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INDUCTION OF SULPHATE TRANSPORT AND HORMONAL CONTROL OF FLUID SECRETION BY MALPIGHIAN TUBULES OF LARVAE OF THE MOSQUITO *AEDES TAENIORHYNCHUS*

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SUMMARY

1. 4th stage larvae of *A. taeniorhynchus* reared in sulphate-enriched sea water drink the medium at the same rate that they do when reared in sulphate-free sea water. They absorb into the haemolymph most of the water and nearly all of the sulphate from the ingested fluid.

2. Larvae are able to keep the concentration of sulphate in the haemolymph at levels well below that of the medium, even when this contains as much as 89 mM sulphate.

3. The Malpighian tubules of larvae reared in sulphate-containing waters soon develop an ability to transport sulphate. The rate of sulphate transport induced varies directly with the sulphate content of the water in which they are reared. This ability is not retained into the adult stage.

4. The rate of fluid secretion by isolated Malpighian tubules is increased by up to 20 times when they are exposed to saline containing 1·5 mM cyclic AMP or concentrations of 5-hydroxytryptamine higher than 10^{-6}M.

5. Tubules isolated from unfed insects into stimulant-free saline secrete fluid only slowly, but similarly treated tubules from feeding insects initially secrete fluid very much faster.

6. Extracts of the brain and of the thoracic ganglia stimulate Malpighian tubules to secrete fluid at a high rate. The brain is about four times as rich a source of stimulant as is the chain of thoracic ganglia. Treatment of the surface of the structures in the head with K-rich saline leads to the release of a factor which stimulates fluid secretion by the Malpighian tubules.

7. The results suggest that the Malpighian tubules in larvae of *A. taeniorhynchus* are under the control of a diuretic hormone which is elaborated in the brain and possibly also in the thoracic ganglia and which reaches high levels in the circulating haemolymph of feeding animals.

8. The rate of sulphate transport by isolated Malpighian tubules is strongly affected by the rate of fluid secretion. This behaviour is compatible with a passive leak of transported sulphate from the lumen back into the haemolymph through the permeable wall of the tubule.

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INTRODUCTION

Larvae of salt-water mosquitoes can thrive in water containing high levels of sulphate; *Aedes taeniorhynchus* lives in sea-water pools (Nayar, 1967) and *Aedes campestris* is found in, among other habitats, inland lakes rich in magnesium and sodium sulphate (Scudder, 1969). The larvae of *A. campestris* ingest and absorb large quantities of the sulphate-rich medium in which they live (Kiceniuk & Phillips, 1974). Appropriately enough, the Malpighian tubules of this insect can carry out rapid active transport of sulphate ions (Maddrell & Phillips, 1975). However, while our calculations showed this would be sufficient to remove all the sulphate absorbed from waters of low or moderate salinity, it seemed that an additional mechanism would be needed to explain the excretion of the large amounts of sulphate absorbed from waters of high salinity. We suggested at that time that secretion of sulphate ions into the rectum might be responsible. However, Bradley & Phillips (1977*a*) have since shown that sulphate ions, unlike all of the other major ions of the external medium, are *not* secreted by the rectum of *A. campestris* or *A. taeniorhynchus*.

In the present paper we have considered the other possible ways in which larvae of *A. taeniorhynchus* might cope with high concentrations of sulphate in the water in which they live. They might, for example, reduce the rate of sulphate absorption by the midgut and/or increase the rate of sulphate excretion by the Malpighian tubules.

We have found that when the larvae feed, they ingest the surrounding fluid at about twice the rate at which ingestion occurs in larvae living in water containing no food. Since much of the water ingested by unfed larvae is to replace that lost by osmosis to the hyperosmotic medium, feeding must greatly increase the rate at which excess fluid has to be removed from the haemolymph by the excretory system. We have therefore looked for evidence of control of fluid secretion by the Malpighian tubules, possibly by a diuretic hormone such as occurs in other insects (Maddrell, 1971).

Finally, because sulphate ions are so rapidly absorbed through the gut wall (Maddrell & Phillips, 1975) we have tested the possibility that they might be absorbed by an active transport mechanism.

MATERIALS AND METHODS

Aedes taeniorhynchus larvae were hatched and reared as previously described (Bradley & Phillips, 1975) at 28 °C in artificial sea water containing different levels of sulphate: 0 mM (sulphate-free sea water), 31 mM (normal sea water) or 89 mM (sulphate-rich sea water). Fourth instar larvae were used in all experiments. Fed larvae were given yeast and powdered dried liver. In experiments involving starvation, larvae were rinsed and transferred to the appropriate artificial sea water which had been freshly made, filtered to remove any particles and to which no food had been added. This external medium was changed daily to remove faecal material and occasional dead larvae.

The *sulphate concentration of body fluids* was determined as previously described (Maddrell & Phillips, 1975) by adding $^{35}SO_4$ to the external medium and following its activity in 1 μl amounts of body fluid with time. A steady level showed that th

specific activity of $^{35}SO_4$ had equilibrated with that of the external medium. Since the absolute concentration of this anion in the sea water was known, levels in body fluids could then be estimated from the relative radioactivities of external and internal fluids. In some experiments, equilibrium was assured by rearing larvae from hatching in $^{35}SO_4$-labelled sea water.

The *rate of uptake of* $^{35}SO_4$ by starved and fed larvae was estimated as previously described (Maddrell & Phillips, 1975). Ligation at the neck with a strand of human hair permitted an estimate to be made of uptake by routes other than drinking.

The *rate of drinking* was estimated from the initial rate of uptake of [^{3}H]inulin by whole larvae as previously described (Bradley & Phillips, 1975).

The study of absorption of ingested material in the gut is complicated by the viscous nature of the midgut contents. Since aliquots of this fluid could not be readily obtained, the volume of the whole contents had to be estimated gravimetrically. Larvae were quickly dissected in their own haemolymph and the whole midgut removed, the midgut being clamped just beyond the posterior end with watch-maker's forceps. If the foregut was cut far enough forward, fluid was not lost from the anterior cut end. Adhering haemolymph was removed by touching the tissue on Kleenex before placing it on a weighed piece of Parafilm. The weight of the midgut and its contents was then rapidly determined on a Cahn microbalance before significant weight loss could occur by evaporation. All the material on the Parafilm was then rinsed off for further treatment. To obtain the weight of midgut contents, the average weight of a whole midgut epithelium under each experimental condition (separately determined) was subtracted from the measured weights of individual midguts and their contents. This method was satisfactory because the weights of the individual midgut epithelia did not greatly vary (see Table 1). Assuming that the midgut contents have a specific gravity of one, the volume of the midgut contents (in μl) can be taken as being the same as its weight (in mg). If the specific gravity were in fact higher than one, the volume of the contents of a midgut would of course be overestimated. This is important in so far as it makes it unlikely that the method of estimating water absorption set out below would give overestimates.

To estimate the *percentage of ingested water absorbed* in the midgut, larvae were transferred to sea water containing [^{3}H]inulin. Inulin is not absorbed from the gut, as indicated by the fact that significant ^{3}H activity did not appear in the haemolymph. Larvae drank an amount of fluid equal to the total midgut contents in about 4–5 h, so that a steady-state level of [^{3}H]inulin in the midgut was approached after this time. The total volume of water taken into the midgut lumen was then obtained from the total ^{3}H activity of whole individual midguts divided by the known ^{3}H activity per unit volume of the external medium. A comparison of the actual volume found with that known to have been ingested yielded an estimate of the percentage of ingested water which had been absorbed.

The *absorption of sulphate* was estimated using different individuals from the same population which were placed for the same period of time in sea water containing $^{35}SO_4$. The ^{35}S activity of whole midguts from individual larvae was determined. To give the quantities of sulphate absorbed, these figures were then subtracted from the amounts of $^{35}SO_4$ ingested (calculated from the volumes ingested and the ^{35}S activity of the external medium). The concentration of $^{35}SO_4$ in the midgut lumen could be

calculated from the total ^{35}S activity in it divided by the volume of the gut contents determined by weighing.

