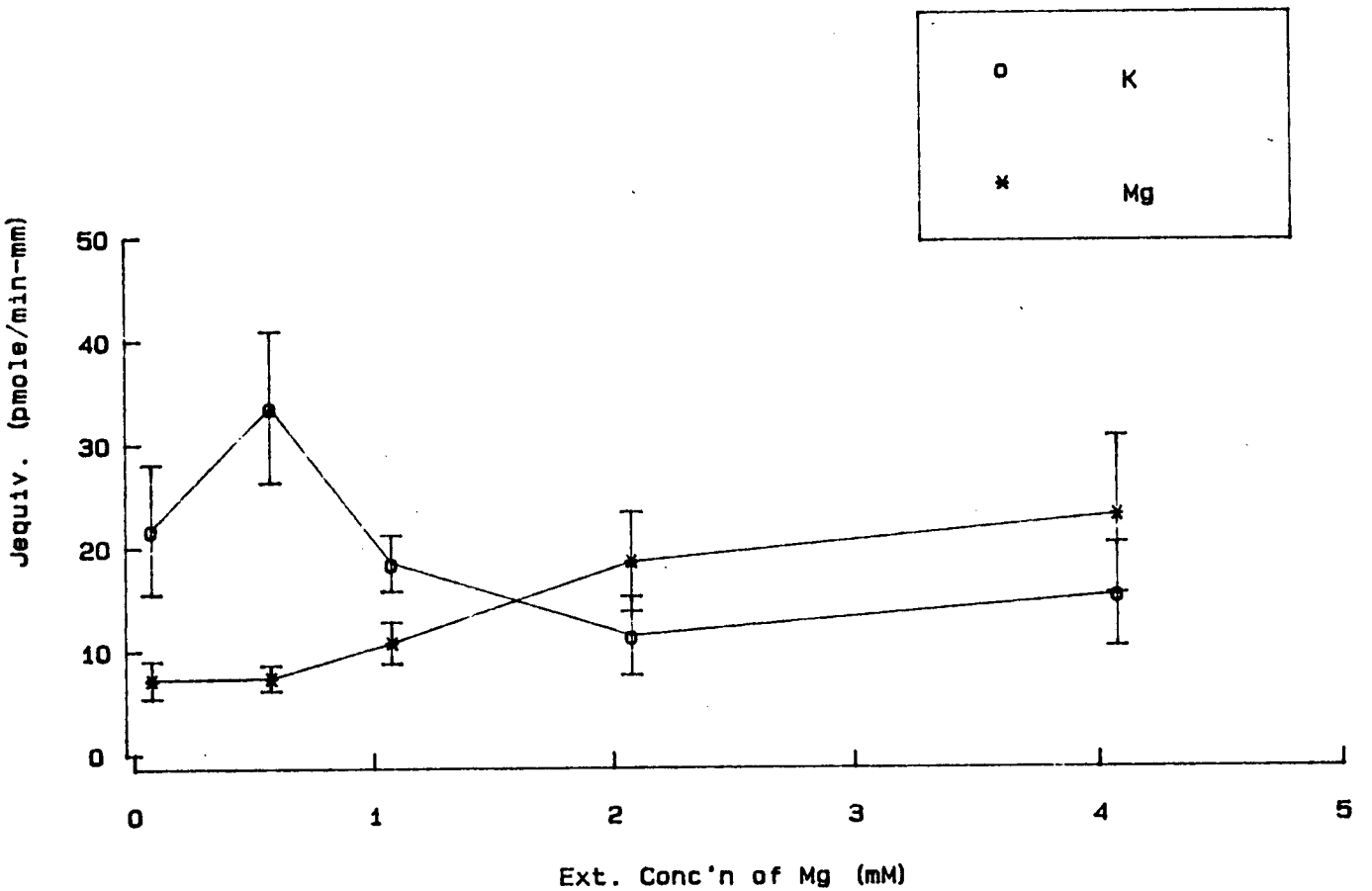

o K
* Mg
Jequiv. (pmole/min-mm)
50
40
30
20
10
0
0
1
2
3
4
5
Ext. Conc'n of Mg (mM)

Figure 13.1. Concentrations of Mg and Na in the secreted fluid versus external concentration of Mg. Mean is average of values at 60' and 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).

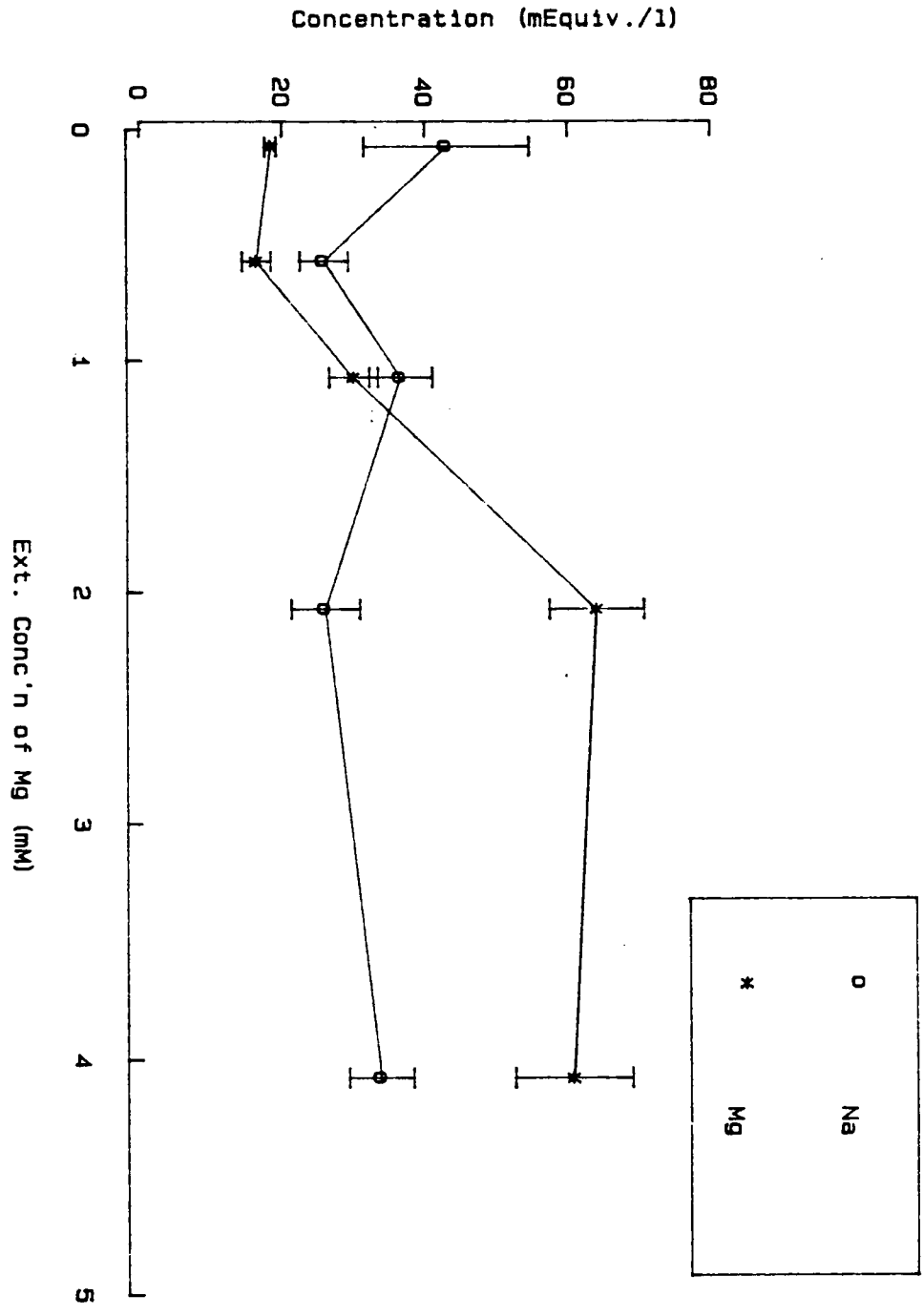

Concentration (mEquiv./l)
0
20
40
60
80
0
1
2
3
4
5
Ext. Conc'n of Mg (mM)
o
Na
*
Mg

Figure 13.2. Secretion rates of Mg and Na versus external concentration of Mg. Mean is average of values at 60' and 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).

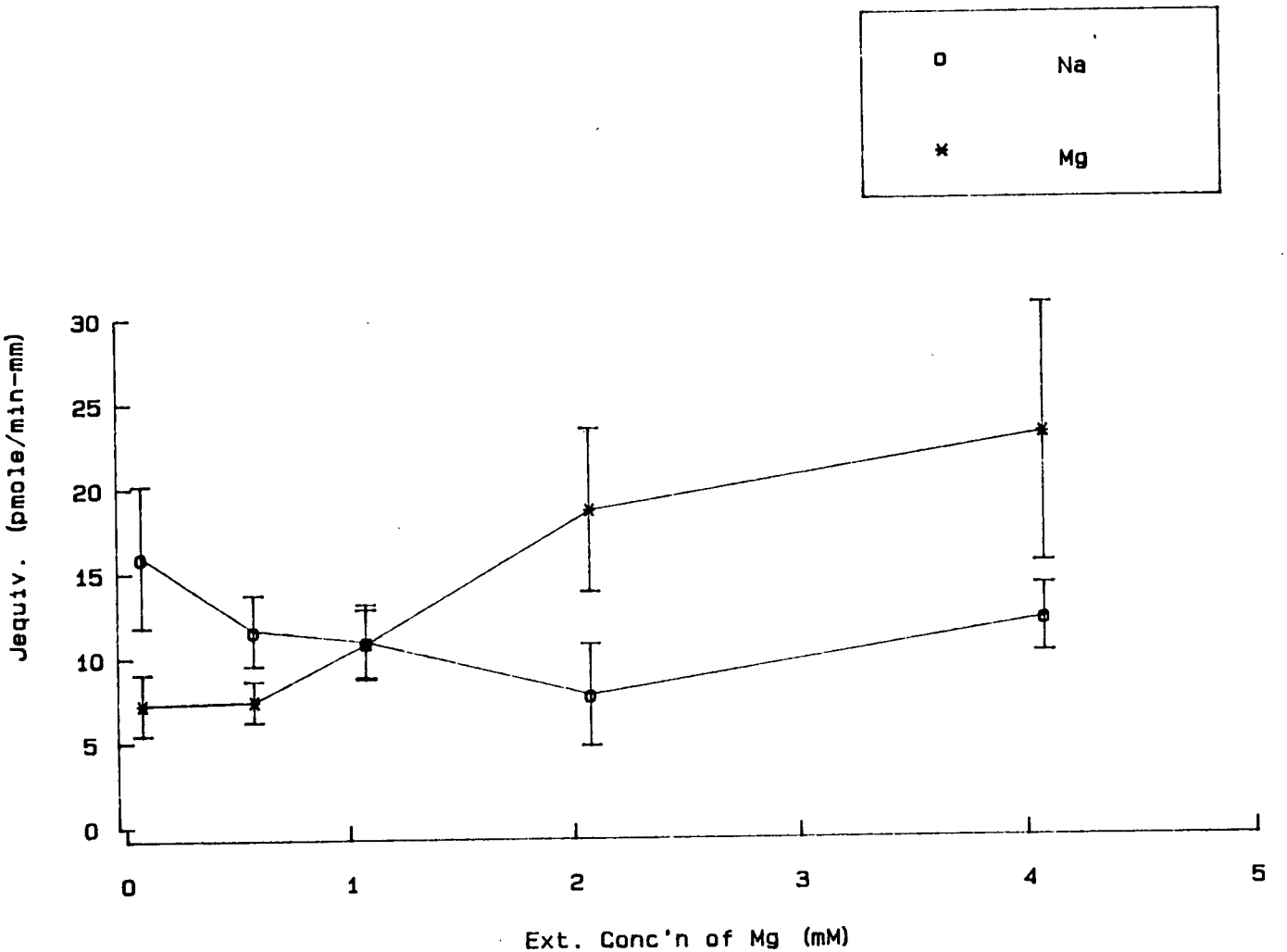

o Na
* Mg
Jequiv. (pmole/min-mm)
30
25
20
15
10
5
0
0
1
2
3
4
5
Ext. Conc'n of Mg (mM)

Figure 14.1. Concentrations of Mg and Cl versus external concentration of Mg. Mean is average of values at 60' and 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).

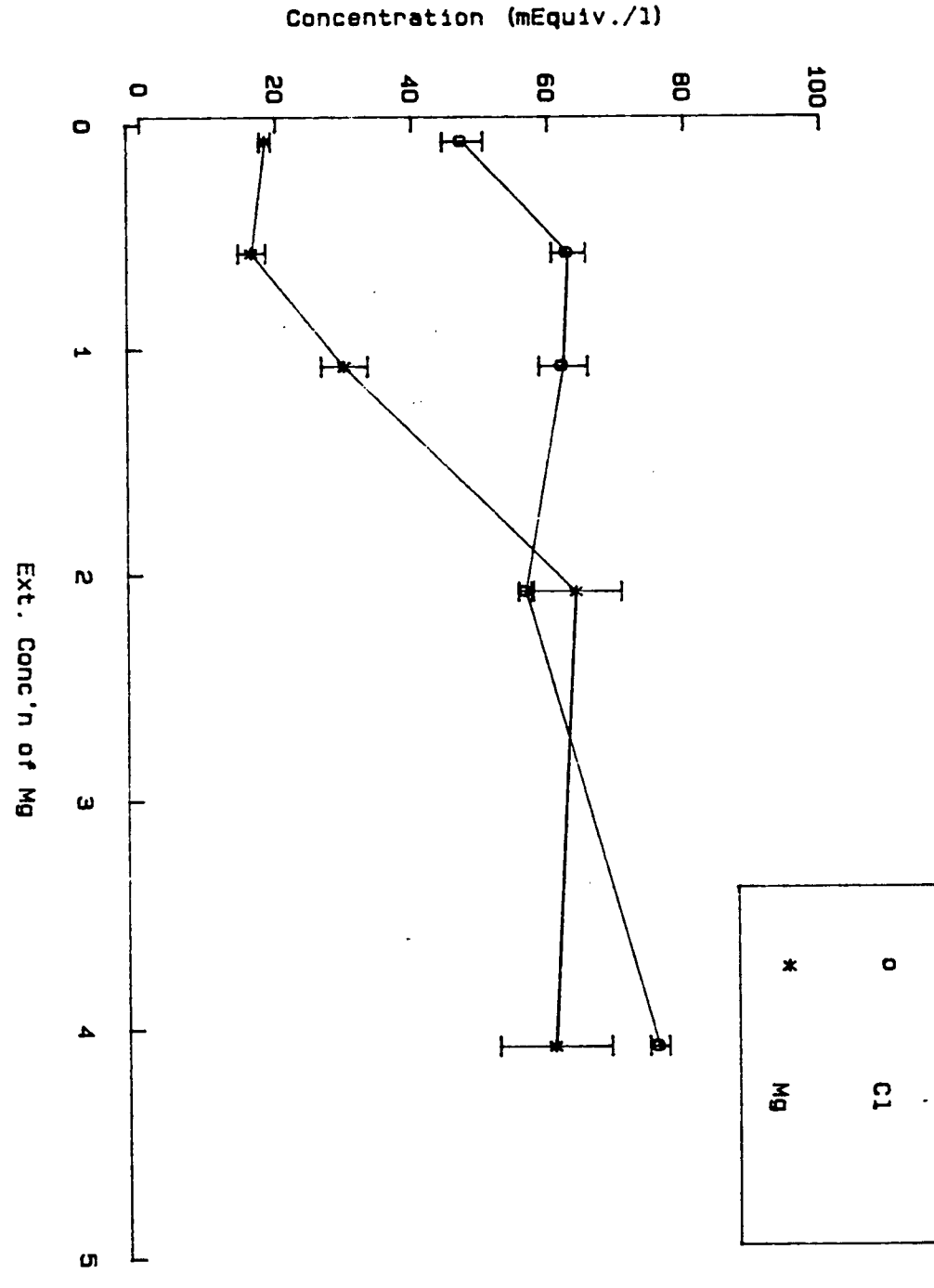
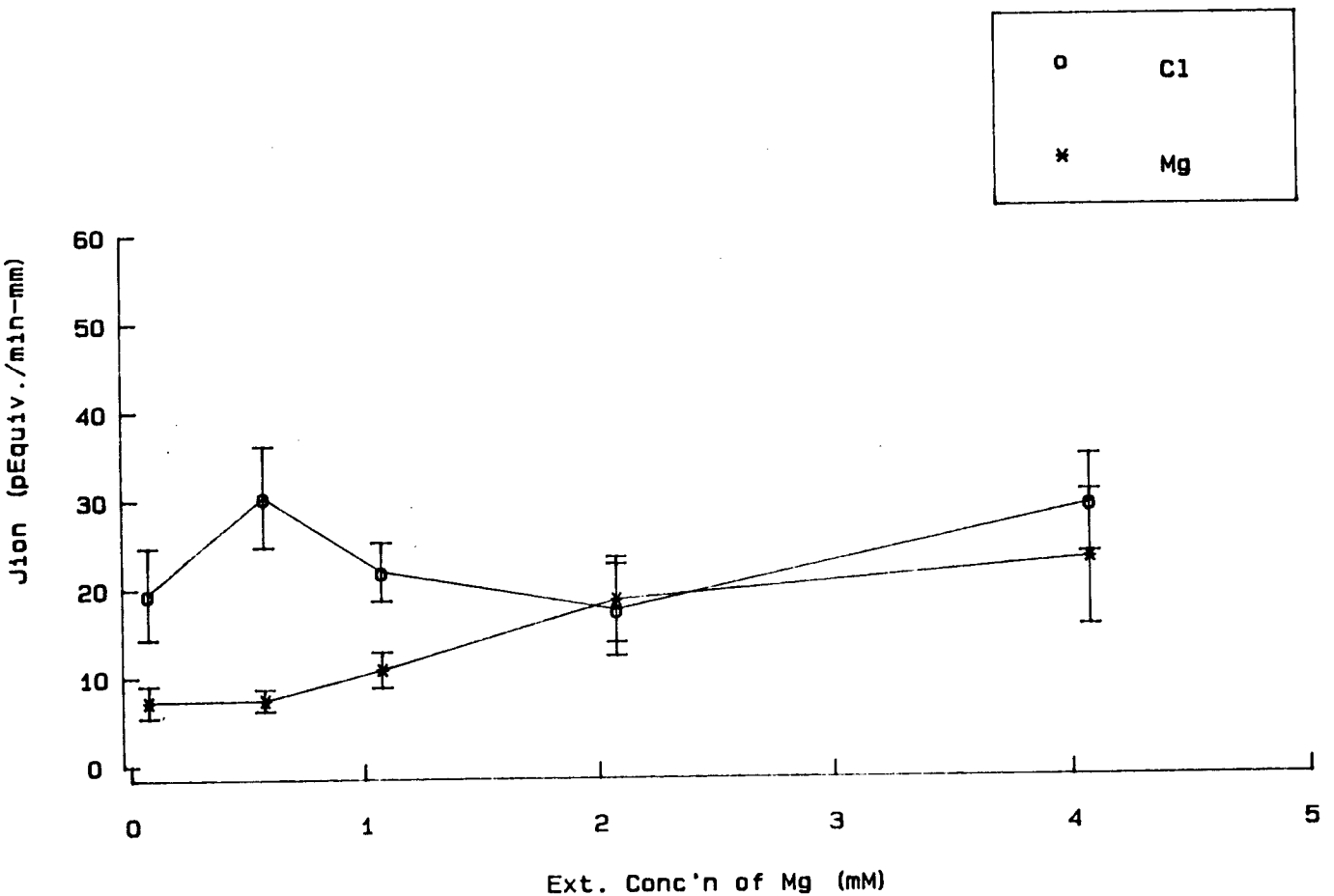

Concentration (mEquiv./l)
0
20
40
60
80
100
0
1
2
3
4
5
Ext. Conc'n of Mg
o
Cl
*
Mg

Figure 14.2. Secretion rates of Mg and Cl versus external concentration of Mg. Mean is average of values at 60' and 90'. Vertical lines attached to points represent ±S.E. of the mean (n=27).


o Cl
* Mg
Jion (pEquiv./min-mm)
60
50
40
30
20
10
0
0
1
2
3
4
5
Ext. Conc'n of Mg (mM)

Effect of Na-free Salines on Mg^{2+} Secretion

Since KCl secretion drives J_v in many insect tubules, it seemed feasible to replace Na^+ in the bathing saline so as to reduce luminal Na^+ to trace levels. If Mg secretion does occur by an apical Na^+/Mg^{2+} exchanger, then such a treatment should abolish J_{Mg}.

a. Choline substitution for Na

When Na was replaced by choline J_v dropped markedly from 0.53 to 0.18 nl/min-mm between 30 and 60 min. (Fig. 15) indicating that either choline or absence of Na^+ does influence secretion rates compared to controls (J_v of control tubules decreased from 0.54 to 0.30 nl/min-mm between 30 and 60 minutes). However, ionic composition of the saline did not change over this period (Fig. 16) except for P and S ($P<0.05$). Mg concentration increased from 19 to 28 mM (Fig.16) and J_{Mg} changed from 10 to 5 pmoles/min-mm (Fig.17) between 30 and 90 minutes, although not significantly. Thus Mg secretion continued against a U/P ratio of 10 to 14 (i.e. only slightly less than controls., Fig. 7,at 2mM ext. Mg) when average Na in the secretion was reduced to 2mM from 30 to 40 mM in controls (Table 6). J_{Na} was only 10% of J_{Mg} (Fig. 17). As seen in Fig. 17, at 30 minutes, J_{Mg}, J_K, J_{Cl} and J_P are all similar or greater than

control values (i.e. Na in saline; Table 7). J_S was reduced by 50% concurrent with decrease (of 50%) in S in saline. In addition, J_{Na} was very low (10% of control values, Table 7) in nominally Na-free saline. J_{Ca} was not decreased in Na-free saline, at 30 minutes (Fig. 17) although its bathing concentration was 25% of control.

