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ACTIVE TRANSPORT OF SULPHATE IONS BY THE MALPIGHIAN TUBULES OF LARVAE OF THE MOSQUITO *AEDES CAMPESTRIS*

BY S. H. P. MADDRELL* AND J. E. PHILLIPS
Department of Zoology, University of British Columbia, Vancouver 8, B.C., Canada

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SUMMARY

1. Larvae of *Aedes campestris* ingest and absorb into their haemolymph large quantities of the sulphate-rich water in which they live, yet they are able to maintain the sulphate content of the haemolymph well below that of the environment.

2. Tracer experiments showed that sulphate regulation was not achieved by deposition of precipitates in the tissues.

3. *In vitro* preparations of Malpighian tubules secrete sulphate ions actively against both a three times concentration gradient and an electrical potential difference of 20 mV. This transport is half saturated at about 10 mM.

4. The rate of sulphate secretion by the Malpighian tubules is sufficient to remove all of the sulphate ingested by larvae living in waters which contain less than 100 mM of this anion. At higher concentrations, sulphate ions are probably also excreted elsewhere, perhaps by the rectum or anal papillae.

INTRODUCTION

Larvae of *Aedes campestris* are able to grow, moult and pupate in naturally occurring hyperosmotic waters of varied composition (Scudder, 1969). These mosquitoes cope with osmotic water loss by drinking and absorbing the water in which they live and then excreting the excess salts (Kiceniuk & Phillips, 1975), a behaviour analogous to that of marine fishes (Hoar, 1966). In magnesium-rich water, for example, they excrete magnesium by active transport through the Malpighian tubules (Phillips & Maddrell, 1975).

Analysis of water from some magnesium-rich hyperosmotic lakes occurring in central British Columbia showed that they are peculiar not only in having high levels of magnesium but are even more remarkable in that the anionic component consists almost entirely of sulphate ions (Fig. 1). Because the larvae absorb into the haemolymph virtually all the water that they ingest (Kiceniuk & Phillips, 1975), it would appear that these insects must face an excess sulphate load considerably

* Present address: A.R.C. Unit of Invertebrate Chemistry and Physiology, Department of Zoology, Downing St, Cambridge CB2 3EJ, England.

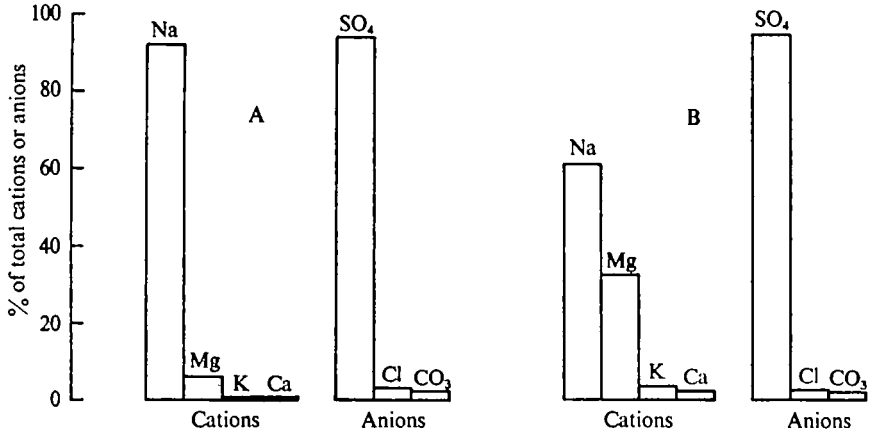

Fig. 1. Diagram to show the composition of two lakes in the Kamloops area of British Columbia typical of those in which larvae of *Aedes campestris* are found: (A) Ctenocladus pond, (B) Ironmask Lake. The contribution of each ion is expressed as the percentage ratio of its concentration (in mM) to the total anionic or cationic concentration. Data from Blinn (1971).

larger even than either that of excess sodium or magnesium. The present paper describes experiments to establish the magnitude of the sulphate load and to find out how the excretory system of these remarkable insects cope with sulphate ions.

MATERIALS AND METHODS

Insect material

Larvae of *Aedes campestris* were collected on 20 May 1974 from Ctenocladus pond in the Kamloops area of British Columbia and held at 10 °C in natural pond water until they were used for experiments. Natural detritus and sediment were provided for food. Analysis of the water showed it to contain 137 mM-Na, 14 mM-Mg, 2·5 mM-K, with 73 mM-SO_4 and 4 mM-Cl; the pH of the water was 8·9 and its osmotic concentration 170 mOsm. The low osmotic concentration of the water compared to the much higher values often recorded was attributable to unusually heavy rainfall in the two preceding months. On 18 May 1968, for example, the same pond contained 706 mM-Na, 21·5 mM Mg, 10·8 mM-K, 4·6 mM-Ca with 375 mM-SO_4 and 13·7 mM-Cl, its osmotic concentration being 536 mOsm (Blinn, 1971).

