ACTIVE TRANSPORT OF MAGNESIUM BY THE MALPIGHIAN TUBULES OF THE LARVAE OF THE MOSQUITO, *AEDES CAMPESTRIS*

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SUMMARY

1. Larvae of *Aedes campestris* can survive in water containing up to 100 mM Mg even though they ingest and absorb into the haemolymph considerable amounts of magnesium-rich fluid.

2. Isolated Malpighian tubules, unlike those of *Rhodnius* and *Carausius* secreted fluid containing elevated concentrations of magnesium. This transport displayed saturation kinetics, the half-maximal rate being at approximately 2·5 mM Mg.

3. Active transport of magnesium was demonstrated by the secretion of this cation against a tenfold concentration gradient and an electrical potential difference of 15 mV.

4. Magnesium ions are not required for fluid transport, which proceeds independently of magnesium transport. As a result fluid which is secreted slowly contains higher concentrations of magnesium than that which is secreted more rapidly.

5. Magnesium is transported by isolated Malpighian tubules fast enough to account for the observed excretion of magnesium in living mosquito larvae.

INTRODUCTION

Larvae of the salt-water mosquito *Aedes campestris* can survive in natural waters containing up to 100 mM Mg. In spite of very high rates of ingestion and assimilation of such water, haemolymph concentrations of this cation are regulated at very low levels (1·5–4 mM Mg). Excretion via the anus appeared to be responsible for removal of ingested magnesium (Kiceniuk & Phillips, 1975). It was also found that whole Malpighian tubules contained several times more magnesium per unit weight than other tissues such as the midgut and rectal epithelium (Kiceniuk, 1971).

This preliminary work strongly suggests that the Malpighian tubules of larvae of *A. campestris* are the site at which magnesium is removed from the haemolymph. Because of the high concentrations of magnesium found in the Malpighian tubules and urine and because of the high rate of magnesium excretion, it seemed a distinct possibility that the Malpighian tubules are able to secrete magnesium, possibly

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against large electrochemical gradients. This paper describes experiments designed to test this possibility. This seemed particularly worth while since there are no reports of magnesium transport in insects, or indeed of the regulation of this cation.

<div align="center">MATERIALS AND METHODS</div>

Insect material

Larvae of *Aedes campestris* were collected on 20 May, 1974 from Ctenocladus pool (near Kamloops, B.C., Canada) and held at 10 °C in natural pond water containing 137 mM Na, 14 mM Mg, 2·5 mM K, 4 mM Cl with the anionic balance presumably being made up by sulphate as previously found (Blinn, 1971); the pH of the water was 8·9 and its osmotic concentration 170 mOsm. The low salinity of the water compared to the much higher values often recorded was attributable to unusually heavy rainfall in the preceding 2 months. Natural detritus and sediment were provided for food.

In one experiment we used larvae of *Aedes taeniorhynchus*, another salt-water mosquito. These we obtained from the Entomological Research Center, Vero Beach, Florida, U.S.A., and they were reared from eggs in 100 % sea water at 25 °C.

All experiments were done using the last larval instar of the species concerned and were carried out at room temperature (24 °C).

Methods

The Malpighian tubules of larvae of *A. campestris* are considerably smaller than those of other insect species which have been successfully used in *in vitro* preparations. None the less by suitable miniaturization of the technique the same methods could be applied to the tubules of these mosquito larvae. Basically, individual Malpighian tubules (of which each larva has five) were dissected free from the alimentary canal of the insect and transferred to a drop of Ringer's solution held in position under liquid paraffin (mineral oil) on a layer of solid wax by a fine glass rod set in the wax. The cut end of each tubule was pulled out from the bathing drop and looped round a very fine glass rod set near to the bathing drop. The fluid secreted by each tubule accumulated as a small drop held by surface tension to the collecting glass rod. To avoid the secreted drop running back into the bathing medium, collecting rods could not be set closer than about 0·8 mm from the bathing drop. As a tubule is only 2·5–3 mm long, this meant in practice that only 50–60% of the length of the tubule was covered by the bathing solution. Not surprisingly such tubules were found to secrete fluid only slowly. In order to collect sufficient secreted fluid for analysis it was found convenient to include up to five Malpighian tubules in each bathing drop, each tubule delivering fluid to one of a row of fine glass collecting rods, set in an arc close to the bathing drop. In this way up to 40 Malpighian tubules could be examined at one time.