Figure 15. Rates of fluid secretion of A. dorsalis Malpighian tubules bathed in Na-substituted with choline saline solution. Vertical lines attached to points represent ±S.E. of the mean (n=6).

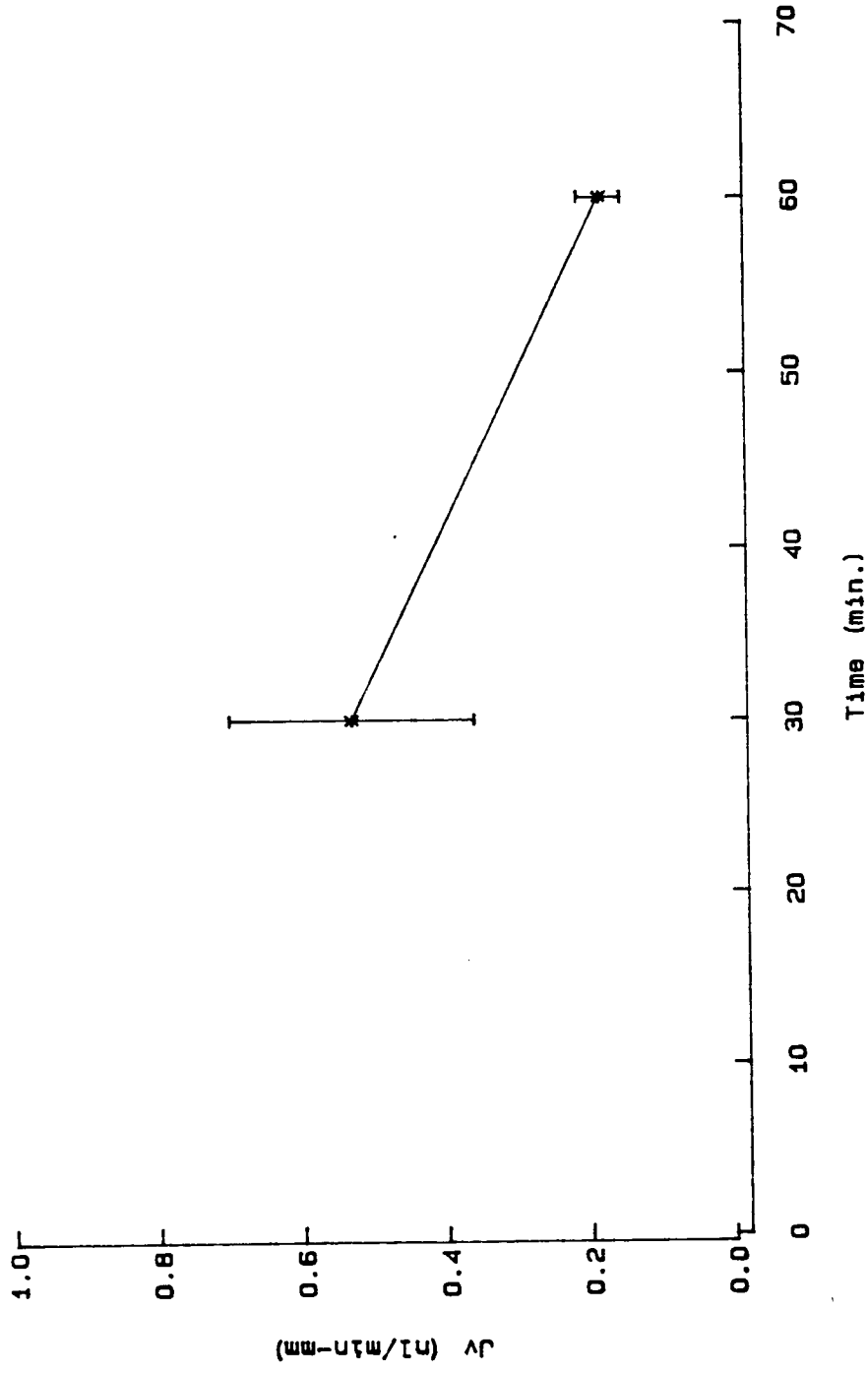

1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Time (min.)
Δv (nl/min-mm)

Figure 16. Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in Na-substituted with choline saline solution. Vertical lines attached to bars represent ±S.E. of the mean (n=6). * indicates significant difference.

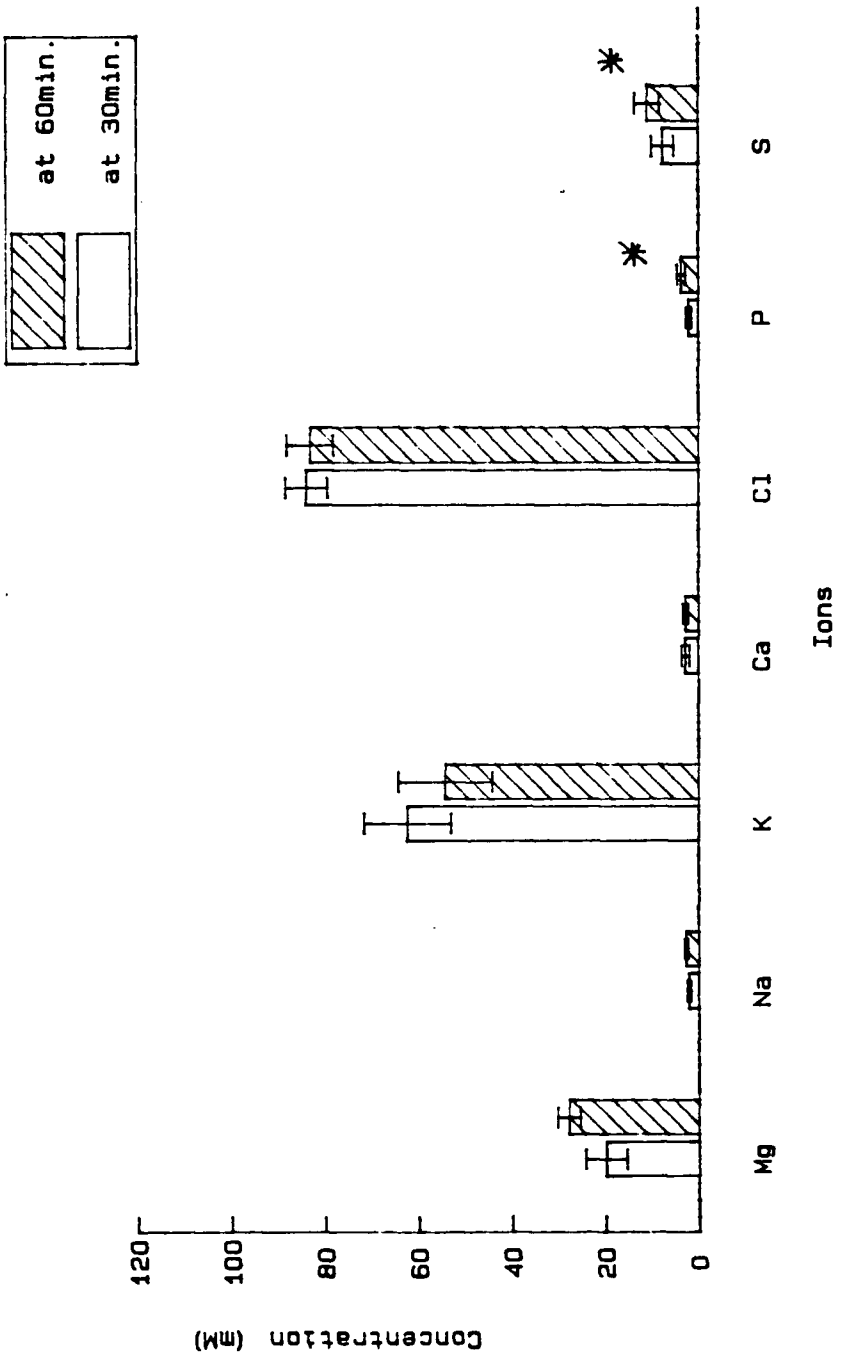
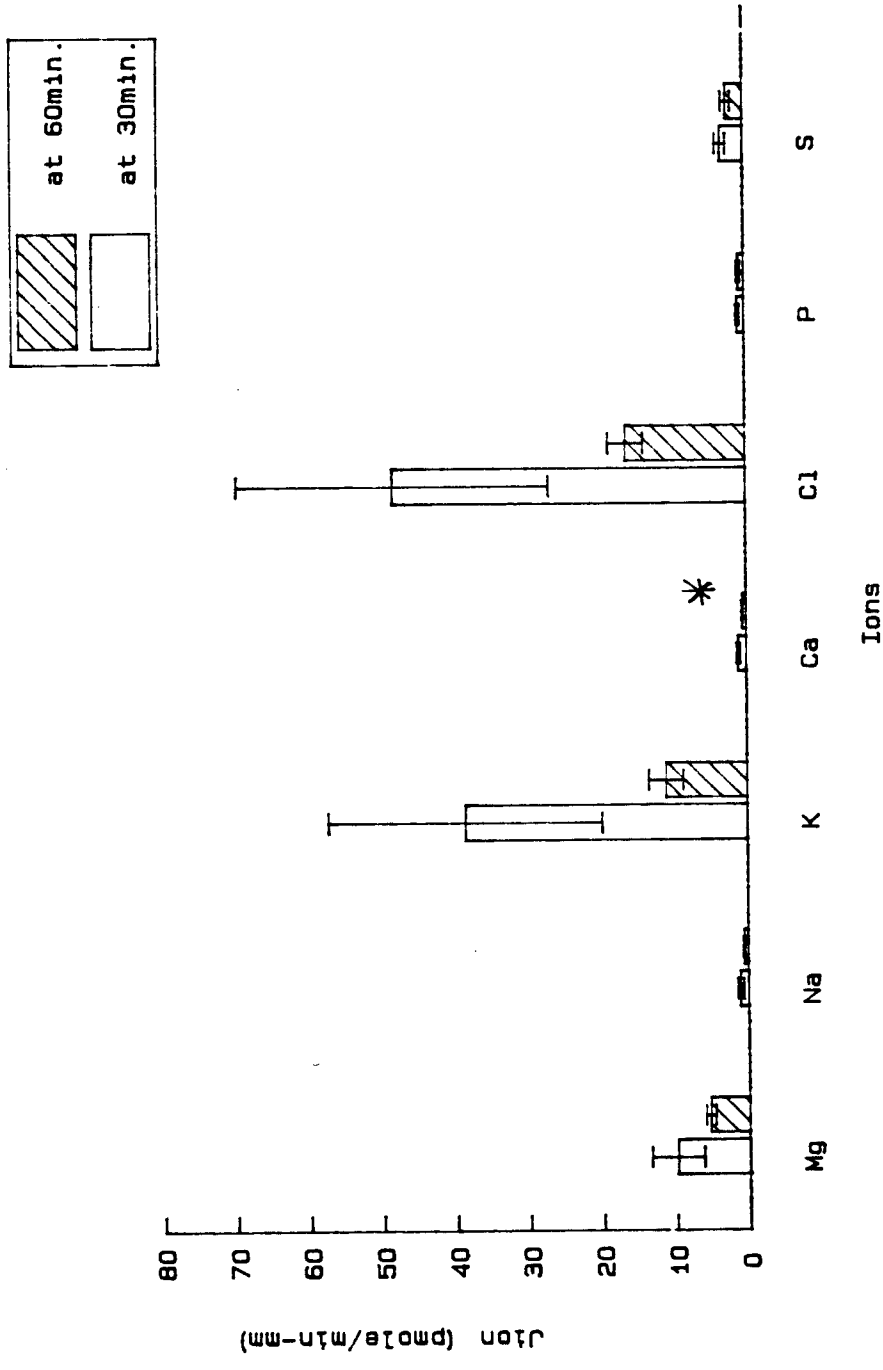

at 60min.
at 30min.
Concentration (mM)
120
100
80
60
40
20
0
Mg
Na
K
Ca
Cl
P
S
Ions

Figure 17. Secretion rates of ions (J_{ion}) of A. dorsalis Malpighian tubules bathed in Na-substituted with choline saline solution. Vertical lines attached to bars represent ±S.E. of the mean (n=6). * indicates significant difference.


at 60min.
at 30min.
Mg
Na
K
Ca
Cl
P
S
Ions
0
10
20
30
40
50
60
70
80
Ion (pmole/min-mm)

b) K^+ substitution for Na^+

When Na was replaced with K, J_v decreased from 1.1 to 0.47 nl/min-mm between 30 and 90 min., similar to control values of 1.32 and 0.64 nl/min-mm respectively (Fig.18). This indicates that substitution of K for Na does not significantly alter the secretion rate over 90 min.($P=0.05$). Analysis of ionic composition of secreted fluid from tubules bathed in 'Na-free' saline (at 60 and 90 min.) indicate that concentrations of S,K,Ca,and Cl are not significantly different from tubules in control saline ($P>0.05$). However, concentrations of Mg and P are significantly increased in secreted fluid of tubules in nominally Na-free saline (1mM). Na concentration in the secreted fluid decreased to 2mM from 15mM in control tubules while Mg secretion increased to 23mM from 6mM in control tubules (Fig.19). In addition, J_{Na} decreased to 0.8 pmole/min-mm from control value of 10 pmole/min-mm, while J_{Mg} increased to 11 pmole/min-mm (from average control value of 4 pmole/min-mm). J_P also increased by 4-fold after Na-replacement whereas J_{Ca} did not change. J_K, J_{Cl}, and J_S all decreased significantly ($P<0.05$; Fig.20).

Figure 18. Rates of fluid secretion of A. dorsalis Malpighian tubules bathed in Na-substituted with K saline solution. Larvae were reared in 126mM $MgSO_4$ solution. Vertical lines attached to points represent ±S.E. of the mean (n=9).

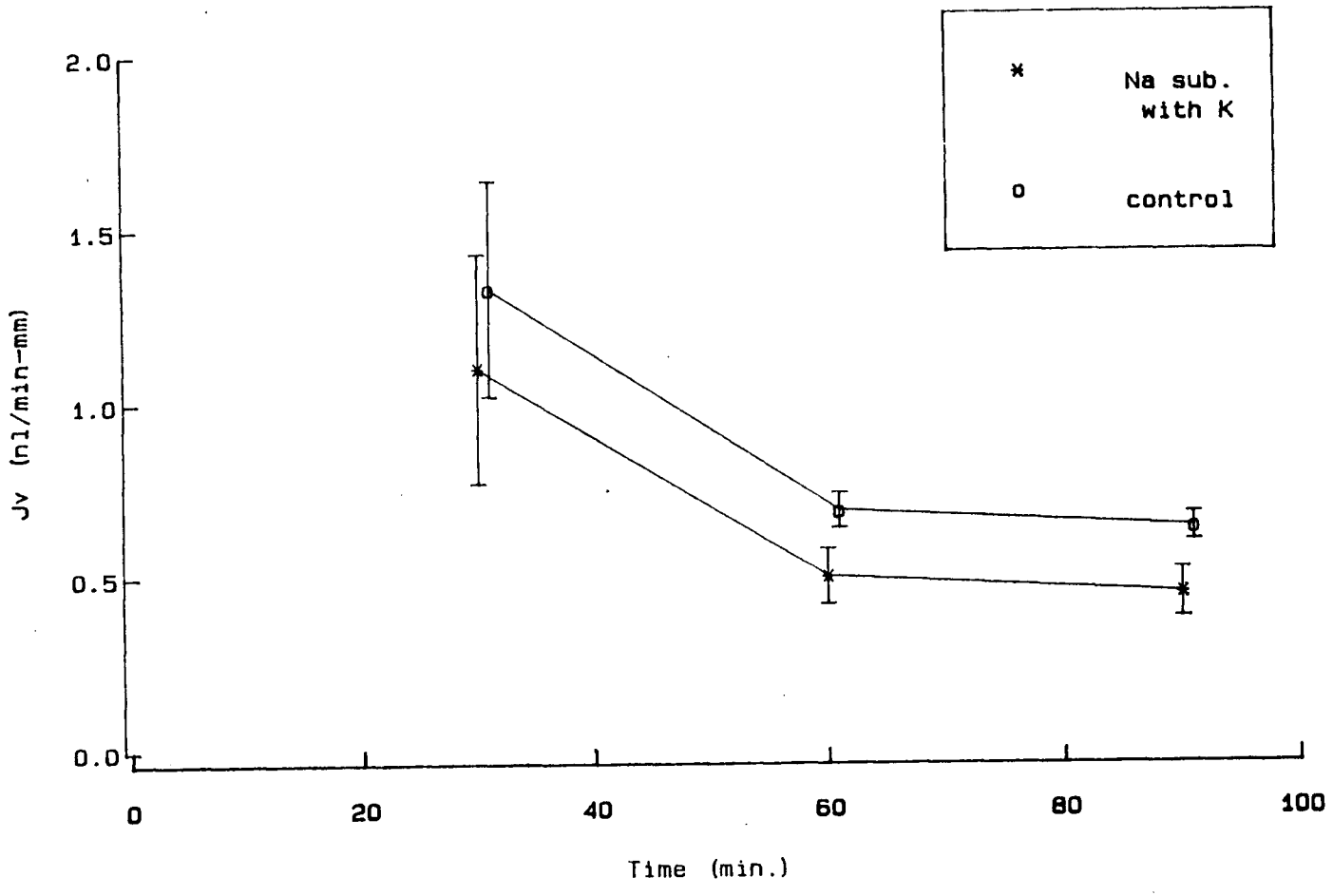

Na sub.
with K
control
2.0
1.5
1.0
0.5
0.0
Jv (nl/min-mm)
0
20
40
60
80
100
Time (min.)