It is important to point out that the methods described must inevitably give underestimates of the extent to which ingested water and sulphate ions are absorbed in the midgut. This is because they do not allow for the fact that, at the moment of sampling the most recently ingested fluid can only have been in the midgut for a short period of time; it will not, therefore, have had its water and sulphate content reduced to the same extent as fluid which leaves the hind end of the midgut.

To estimate ^{3}H or ^{35}S activities, whole midguts were placed individually in liquid scintillation vials and digestion was carried out by adding 0·4 ml of 10% KOH and heating at 52 °C for 16 h. The material was neutralized with 0·8 ml of 10NH_2SO_4, 10 ml of 'Scintiverse' (Fisher Scientific Co.) was added, and radioactivity was determined by liquid scintillation counting as previously described (Maddrell & Phillips, 1975).

To investigate the ability of Malpighian tubules of larvae of *A. taeniorhynchus* to secrete sulphate ions, they were isolated into drops (*ca.* 50 μl) of bathing solution essentially as we described for the tubules of *A. campestris* (Phillips & Maddrell, 1974). The Malpighian tubules of *A. taeniorhynchus* are even smaller than those of *A. campestris* and so the rates at which they secrete fluid tend to be lower. However, we discovered that as with Malpighian tubules of *Rhodnius* and *Carausius* (Maddrell, Pilcher & Gardiner, 1969), fluid secretion by those of *A. taeniorhynchus* can be stimulated by the inclusion of 5-hydroxytryptamine in the bathing medium (see p. 193). In addition, we found that fluid secretion can be maintained longer if the tubules are isolated into a more complex medium containing various amino acids and organic materials. We used the physiological saline developed by Bradley & Phillips which contains 148 mM-Na^+, 17 mM-K^+, 12 mM-Mg^{2+} and 97 mM-Cl^- as well as citrate, succinate, malate, glutamate, glucose, maltose, trehalose, glycine, proline, glutamine and sucrose (see Bradley & Phillips, 1975 for full details). The pH of the solution was pH 6·8 and its osmotic concentration was 315 m-osmol.

Extracts of different parts of the central nervous system were prepared by homogenizing them in a small amount (*ca.* 0·5 μl) of distilled water in a suitably miniaturized version of the glass pestle and mortar described by Maddrell (1963). The resulting brei was taken up in an equal volume of twice normally concentrated saline before use.

All values are quoted as mean ± S.E. (number of observations).

RESULTS

Sulphate excretion by the Malpighian tubules

Sulphate assimilation in the midgut; the influence of pre-adaptation

Larvae of *A. taeniorhynchus* might owe their ability to survive in sulphate-rich water to a reduction in the rate of ingestion and/or absorption of sulphate ions. Either process would lessen the sulphate load put on the excretory system. To see if the larvae do so regulate their sulphate load, we compared the rate of ingestion and absorption of sea water by larvae hatched from the same population but reared either in sulphate-free or sulphate-rich water. Both sets of larvae were transferred in the

Table 1. *Uptake of water and sulphate after transfer into normal sea water by 4th instar larvae of* A. taeniorhynchus *reared in sulphate-free or sulphate-rich water*

	Larvae reared in sulphate-free water		Larvae reared in sulphate-rich water	
	4 h after transfer	23 h after transfer	4 h after transfer	23 h after transfer
	Water uptake			
Water ingested (μl)	0·56 ± 0·07	1·10 ± 0·26	0·61 ± 0·07	1·20 ± 0·10
Water absorbed (μl)	0·20 ± 0·07	0·78 ± 0·26	0·42 ± 0·08	0·98 ± 0·12
% absorbed	36	71	69	82
n	7	7	6	10
	Sulphate uptake			
Sulphate ingested (nmol)	17	35	19	37
Sulphate absorbed (nmol)	13 ± 0·6	32 ± 0·6	14 ± 0·45	32 ± 0·85
% absorbed	78	92	74	87
n	7	8	7	8

The weight of the midgut epithelium was 0·32 ± 0·04 mg ($n = 7$) and 0·34 ± 0·03 mg ($n = 7$) for larvae reared in sulphate-free and sulphate-rich sea water respectively.

4th instar to normal sea water containing either $^{35}SO_4$ or [^{3}H]inulin. The percentages of ingested water and sulphate which had been absorbed after 4 h and 23 h are shown in Table 1 together with the volumes of the contents of the midgut. Although larvae which had been reared in sulphate-free water contained somewhat more fluid in the gut after 23 h than did ones reared in sulphate-rich water, it is clear from Table 1 that, by 23 h, both sets of larvae had absorbed about 90% of the sulphate from the water that they ingested. Bearing in mind that the methods used underestimate the extent of absorption of water and sulphate (p. 184), it is plain that just like larvae of *A. campestris* from sulphate-rich water (Kiceniuk & Phillips, 1974; Maddrell & Phillips, 1975), larvae of *A. taeniorhynchus* absorb most of the water and nearly all of the sulphate (and probably the other ions as well) from the fluid that they ingest.

Is sulphate absorption by the midgut active?

Table 1 shows that sulphate ions can be absorbed from the midgut relatively more rapidly than water, which raises the possibility that uptake of sulphate ions might be active. We followed the concentration of ^{35}S-labelled sulphate in the midgut contents and haemolymph of insects treated in a similar manner to those described above and in Table 1. Fig. 1 sets out the results; it shows that after about 3 h the ^{35}S activities were constant, suggesting the specific activity of $^{35}SO_4$ was now the same as that in the external normal sea water. The estimated haemolymph concentration of sulphate (2 mM) for larvae in normal sea water was not significantly different from that of animals which had been either in sulphate-free or in sulphate-rich sea water. The sulphate levels in midgut contents were significantly different (10 and 22 mM respectively) for these two latter groups, but both values were below the external concentration (31 mM) and both were well above haemolymph levels. Note that the higher concentration of sulphate (22 mM) in the midgut contents of insects reared in sulphate-rich water reflects not a slower uptake of sulphate but a faster uptake of water (see

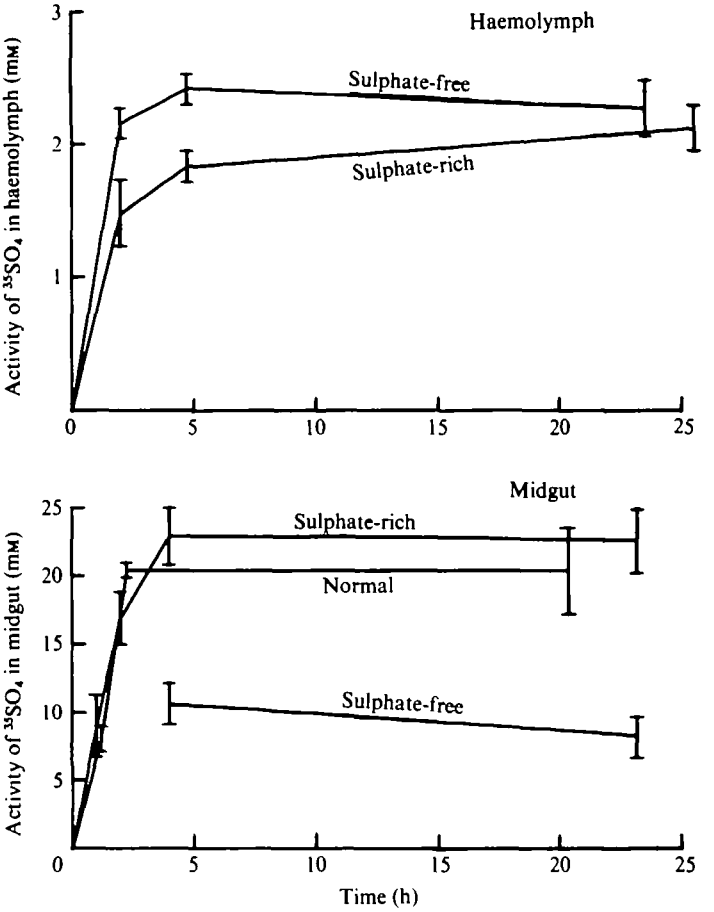

Fig. 1. The activity of $^{35}SO_4$ taken up from sea water into the haemolymph (upper graph) or midgut (lower graph) by 4th stage larvae of *A. taeniorhynchus* reared in sulphate-rich sea water, normal sea water or sulphate-free sea water. Each point is the mean of not less than eight determinations and the attached vertical lines represent ±S.E. of the mean.