Secreted fluid was harvested from the tubules by carefully detaching the secreted drops from the collecting glass rods with another glass rod of slightly larger diameter. The volume of these drops was determined from measurements of their diameter and the rate at which they had been secreted from a knowledge of the time interval during which they had been produced.

The Ringer's solution used for our initial experiments was one originally devised for use with isolated Malpighian tubules of *Rhodnius*; it contained 147 mM Na^+, 4 mM K^+, 8·5 mM Mg^{2+}, 2 mM Ca^{2+}, 155 mM Cl^-, 10·2 mM HCO_3^-, 4·3 mM $H_2PO_4^-$ and 34 mM glucose. Later analysis of 1 μl samples of the haemolymph of larvae of *A. campestris* showed it to contain 129 mM Na^+, 6 mM K^+ and 3·5 mM Mg^{2+}, sufficiently close to the concentrations contained in the *Rhodnius* Ringer's solution to justify its use. In this Ringer's solution, Malpighian tubules of *A. campestris* secrete fluid on average at about 250 picolitres* a minute.

Many insect Malpighian tubules secrete fluid faster in K-rich media (Maddrell, 1971). Accordingly, we set tubules of *A. campestris* to secrete in a Ringer's solution containing 76 mM K (with 76 mM Na); the average rate of secretion rose to nearly 1nl min^{-1}. We were able to use this acceleratory effect in experiments calling for relatively large amounts of fluid secretion or for a higher rate of secretion.

It is worth recording in passing that the secreted fluid produced in a 4 mM K bathing solution contained 31 mM K and in a bathing solution with 76 mM K and 76 mM Na the secreted fluid contained 128 mM K and 36 mM Na. These tubules seem to be similar to most other Malpighian tubules in preferentially secreting K rather than Na (Maddrell, 1971).

The osmotic concentration of fluid secreted by tubules isolated into Ringer's solutions of different ionic compositions was always closely similar to that of the bathing medium; six samples of secreted fluid had an average osmotic concentration of 340 mOsm and the bathing medium an average osmotic concentration of 345 mOsm.

Aliquots, 50–300 nl in volume, were diluted in 1 cm^3 of 1·5% disodium EDTA contained in capped polythene vials for magnesium determinations using a 'Techtron Model AA 120' atomic absorption spectrophotometer (Willis, 1960). Aliquots of similar size were used for Na, K and Cl ion determinations as previously described (Kaufman & Phillips, 1973). Osmotic concentration was determined with a 'Clifton Technical Physics' nanolitre osmometer.

To measure potential differences, two agar 3 M KCl bridges (in P.E. 10 tubing) were brought into contact with the bathing medium and secreted droplets respectively using micro-manipulators. The potential difference (P.D.) across the Malpighian tubule epithelium was determined by connecting the two salt bridges through calomel half-electrodes to a Keithley Model 602 electrometer. The asymmetry potential was determined with both salt bridges in the bathing droplet and was subtracted from transepithelial readings.

<div align="center">RESULTS</div>

The demonstration of magnesium transport

Our first concern was to discover whether the Malpighian tubules of *A. campestris* can concentrate magnesium in the fluid which they secrete. To test this, we isolated Malpighian tubules into solutions of varying magnesium concentration, collected the fluid they secreted during the next 2–3 h and measured the concentration of magnesium in it. The results of these experiments involving a total of 330 isolated tubules are set out in Figs. 1 and 2. Clearly the Malpighian tubules of these insects can achieve a considerable concentration of magnesium in the secreted fluid. This concentrating