Figure 19. Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in control and "Na-free" saline. Larvae were reared in 126mM $MgSO_4$ solution. Mean is average at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=9).
* indicates significant difference.

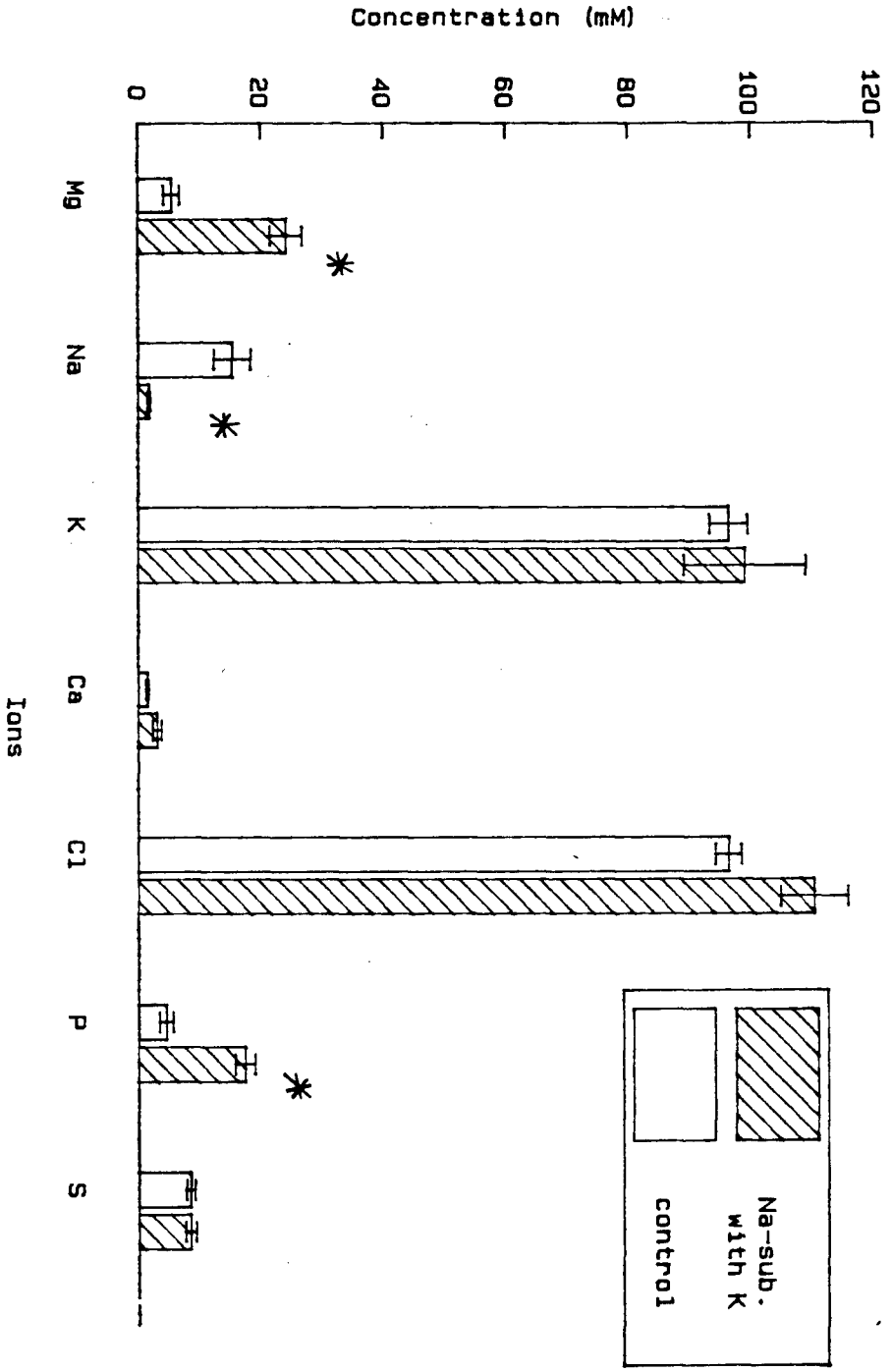
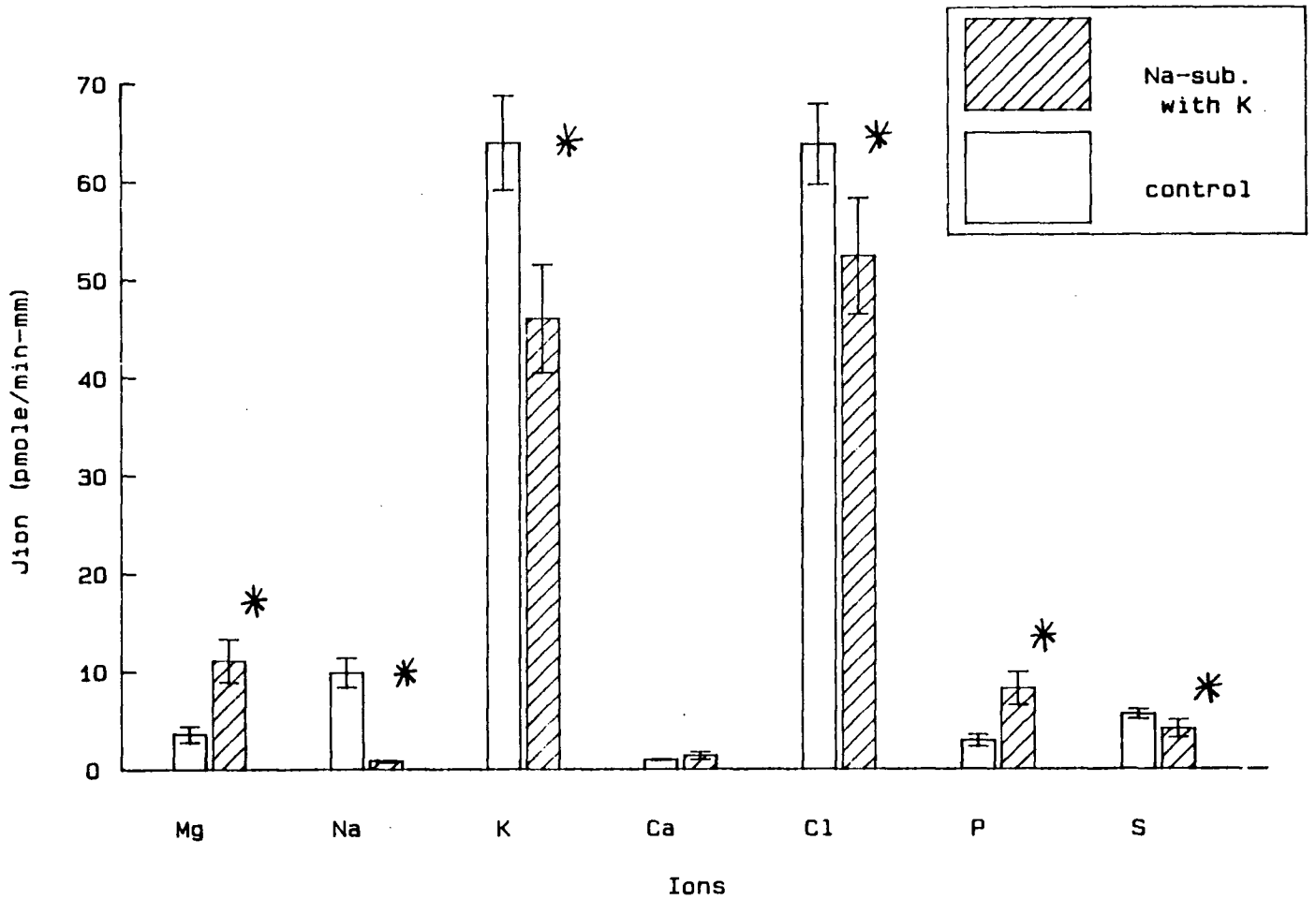

Concentration (mM)
0
20
40
60
80
100
120
Mg
Na
K
Ca
Cl
P
S
Ions
Na-sub. with K
control

Figure 20. Secretion rates of ions (J_{ion}) of A. dorsalis Malpighian tubules bathed in control and "Na-free" saline. Larvae were reared in 126mM $MgSO_4$ solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=9). * indicates significant difference.


Na-sub.
with K
control
70
60
50
40
30
20
10
0
Jion (pmole/min-mm)
Mg
Na
K
Ca
Cl
P
S
Ions

Inhibitor Studies

The influences of known inhibitors of Na^+ transfer mechanisms in biological membranes on tubular secretion was investigated as another means to explore Na coupling of J_{Mg}, J_v and other J_{ions}.

a) Amiloride

Amiloride inhibits Na channels (uM range) and Na^+-H^+ exchangers (mM range) in various epithelia (Gee, 1976). Therefore, this agent might decrease entry of Na^+ into tubule cells and hence the lumen, thereby reduce luminal Na available for apical exchange for cellular Mg, and consequently reduce J_{Mg} if a Na-Mg exchanger is involved. By blocking basolateral Na^+ channels, amiloride might also conceivably stop basal Na recycling via a Na/K ATPase and thereby indirectly inhibit KCl-driven tubular secretion if K entry occurs in exchange for cellular Na. This idea has not previously been tested in any KCl-secreting insect tubule.

As shown in Fig. 21, 1mM amiloride does reduce KCl-driven fluid secretion by 80% as predicted by the basal Na-recycling hypothesis. Moreover as shown in Fig. 22, K^+ levels in the secretion is reduced 50% ($P<0.05$) while Na^+, Ca^{2+}, Cl^-, and S concentrations are unchanged by this inhibitor. Remarkably, concentration of Mg^{2+} increases by 240% ($P<0.05$) and phosphorus by 200% ($P<0.05$; Fig.22). This can be explained if J_{Mg} and J_P do not decrease substantially

or significantly when amiloride inhibits J_K by 90% ($P<0.05$), J_{Na} by 81% ($P<0.05$), J_{Cl} by 80% ($P<0.05$), J_{Ca} by 66% ($P<0.05$),and J_S by 44% ($P<0.05$; Fig. 23).

Figure 21. Fluid secretion rates of A. dorsalis Malpighian tubules bathed in saline with and without 1mM amiloride (in the presence of 1mM cAMP). Larvae were reared in 126mM $MgSO_4$ solution. Vertical lines attached to points represent ±S.E. of the mean (n=10).
* indicates significant difference.

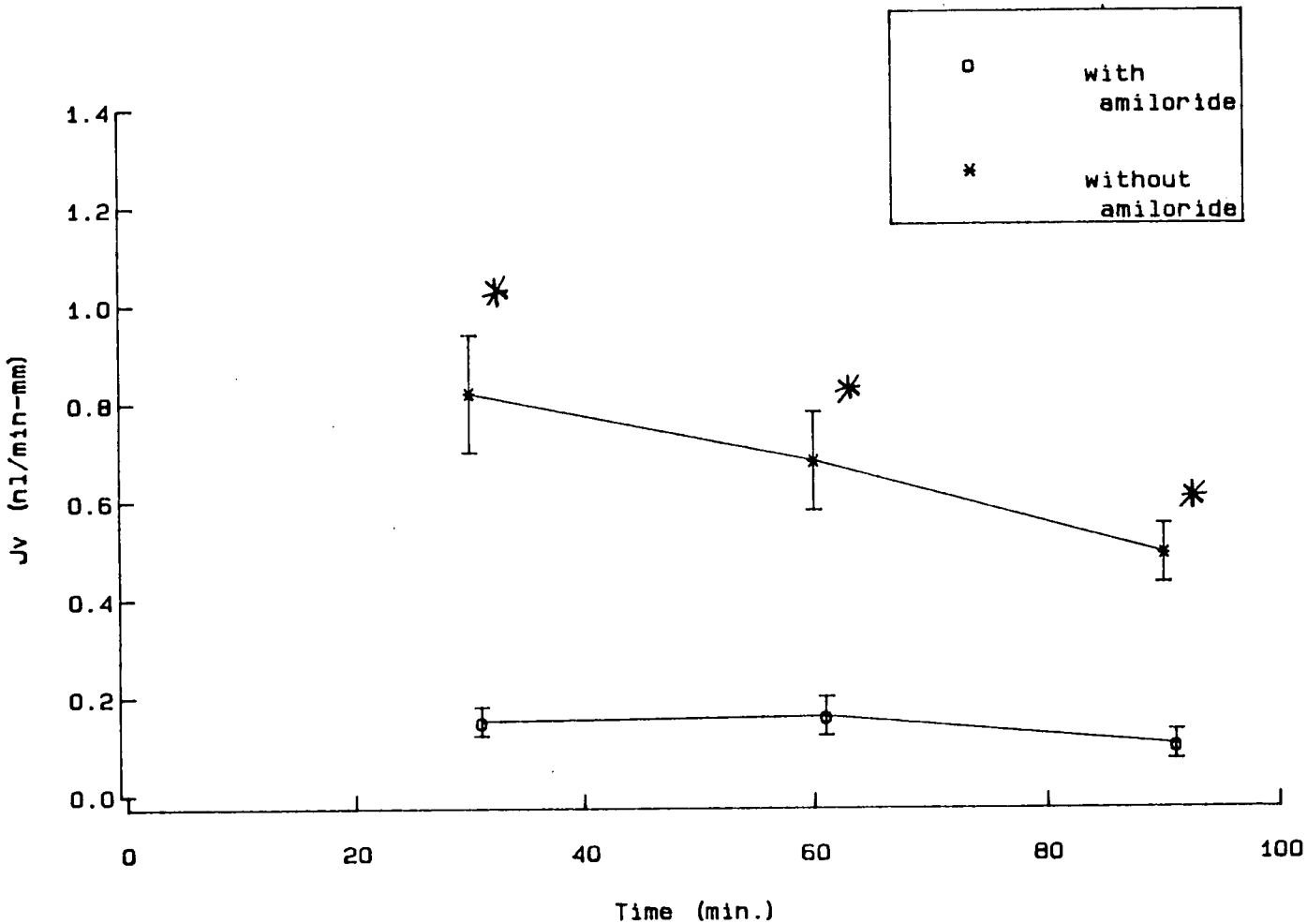

o
with amiloride
*
without amiloride
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Jv (nl/min-mm)
0
20
40
60
80
100
Time (min.)

Figure 22. Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in saline with and without 1mM amiloride (in the presence of 1mM cAMP). Larvae were reared in 126mM $MgSO_4$ solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=10).
* indicates significant difference.

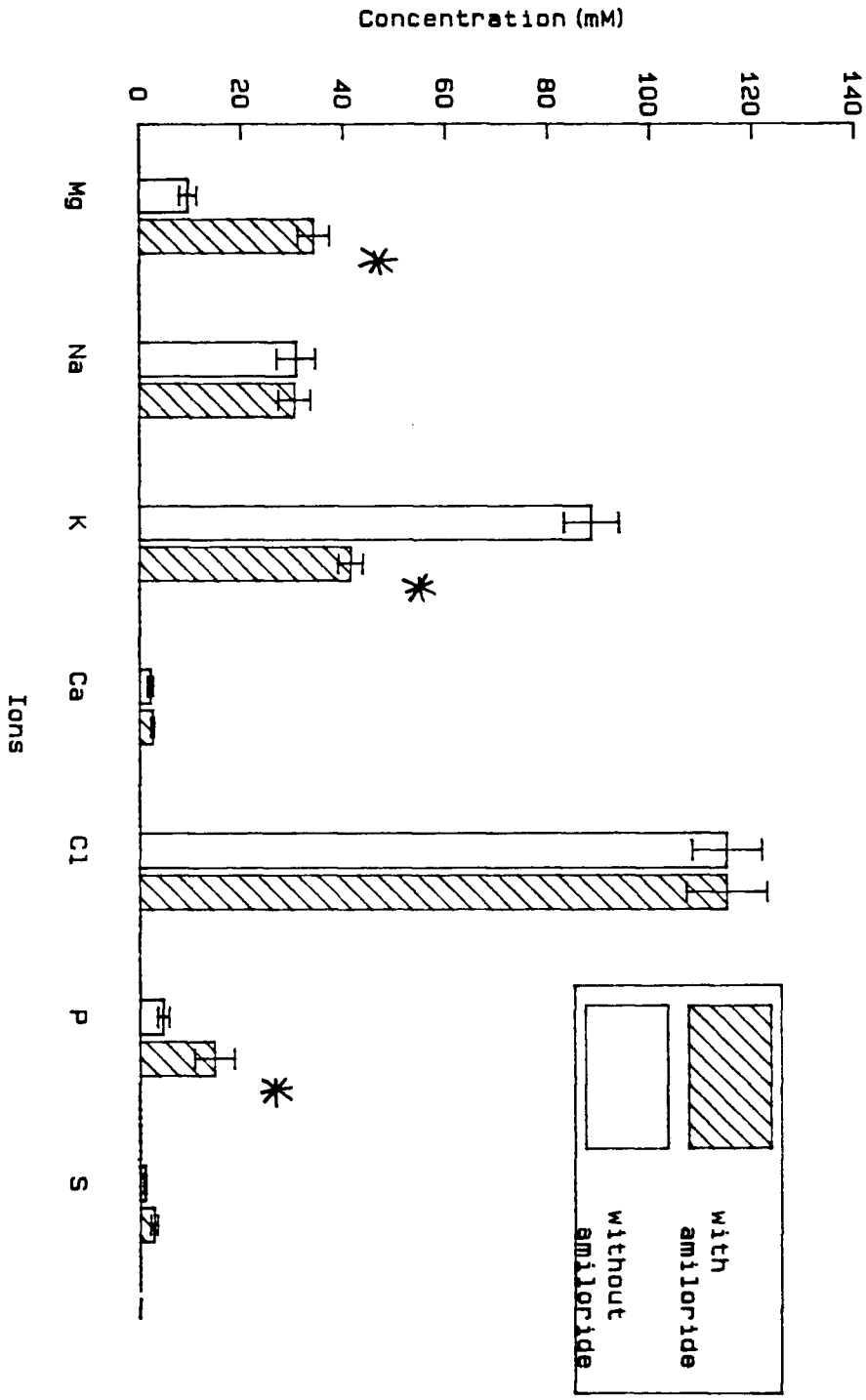
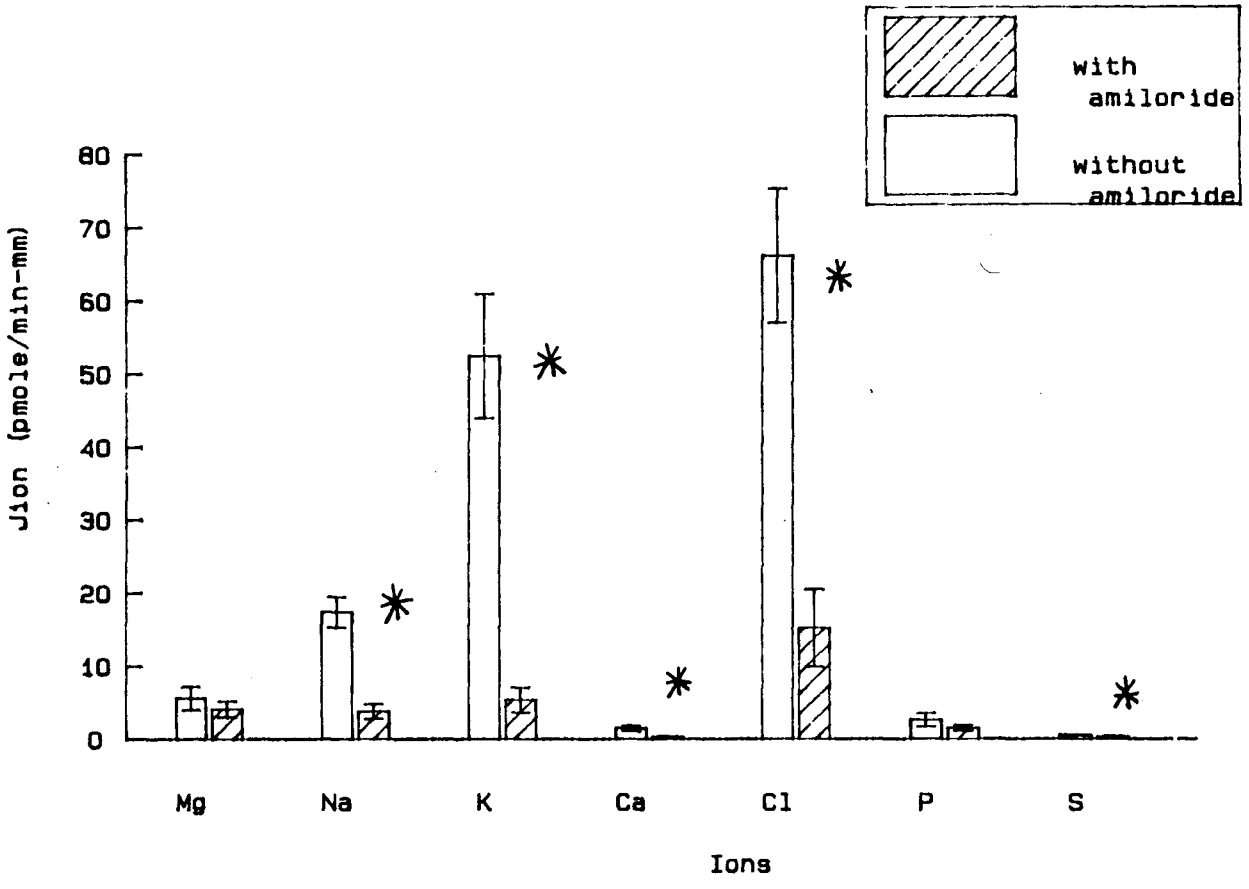