Table 1). These results are similar to those for *A. campestris* (Maddrell & Phillips, 1975). They indicate (1) that there is normally a favourable gradient for passive downhill absorption of sulphate from the midgut lumen and (2) that sulphate absorption occurs proportionally more quickly (or at least as quickly) as does that of water. Since most epithelia are relatively impermeable to ions as large a sulphate, these findings indicate that its uptake across the midgut epithelium probably involves some form of facilitated transfer. The question naturally arises as to whether its uptake might be an active process capable of occurring against a concentration gradient.

We devised the following experiment to discover whether sulphate absorption could occur against a concentration difference when water absorption was prevented. Seven larvae were reared in normal sea water containing $^{35}SO_4$ and were then transferred to a further sample of the same labelled sea water which had been diluted tenfold with a 21% solution of sucrose. After 24 h the total $^{35}SO_4$ content and the $^{35}SO_4$ concentration of the gut contents were determined as described above. Seven

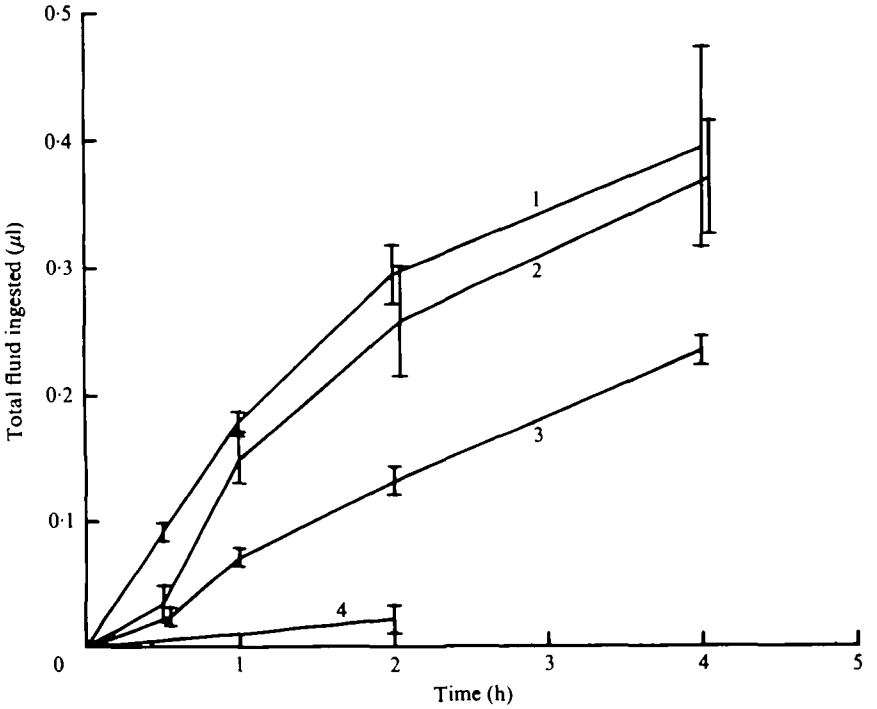

Fig. 2. The ingestion of sea water by 4th stage larvae of *A. taeniorhynchus*. The uppermost line (1) is for insects allowed to feed after 1 day without food. Line 2 shows the drinking behaviour of insects fed continuously. Line 3 shows the reduced rate of drinking by insects deprived of food during the experiment and for 1 day previously. Line 4 is for insects ligated at the neck; they take up only insignificant amounts of the medium. Each point is the mean of not less than ten determinations and the attached vertical lines represent ±S.E. of the mean.

further larvae from the same egg batch were treated identically except that $^{35}SO_4$ was omitted from the sea water and the larvae were transferred to sea water again diluted ten times with 21% sucrose but containining [^{3}H]inulin so that water movement across the gut could be estimated as described in previous experiments. It was evident on dissection that the inclusion of substantial levels of sucrose in the external medium had not only prevented but had indeed reversed the normal absorption of water from the midgut. The latter was swollen with clear fluid which now could be collected directly by micropipette. The concentration of [^{3}H]inulin in this fluid showed that it had been diluted by 2·3 times, due to water movement from the haemocoel into the lumen. Likewise, the sulphate level in the fluid consumed (3·1 mM) which was initially more concentrated than that in the haemolymph (2·3 ± 0·03 mM), had fallen after 24 h to a value of only 1·5 ± 0·03 mM. This was almost identical to the value (1·4 mM) that one would expect as a result of the net fluid movement into the lumen indicating that no net absorption of sulphate had occurred. Clearly, sulphate absorption does not proceed even against such a small concentration difference. We therefore propose that the normal rapid assimilation of this large anion probably involves some form of facilitated diffusion but does not involve uptake by an active mechanism.

Table 2. *Concentrations of sulphate in the haemolymph of 4th instar larvae of* A. taeniorhynchus *reared in waters of different sulphate content. The values are based on a minimum of seven determinations*

	Concentration of sulphate in water (mM)	Concentration of sulphate in haemolymph (mM)
Sulphate-free water	0·003	< 0·01
Normal sea water	31	1·8±0·2
Sulphate-rich water	89	34±7

The influence of feeding on the ingestion of sulphate-containing water

As we have seen, nearly all of the sulphate ingested by the larvae is absorbed in the midgut. Any increase in ingestion of water containing this anion will clearly impose an increased load on the secretory mechanism in the Malpighian tubules, the only known regulatory site responsible for excretion of SO_4 (Bradley & Phillips, 1977*a*). Ingestion rates appear to be independent of external salinity but are proportional to weight and surface area of larvae (Bradley & Phillips, 1977*b*). We considered the possibility that increased amounts of water and sulphate might be ingested during feeding on particulate matter. We therefore followed the uptake of $^{35}SO_4$ and [^{3}H]-inulin by larvae from the same egg batch which had been fed continuously, left unfed for 1 day, or fed after starvation for 1 day, all in normal sea water. To check the assumption that the larvae accumulated sulphate only by drinking, we followed the ^{35}S activity of larvae, which had been ligated at the neck. As Fig. 2 shows. such larvae accumulate only negligible amounts of ^{35}S. Further confirmation is provided by the finding that the rates of fluid ingestion by larvae as estimated by the increase in whole body content of [^{3}H]inulin were identical to the values obtained from the increase in ^{35}S activity. Fig. 2 also shows the data from experiments on ingestion of ^{35}S-containing water. The most important finding, shown very clearly in Fig. 2, is that feeding animals ingest the medium at about twice the rate of non-feeding animals. Since nearly all the ingested fluid and its content of sulphate ions are absorbed in the gut, feeding animals clearly face a bigger problem in maintaining the concentration of sulphate in the haemolymph at a low level.

Essentially, then, there are two methods of affecting the rate at which these larvae ingest sulphate; the larvae can be reared in waters containing different concentrations of sulphate ions and the rate at which they ingest that fluid can be changed by giving or withholding food.