Some experiments on regulation of sulphate were carried out in 1971 when conditions in Ctenocladus pond (described by Kiceniuk & Phillips, 1975) were very different from those observed in 1974. These 1971 larvae were from the same population as used by Kiceniuk & Phillips (1975) and may represent a selected group of very able hypo-regulators. They are referred to as 1971 larvae. Otherwise all results are for 1974 larvae.

All experiments were made with the last larval instar and were carried out at room temperature (24 °C).

Methods

To study sulphate regulation, groups of larvae were placed in Ctenocladus pond water, or dilutions of it, for at least 1 week. Haemolymph levels of sulphate were then estimated by adding trace amounts of radioactive sulphate (^{35}S) to the external medium and following the increase in haemolymph ^{35}S activity with time. The procedure for obtaining samples of haemolymph has been described (Kiceniuk & Phillips, 1975). The ^{35}S activity of body fluids was estimated by placing 1 μl aliquots in vials containing 'ScintiVerse' (Fisher Co.) and counting these in a Nuclear Chicago 'Isocap' liquid scintillation counter, using the channels ratio method for quench correction. When ^{35}S activity in the haemolymph became constant with time the specific activity of the radiosulphate was presumed to be the same as that of the external medium. Since external sulphate concentrations were known, haemolymph levels could then be calculated from the haemolymph/medium ratio of ^{35}S activity.

To confirm that ^{35}S activity remained in the form of sulphate, 1 μl aliquots of haemolymph were placed in 10 μl of cold 100 mM sodium sulphate solution and an excess of barium chloride solution (20 μl of 100 mM) was added to precipitate SO_4^{2-} ions as barium sulphate. The fluid was taken up in haematocrit capillary tubes, the ends were sealed with 'Seal-ease' (Clay Adams, Inc.) and the precipitate centrifuged down in an 'Adams' micro-haematocrit centrifuge (5 min). Ten μl aliquots of supernatant were used for determinations of ^{35}S activity as described above. The absence of radioactivity in the supernatant indicated that all the ^{35}S activity was in the form of sulphate.

A variation of the latter procedure was used to estimate the sulphate concentration of samples of pond water. Ten μl of 100 mM barium chloride was added to a larger amount of unknown or standard sodium sulphate solutions (30 μl of 40–150 mM) to which 1 μl of a standard $^{35}SO_4$ solution had been previously added. The sulphate concentration could then be calculated from the percentage of ^{35}S activity remaining in the supernatant after centrifugation. The error for this method, as determined with standard solutions, was less than 5% of the mean. The value for Ctenocladus water determined in this way agreed closely with the value calculated from the observation that sulphate ion concentration makes up about 95% of total anion levels in Ctenocladus pond water (Blinn, 1971).

The total ^{35}S activity in whole larvae, or the midgut and its contents, was estimated by placing the weighed material in 1 ml of distilled water in polythene vials and macerating it with needle-pointed forceps. To this was added 50 μl of toluene to prevent bacterial growth and 50 μl of cold 100 mM sodium sulphate to free adsorbed $^{35}SO_4$ ions. After 1 day the whole content of each vial was analysed for S^{35} activity as previously described.

To investigate the ability of the Malpighian tubules to secrete sulphate ions, they were isolated into small drops (50 μl) of bathing solution as previously described (Phillips & Maddrell, 1975). Bathing solutions were made by adding appropriate amounts of a ^{35}S-containing 160 mM-Na_2SO_4 solution to a base solution containing 76 mM-KCl, 62 mM-NaCl, 8·5 mM-$MgCl_2$, 2 mM-$CaCl_2$ and 34 mM glucose with 10·2 mM-$NaHCO_4$ and 4·3 mM-NaH_2PO_4 to buffer the solution at pH 6·7. For