Concentration (mM)
0
20
40
60
80
100
120
140
Mg
Na
K
Ca
Cl
P
S
Ions
with amiloride
without amiloride

Figure 23. Secretion rates of ions (J_{ion}) of A. dorsalis Malpighian tubules bathed in saline with and without 1mM amiloride (in the presence of 1mM cAMP). Larvae were reared in 126mM $MgSO_4$ solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=10). * indicates significant difference.


with amiloride
without amiloride
Jion (pmole/min-mm)
80
70
60
50
40
30
20
10
0
Mg
Na
K
Ca
Cl
P
S
Ions

b) Bumetanide

There is evidence in Rhodnius Malpighian tubules (Maddrell, 1969) that Cl^- enters cells by a NaCl or (Na,K,2Cl) cotransport mechanism as commonly found in various vertebrate epithelia and which is inhibited by such diuretics as furosemide and bumetanide (Schlatter et al.,1983). Addition of bumetanide, at $5x10^{-5}M$ to bathing solution, reduced secretion rate significantly (P=0.05) from 0.72±0.12 (control tubules,n=4) to 0.41±0.06 nl/min-mm (n=4) at 90 minutes (Fig.24). Analysis of average ionic concentrations of secreted fluid at 60 and 90 minutes (Fig. 25) indicate significant (P<0.05) decreases in Mg (of 10mEquiv), and K (of 16mEquiv) and P (of 4mEquiv). Concentration of Na in the secreted fluid increased significantly from control value of 26mM to 47mM (P<0.05). Concentrations of Ca and Cl remain unchanged from control values (Fig.25). Concentration of S in secreted fluid was approximately 2.5-fold greater than control value of 2.0mM. J_{Na} and J_{Ca} are not significantly different from control values while significant decreases in J_{Mg}, J_K, J_{Cl}, and J_P are evident. J_{Mg} decreased 60% from its control value, while J_K and J_{Cl} decreased 45% and 34% from their control values, respectively (P=0.05; Fig.26).

This experiment should be repeated using higher concentrations of bumetanide. Since J_{Na} was not decreased

by this treatment whereas J_K and J_{Cl} were, these results do not suggest the presence of a basal K, Na, Cl cotransporter unless a basal Na recycling occurs which is independent of transepithelial net Na flux. Regardless, in this experiment the reduction in J_{Mg} is associated with increased rather than decreased luminal Na which is not the expected result if and apical Na/Mg exchange is involved.

Figure 24. Rates of fluid secretion of A. dorsalis Malpighian tubules bathed in saline with and without $5X10^{-5}M$ bumetanide (in the presence of 1mM cAMP). Larvae were reared in 126mM $MgSO_4$ solution. Vertical lines attached to points represent ±S.E. of the mean (n=8).
* indicates significant difference.

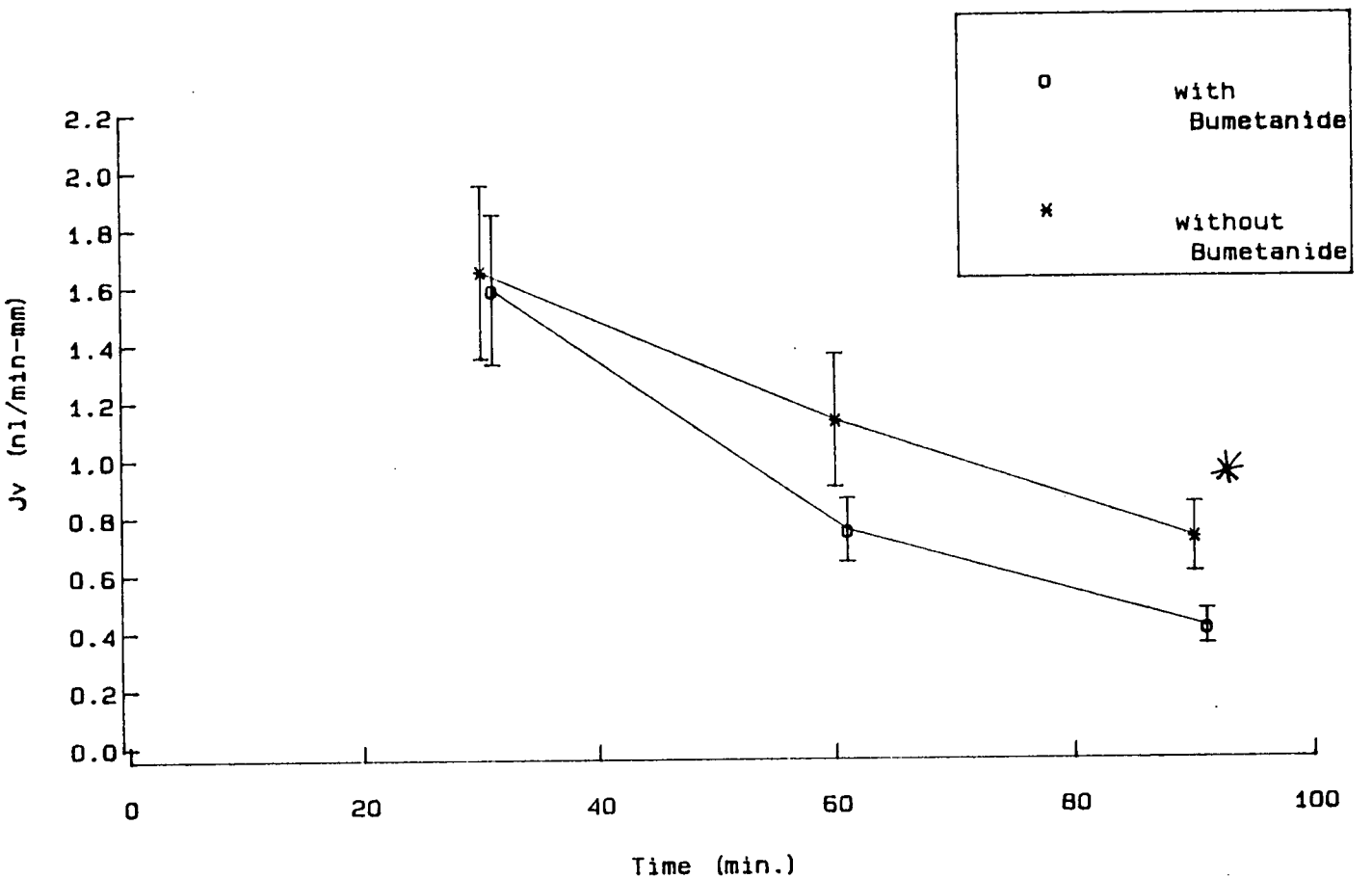

o
with
Bumetanide
*
without
Bumetanide
Jv (nl/min-mm)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (min.)

Figure 25. Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in saline with and without $5X10^{-5}M$ bumetanide (in the presence of 1mM cAMP). Larvae were reared in 126mM $MgSO_4$ solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=8).
* indicates significant difference.

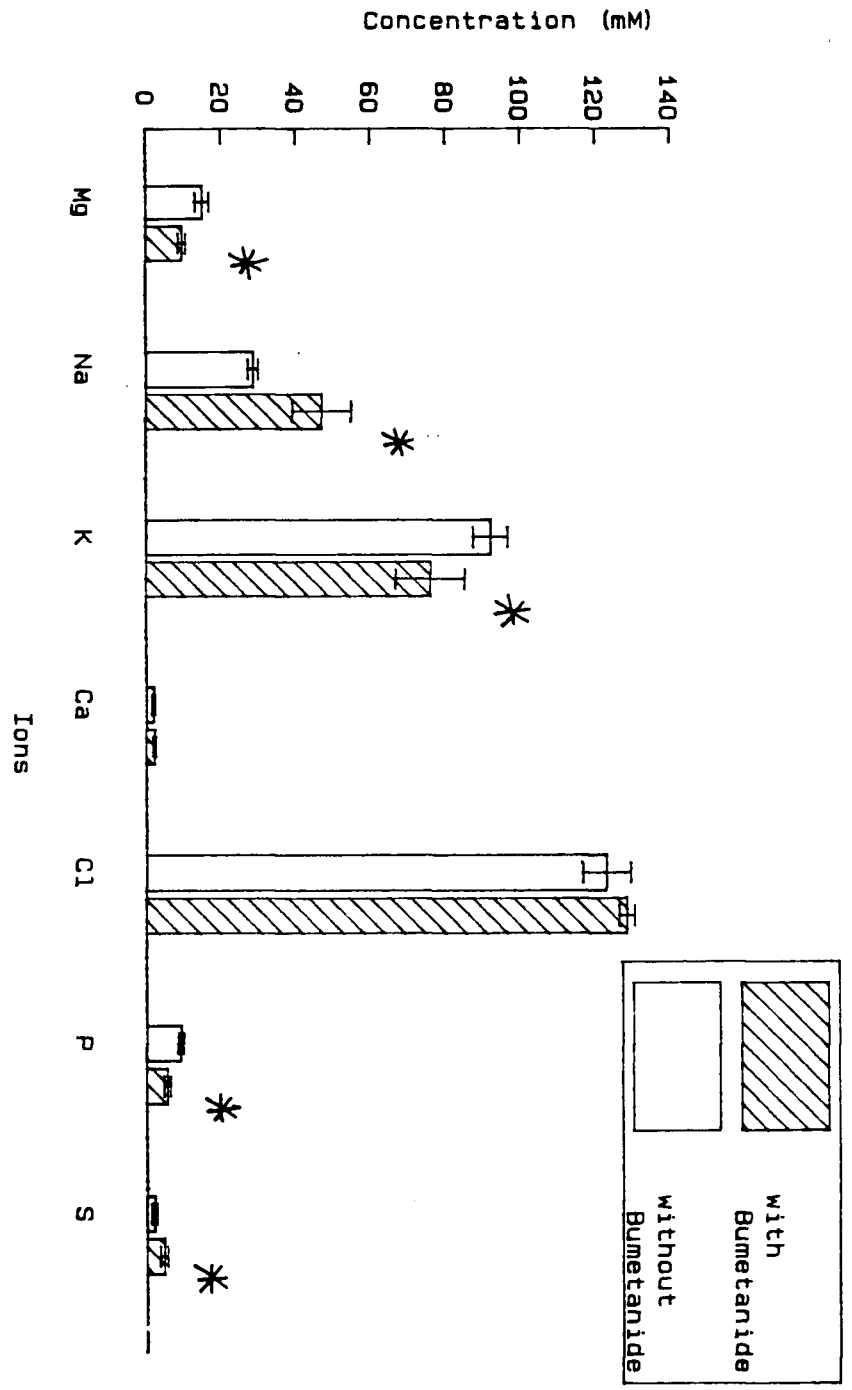
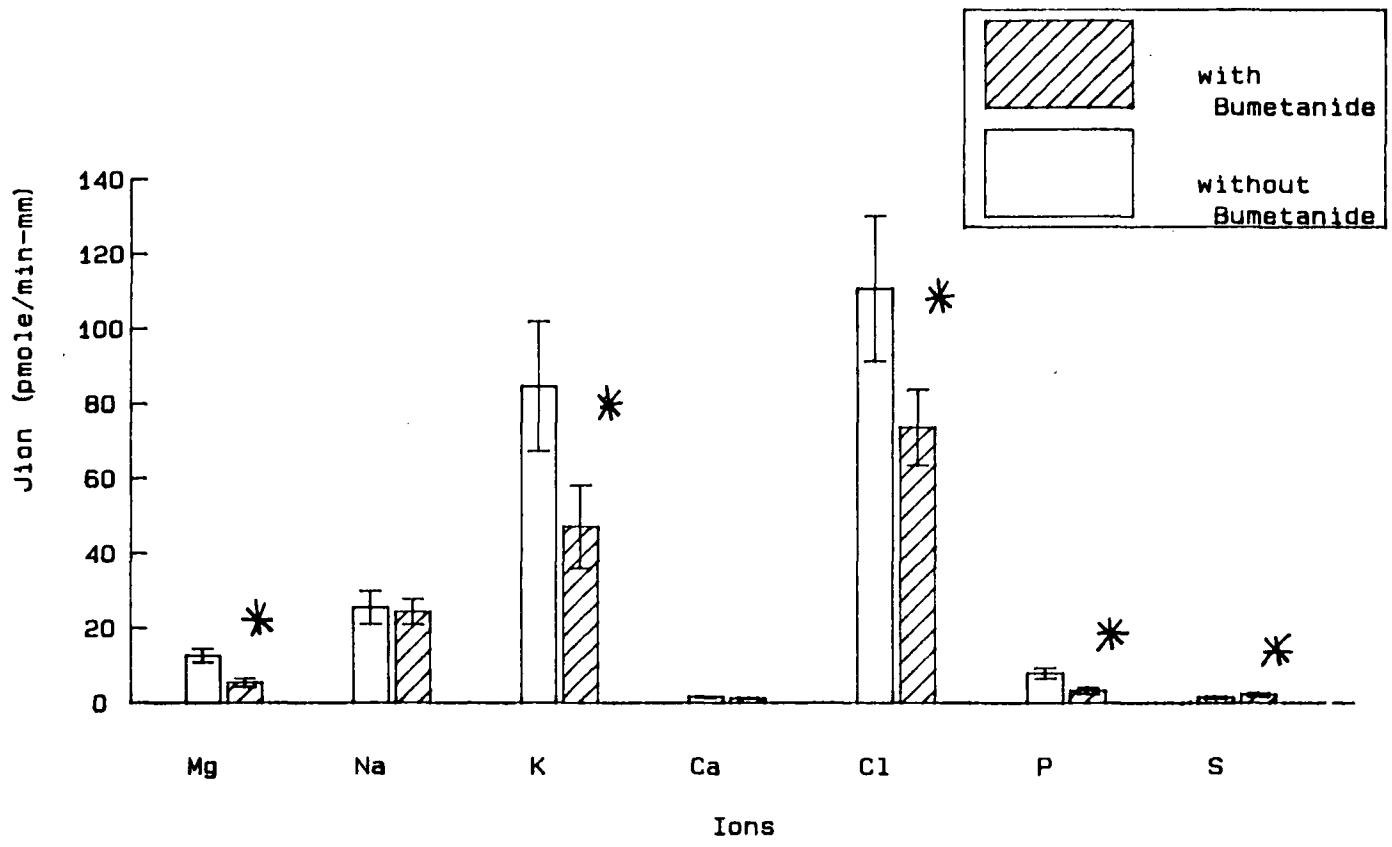

Concentration (mM)
0
20
40
60
80
100
120
140
Mg
Na
K
Ca
Cl
P
S
Ions
with Bumetanide
without Bumetanide

Figure 26. Secretion rates of ions (J_{ion}) of A. dorsalis Malpighian tubules bathed in saline with and without $5X10^{-5}M$ bumetanide (in the presence of 1mM cAMP). Larvae were reared in 126mM $MgSO_4$ solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=8).
* indicates significant difference.


with Bumetanide
without Bumetanide
Jion (pmole/min-mm)
140
120
100
80
60
40
20
0
Mg
Na
K
Ca
Cl
P
S
Ions

DISCUSSION

Characterization and Viability of Malpighian Tubules

The experiments described in this thesis clearly show that isolated Malpighian tubules of A. dorsalis can transport magnesium actively at high rates against a considerable electrochemical gradient. Concentration gradients as large as 16-fold were developed and the average transepithelial potential difference was -15mV (calculated equilibrium value of -35mV for Mg) when tubules were bathed in saline containing 2mM Mg (iv,Table 2). Moreover, the lumen is actually positive in some tubules, as reported for A. campestris (Maddrell and Phillips, 1974).