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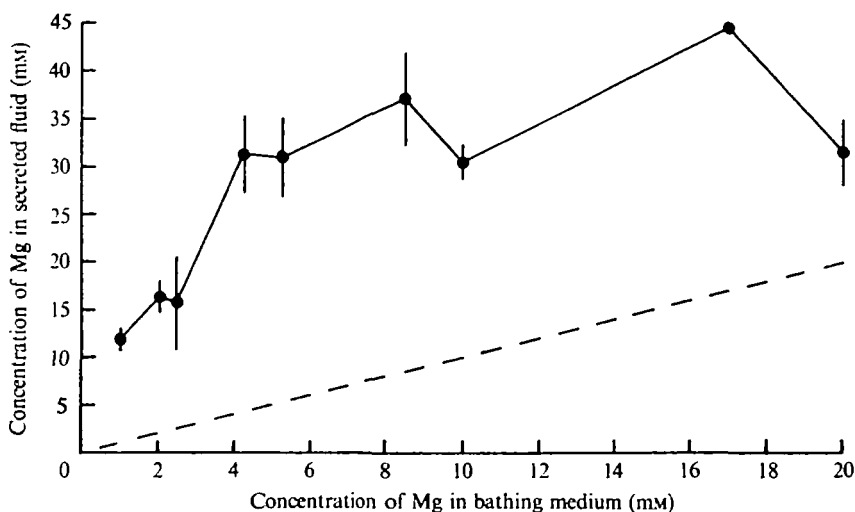* 1 picolitre (pl) = 10^{-3} nanolitres (nl) = 10^{-6} μl.

Fig. 1. The dependence of the magnesium concentration in the fluid secreted by isolated Malpighian tubules of *A. campestris* on the concentration of magnesium in the bathing medium. The dotted line is that of a relationship where the secreted fluid and the medium contain equal concentrations of magnesium. The vertical lines attached to the points indicate the extent of the standard error of the mean of several determinations.
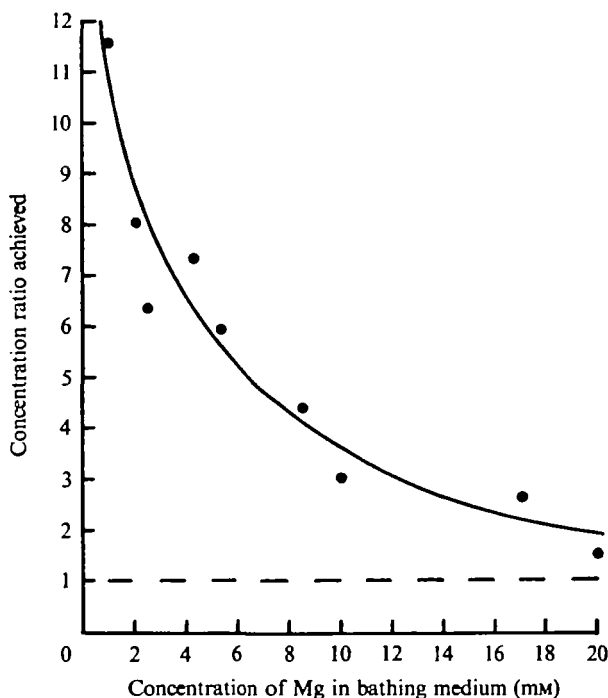