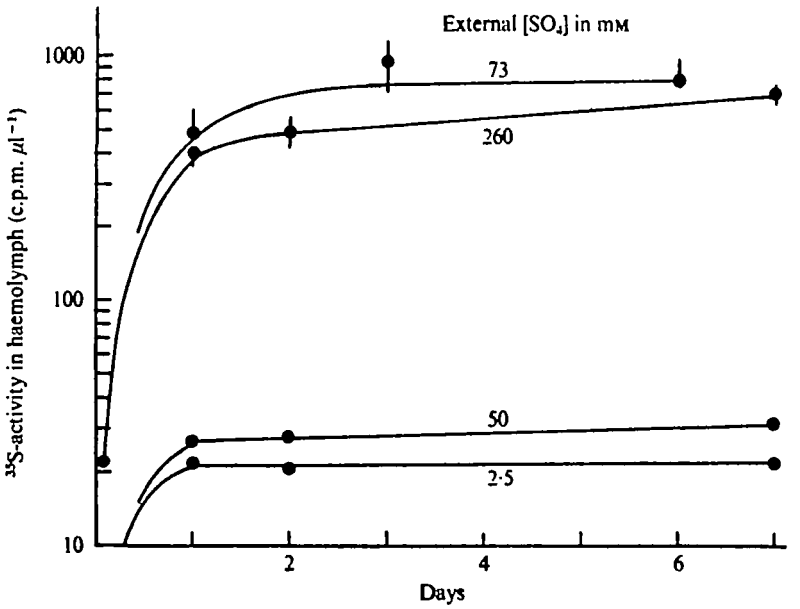

Fig. 2. The increase in radiosulphate activity in the haemolymph with time after placing this isotope in the external media. The sulphate concentration of the media was varied by diluting natural pond water (260 mM in 1971). The experiment in 73 mM water was carried out in 1974 with a higher specific activity of $^{35}SO_4$ in the natural pond water. Vertical lines indicate ±S.E. of the mean for 5–15 larvae, except where this was smaller than the size of the point.

solutions with a higher total sulphate content appropriate amounts were added of a version of the base solution in which KCl and NaCl were replaced by 60 mM-K_2SO_4 and 52 mM-Na_2SO_4.

RESULTS

Absorption and regulation of sulphate

The very rapid increase in ^{35}S activity in the haemolymph during the first day of exposure to radiosulphate (Fig. 2) confirms that this anion enters the body quickly. Presumably this occurs by ingestion and assimilation since it has been shown that larvae ingest 17–300% of their body weight per day (Kiceniuk & Phillips, 1975) and yet the radiosulphate level in the midgut contents is low (discussed below). Steady-state levels of ^{35}S activity in the haemolymph were achieved within 1–2 days of exposure, except at the highest external sulphate concentration (260 mM) where a slow increase continued for at least 7 days. All of the ^{35}S activity in the haemolymph from larvae living in the 73 mM sulphate medium could be precipitated with barium chloride, indicating that it remained in the form of sulphate.

The steady-state concentrations (6–7 days) of radiosulphate in the haemolymph were maintained well below the external level of this anion (Fig. 3). When external concentration of this anion were increased 30-fold from 2·5 to 73 mM, the haemolymph concentration showed only a four-fold increase from 1·5 to 6·6 mM. While more data is required at higher external concentrations, it would appear that haemolymph levels increase sharply and tend to parallel external levels when external sulphate

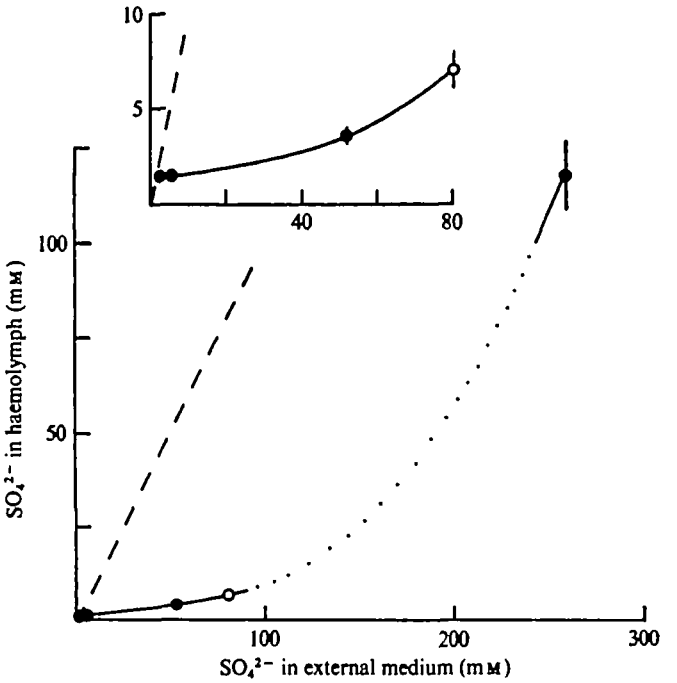

Fig. 3. The steady-state levels of sulphate in the haemolymph as a function of external concentration of this anion. ●, Larvae and pond water (260 mM-SO_4) collected in 1971. ○, For larvae and pond water (73 mM-SO_4) collected in 1974. Vertical lines indicate ±S.E. of the mean unless this is included within the symbol itself.

exceeds 100 mM. Larvae able to survive in 260 mM -SO_4 water (Ctenocladus pond water in 1971) have to tolerate unusually high levels of this anion in their haemolymph (117 mM). In summary, the strategy employed by these larvae appears to be regulation at low and tolerance at high external sulphate concentrations.