Fluid secretion rates, determined over 90 minute periods, in the presence and absence of cAMP, indicated that A. dorsalis tubules used in this study were viable and had secretion rates of the same magnitude as those reported for A. taeniorhynchus tubules (Maddrell and Phillips, 1978). Rapid hormonal stimulation of secretory processes commonly involves increases in intracellular concentrations of cAMP (Berridge,1975) and this nucleotide stimulates fluid secretion of all insect Malpighian tubules studied to date (Maddrell et al., 1971). Moreover, tubules from fed A. taeniorhynchus larvae secrete fluid at higher rates (1.95±0.16 nl/min-min, n=10) than tubules from unfed larvae (0.26±0.04 nl/min-min, n=22) suggesting hormonal stimulation

during feeding *in situ*; secretion rates of tubules from fed larvae are comparable to tubules bathed in saline containing 5-hydroxytryptamine which mimics the actions of diuretic hormone in several insects. Moreover, homogenates of the brain and the thoracic ganglia of *A. taeniorhynchus* stimulate tubular secretion, suggesting they are the source of a diuretic neurohormone which is released after larvae feed (Maddrell and Phillips, 1978). To exclude differences between secretion rates of fed and unfed larvae, larvae used in all experiments in the present study were fed an hour prior to experiments. Although evidence for a neurohormone causing changes in tissue cAMP (and hence increase fluid and ion secretion rates) was not tested in this study, the addition of 1mM cAMP did significantly increase J_K, J_{Na}, J_{Cl}, J_{Ca}, J_P, and J_S. Experiments on *Rhodnius* suggest that cAMP acts first on an electrogenic cation pump in the apical membrane and later on the uphill transport of Cl^- across the basal plasma membrane (Maddrell, 1971). This theory agrees with the present observation of increased J_{Cl}, J_K, and J_{Na} after stimulating *A. dorsalis* tubules.

Maddrell (1977) has hypothesized that Na^+ and K^+ transport in insect Malpighian tubules occurs by a common apical pump and the predominant ion transported depends on relative intracellular levels. As most insects secrete fluid with high K^+-to-Na^+ ratios, J_K should increase more than J_{Na} after stimulation so that transport of KCl would be

primarily responsible for driving fluid secretion (Phillips, 1981). The present results agree with this pattern in that J_K and J_{Cl} increased more that J_{Na} (Fig. 6). Of interest is the increased J_S after addition of 1mM cAMP. This finding is similar to that of Maddrell and Phillips (1978) for A. taeniorhychus tubules which show increased rate of SO_4^{2-} transport as rate of fluid secretion is increased. This indicates the possibility that the increased fluid secretion rate reduces the concentration difference driving back diffusion of S from lumen to the bathing solution;this has been hypothesized for other tubules (Phillips,1981).

Similarly J_P and J_{Ca} are significantly increased in the presence of 1mM cAMP in the bathing medium, suggesting that at low rates of fluid secretion, the concentrations of P and Ca^{2+} rise (in the secreted fluid) so that net diffusion of these ions through the tubule wall into the bathing solution is decreased, thereby reducing the rate of elimination of P and Ca^{2+}. Changes in J_{Mg} in A. dorsalis tubules is negligible after addition of 1mM cAMP to the bathing medium which is suggestive that back flux (i.e. permeability) is normally low.

As mentioned previously, deterioration of fluid secretion rates from 30 to 90 minutes (Fig. 3) indicates reduced viability of A. dorsalis Malpighian tubules or decreased stimulation. It has been postulated that 5-Hydroxytryptamine, a potent stimulator of secretion

through mediation of cAMP, is broken down or eliminated by Rhodnius tubules after 30 minutes when added at concentrations of 10^{-7} M to the bathing droplet. It is known that Malpighian tubules break down the diuretic hormone (Maddrell,1964; Pilcher, 1969). Possibly, the reduction of fluid secretion rate at 90 minutes may be due to the fact that externally applied cAMP is broken down, similarly to 5-Hydroxytryptamine. The deterioration of secretion rate at 90 minutes in tubules not exposed to cAMP may be due to elimination of DH present on membrane sites at the beginning of the experiment.

Lack of a consistent decline in transepithelial potential readings with time (Table 5) also indicated that tubules were viable over the 90 minute experimental period. Electropotential measurements at 90 minutes did not deviate more than 6mV for any tubule from measurements taken at 30 minutes. As observed by Phillips and Maddrell (1974) on A. campestris tubules, A. dorsalis tubules had a trans-wall potential which was on average lumen negative but variable. However, as the medium bathing A. campestris tubules was altered to a K-rich Cl-poor one, the tubules' lumina became more positive with respect to the bath. The ability of A. campestris tubules to transport Mg against a large concentration gradient and against an electrical gradient indicates that an active transport mechanism is involved. It would be of interest to perform similar alterations in

KCl levels in the medium bathing A. dorsalis tubules to substantiate that transport occurs against both a concentration as well as an electrical gradient simultaneously, although it apparently did in those few tubules with lumen positive potentials.

Properties of Mg Transport

Having characterized the in vitro preparation of Malpighian tubules ,including their capacity to transport Mg, I tested my main hypothesis that Mg^{2+} transport is driven predominantly by counter transport with Na^{+}. This hypothesis was not supported by kinetic, Na-substitution , or inhibitor studies. The data in Table 7 suggest that the increased J_{Mg} which occurs when saline Mg levels are raised could not occur by a $2Na^{+}/Mg^{2+}$ neutral, or a $1Na^{+}/1Mg^{2+}$ electrogenic exchanger at the luminal border. J_{Na} is 12 to 20 pmole/min-mm when J_{Mg} is low (eg. baseline 3.7 at 0 or 0.5 mM external Mg^{2+} in the saline; Table 7). Raising saline Mg to 4mM (series 6, Table 7) causes an increase in J_{Mg} of 8pmole/min-mm or 16 pEquiv./min-mm but at most J_{Na} may decline by 4 pEquiv./min-mm (Fig. 13.2). However, the maximum possible decline in J_{K} of 11 pEquiv./min-mm (see 0.5 mM, series b to 4mM in Table 7; Fig. 12.2) would be sufficient for a $2K^{+}/Mg^{2+}$ neutral exchanger. Although corresponding decreases in J_{K} does not occur with increases in J_{Mg} at each concentration of Mg tested in the external

solution (Fig. 12.2), general trends are evident over a 90 minute period (i.e. as J_{Mg} decreases, J_K increases). At each concentration of external Mg, K concentration decreases as Mg concentration increases in the secretion (Fig. 12.1). An inverse relationship, however, does not exist between Na and Mg concentrations at all concentrations of external Mg tested (Fig. 13.1). In addition, decreases in Na (of 10 mEquiv./L) is not sufficient to explain increase in Mg (of 40mEquiv./L) over the range of external Mg tested. Thus data in this experimental series suggests that a Na/Mg^{2+} countertransport process is unlikely in A. dorsalis tubules because the expected increased recovery of luminal Na^+ from the baseline secretion to balance increase J_{Mg} is not observed. However, there is a driving force for back flux of luminal K^+ (high) to the hemocoel side (low K^+) and luminal K^+ may decline enough to account for observed increases in J_{Mg}. However, there is no consistent relationship between J_{Mg} and J_K or luminal K levels in other types of experiments. The only other evidence suggesting backflux of K drives J_{Mg} is shown in the bumetanide study (Fig. 26) where J_{Mg} and J_K both decrease significantly and to the same magnitude (2-fold) in tubules treated with bumetanide. Other studies do not suggest that backflux of K drives J_{Mg}. When tubules were treated with amiloride, J_K

decreased significantly whereas J_{Mg} did not decrease substantially or significantly. In Na-substituted with choline study, J_K and J_{Mg} did not decrease significantly; therefore, no conclusions can be made about the effect of decreased J_K on J_{Mg}. In the presence of cAMP in the bathing solution, J_K increased while no significant changes in J_{Mg} was noted. Furthermore, in Na-substituted with K study, J_K decreased significantly and J_{Mg} increased significantly suggesting that a K/Mg exchange mechanism is unlikely since J_{Mg} increased rather than decreased along with J_K.

To obtain a more accurate correlation of ion fluxes with Mg flux, the kinetics of Mg would have to be studied further at more concentrations to obtain an accurate kinetics plot.

When Na was substituted with choline, J_{Na} was reduced to 10% or control values (Table 7) while J_{Mg}, J_K, J_{Cl}, and J_P were all similar or greater than control values (Table 7). This does not support the hypothesis of a Na/Mg exchange mechanism since decreases in J_{Mg} would be expected with corresponding decreases in luminal Na levels and J_{Na}. Any deterioration of tubules over the 60 minute period did not affect rate of fluxes significantly, except for Ca

(Fig. 17). In addition, the concentration of ions (except P and S) in the secreted fluid did not change significantly over the experimental period (Fig. 16). However, to obtain a more significant correlation between ions, a proper control would have to be used in this experiment with concentrations of solutes similar to experimental saline except for Na; in addition cAMP can be added to keep rates of fluid secretion constant over a longer period of time.

When Na was substituted with K, fluid secretion rates did not alter significantly, indicating similar viability as control tubules (Fig. 18). When Na concentration in the secreted fluid was decreased to 2mM from 15mM, Mg levels in the secretion increased significantly to 23mM from 6mM (Fig. 19). In addition, as J_{Na} decreased significantly by 12-fold, J_{Mg} increased significantly by 3-fold. This strongly suggests that a Na/Mg exchange mechanism is unlikely since Mg increases rather than decreases in response to decreased J_{Na}. It is interesting that J_K and J_{Cl} in Na substituted with K study are less that J_K and J_{Cl} in control study although higher concentrations of K and Cl are found in the bathing solution of the former. Perhaps Na was not present in high enough concentration to stimulate KCl transport (i.e. 2mM Na in the bathing saline is lower than the K_m required to stimulate KCl transport). It has been shown in previous experiments

that Na is required (in the bathing medium) to drive K transport when concentration of K is low in the bathing medium (Phillips, 1981). Perhaps a similar mechanism exists for A. dorsalis Malpighian tubules and Na is required in the bathing medium to stimulate KCl transport even when concentration of K is high in the medium. To further study involvement of K^+ in Mg^{2+} transport, K^+ concentrations should be varied perhaps by using PAH transport to drive fluid secretion to study the correlation between J_K and J_{Mg}.

When amiloride (1mM) is added to the bathing solution, J_{Na} is inhibited by 81%, J_K by 90%, J_{Cl} by 80%, J_{Ca} by 66%, J_P by 44%, J_v by 80% whereas J_{Mg} and J_S are not decreased substantially or significantly (Fig. 21, 23). It has been previously shown that amiloride inhibits J_v of tubules of the tsetse fly which like other blood feeding insects, secretes NaCl-rich rather than KCl-rich fluid. However, even KCl secreting tubules often are stimulated by low levels of Na when external K^+ is low (Phillips, 1981). One explanation of this effect is that K^+ is pumped into tubule cells from the hemolymph in exchange for cellular Na^+ by a typical Na^+/K^+-ATPase: Na^+ then leaks down a large electrochemical gradient from lumen to cell (i.e. is recycled). In this way Na that is recycled can be exchanged for Mg. However, Mg secretion is seen again to be independent of changes in J_{Na} and in this case also J_K.

Furosemide, bumetanide, and piretanide are known to inhibit Na:K:2Cl transport process in erythrocytes (Palfrey et al., 1980), Ehrlich ascites cell (Geck et al.,1981), shark rectal gland (Palfrey et al.,1979), and cortical thick ascending limb of rabbit (Schlatter et al., 1983). The concentrations of furosemide and bumetanide for half-maximal inhibition of active transport in rabbit cortical thick ascending limb was $5x10^{-4}$ M and $2x10^{-7}$ from the lumen and bath respectively. In the shark rectal gland these inhibitors are less efficient than they are in the cTAL segment by a factor of about 100. In addition, it is known that furosemide,at 10^{-3} M, causes inhibition of glycolysis and Na/K-ATPase (Klahr et al.,1971) so higher levels of these 'diuretics' may not give meaningful information. Although these actions have not been investigated for bumetanide, this possibility remains. On this basis, a concentration of $5x10^{-5}$ M bumetanide was chosen for this study. At the concentration of bumetanide used, J_{Na} was not significantly decreased (P=0.05) although J_{Mg}, J_K, J_{Cl}, and J_P were significantly decreased (P=0.05); (Fig. 26). This again suggests Na independence of Mg flux. It has been proposed in Rhodnius tubules (Maddrell,1969) that Na^+ drives Cl^- entry into cells across the basolateral membrane by a cotransport mechanism and Na^+ is then pumped out across the basolateral membrane in exchange for K^+. In this way Na^+ is recycled and may stimulate Cl^- and K^+ secretion (Phillips,

1981) and also Mg^{2+} secretion. Schlatter et al. (1983) have proposed a similar mechanism for (Na:K:2Cl transport) across the TAL of rabbit tubules. Results from these vertebrate studies indicate that furosemide causes a greater inhibition of magnesium and calcium transport over that of sodium (Quamme,1978). Therefore, a Na-independent component is suggested again. A higher concentration of bumetanide should be tested to see if it might inhibit J_{Na} since at low concentrations I found only inhibition of J_K, J_{Cl}, and J_{Mg} (Fig. 21). Therefore, the action of bumetanide found in this study is difficult to interprete in terms of past studies mentioned above. The decrease in J_{Mg} may be explained by a decrease in J_K; possibly backflux of K from the secreted fluid into the cell drives Mg secretion. Therefore, if J_K is decreased and luminal K concentration is lower, less K is available to diffuse from the secreted fluid into the cell to drive Mg secretion and therefore J_{Mg} would decrease.

It seems unlikely that transport of an anion (besides Cl^-) accompanies Mg^{2+} because inconsistent correlations between P, S, and Mg are observed in kinetic studies (Table 7). Organic anions and HCO_3^- also are probably not major components in the secretion because the difference between total cations and anions in the secreted

fluid did not vary more than 10mM in all experiments, except controls used in amiloride and bumetanide experiments.

The amount of bound as opposed to free Mg^{2+} in the secretion is also uncertain. To determine the amount of "free" Mg, Mg-selective microelectrodes would be required to determine the activity of Mg. However, the possibility of an intracellular pool of bound Mg^{2+} which can be mobilized to free Mg^{2+} is possible. Indeed, the fact that tubules bathed in saline containing 0mM Mg^{2+} continued to secrete substantial amounts of this cation suggests that such a pool exists (Fig. 10). Previous studies (Scarpa, 1974) indicate that Mg^{2+} as well as Ca^{2+} exist largely as "bound" fractions in concretions found in Malpighian tubule cells. This would alter the actual value of Mg found in secreted fluid.

An induction of Mg transport in A. dorsalis tubules was not convincingly shown in this study because Mg transport only increased significantly over controls in tubules from larvae reared in 126mM Mg-waters when bathed in vitro in 1mM Mg saline. Possibly the 26mM Mg in the control saline was sufficient to activate tubular Mg secretion. Therefore, comparisons between larvae reared in Mg-free and 126mM Mg-waters might give a clearer indication of induction similar to that found for SO_4^{2-} transport in A. taeniorhynchus. This would be of interest as a carrier-mediated mechanism is suggested by kinetics of Mg transport (Fig. 10). J_{Mg} increases as the external

concentration of Mg increases, and J_{Mg} approaches a maximum at 4mM Mg (external) for larvae reared in 26 and 126mM Mg waters. For larvae reared in 126mM Mg medium, V_{max} was 14.92±2.89 pmole/min-mm and K_m was 1.57±0.16. For larvae reared in 26mM Mg medium, V_{max} was 9.17±8.16 pmole/min-mm while K_m was 1.40±0.77. Regression analysis indicate V_{max} and K_m of larvae reared in 26mM $MgSO_4$ medium are not significantly different from those of larvae reared in 126mM $MgSO_4$ medium. These results suggest that magnesium transport is not induced across Malpighian tubules of A. dorsalis larvae when transferred from 26mM to 126mM $MgSO_4$ medium. However, only 4 sets of values were compared. If more external Mg concentrations were tested for larvae reared in low and high $MgSO_4$ media, more data points would be generated; this would give more accurate conclusions of differences (of V_{max} and K_m) between larvae reared in 26mM $MgSO_4$ and 126mM $MgSO_4$ media.

This thesis indicates that Mg transport in A. dorsalis tubules is active, carrier mediated and largely Na-independent. There is no consistent evidence for Mg secretion by apical exchange for luminal K^+. Perfusion of the tubular lumen with Na-free salines would clearly eliminate any remaining doubt that Mg secretion is completely Na-independent.

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APPENDIX A

INFLUENCE OF CAMP ON CONCENTRATIONS OF IONS IN THE SECRETED FLUID

Appendix A: Concentrations of Ions in Secreted Fluid (mM)

Time (min.)	Mg^{2+}	Ca^{2+}	K^{+}	Na^{+}	Cl^{-}	P	S
30′	i)30.51±2.12	2.39±0.39	42.62±2.10	20.25±4.04	64.57±1.44	2.19±0.98	34.00±1.91
	ii) 8.82±2.94	2.14±0.63	73.71±7.69	19.59±2.75	66.79±8.55	5.09±2.09	14.74±0.85
60′	i)34.26±3.73	2.43±0.37	36.76±1.58	23.16±5.74	59.79±0.55	4.34±1.90	34.98±1.68
	ii)7.77±3.51	1.51±0.44	70.22±6.95	17.64±2.79	68.76±7.47	4.01±2.34	14.78±0.86
90′	i)29.79±2.76	2.98±0.36	34.29± 2.30	28.92±3.88	53.33±1.59	5.00±2.53	35.35±1.95
	ii)8.06±3.93	1.72±0.38	64.13±10.72	22.06±6.22	65.99±7.91	4.65±2.69	16.72±1.49

i=unstimulated tubules

ii=stimulated tubules(with 1mM cAMP)

APPENDIX B

JIONS AND CONCENTRATIONS OF IONS IN SECRETED FLUID

Figure B.1 Rates of fluid secretion by A. dorsalis Malpighian tubules bathed in 0mM Mg, 0mM cAMP solution. Larvae were reared in 126mM MgSO4 solution. Vertical lines attached to points represent ±S.E. of the mean (n=4).