Fig. 2. The dependence of the ratio of the magnesium concentrations in the secreted fluid to the magnesium concentration in the bathing medium on the magnesium concentration of the bathing fluid. The values are calculated from those shown in Fig. 1. The curve drawn through the points was fitted by eye. The dotted line is again that of a relationship where the secreted fluid and the medium contain equal concentrations of magnesium.
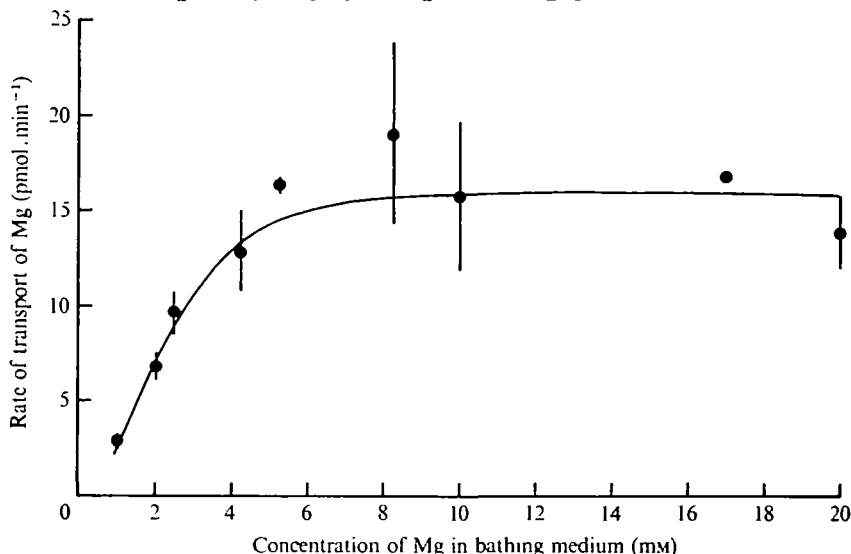
Fig. 3. The dependence of the rate of secretion of magnesium by isolated Malpighian tubules of *A. campestris* on the concentration of magnesium in the bathing medium. The curve drawn through the points was fitted by eye. The vertical lines attached to the points indicate the extent of the standard error of the mean of several determinations.

ability is particularly marked at low concentrations of magnesium in the bathing medium (Fig. 2). In no case did the Malpighian tubules produce fluid containing a lower concentration of magnesium than the bathing medium.

The ability of *A. campestris* tubules to concentrate magnesium contrasts with the behaviour of Malpighian tubules of *Carausius morosus* where the concentration of magnesium in the secreted fluid is always much lower ($< 10\%$) than in the bathing medium (Ramsay, 1956). To provide further information on this point we carried out experiments on isolated Malpighian tubules of *Rhodnius*. Five such tubules in a bathing medium containing 8·5 mM Mg and stimulated to secrete fluid at a high rate by 5-hydroxytryptamine (Maddrell, Pilcher & Gardiner, 1971) secreted fluid containing only 0·53 mM Mg. However, at the high rates of fluid secretion induced by 5-hydroxytryptamine, magnesium transport might not be able to achieve an elevated concentration of magnesium in the secreted fluid. The experiment was therefore repeated using seven unstimulated Malpighian tubules, which secrete about 100 times more slowly, immersed in a meduim containing 2 mM Mg. Under these conditions and although the lumina of such tubules is at a potential negative with respect to the bathing medium (Maddrell, 1971) which would favour magnesium entry, the secreted fluid contained, on average, only 0·44 mM Mg. By contrast, five Malpighian tubules from larvae of *A. taeniorhynchus* secreted fluid containing 12·8 mM Mg when immersed in the same 2 mM Mg medium.

Because the rate of fluid secretion of *A. campestris'* tubules varies, depending for example on the potassium concentration of the bathing fluid, the magnesium concentration in the secreted fluid gives no more than an indication of the rate of magnesium transport. From measurements of the rate of fluid secretion and a knowledge of the magnesium concentrations in the fluid we were able to calculate the rate at which magnesium was transported. Fig. 3 shows how the rate of magnesium

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Table 1. *Analysis of fluid secreted by 40 isolated Malpighian tubules of*
A. campestris

	Secreted fluid (1·74 μl)	Bathing fluid
O.P. (mOsm)	344	342
Na+ (mM)	36	76
K+ (mM)	128	76
Mg²+ (mM)	21	5
Cl⁻ (mM)	171	155
Ca²+ (mM)	Not measured	2
HCO₃⁻(mM)	Not measured	10
H₂PO₄ (mM)	Not measured	4
Glucose (mM)	Not measured	34

transport depends on the magnesium concentration in the bathing fluid. What emerges is that magnesium is transported at a rate which reaches saturation at a concentration of magnesium in the bathing medium of about 5–6 mM and which is half maximal at around 2·5 mM.