Sulphate assimilated by *Aedes campestris* larvae is not removed by storage as precipitates in the tissues. For example, the ^{35}S activity per unit weight of whole larvae in 260 mM-$^{35}SO_4$ water was only 17% of that in the external medium (Fig. 4*b*) compared to 45% for the haemolymph. This suggests that intracellular levels of sulphate are maintained at very low levels even when the haemolymph concentrations of this anion reaches exceptionally high values.

The ^{35}S activity in the midgut contents increased rapidly during the first day after transfer to 73 mM-$^{35}SO_4$ water (normal Ctenocladus water in 1974) and remained unchanged for 6 days thereafter (Fig. 4*a*). Larvae consume considerable quantities of pond water each day and assimilate at least 95% of the water so ingested (Kiceniuk & Phillips, 1975). Clearly sulphate must also be rapidly absorbed in the midgut because the ^{35}S activity per unit weight of midgut and contents is only 13% of that in the external medium, compared to 9% for the haemolymph (Figs. 4(*a*) and 3 respectively). This suggests, in fact, that sulphate is absorbed proportionally more quickly than water down a small concentration difference across the midgut wall. Considering the large size of the SO_4^{2-} ion, its passage across the gut wall must at least be facilitated, possibly by a carrier or by a series of fixed charge sites. Indeed,

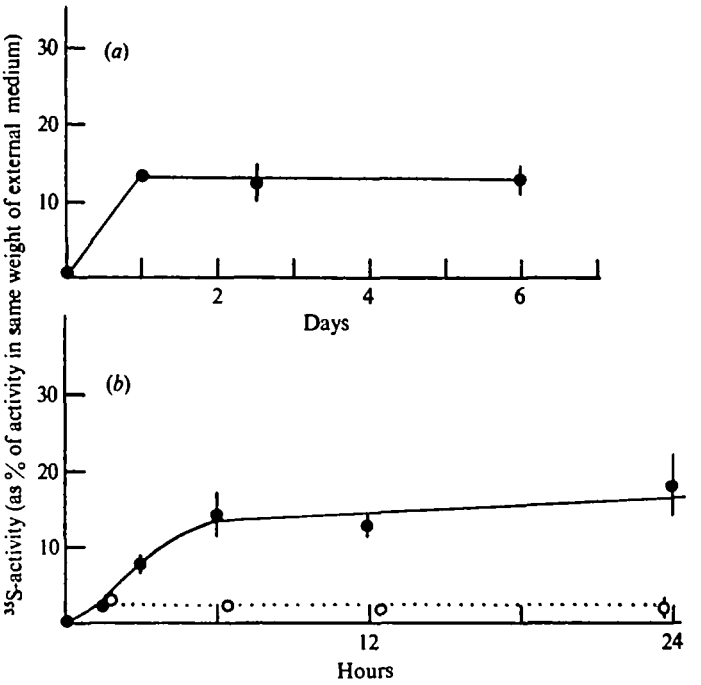

Fig. 4. The increase in ^{35}S activity as a percentage of that in the same weight of external medium with time after exposing larvae to $^{35}SO_4$ in natural pond water (73 mM-SO_4 in 1974, ▲; 260 mM-SO_4 in 1971, ○) or dilutions of the latter (2·5 mM-SO_4 in 1971, ●). (*a*) The whole midgut and its contents. (*b*) Whole larvae. Vertical lines indicate ±S.E. of the mean for 5–15 animals, unless this was smaller than the symbol itself. Activity was so low in the 2·5 mM-SO_4 experiment (●) that results are only an approximation.

since these larvae in hyperosmotic media (like marine fish) must acquire water by drinking fluid in which sulphate constitutes 95% of the anions, it is conceivable that fluid absorption in the midgut is in fact driven by active transport of sulphate. This suggestion is all the more appealing because, as shown in the next section, we have been able to demonstrate active transport of this anion by the Malpighian tubules of these larvae.

In summary then, it is clear that in larvae of *A. campestris* living in sulphate-rich water, large amounts of sulphate ions are absorbed from the midgut into the haemolymph, and yet the level of sulphate ions in the haemolymph is normally kept about 7 mM. Only when the external sulphate is very high does regulation appear to break down and the haemolymph content increase. Even under these conditions the insects can survive several days with very little further rise in the haemolymph sulphate level. Plainly, these mosquito larvae must have a system capable of removing sulphate ions from the haemolymph at a high rate. Since we had recently established that the somewhat similar problem of magnesium excretion could largely be explained by the ability of the Malpighian tubules actively to transport magnesium out of the haemolymph (Phillips & Maddrell, 1975) we examined the ability of the Malpighian tubules to secrete sulphate ions.