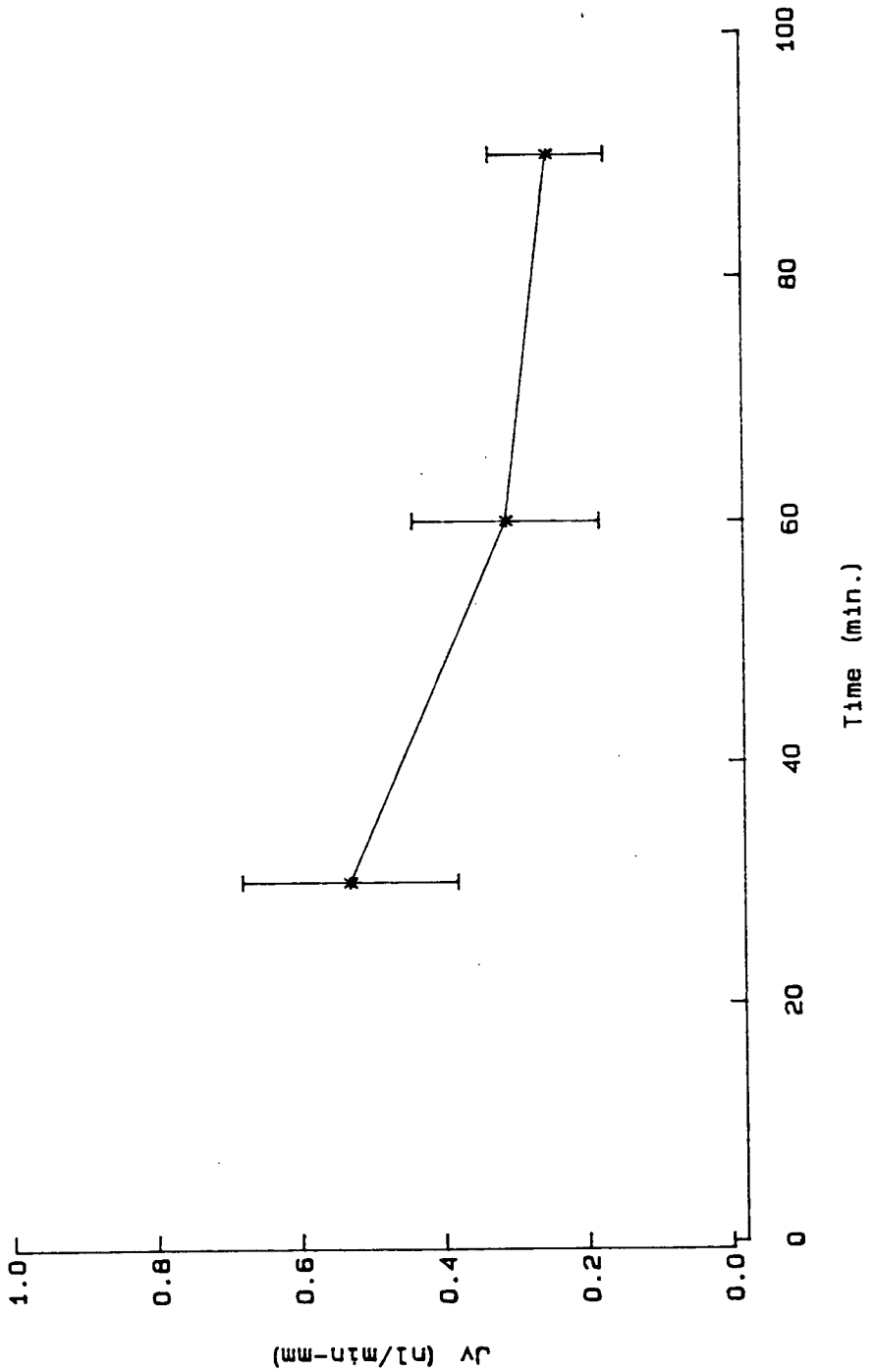

1.0
0.8
0.6
0.4
0.2
0.0
ΔV (ml/min-mm)
0
20
40
60
80
100
Time (min.)

Figure B.2 Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in 0mM Mg, 0mM cAMP solution. Larvae were reared in 126mM MgSO4 solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=4).

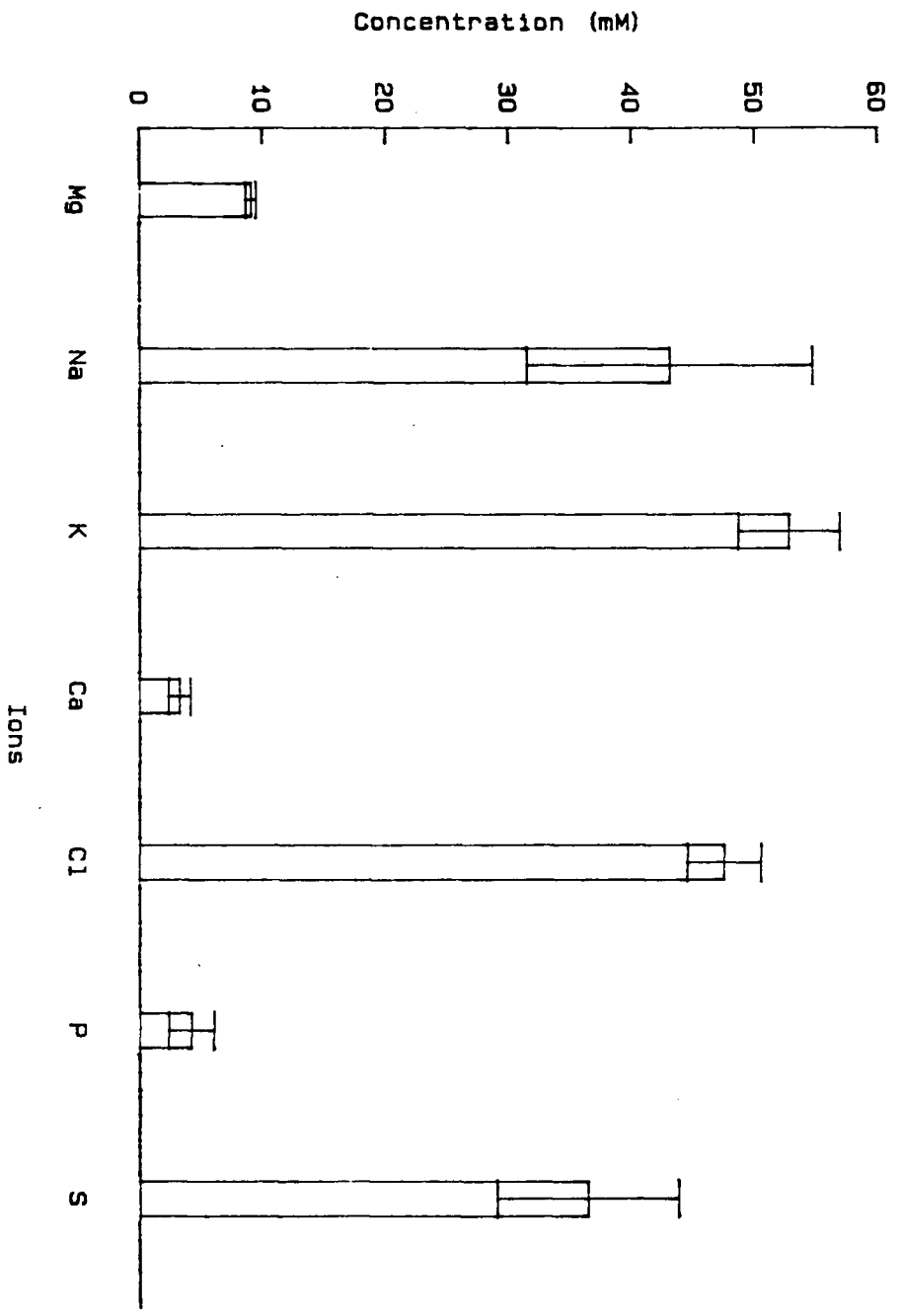

Concentration (mM)
0
10
20
30
40
50
60
Mg
Na
K
Ca
Cl
P
S
Ions

Figure B.3 Secretion rates of ions (J_{ion}) of A. dorsalis Malpighian tubules bathed in 0mM Mg, 0mM cAMP solution. Larvae were reared in 126mM MgSO4 solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=4).

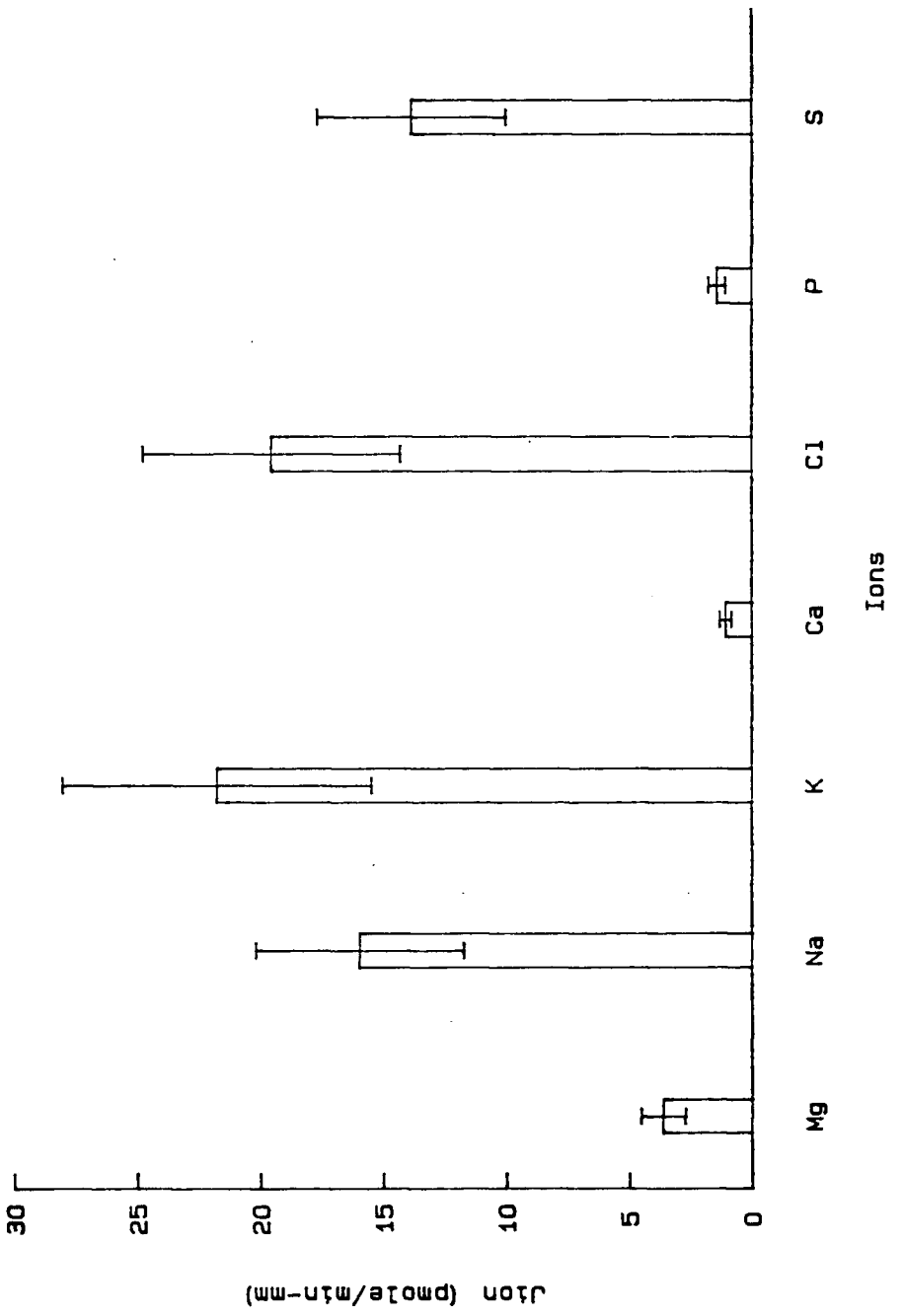

Mg
Na
K
Ca
Cl
P
S
Ions
0
5
10
15
20
25
30
Ion (pmole/min-mm)

Figure B.4 Rates of fluid secretion by A. dorsalis Malpighian tubules bathed in 0.5mM Mg, 0mM cAMP solution. Vertical lines attached to points represent ±S.E. of the mean (n=10).

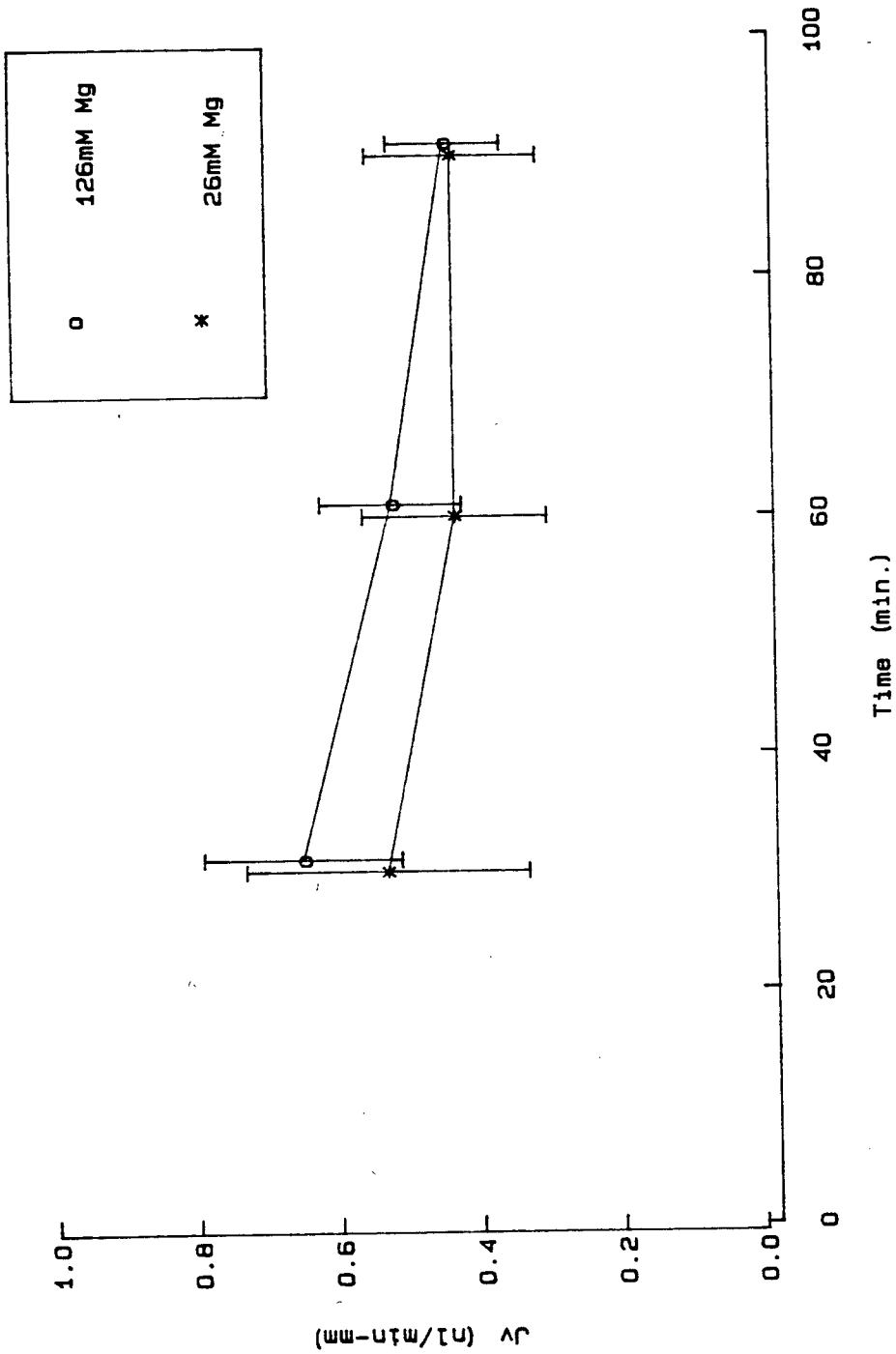

126mM Mg
26mM Mg
o
*
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (min.)
dv (nl/min-mm)

Figure B.5 Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in 0.5mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to points represent ±S.E. of the mean (n=10).
* represents significant difference.

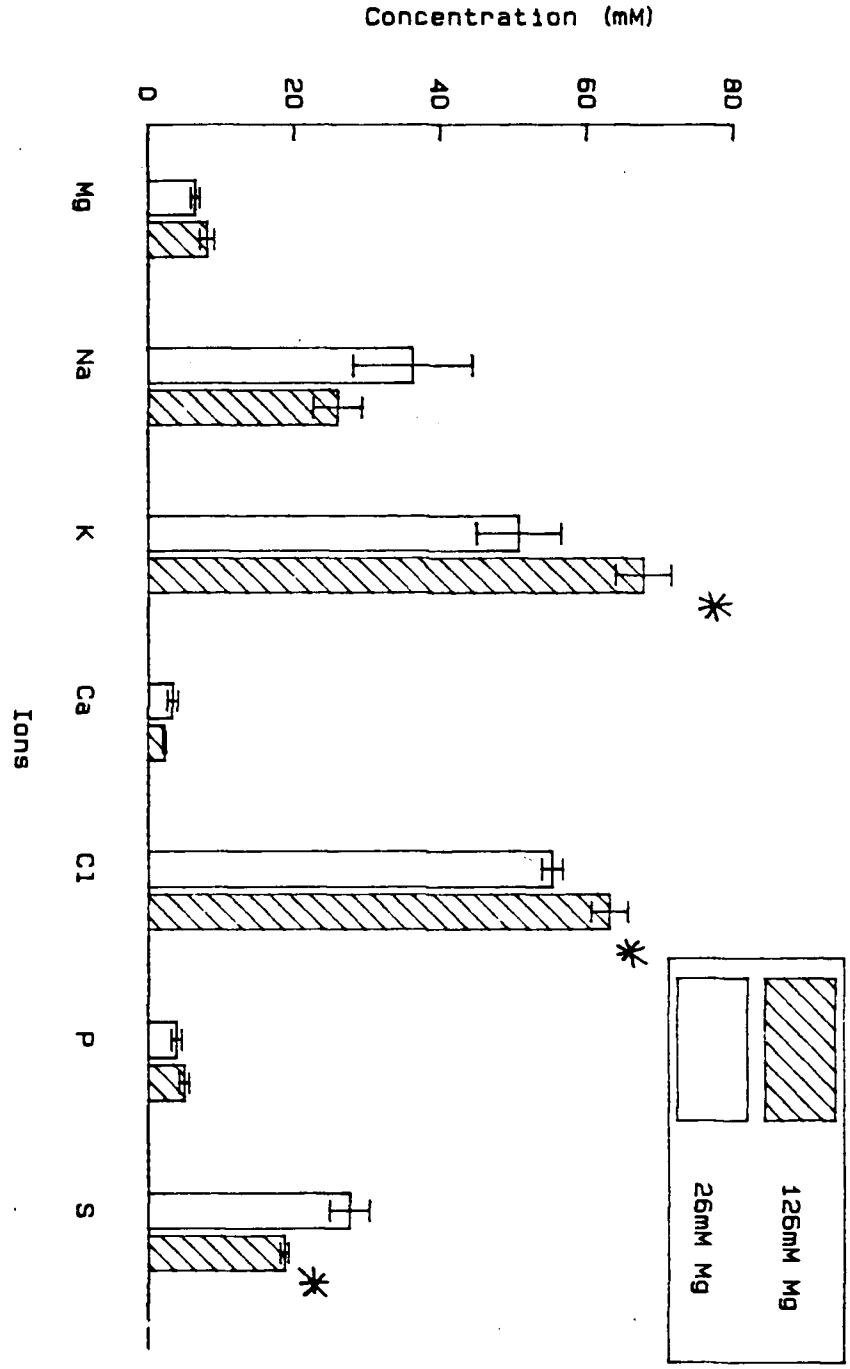

Concentration (mM)
0
20
40
60
80
Mg
Na
K
Ca
Cl
P
S
Ions
126mM Mg
26mM Mg

Figure B.6 Secretion rates of ion (J_{ion}) of A. dorsalis Malpighian tubules bathed in 0.5mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=10).