Having demonstrated that the Malpighian tubules of *A. campestris* can transport magnesium at high rates, the question arises as to whether this transport is an active one. The findings that the transport achieves a high concentration of magnesium in the secreted fluid and displays saturation kinetics do not, of course, establish that this transport is active although it suggests mediation by a carrier. For example, one way in which an elevated concentration of magnesium in the secreted fluid might be achieved would be if there were an accompanying transport of polyvalent anions which would reduce the activity of Mg²⁺ ions in the secreted fluid and so promote their passive entry down an activity gradient. It seemed to us unlikely that this could explain, for example, the more than ten times concentration ratio which the tubules achieve in 1 mM Mg. However, we set 40 isolated tubules to secrete in a solution containing 5 mM Mg; its full composition is shown in Table 1. The fluid secreted by these tubules was pooled and found to contain 21 mM Mg and 171 mM-Cl; full details of its osmotic and ionic concentrations are set out in Table 1. From the figures it is plain that chloride accounts for so much of the total possible anionic contribution to the osmotic concentration that polyvalent anions are not secreted at sufficiently high concentration significantly to reduced the activity of Mg²⁺ ions in the secreted fluid. The explanation of the ability of these Malpighian tubules to concentrate magnesium must be sought elsewhere.

Passive magnesium transport against a concentration gradient might be achieved if the electrical gradient favoured it. We therefore measured the trans-wall potential difference of 30 Malpighian tubules isolated in a bathing solution containing 2 mM Mg²⁺ (and 76 mM K⁺, 76 mM Na⁺, 2 mM Ca²⁺, 155 Cl⁻, 10·2 mM HCO₃⁻, 4·3 mM H₂PO₄⁻). Rather surprisingly and unlike the Malpighian tubules of most other insects (Maddrell, 1971), these tubules of *A. campestris* mostly had a trans-wall potential difference in which the lumen was negative with respect to the bathing solution. Nevertheless, the tubules from some individual insects had lumina positive to the bathing solution and these tubules did not reveal any lesser ability to concentrate magnesium than did the other tubules (Fig. 4). However, as a further test 40 more
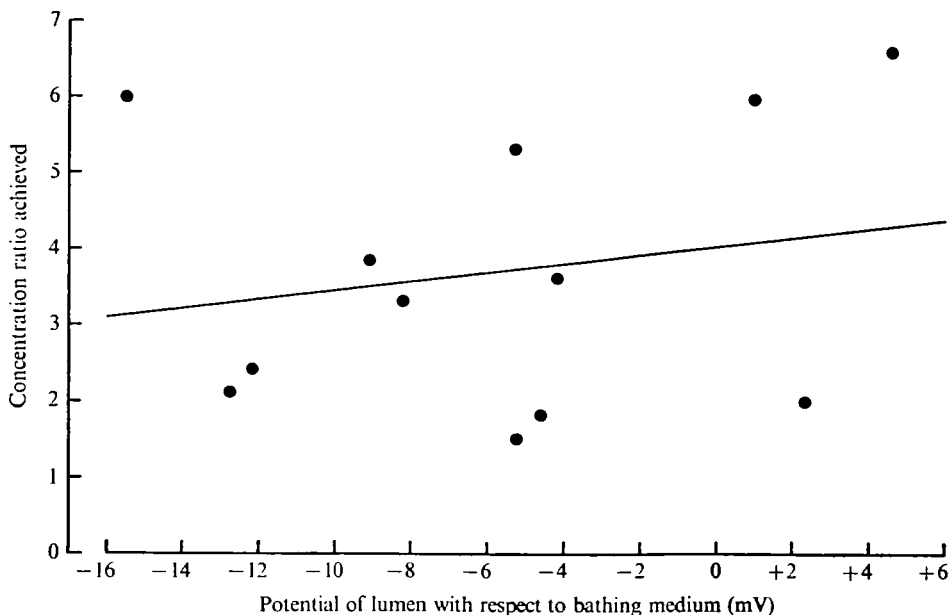
Fig. 4. The dependence of the magnesium concentrating ability of isolated Malpighian tubules of *A. campestris* on the trans-epithelial potential difference. The line drawn through the points is the linear regression line calculated by the least-squares method. The bathing fluid contained 2 mM Mg.