Regulation of sulphate levels in the haemolymph

The first question which arises is how successful the larvae are in keeping the concentration of sulphate in the haemolymph at a low level. Larvae of *Aedes campestris* kept in sulphate-rich water are known to be able to keep the level of sulphate in the haemolymph below 6 mM when the external fluid contains 73 mM sulphate or less; in water containing 260 mM sulphate they are not so successful, the concentration in the haemolymph rising to 117 mM (Maddrell & Phillips, 1975). Table 2 shows the levels of sulphate found in the haemolymph of larvae of *A. taeniorhynchus* reared

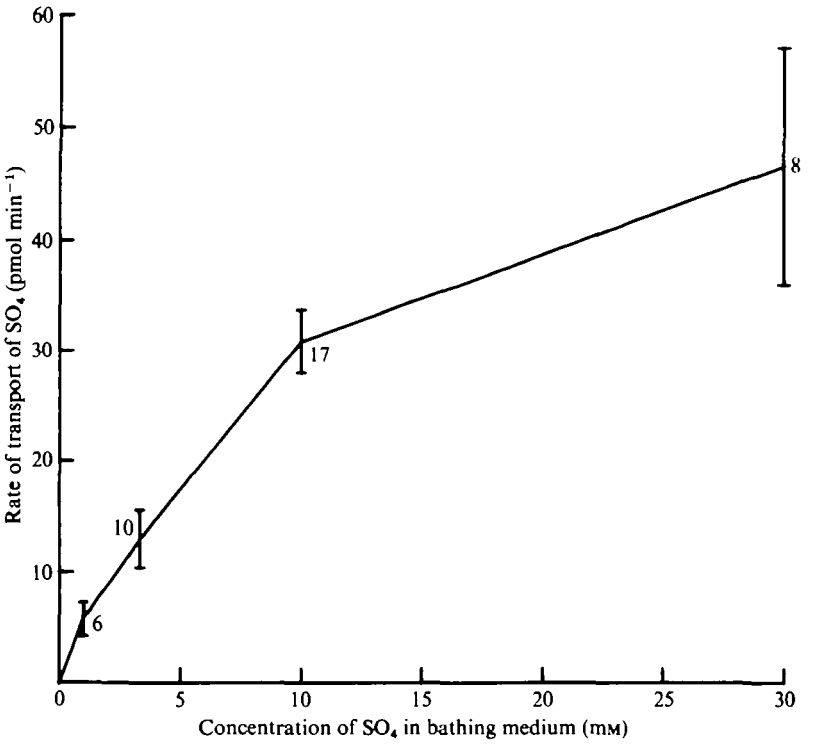

Fig. 3. The characteristics of secretion of sulphate by isolated Malpighian tubules of 4th stage larvae of *A. taeniorhynchus* reared in normal sea water. Each point is the mean of the number of determinations indicated; the vertical lines represent ±S.E. of the mean.

the three different waters we have used. Clearly, good regulation is possible in normal sea water. Regulation is not so successful in the sulphate-rich water but even here the haemolymph comes to contain only about a third of the concentration of the medium. How is this regulation achieved? In particular, can the properties of the Malpighian tubules be altered in such a way as to cope with the variable sulphate load imposed on the animal when it is reared under the different experimental conditions?

Sulphate transport by the Malpighian tubules

We needed first to establish that Malpighian tubules of *A. taeniorhynchus* can transport sulphate ions as this was previously only known for tubules of *A. campestris*. Accordingly, we isolated Malpighian tubules of *A. taeniorhynchus* (reared in normal sea water) into a bathing medium containing 1 mM sulphate made radioactive by the addition of $^{35}SO_4$. Ten such tubules secreted fluid containing $4{\cdot}03 \pm 0{\cdot}91$ mM-SO_4. Plainly the Malpighian tubules from these insects can concentrate sulphate ions to a considerable extent in the fluid they secrete. Fig. 3 shows how the rate of sulphate transport by Malpighian tubules from larvae reared in sea water depends on the concentration of sulphate in the bathing medium. As with tubules from *A. campestris*, tubules from *A. taeniorhynchus* appear to have a sulphate transporting system with a high capacity and low affinity, transport being half saturated somewhere in the range 5–10 mM.

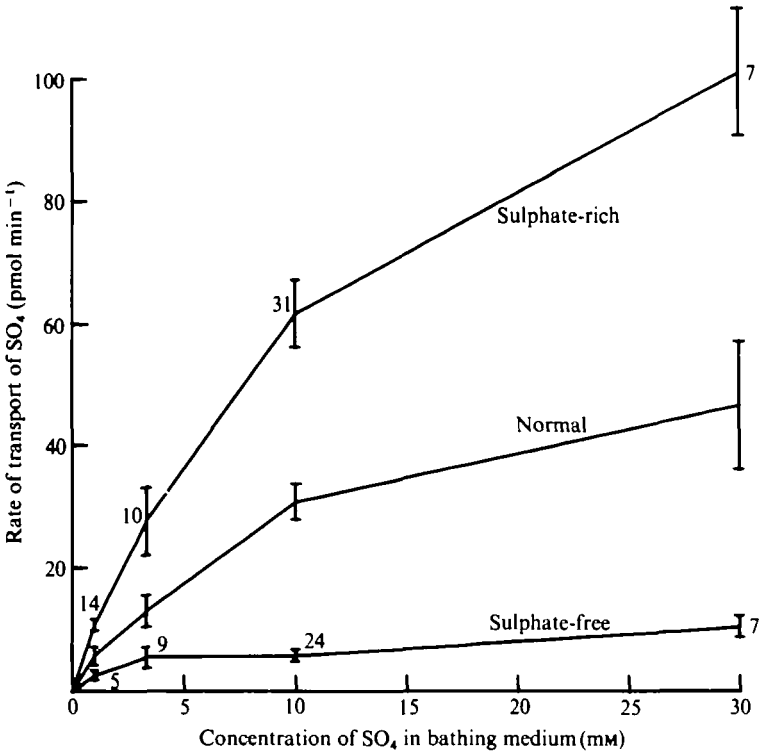

Fig. 4. The characteristics of sulphate transport by isolated Malpighian tubules of 4th stage larvae of *A. taeniorhynchus* reared in sulphate-rich sea water (upper line), normal sea water (middle line) or sulphate-free sea water (lower line). The values for insects reared in normal sea water are those shown in Fig. 3. For insects reared in sulphate-rich or sulphate-free sea water the number of determinations used to calculate the mean values is shown alongside each point. The vertical lines attached to the points represent ±S.E. of the mean.

Induction of sulphate transport

Malpighian tubules from insects reared in sulphate-free water or sulphate-rich sea water behave differently. Fig. 4 summarizes the results from experiments on the tubules from 150 insects. Tubules from insects reared in sulphate-free water have only a weak ability to transport sulphate ions; indeed the fluid they secrete was scarcely if ever found to contain sulphate ions at a higher concentration than in the bathing medium. Since insect Malpighian tubules are permeable structures (Maddrell & Gardiner, 1974) much of the sulphate content of the fluid secreted by these animals could have crossed the tubule wall passively. In contrast, Malpighian tubules from insects reared in sulphate-enriched sea water containing 89 mM sulphate were able to transport sulphate ions on average about twice as fast as those from insects reared in normal sea water. The fluid secreted contained sulphate ions at concentrations several times higher than in the bathing medium. For example, tubules bathed in fluid containing 3·33 mM sulphate secreted fluid containing an average of 18·45 mM sulphate (n = 10). Most impressive was the finding that several tubules bathed in fluid containing 30 mM sulphate secreted fluid containing more than 130 mM sulphate

Evidently exposure of these animals to sulphate-containing water leads to the induction of a pronounced ability of the Malpighian tubules to transport sulphate. The curves shown in Fig. 4 for the three populations of insects have distinctly similar shapes; this suggests that it is not the affinity of the transport system which alters but the capacity. We also looked briefly at the performance of Malpighian tubules from 4th instar larvae able to survive in water containing 160 mM sulphate. These larvae had to be reared at first in water containing 120 mM sulphate and transferred during the 3rd instar to water whose sulphate content was 160 mM. Rather few insects survived this treatment. However, four tubules from two such insects were isolated into saline containing 10 mM sulphate; they had sulphate transport rates which averaged $95{\cdot}6 \pm 8{\cdot}5$ pmol min^{-1}, significantly higher than in tubules from insects reared in water containing 89 mM sulphate ($61{\cdot}8 \pm 5{\cdot}5$ pmol min^{-1}, $n = 31$).

The results show that, appropriately enough, Malpighian tubules from insects reared in sulphate-containing waters develop an ability to transport sulphate at a rate which varies directly with the sulphate content of the environment.