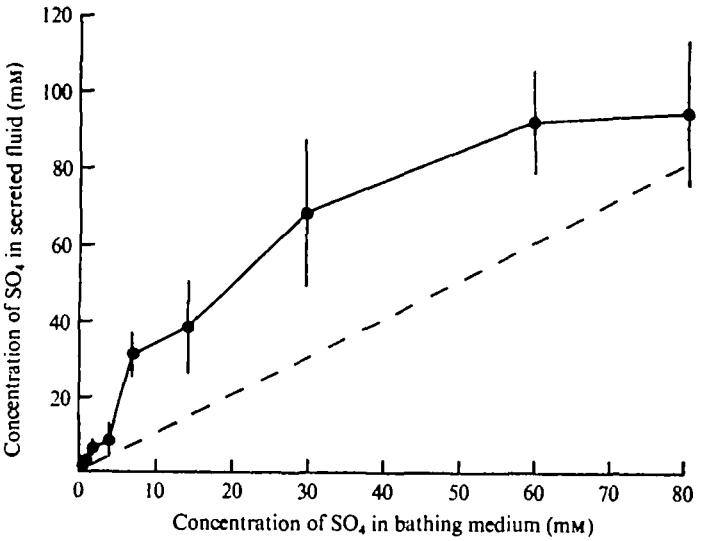

Fig. 5. The dependence of the sulphate concentration in the fluid secreted by isolated Malpighian tubules of *A. campestris* on the concentration of sulphate in the bathing medium. The dotted line is that of a relationship where the secreted fluid and the medium contain equal concentrations of sulphate. The vertical lines attached to the points indicate the extent of the standard error of the mean of several determinations.

Transport of sulphate ions by the Malpighian tubules

Isolated Malpighian tubules were placed in solutions containing concentrations of SO_4^{2-} ions varying from 0·2 to 80 mM and containing $^{35}SO_4$ ion as a tracer. We collected the drop of fluid that each tubule secreted during the next 60–90 min, and after calculating its volume from measurements of its diameter, measured its ^{35}S content. To establish that the ^{35}S in the secreted fluid was wholly associated with SO_4^{2-} ions, samples were treated with 100 mM-$BaCl_2$ solution and centrifuged. This treatment removed more than 99·7% of the radioactive content of the fluid. It is reasonable, therefore, to conclude that ^{35}S in the fluid secreted by the Malpighian tubules is largely in the form of SO_4^{2-} ions. Our measurements of the ^{35}S content of secreted fluid samples thus gave us a direct measure of their sulphate content. The results of such measurements made on fluid collected from a total of 130 Malpighian tubules are set out in Figs. 5 and 6. Quite clearly the tubules are able to secrete sulphate ions into the lumen at a high rate and achieve concentrations in the secreted fluid which are always higher than in the bathing medium even when this is as high as 60 mM. Only at a concentration of sulphate in the bathing medium of 80 mM did some Malpighian tubules secrete fluid containing less sulphate than in the medium.

The rate of sulphate transport was calculated from the time taken to secrete the drops collected, its dependence on the concentration of sulphate ions in the bathing medium being shown in Fig. 7. The Malpighian tubules evidently possess a sulphate transporting system which has a high capacity but a rather low affinity, transport being half saturated at about 10 mM.

The question now arises as to whether sulphate transport is achieved by an active mechanism. One way in which high sulphate concentrations in the secreted fluid

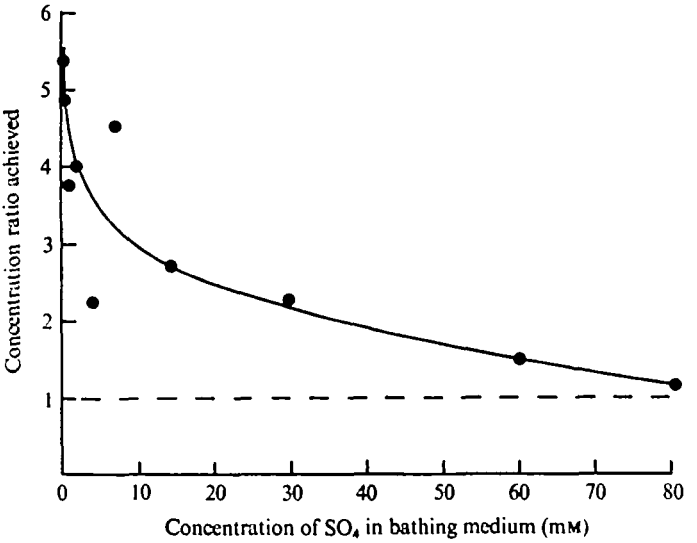

Fig. 6. The dependence of the ratio of the sulphate concentration in the secreted fluid to the sulphate concentration in the bathing medium on the sulphate concentration of the bathing fluid. The values are calculated from those shown in Fig. 5. The curve drawn through the points was fitted by eye. The dotted line is again that of a relationship where the secreted fluid and the medium contain equal concentrations of sulphate.