tubules were isolated, this time into a K-rich Cl-poor medium in an attempt both to make the tubules' lumina more positive with respect to the medium and to slow the rate of fluid secretion so that the concentration gradient for magnesium would be increased; the medium contained 1 mM-Mg. The results are shown in Fig. 5, from which it is clear that both objectives were achieved. Measurements of potential difference were taken before and after samples of secreted fluid were collected. Of 27 tubules which gave initial lumen-positive readings, none gave other than a lumen-positive reading after they had secreted the fluid whose magnesium content was measured. These tubules produced fluid containing an average of 10·3 mM Mg at an average trans-wall potential difference of 15·8 mV, lumen positive. This clear finding of transport against a concentration gradient of more than ten times and against an electrical potential difference established that Malpighian tubules of *A. campestris* can carry out active transport of magnesium. As with the earlier experiment, we again found that the concentration of magnesium in the secreted fluid was not obviously affected by the trans-wall potential difference; this suggests that the transport process has no large passive element.

Magnesium transport and fluid secretion

An obvious question which arises from this work is whether magnesium transport in *A. campestris* plays any large part in the secretion of fluid by the Malpighian tubules. Two lines of evidence strongly suggest that the two processes are to a large degree independent.

In the first experiments the magnesium concentration of the bathing solution was varied to test the effect that this had on the rate at which fluid was secreted. For
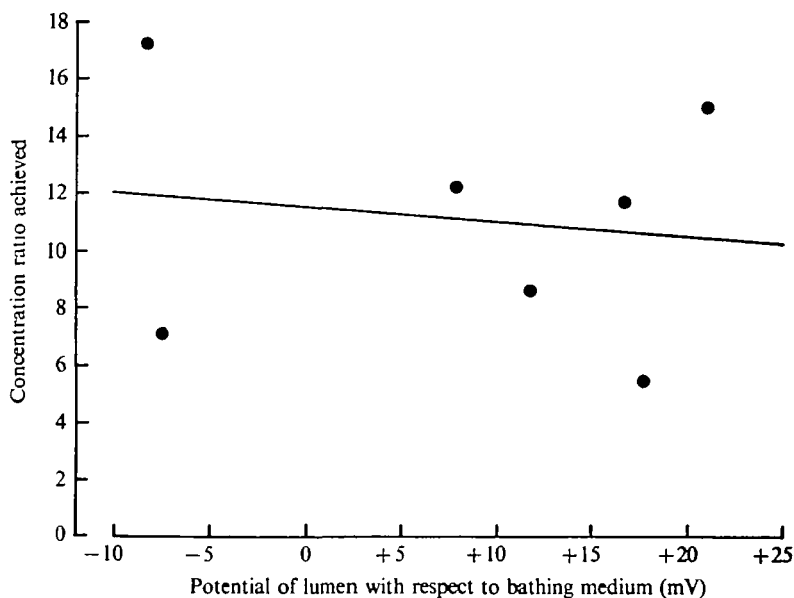
Fig. 5. The ability of isolated Malpighian tubules of *A. campestris* to concentrate magnesium as a function of the trans-epithelial potential difference, when the lumen had been made positive with respect to the bathing medium by increasing the potassium and sulphate content of the medium. The line drawn through the points is the linear regression line calculated by the least-squares method. The medium contained 1 mM Mg.
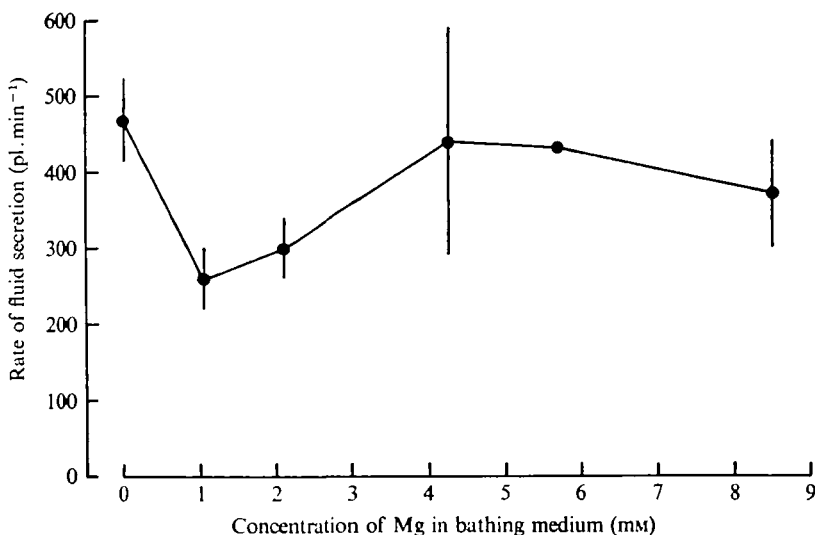