Sulphate transport by Malpighian tubules of adult A. taeniorhynchus

Four Malpighian tubules, two from each of two adult insects, one male and one female, secreted sulphate at an average rate of only $5{\cdot}6 \pm 1{\cdot}0$ pmol min^{-1} when bathed in fluid containing 10 mM sulphate. These adults were from larvae reared in water containing 120 mM sulphate. Evidently, adult insects do not retain the ability to transport sulphate which they had as larvae.

Time-scale of induction of sulphate transport

Since larvae of *A. taeniorhynchus* may, *in vivo*, face an environment in which, because of evaporation, the sulphate levels are increasing, it is of interest to discover how rapidly the sulphate-transporting ability of their Malpighian tubules can increase. We reared larvae in sulphate-free water to the 4th instar and then transferred the larvae 1–2 days after their last moult to sulphate-rich water (containing 89 mM sulphate). After 16 h, ten Malpighian tubules from five insects (which were not fed after the transfer) secreted sulphate from a solution containing 33·3 mM sulphate at a rate of $41{\cdot}38 \pm 4{\cdot}55$ pmol min^{-1}, nearly four times faster than those from insects left in the sulphate-free water ($10{\cdot}47 \pm 1{\cdot}72$ pmol min^{-1} ($n = 9$)). In another experiment the insects were fed after transfer, which treatment increases their sulphate load (p. 188). The same 16 h exposure now led, again in the case of ten tubules from five different larvae, to a six times increase in the rate of sulphate transport; in a bath containing 10 mM sulphate, the tubules secreted sulphate at an average rate of $34{\cdot}23 \pm 4{\cdot}24$ pmol min^{-1} compared with $5{\cdot}70 \pm 0{\cdot}85$ pmol min^{-1} ($n = 24$) for tubules from control insects not exposed to sulphate-containing water. These increases can be compared with the increase seen after continuous exposure from hatching to the same sulphate-rich water. Tubules from such larvae transport sulphate approximately 9–10 times faster than those from control larvae not exposed to sulphate-containing water. In other words a 16 h exposure to sulphate-containing water leads to a development in the Malpighian tubules of a sulphate transporting ability which is at about half the level it reaches after continuous exposure.

In another experiment eight tubules were taken from four 4th stage insects

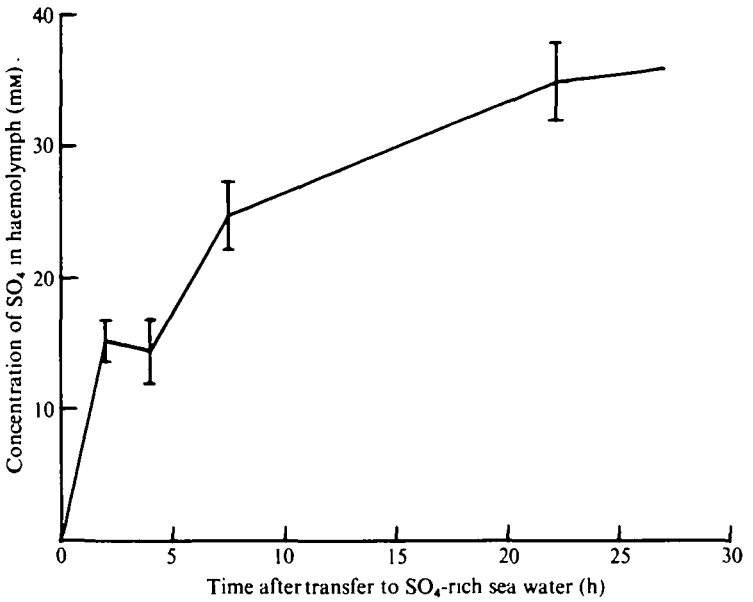

Fig. 5. The increase in concentration of sulphate in the haemolymph of 4th stage larvae of *A. taeniorhynchus* on transfer to sulphate-rich sea water after being reared in sulphate-free sea water. Each point is the mean of not less than five determinations and the vertical lines attached to the points represent ±S.E. of the mean.

Table 3. *Concentrations of sulphate in the haemolymph of 4th instar larvae of* A. taeniorhynchus *reared in sulphate-free water and then transferred either to normal sea water or to sulphate-rich sea water*

Sulphate content of water (mM)	Feeding status of animals	Time after transfer (min)	Sulphate level in haemolymph (mM)
31	Fed	195	2·26
	Fed	330	3·54
	Fed but starved for 3 days previously	240	3·08
89	Fed	240	10·57
	Fed but starved for 3 days previously	240	18·39

which had been reared in sulphate-free water before being transferred for 330 min into sulphate-rich water (89 mM sulphate). These tubules, in fluid containing 10 mM sulphate, transported sulphate at an average rate of 11·22 ± 1·01 pmol min^{-1}, significantly faster than tubules from control insects (t = 3·46 for 30 degrees of freedom; $P < 0{\cdot}01$).

Possible mechanism of induction

What is the stimulus for the induction of sulphate transport by the Malpighian tubules? In particular, does the sulphate concentration of the haemolymph rise fast enough for this to be used as a stimulus? We measured the sulphate concentration in

the haemolymph of 4th stage insects transferred from sulphate-free water to sulphate-containing waters (Table 3 and Fig. 5). The results show that the sulphate levels rise very quickly after a transfer to sulphate-containing water, fast enough for this to act as the stimulus for induction of sulphate transport by the Malpighian tubules.

With this in mind we tried to induce sulphate transport *in vitro* in Malpighian tubules from sulphate-naive insects by isolating 22 tubules from six insects into Ringer's solution containing sulphate at concentrations of 10 mM (six tubules) or 20 mM (16 tubules) and measuring the rates of sulphate transport and fluid transport during the following 8 h. In all but one case the rate of sulphate transport declined more or less in parallel with the slowing of fluid secretion which on average fell in rate by 50%. In one case, of a tubule in the 10 mM sulphate solution, the rate of sulphate transport rose from 2·12 pmol min^{-1} to 4·17 pmol min^{-1}, but as the concentration of sulphate in the secreted fluid even then only reached 60% of that in the bathing fluid, it is possible that the apparent rise in sulphate transport was due to an increase in passive permeability, perhaps induced by accidental rough handling. The results show no clear evidence of induction of sulphate transport under these *in vitro* conditions. The significance of this finding is discussed on p. 199.

The control of the rates of fluid secretion of the Malpighian tubules

As mentioned earlier (p. 184) Malpighian tubules of larvae of *A. taeniorhynchus* secrete fluid much faster in the presence of 5-hydroxytryptamine. This discovery arose from attempts to overcome the difficulty of working with the small tubules of *A. taeniorhynchus*; the unstimulated rate of fluid secretion by such tubules is only of the order of 0·2 nl min^{-1}.

To test the idea that the rate of fluid secretion might, *in vivo*, be under hormonal control, we isolated seventeen Malpighian tubules from five 4th instar larvae into saline containing 1·5 mM of cyclic AMP. The reasoning here was that nearly all rapid hormonal stimulation of secretory processes involves increases in the intracellular levels of cyclic AMP (Berridge, 1975) and that it stimulates secretion by the Malpighian tubules of other insects that are under hormonal control (Maddrell, Pilcher & Gardiner, 1971). Under these conditions the tubules secreted fluid at an average rate of 2·64 ± 0·32 nl min^{-1}, i.e. at least ten times faster than unstimulated tubules. Since it was known that some Malpighian tubules (e.g. those of *Carausius* and *Rhodnius*) are stimulated by 5-hydroxytryptamine (5-HT) we tried the effect of adding 10^{-5}M 5-HT to tubules bathed in saline. The rate of secretion again rapidly increased to a high level as shown in Fig. 6. From a series of such tests we were able to construct the dose/response curve for treatment with 5-HT shown in Fig. 7.