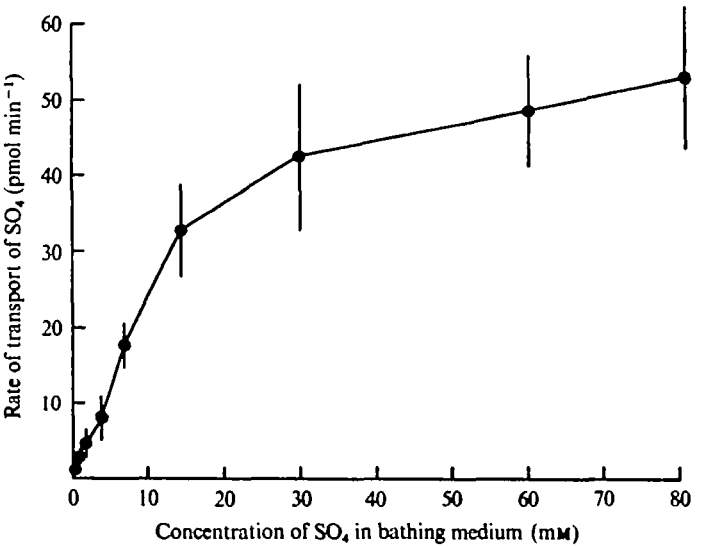

Fig. 7. The dependence of the rate of secretion of sulphate by isolated Malpighian tubules of *A. campestris* on the concentration of sulphate in the bathing medium. The vertical lines attached to the points indicate the extent of the standard error of the mean of several determinations.

might passively be obtained would be by the accompanying transport of divalent cations which, conceivably, might lower the activity of SO_4^{2-} ions to such an extent that passive entry down an activity gradient could occur. Since we had already shown that these Malpighian tubules can carry out rapid active transport of Mg^{2+} ions (Phillips & Maddrell, 1975), this was a distinct possibility. However five Malpighian

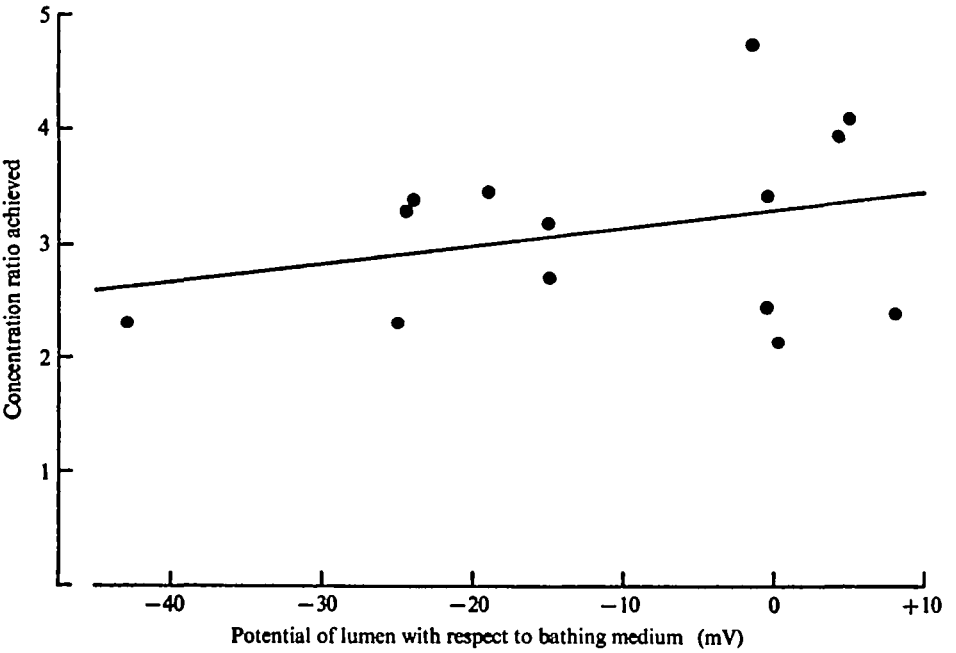

Fig. 8. The dependence of the sulphate-concentrating ability of isolated Malpighian tubules of *A. campestris* on the trans-epithelial potential difference. The line drawn through the points is the linear regression line calculated by the least squares method. The bathing fluid contained 1·5 mM sulphate.