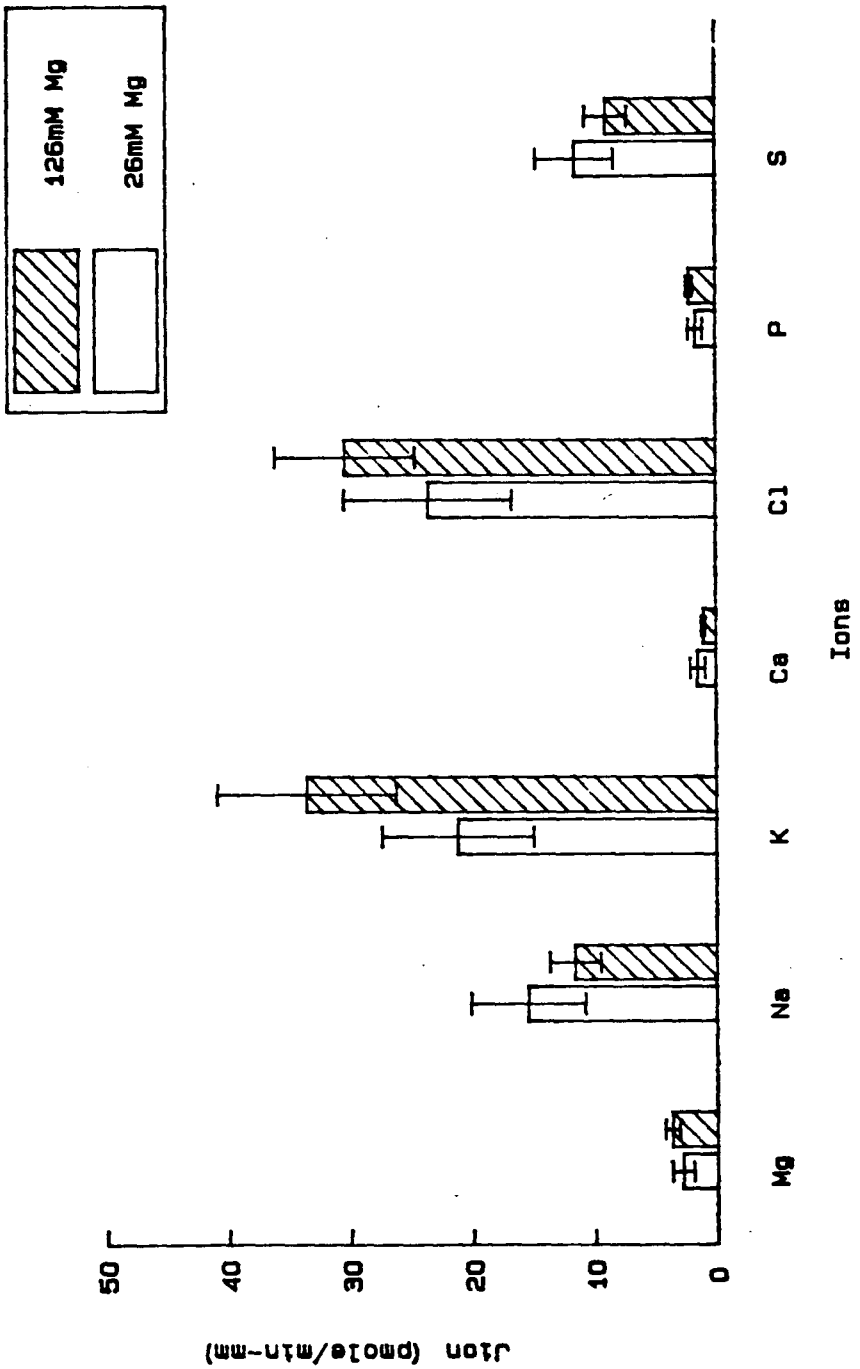

126mM Mg
26mM Mg
Mg
Na
K
Ca
Cl
P
S
Ions
0
10
20
30
40
50
Flux (pmole/min-mm)

Figure B.7 Fluid secretion rates of A. dorsalis Malpighian tubules bathed in 1mM Mg, 0mM cAMP solution. Vertical lines attached to points represent ±S.E. of the mean (n=13).

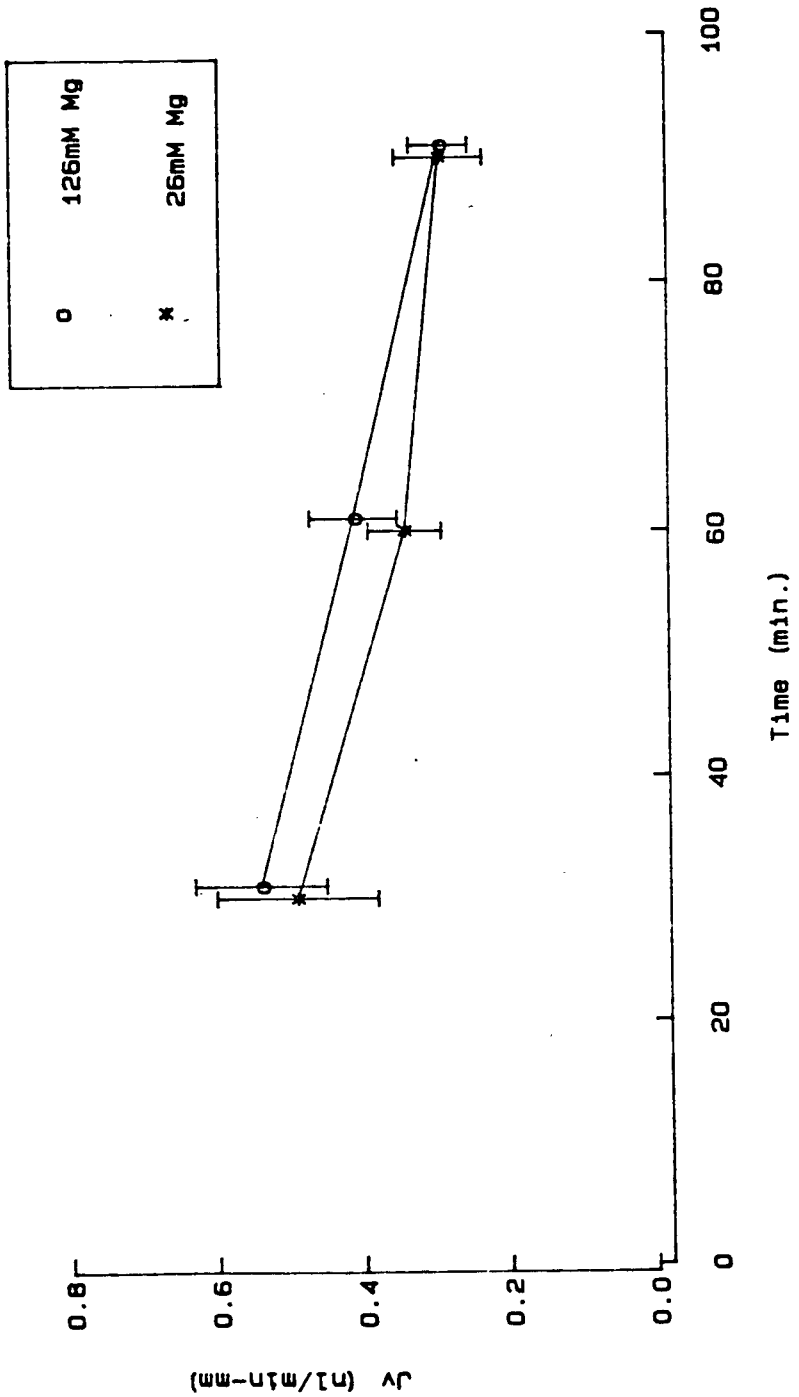

126mM Mg
26mM Mg
0
*
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (min.)
ΔV (nl/min-mm)

Figure B.8 Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in 1mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=13). * indicates significant difference.

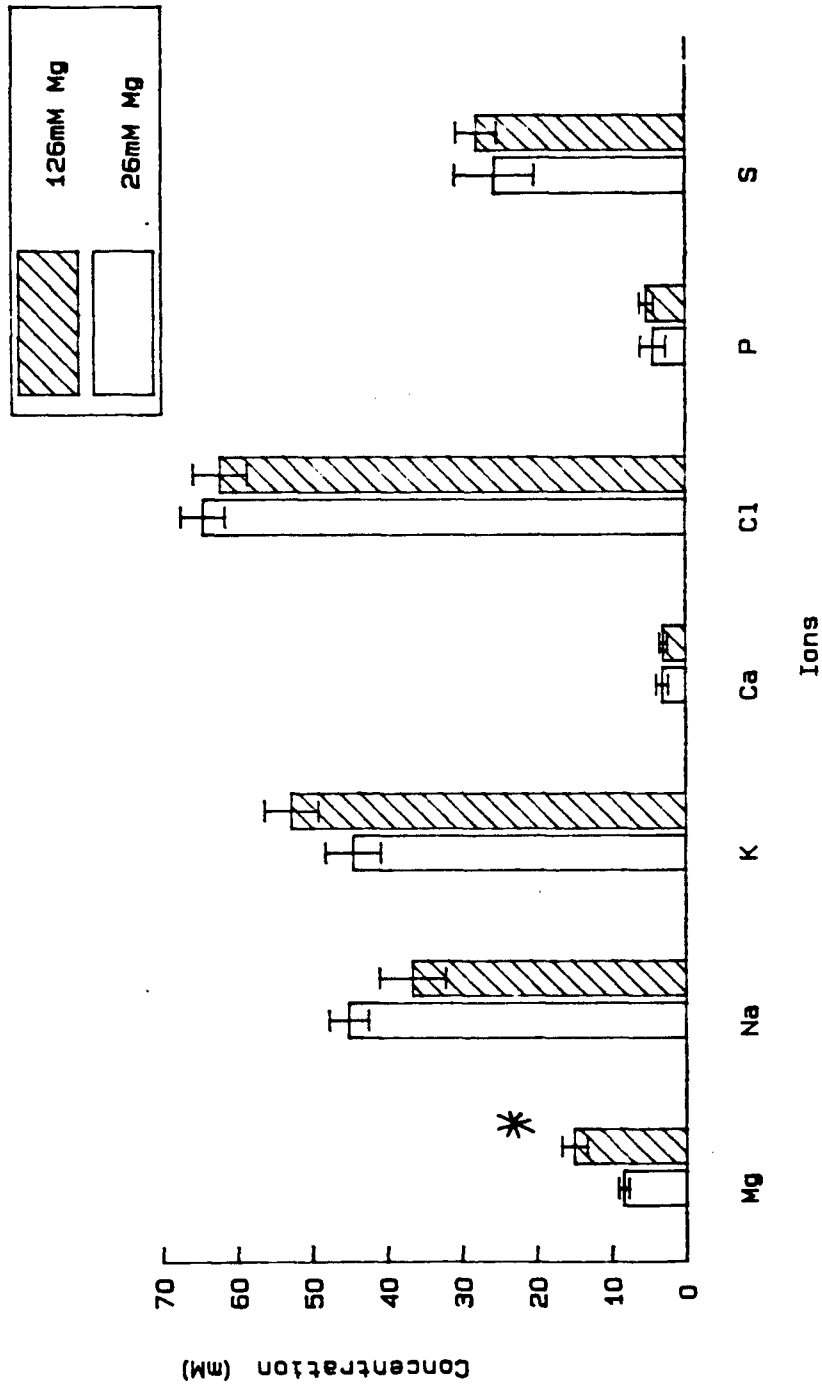

126mM Mg
26mM Mg
Concentration (mM)
70
60
50
40
30
20
10
0
Mg
Na
K
Ca
Cl
P
S
Ions

Figure B.9 Secretion rates of ion (J_{ion}) of A. dorsalis Malpighian tubules bathed in 1mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=13).
* indicates significant difference.

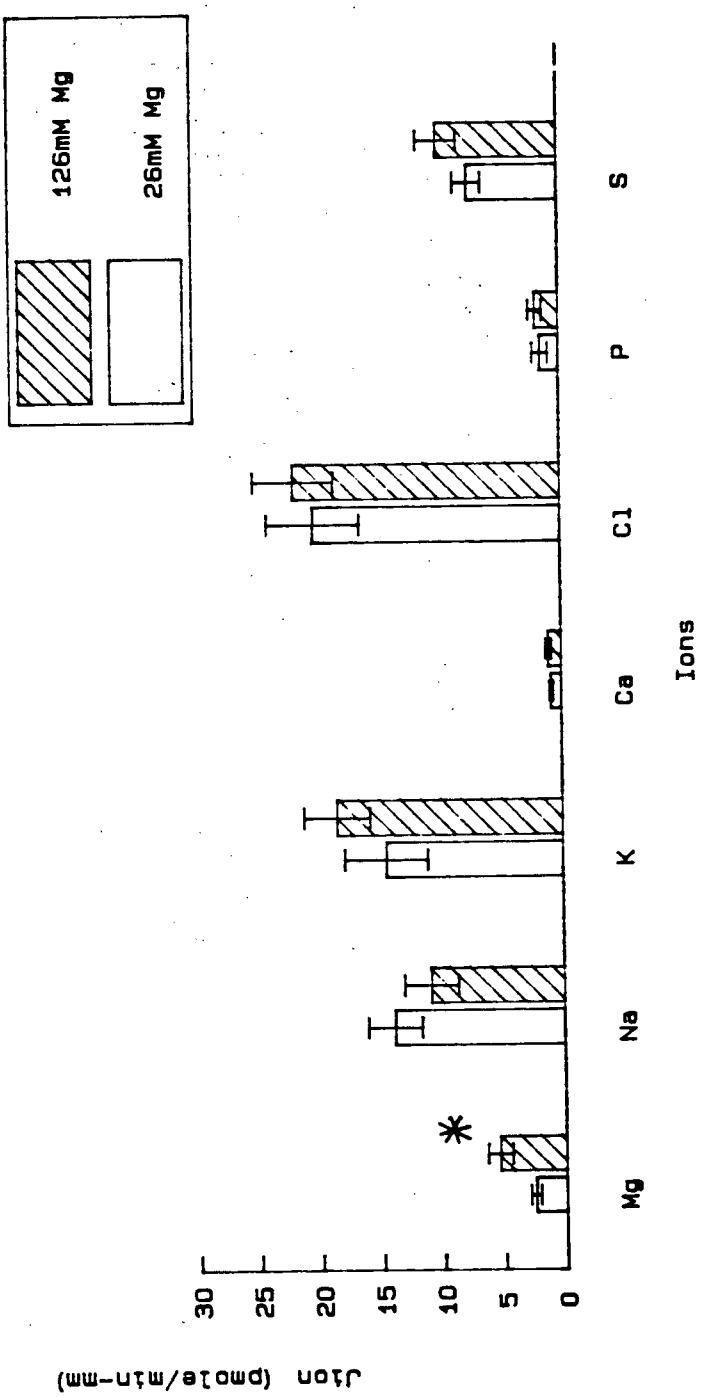

126mM Mg
26mM Mg
Mg
Na
K
Ca
Cl
P
S
Ions
0
5
10
15
20
25
30
*

Figure B.10 Fluid secretion rates of A. dorsalis Malpighian tubules bathed in 2mM Mg, 0mM cAMP solution. Vertical lines attached to points represent ±S.E. of the mean (n=11).

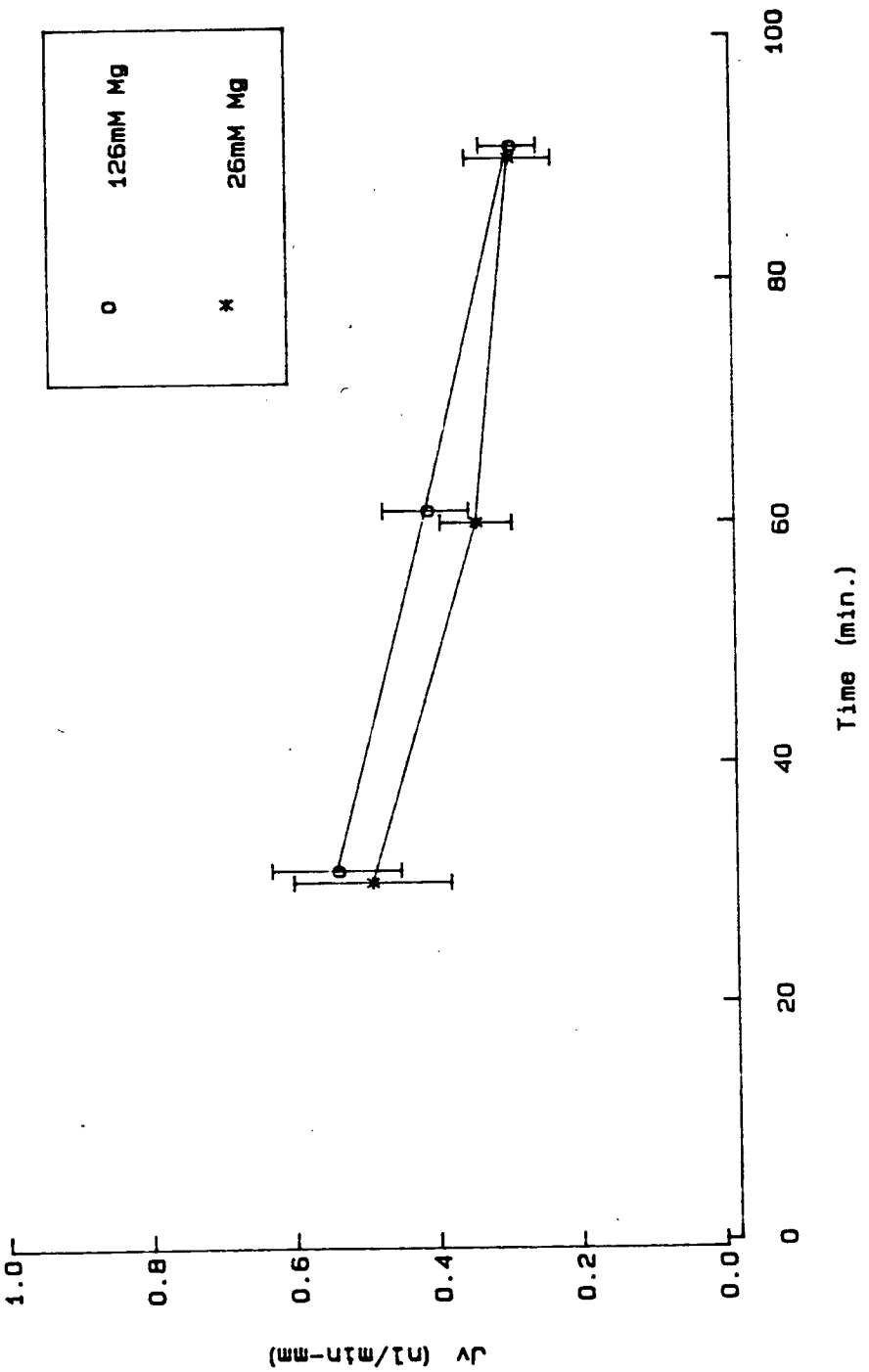

126mM Mg
26mM Mg
o
*
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (min.)
ΔV (nl/min-mm)

Figure B.11 Concentrations of ions secreted by A.dorsalis Malpighian tubules bathed in 2mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=11).