These pieces of evidence suggest that the rate of fluid secretion *in vivo* might well be under hormonal control. There are two further lines of evidence which provide rather more direct support for this view. First, it was repeatedly observed that tubules taken from fed larvae would initially secrete fluid at a high rate but would then slow down to a low level; the rate of secretion by such tubules was found to be 1·95 ± 0·16 nl min^{-1} (n = 10) during the first 5 min after isolation, falling to 0·98 ± 0·06 nl min^{-1} (n = 27) after 15 min and to 0·41 ± 0·05 nl min^{-1} (n = 10) during the subsequent 15 min. For comparison, the rates of secretion by tubules from unfed insects were 0·26 ± 0·04 nl min^{-1} (n = 22) and 0·35 ± 0·06 nl min^{-1} (n = 15) during,

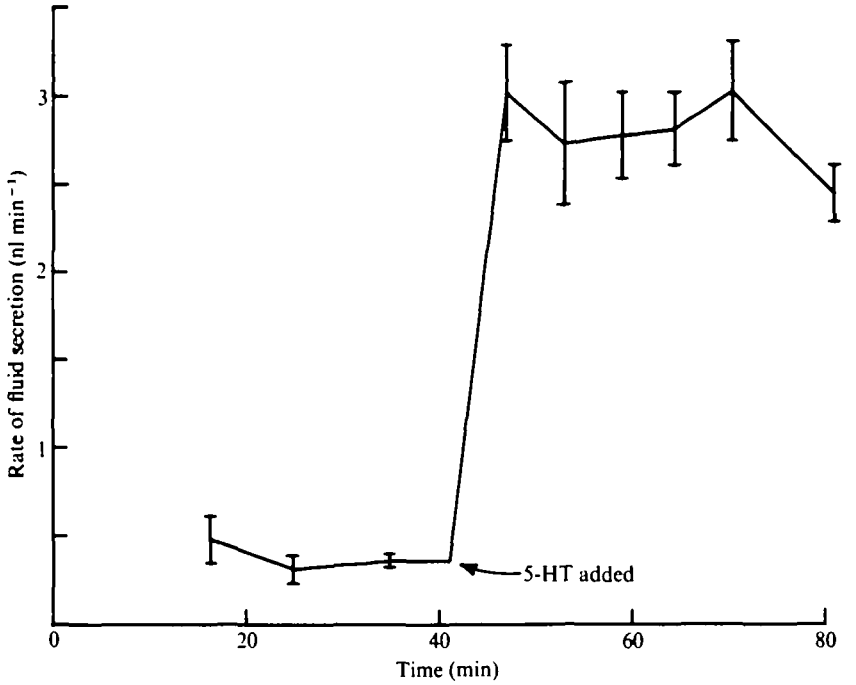

Fig. 6. The effect on the rate of fluid secretion by a set of five Malpighian tubules isolated from a fed 4th instar larva of *A. taeniorhynchus* of adding 5-HT to the bathing medium at a final concentration of 10^{-5} M. The vertical lines attached to the points represent ±S.E. of the mean.

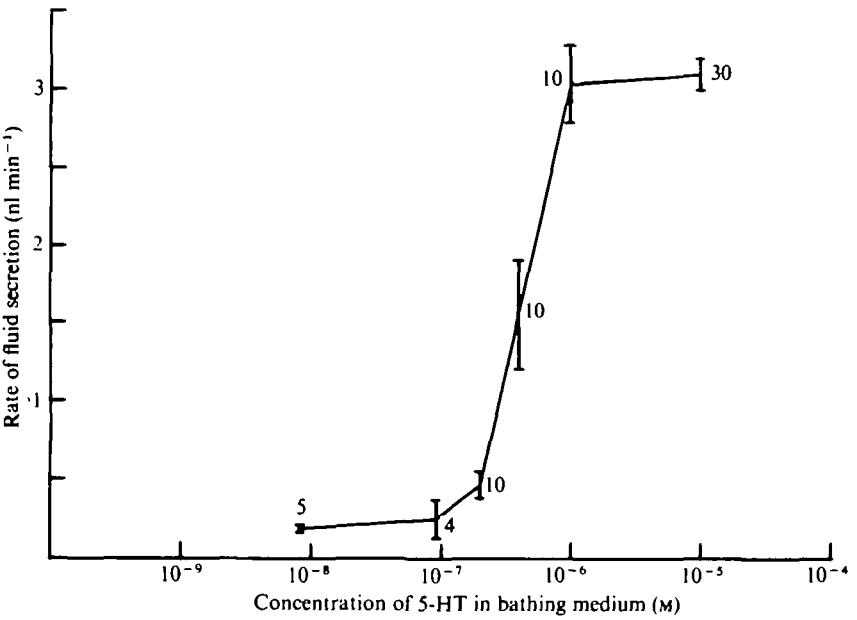

Fig. 7. Dose/response curve for the effects of 5-HT on the rate of fluid secretion by Malpighian tubules isolated from 4th stage larvae of *A. taeniorhynchus*. Each point is the mean of the number of determinations indicated; the vertical lines attached to the points represent ±S.E. of the mean.

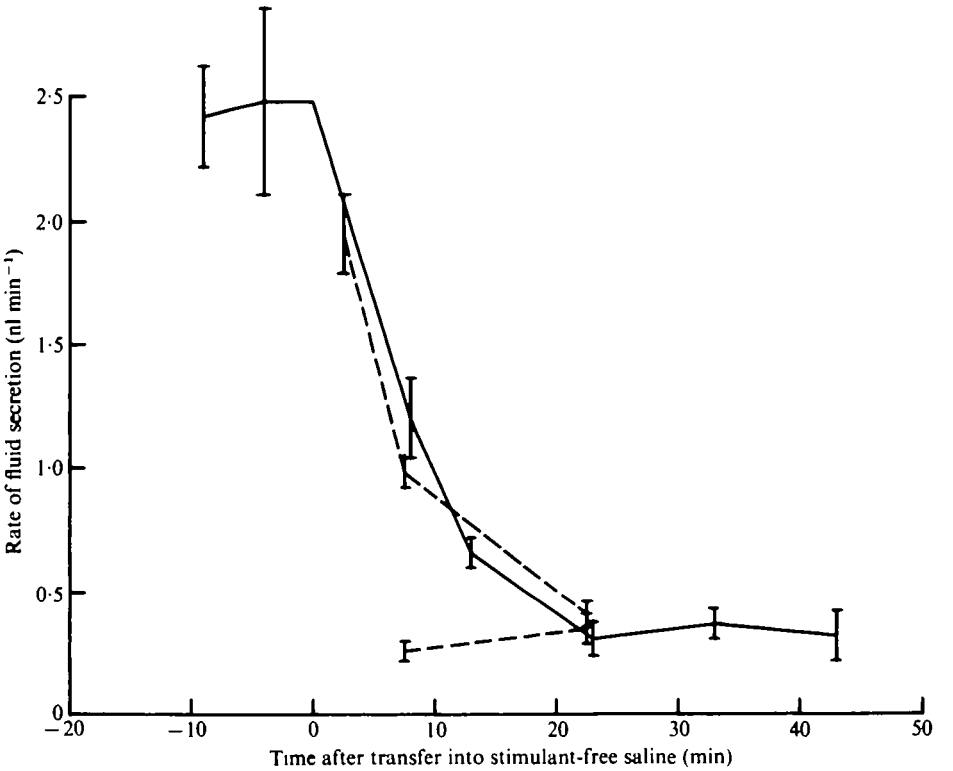

Fig. 8. The rates of fluid secretion by Malpighian tubules of *A. taeniorhynchus* after transfer to stimulant-free saline. The continuous line shows the fall in rate of fluid secretion of six tubules after removal from a 5-HT containing solution. The broken lines are for tubules removed from feeding larvae (upper line) and for larvae kept without food for 1 day prior to the experiment (lower line). The vertical lines attached to the points represent ±S.E. of the mean.

respectively, the first and second 15 min periods after removal from the larvae. When transferred from a solution containing 5-HT the rate of fluid secretion by isolated Malpighian tubules was found to fall at a rate rather similar to that seen in tubules removed from fed insects (Fig. 8). The secretory behaviour of the tubules after these different histories is compared in Fig. 8. As can be seen, the behaviour of the tubules isolated from fed larvae is consistent with their having been removed from an environment in which they had been stimulated to secrete at a high rate.