tubules set to secrete in a magnesium-free fluid containing 6 mM-SO_4 all secreted fluid containing concentrations of SO_4 in the 10–20 mM range so that sulphate secretion does not depend on an accompanying secretion of Mg^{2+} ions. If Ca^{2+} ions were to be concentrated in the secreted fluid this could lead to precipitation of $CaSO_4$ and the lowered concentration of sulphate ions in solution would favour their passive entry from the bathing medium. That such a mechanism does not operate was shown by an experiment in which samples of sulphate-rich secreted fluid were centrifuged. In no case was the sulphate content of the fluid significantly reduced by such treatment. This result effectively excludes any transport mechanism for sulphate involving its appearance in the lumen in an insoluble form.

High activities of sulphate ions in the secreted fluid might arise of course merely from an electrical potential gradient favouring their entry. To discover whether the tubules could concentrate sulphate ions against an electrical potential gradient we set tubules to secrete in a relatively potassium poor fluid,* which, from our earlier work (Phillips & Maddrell, 1975), we knew would tend to make the lumen negative with respect to the bathing solution. For each tubule we measured its trans-wall potential difference before collecting a sample of secreted fluid; we then took a further reading of the trans-wall potential gradient to check that no large change had occurred. In only two cases did the potential measurements made before and after the secretion of a drop differ by more than 10 mV; both of these were for tubules

* The fluid contained 6 mM-K^+, 145 mM-Na^+, 7 mM-Mg^{2+}, 2 mM-Ca^{2+}, 154 mM-Cl^-, 10·2 mM-HCO_3^-, 4·3 mM-$H_2PO_4^-$, and 1·5 mM-$^{35}SO_4^{2-}$.

initially having lumina very negative with respect to the bathing solution so that the second readings were still markedly negative. Fig. 8 records the results of experiments on 14 Malpighian tubules. It shows clearly that sulphate secretion is not greatly affected by the trans-wall potential. More importantly, seven tubules secreted fluid containing elevated concentrations of SO_4^{2-} ions against average potential gradients of 15 mV or more. These findings show that Malpighian tubules of *A. campestris* can carry out active transport of SO_4^{2-} ions. This behaviour is in marked contrast to that of Malpighian tubules of *Calliphora* and *Rhodnius*, neither of which seem to be able to transport sulphate ions (Berridge, 1969; Maddrell, 1969, 1971).

DISCUSSION

It is clear from these experiments described that the Malpighian tubules of larvae of *A. campestris* can secrete sulphate ions at a high rate. Before one can assert that the tubules are the main sulphate-excreting organs, one must question whether the rate at which they can transport sulphate ions is high enough to match the rate at which these ions are known to enter the haemolymph from the lumen of the midgut. In water containing 73 mM-SO_4^{2-} ions, the haemolymph sulphate level is 6·6 mM (Fig. 3). In fluid of this sulphate content, a single isolated tubule secretes about 17·5 p-mol min^{-1} of SO_4^{2-} ions averaged over the first hour or so (Fig. 7). Allowing for the fact that only 50–60% of the tubule can be immersed in the bathing drop and that the rate of secretion declines relatively rapidly after isolation (Phillips & Maddrell, 1975), it is probable that a single tubule can in fact secrete sulphate ions at a rate of about 70 p-mol min^{-1}. The full complement of five Malpighian tubules would be expected, therefore, to be able to rid the haemolymph of about 500 nmol SO_4^{2-} in a day. This means that 6–8 mg larvae living in water containing 73 mM-SO_4^{2-} ions could daily drink and absorb 6 μl of the pond water without any increase in the sulphate content of the haemolymph. This capability is in line with estimates of the drinking rate in this water. Similar calculations indicate that the rate of sulphate secretion by Malpighian tubules of larvae living in more dilute media equals or exceeds the estimated rate of sulphate ingestion. This is not the case, however, for larvae surviving in 260 mM-SO_4 water (Ctenocladus pond in 1971). The rate of sulphate ingestion for these larvae (4 μmol day^{-1}) is 2·5 times the calculated secretion rate. However, these larvae may represent a select group of the best hyporegulators with better than average sulphate transporting abilities (discussed by Phillips & Maddrell, 1975; Kiceniuk & Phillips, 1975). Alternatively, higher external levels of sulphate may induce greater transport of this anion either in the Malpighian tubules or in other organs such as the rectum or anal papillae.

The possibility exists that regulation of haemolymph sulphate level is directly due to, and can be explained by, the kinetics of the sulphate pump in the Malpighian tubules. Increased ingestion of sulphate and hence slight increases in haemolymph levels lead to large increases in Malpighian tubule secretion of this ion until external concentration exceeds 100 mM (equivalent to 10 mM in blood). Above this point the transport process begins to saturate (Fig. 7) and haemolymph levels of sulphate would be expected to increase rapidly, as is observed (Fig. 3).