Fig. 6. The effect on the rate of fluid secretion by isolated Malpighian tubules of *A. campestris* of changes in the magnesium concentration of the bathing medium. Vertical lines attached to the points indicate the extent of the standard error of the mean of several determinations.

these experiments we used thirteen insects, a total of eighty-five Malpighian tubules. Each of the tubules from any particular insect was put into a bathing drop of a different magnesium content to minimize the effects of variation between insects. The results of these experiments are shown in Fig. 6. Two points are clear. First, tubules secrete

fluid at an undiminished rate in the absence of magnesium from the bathing fluid. Secondly, changes in the magnesium concentration have no effects of the rate of fluid secretion.

In a second series of experiments we investigated the effect of varying the rate of fluid secretion on the rate of magnesium transport. Malpighian tubules were set to secrete in fluid containing 2 mM Mg but having either 4 mM K or 76 mM K to elicit respectively low and high rates of fluid secretion. Thirty-five Malpighian tubules in the K-rich medium secreted fluid at an average rate of 852 pl min^{-1}, the fluid contained on average 8·15 mM Mg and the average rate of magnesium transport was 6·95 pmol min^{-1}. Twenty-four tubules in the K-poor medium secreted fluid at an average rate of only 260 pl min^{-1}, but it contained on average much more magnesium, 25·47 mM, while the average rate of magnesium transport was scarcely changed at 6·72 pmol min^{-1}. This result shows that the rate of transport of magnesium is very little affected by changes in the rate of fluid secretion; the tubules produce Mg rich fluid at low rates of fluid secretion and vice versa.

DISCUSSION

The experiments described in this paper clearly show that isolated Malpighian tubules of *A. campestris* can transport magnesium at high rates against considerable electrochemical potential gradients. The question arises as to whether this transport is fast enough to account for the excretory rate observed *in vivo*. Larvae of *A. campestris* from waters of intermediate salinities weigh about 8 mg. Such larvae may daily absorb 17–100 % of their body weight of pond water, depending on salinity (Phillips, unpublished observation). The highest tolerable external level of magnesium is 90 mM (Kiceniuk & Phillips, 1975). This means that the excretory system may rid the haemolymph of up to 700 nmoles of magnesium per day. As shown in Fig. 3, single isolated Malpighian tubules, in a solution containing magnesium at a concentration comparable to that found in the haemolymph, transport magnesium on average at a rate of more than 12 pmol min^{-1} for 2–3 h. By the end of experiments of this duration, the secretory performance of the tubules has inevitably declined, the rate of fluid secretion, for example, had often fallen to 50 % or less of its initial value. In the few cases where we measured the rate of magnesium transport during the first hour after the tubules had been isolated, we found some magnesium transport rates as high as 50 pmol min^{-1}. Taking into account the fact that isolated Malpighian tubules have only 50–60 % of their length immersed in bathing solution (p. 762), whole tubules might well, therefore, be able to secrete magnesium at rates of the order of 100 pmol min^{-1}. At this rate the full complement of five tubules could excrete more than 700 nmoles of magnesium daily – enough to meet the excretory need of the insect.