If the tubules are under the control of a neurohormone it might be possible to stimulate them to secrete by adding extracts of the nervous system to the fluid bathing them. Fig. 9 shows the stimulatory effect exerted by an extract of the brain; nine further experiments gave essentially similar results. Extracts of the thoracic ganglia also have a stimulatory effect (five experiments) but in none of four trials did extracts of the abdominal ganglia have any effect. The brain is the richest source of stimulant; stimulation still occurs at a dilution of one brain in 20 μl, whereas if the material from the three thoracic ganglia is extracted into more than 5 μl, stimulation fails. These findings suggest that the brain and/or the thoracic ganglia might be the source of a diuretic neurohormone. If this is the case then treatment of the surfaces of these solutions should lead to the release of the hormone from the appropriate neurohaemal

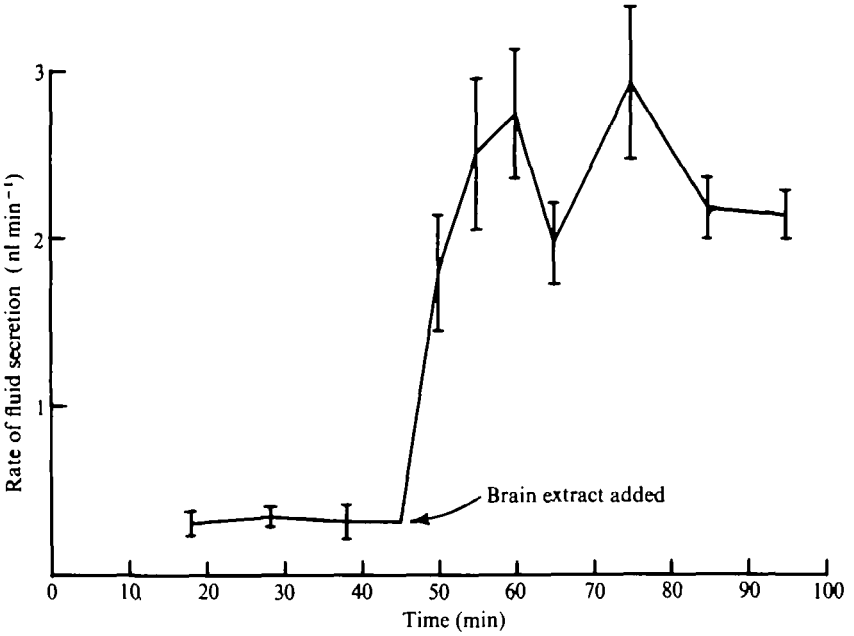

Fig. 9. The effect on the rate of fluid secretion by Malpighian tubules of *A. taeniorhynchus* of adding to the bathing medium an extract of the brain. In this experiment seven tubules were used. Each point represents the mean value at a particular time and the attached vertical lines indicate ±S.E. of the mean.

areas. To do this we placed 5 μl drops of saline containing 95 mM-K^+ on isolated heads and thoraces which had been dissected open to allow the fluid access to their contents. (K-rich solutions are known to depolarize accessible neurosecretory axon endings in neurohaemal structures and so to induce hormone release (Maddrell & Gee, 1974).) In three such experiments, a diuretic factor appeared in K-rich saline after it had been in contact with a head for 7 min. Fig. 10 shows the results of one of these experiments. Three similar experiments using the thorax gave negative results. The evidence thus supports the idea that a diuretic hormone is released at some site within the head. That no measurable release was obtained from the thorax is not particularly informative; it might, for example, have been that hormone was released but not in sufficient quantity to activate the test tubules.

Taken together the results suggest that the Malpighian tubules of *A. taeniorhynchus* are under the control of a stimulatory hormone synthesized in the brain and possibly also in the thoracic ganglia. This hormone is released in the head and from the initially high rates of fluid secretion of tubules taken from feeding insects it is likely that the levels of hormone in circulation are highest in feeding animals.

Effects of changing rates of fluid secretion on sulphate elimination

The preceding section provides evidence that the rate of fluid secretion of the Malpighian tubules is under hormonal control. How might changes in the rate of fluid secretion affect sulphate elimination by the tubules? This question arises because it is known that other Malpighian tubules are permeable to relatively small molecules

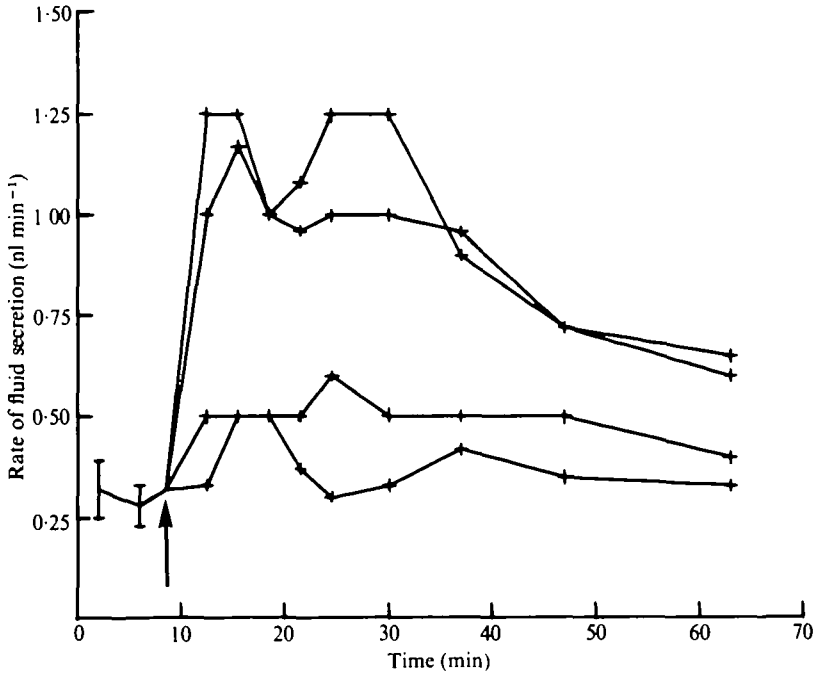

Fig. 10. The stimulatory effect on the rate of fluid secretion by Malpighian tubules of larvae of *A. taeniorhynchus* of adding to the bathing medium K-rich fluid which had been in contact with the tissues of the head. Four tubules, which had been secreting fluid at the low rates indicated, were treated with K-rich fluid at the time indicated by the arrow. As a control, two tubules were exposed to K-rich fluid which had not been in contact with tissue; the rate of secretion increased slightly (lower 2 lines) as expected from the increase in K level (Phillips & Maddrell, 1974). The other two tubules were treated with K-rich fluid which had bathed the tissues of the head of a larva for 7 min; the rate at which they secreted fluid increased by about four times (upper 2 lines).

and ions (Maddrell & Gardiner, 1974). As a result, active transport that produces high concentrations of transported material in the lumen can often be reduced in its effectiveness by diffusion of the transported substance back through the tubule wall into the haemolymph. This has been found to occur in the transport of organic anions such as acylamides (Maddrell *et al.* 1974) and in the transport of organic cations such as nicotine (Maddrell & Gardiner, 1976).