Isolated Malpighian tubules of larvae of *A. campestris* from Ctenocladus pond

water can each secrete magnesium ions at a rate of about 15 p-mol min^{-1} and with a K_m at about 2·5 mM (Phillips & Maddrell, 1975). Although sulphate ions are present in the water ingested by these insects in amounts 5–30 times greater than are magnesium ions (Blinn, 1971), their Malpighian tubules when isolated can each secrete sulphate ions at a rate of only about 50 p-mol min^{-1} and with a K_m at about 10 mM. This may explain why the insects are better able to maintain lower levels of magnesium in the haemolymph than they can of sulphate ions. This of course makes good sense for it is likely that high levels of magnesium ions in the haemolymph would be toxic. By contrast, the larvae can clearly tolerate high levels of sulphate in the haemolymph (p. 371). The low intracellular level of sulphate under these conditions (p. 371) can probably be attributed to anion exclusion by a Gibbs–Donnan equilibrium. This raises the question of why these insects need a transporting system to remove sulphate ions from the haemolymph. The answer must lie in the fact that, to absorb large amounts of the sulphate-rich external medium, considerable amounts of this anion must be transported into the haemolymph from which it must in turn be removed.

From the point of view of the osmotic balance of the insect, the secretion by the Malpighian tubules of sulphate (and magnesium) ions in a fluid iso-osmotic with the haemolymph is of little help. The insect still has either to reabsorb water from the fluid produced by the Malpighian tubules or to secrete ions without osmotically compensating amounts of water elsewhere in the body – perhaps in the rectum or at the anal papillae. It is arguable that the high rates at which these larvae ingest the external medium is only partly forced on them by their usually hyperosmotic environment (Kiceniuk & Phillips, 1975). Their food is in the form of a rather loosely packed organic material and it may well be that the removal of water from the midgut concentrates the food material prior to its enzymic degradation and assimilation. Plant sucking Homopterans resort to rather similar mechanisms in concentrating the sap on which they feed (Wigglesworth, 1972). That this interpretation of the drinking behaviour of *A. campestris* is an accurate one is supported by the fact that the larvae still drink the medium, albeit at a reduced rate, when its osmotic concentration is the same, or lower than that of the insects' haemolymph (Kiceniuk & Phillips, 1975).

One can now suggest why sulphate secretion by the Malpighian tubules seems to be insufficient when the sulphate levels in the water in which the larvae live rises to such high levels as 260 mM. If, under hyperosmotic conditions, a significant contribution to osmoregulation is made by ion secretion elsewhere than in the Malpighian tubules, such a mechanism could only have much effect if it used sulphate because this is the major anion both in the ingested fluid and in the haemolymph. It is of great interest, therefore, that in larvae of another mosquito, *Aedes taeniorhynchus*, which can also live under hyperosmotic conditions, Bradley (1975) has recently demonstrated that the rectum can secrete into the lumen a markedly hyperosmotic fluid, as originally hypothesized by Phillips & Meredith (1969) and Meredith & Phillips (1973). A similar mechanism in *Aedes campestris* could well be responsible for an increasing proportion of sulphate excretion as the osmotic concentration of the external medium rises and this would explain why the sulphate-excreting ability of the Malpighian tubules falls short of that required

under hyperosmotic conditions. It is already known that the rectum of *A. campestris* has an extra posterior region which is lacking in its freshwater relative *A. aegypti* (Meredith & Phillips, 1973) so that it is quite likely that this part of the rectum can excrete sulphate ions under hyperosmotic conditions.

We would like to advance the hypothesis that in hypo-osmotic and iso-osmotic environments the Malpighian tubules are capable of ridding the haemolymph of sulphate ions by active transport; under hyperosmotic conditions, a significant fraction of the total sulphate excretion may be achieved elsewhere, probably in the rectum.

Recently it has become obvious that insect Malpighian tubules not only actively transport sodium and potassium chlorides but also a variety of other substances. They secrete organic anions such as acylamides and sulphonates (Maddrell *et al.* 1974), organic cations such as nicotine and its derivatives (Maddrell & Gardiner, unpublished results), and now it has been found that the inorganic divalent ions magnesium and sulphate can actively be secreted by mosquito Malpighian tubules. In each case it is arguable that such transport is related to the feeding activity of the insects concerned. It seems that if feeding leads to the appearance in the haemolymph of a substance in amounts too high to be catered for by the normal diffusive loss through the permeable Malpighian tubules wall, then the Malpighian tubules develop an ability actively to secrete the substance in question.

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