It is worth noting that the steady-state level of magnesium in the haemolymph of *A. campestris* increases only slightly from 1·5 to 4 mM as the external medium concentration of magnesium is raised from 4 to 95 mM (Kiceniuk & Phillips, 1975). Our present results can explain this regulation of haemolymph magnesium level. The observed haemolymph values fall in the region where the rate of magnesium secretion by the Malpighian tubules (K_m = 2·5 mM) is more or less directly proportional to magnesium concentration of the bathing medium. Thus rather small increases in

haemolymph magnesium levels due to ingestion of concentrated magnesium solutions would automatically lead to increased rates of magnesium secretion as a direct consequence of the kinetic properties of the magnesium pump in the the Malpighian tubules. The concentrations of magnesium in fluid secreted by Malpighian tubules isolated in normal (i.e. high Na, low K) medium we found to be about 20–25 mM. This is in good agreement with the magnesium concentration (Kiceniuk & Phillips, 1975) in urine collected at the anus of this species reared in media of magnesium content similar to that of the pond water in which our sample of *A. campestris* was living when collected. This suggests that, at relatively low external magnesium levels, there is no need to postulate a concentration of magnesium by fluid reabsorption in the rectum. However, at high external levels of magnesium, the excreta may contain over 90 mM Mg, about twice the maximum concentration observed in Malpighian tubules secretion in the present experiments. Moreover, because drinking rates increase drastically to several times body weight per day at the upper tolerance limits, ingestion rates of 1300 nmoles Mg per day are observed in the few surviving larvae (Kiceniuk & Phillips, 1975). Three explanations seem possible:

(*a*) When magnesium levels in the pond approach 90 mM, only a few (*ca.* 5%) of the larvae survive (Kiceniuk & Phillips, 1975) and it is possible that these individuals have Malpighian tubules with an unusually pronounced ability to secrete magnesium. The experiments described in this paper were for animals growing in relatively dilute media where mortality was minimal.

(*b*) The development of larvae in water with a very high magnesium content might induce a greater transport capacity by the Malpighian tubules. Just such an induction has been shown for NaCl absorption by anal papillae of *A. campestris* (Phillips & Meredith, 1969).

(*c*) The magnesium concentration in the rectal fluid of larvae reared in very concentrated water may rise due to water reabsorption or further magnesium secretion.

The high level of Mg^{2+} ions in sea water (52 mM; Prosser, 1973) led us to look very briefly at a salt water species of mosquito, *A. taeniorhynchus*, which lives in salt pools fed by high tidal levels of sea water (Nayar, 1969). Like *A. campestris* and in a rather similar way to the magnesium secretion by the kidney tubules of marine fishes (reviewed by Hickman & Trump, 1969) this species also appears to secrete Mg^{2+} ions via the Malpighian tubules. We would like to advance the hypothesis that magnesium secretion by Malpighian tubules may occur generally in insect larvae which develop in sea water.

At present we do not wish to distinguish between primary and secondary active transport of magnesium by Malpighian tubules of *A. campestris*. There is increasing reason to believe that, in spite of the much higher intracellular contents of magnesium in most cells compared to extracellular fluid, the activity gradient for this cation is in the reverse direction (Palaty, 1971). Active extrusion of magnesium ions may therefore be a general phenomenon amongst animal cells. In the case of the squid giant axon at least, there is good evidence that the electrochemical gradient for sodium entry may provide part of the energy for magnesium transport out of this cell (Baker & Crawford, 1973). The potential usefulness of the *A. campestris* tubules could be in providing a convenient system for investigating the membrane asymmetries which give rise to transport of magnesium across epithelial membranes.

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