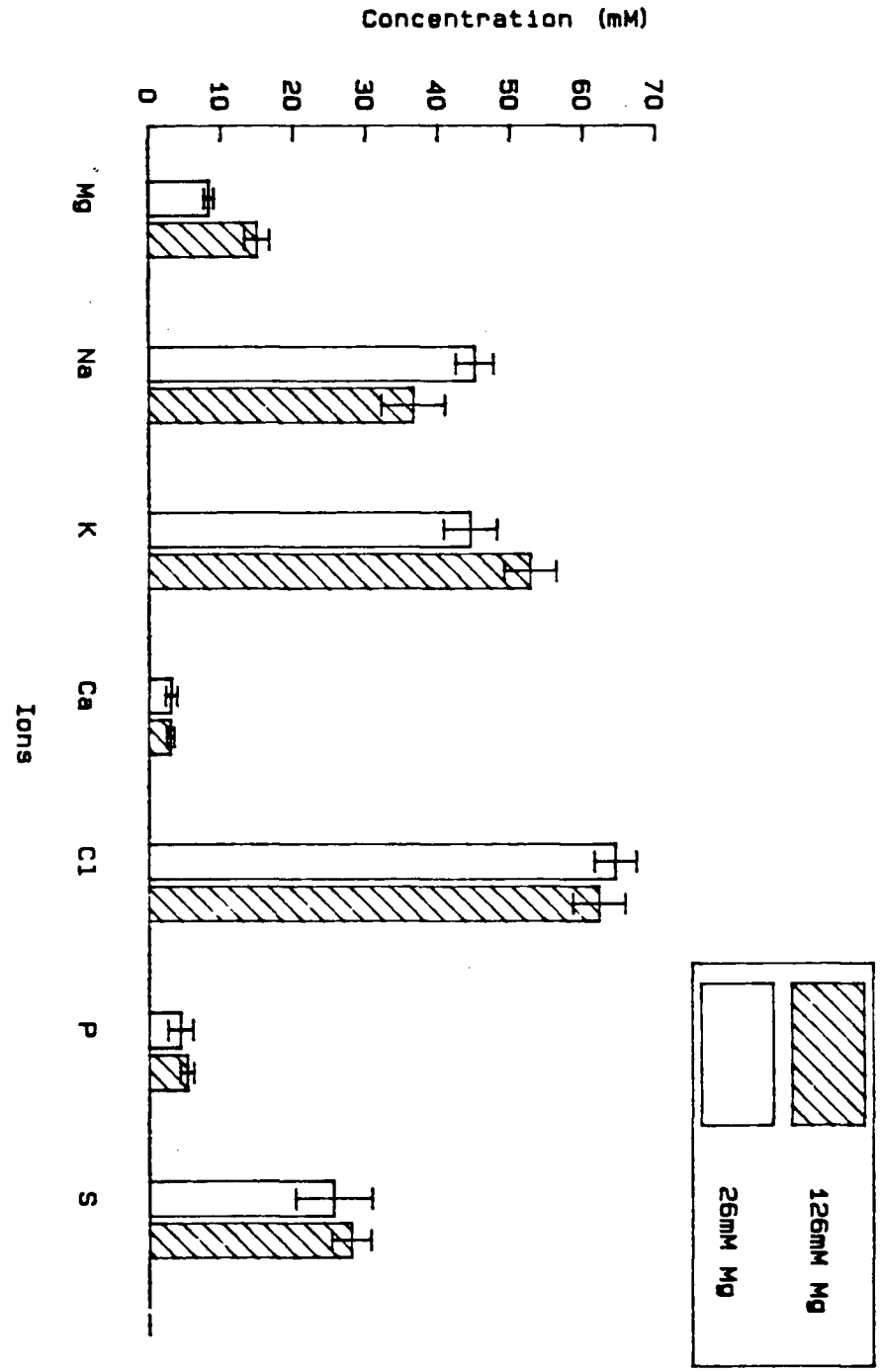

Concentration (mM)
0
10
20
30
40
50
60
70
Mg
Na
K
Ca
Cl
P
S
Ions
126mM Mg
26mM Mg

Figure B.12 Secretion rates of ion (J_{ion}) of A. dorsalis Malpighian tubules bathed in 2mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=11).

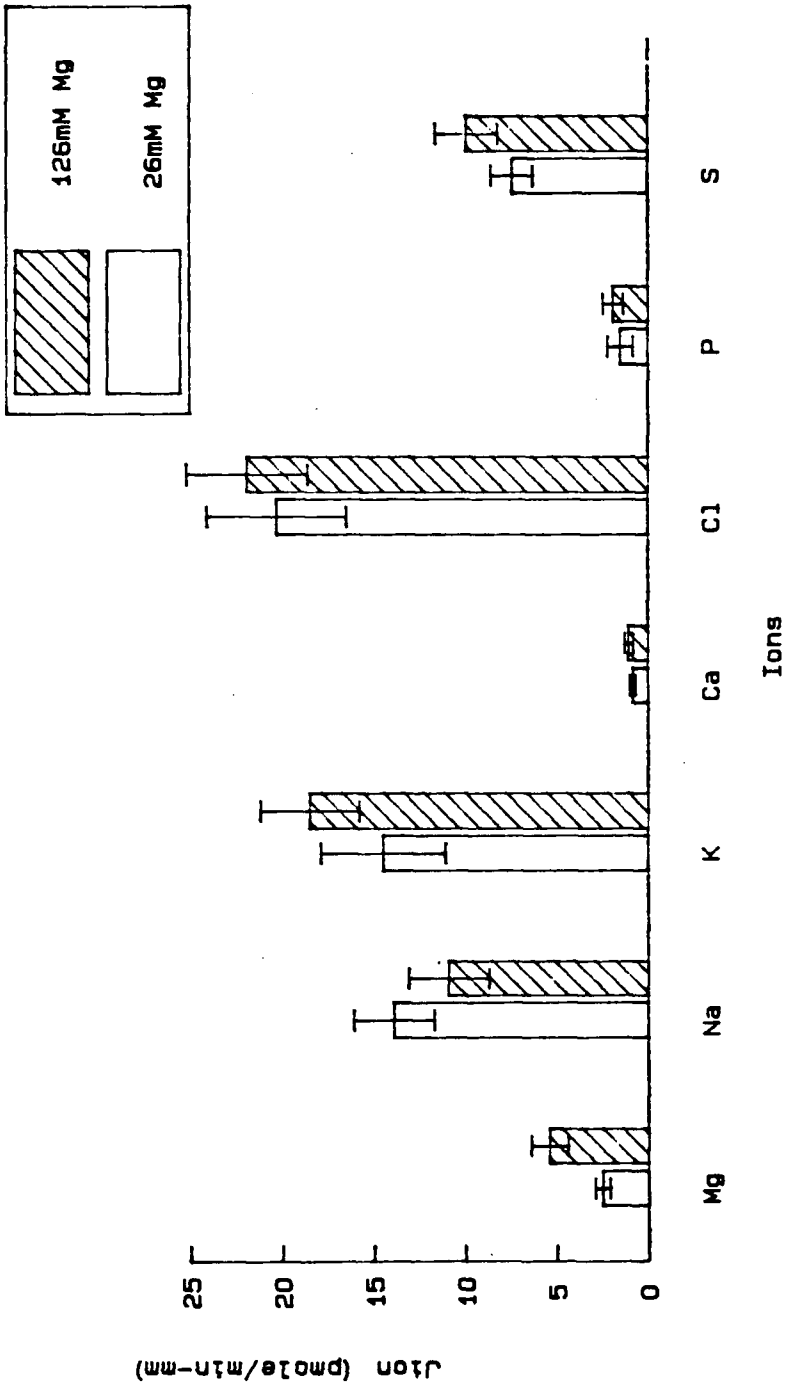

126mM Mg
26mM Mg
Mg
Na
K
Ca
Cl
P
S
Ions
0
5
10
15
20
25
Jion (pmole/min-mm)

Figure B.13 Fluid secretion rates of A. dorsalis Malpighian tubules bathed in 4mM Mg, 0mM cAMP solution. Vertical lines attached to points represent ±S.E. of the mean (n=14).

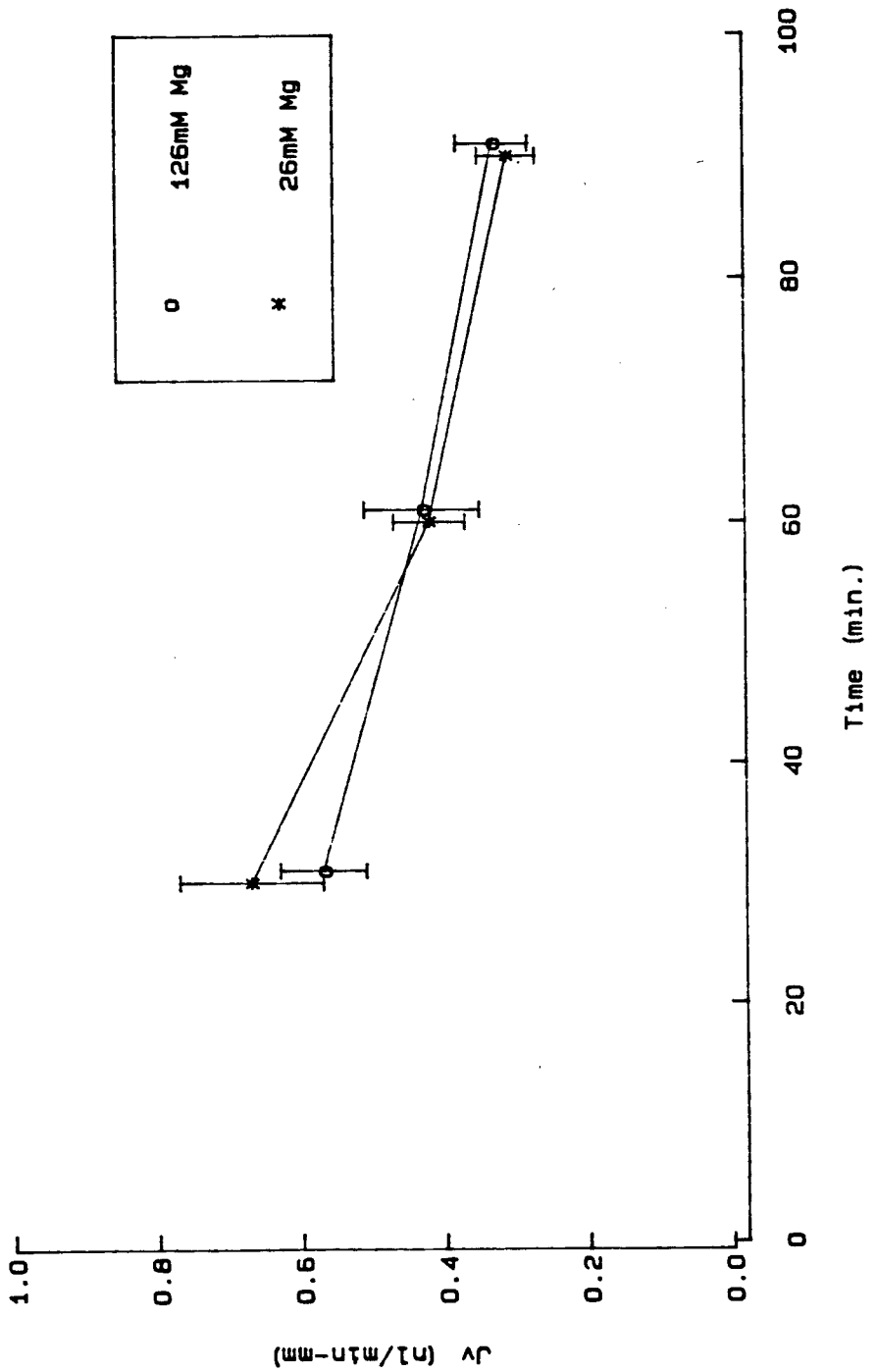

126mM Mg
26mM Mg
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (min.)
dv (nl/min-mm)

Figure B.14 Concentrations of ions secreted by A. dorsalis Malpighian tubules bathed in 4mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=14). * represents significant difference.

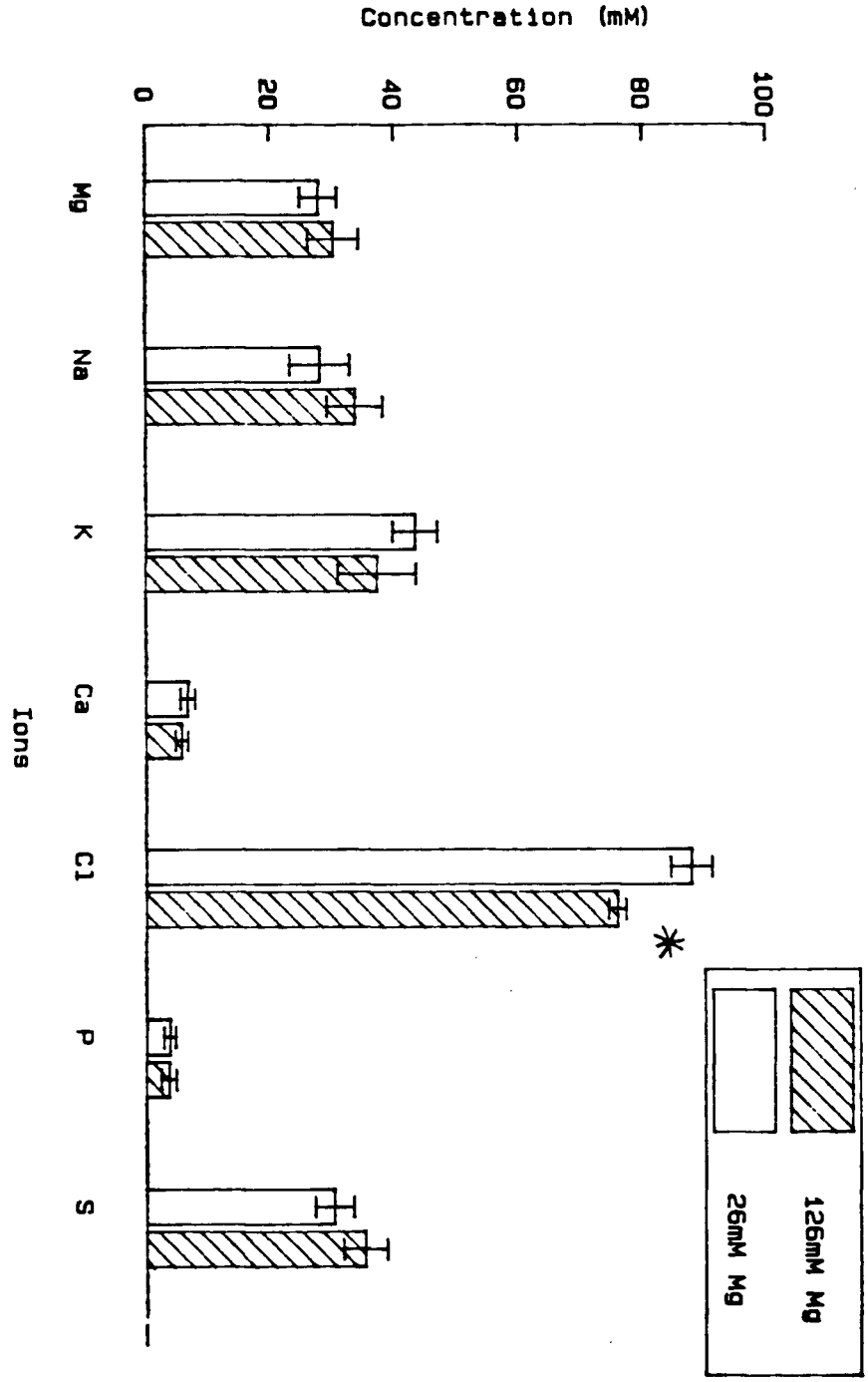
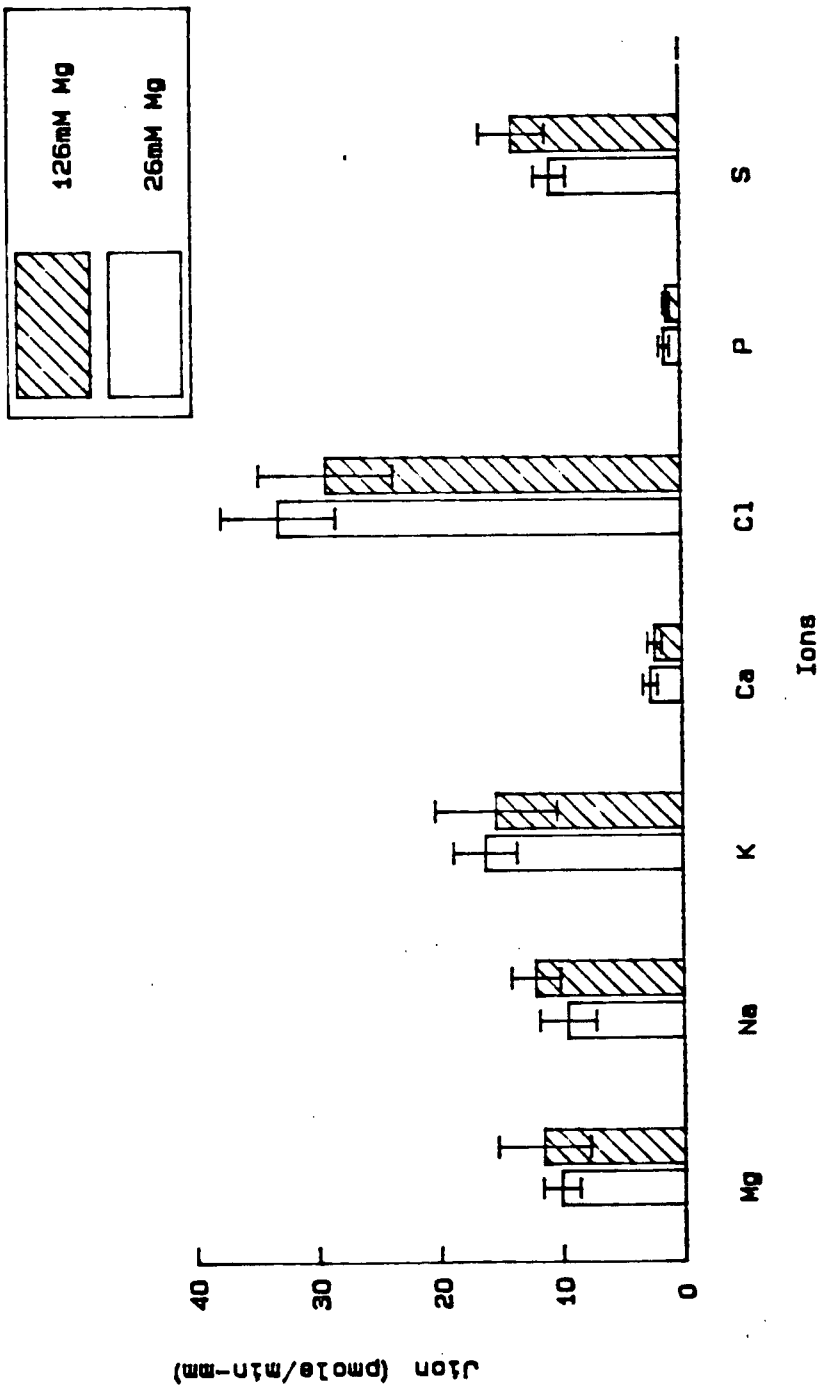

Concentration (mM)
0
20
40
60
80
100
Mg
Na
K
Ca
Cl
P
S
Ions
126mM Mg
26mM Mg

Figure B.15 Secretion rates of ion (J_{ion}) of A. dorsalis Malpighian tubules bathed in 4mM Mg, 0mM cAMP solution. Mean is average of values at 60' and at 90'. Vertical lines attached to bars represent ±S.E. of the mean (n=14).


126mM Mg
26mM Mg
Mg
Na
K
Ca
Cl
P
S
Ions
0
10
20
30
40
Ion (pmole/min-mm)