To test the possibility that sulphate elimination by Malpighian tubules of *A. taeniorhynchus* might be affected similarly, we set tubules from insects reared in full strength sea water to secrete in fluid containing 10 mM-SO_4 and 10^{-6} M 5-HT. Under these conditions the rate of fluid secretion falls away from initially higher rates, probably as 5-HT breaks down under the influence of light (as described in the Merck Index, 8th edition). Fig. 11 shows how the rate of sulphate transport depends on the rate of fluid secretion. Plainly, sulphate ions are removed more slowly from the bathing medium at the lower rates of fluid secretion. This relationship is very similar to that seen in the transport of organic anions and cations by Malpighian tubules of other insects (Maddrell *et al.* 1974; Maddrell & Gardiner, 1976), where it is known that the passive permeability of the tubule wall does reduce the effectiveness of

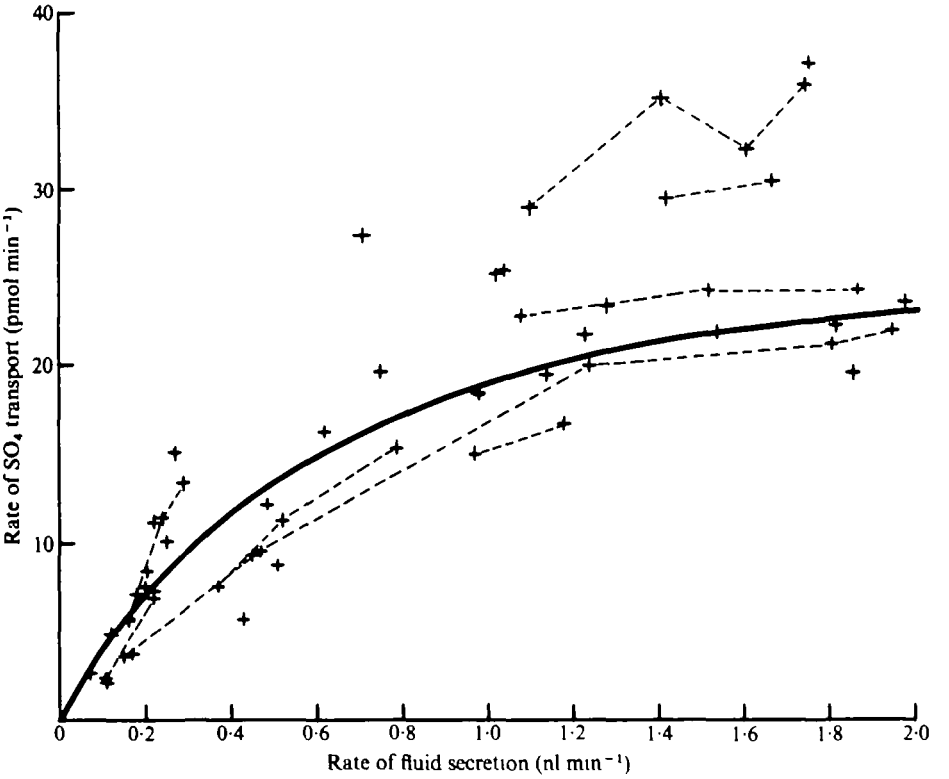

Fig. 11. The relationship between the rate of sulphate transport and the rate of fluid secretion by Malpighian tubules of 4th stage larvae of *A. taeniorhynchus*. Points joined by dotted lines are determinations made on the same tubule. The solid line is that of a relationship where sulphate is transported into the lumen at a constant rate but diffuses back out at a rate dependent on its concentration in the lumen. The tubules were isolated into saline containing 10 mM sulphate and were taken from insects reared in normal sea water.

transport at the lower rates of fluid secretion. Fig. 11 includes a line drawn to show the relationship between the rates of sulphate transport and fluid secretion which would occur if inward active transport of sulphate occurred at 30·9 pmol min^{-1} (taken from Fig. 4) and if the permeability of the wall to sulphate ions were 0·60 nl mm^{-2} min^{-1}. The measured values are at least compatible with such a relationship. Also a value of 0·60 nl mm^{-2} min^{-1} as the permeability of the tubule wall to sulphate ions is not an unreasonable one; it compares, for example, with a figure of 0·62 nl mm^{-2} min^{-1} for the permeability of the Malpighian tubules of *Rhodnius* to glutamate (Maddrell & Gardiner, 1974).

DISCUSSION

The main purpose of the present paper can be said to have been achieved in the results shown in Fig. 4. They show that rearing the larvae of *Aedes taeniorhynchus* in waters containing increased concentrations of sulphate ions leads to the induction of increased rates of sulphate transport by the Malpighian tubules.

This induction is significantly advanced within 6 h of transfer to sulphate-rich

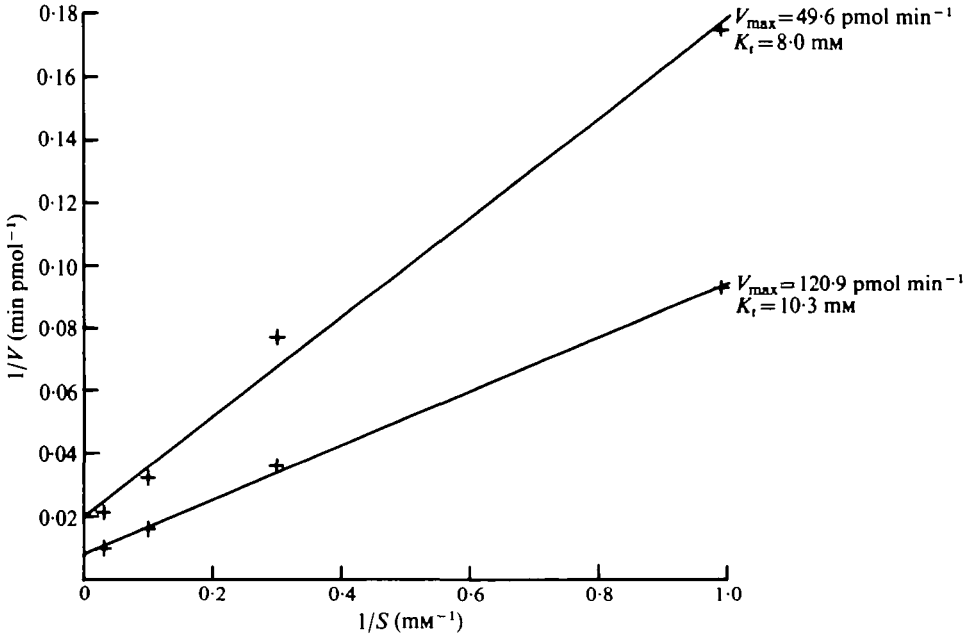

Fig. 12. Lineweaver–Burke plot of sulphate transport by Malpighian tubules of 4th stage larvae of *A. taeniorhynchus* reared in sulphate-rich water (lower line) or in normal sea water (upper line).

water and is about 50% complete after a further 10 h. As was pointed out (p. 191), the characteristics of the sulphate transport system in insects reared in waters of different sulphate content are such as to suggest that the induction might involve only a change in capacity with no change in affinity. Replotting the results from Fig. 4 as a Lineweaver–Burke plot supports this suggestion (Fig. 12). The tubules behave as if they had a transport system half-saturating at a concentration of sulphate in the medium of about 8–10 mM.

In response to a transfer to water richer in sulphate, the tubules are soon able to transport sulphate at a higher rate. If sulphate transport depends on a sulphate pump then the induction might involve synthesis of more pump molecules, the incorporation of more such molecules into the cell membranes or the activation of inactive pumps already in the membranes, or a combination of these. The stimulus for the induction process might be the direct one of an increase in the sulphate content of the haemolymph. The scope for investigating any of these possibilities is limited by our finding that we could not induce an increased rate of sulphate transport by Malpighian tubules *in vitro*. This failure might have a variety of explanations; for example, isolated Malpighian tubules might lose an ability to carry out protein synthesis or it might be that induction involves some hormonal mediator.

Although the mechanism of induction may not be certain its significance is clear; larvae growing in sulphate-containing water can thereby eliminate sulphate taken up into the haemolymph by the midgut. This is important because the posterior part of the rectum, which is able to remove other ions from the haemolymph, cannot so excrete sulphate ions. Fig. 2 shows that feeding larvae of *A. taeniorhynchus* ingest

the external medium at about 0·15 μl h^{-1}. Since the rate of ingestion is not affected by salinity (Bradley & Phillips, 1977*b*), it follows that larvae reared in water containing sulphate ions at a concentration, say of 89 mM, would ingest sulphate ions at a rate of about 13·5 nmol h^{-1}. These ions are absorbed into the haemolymph (p. 185) but the insects succeed in keeping the haemolymph level of sulphate down to about 35 mM (Table 3). The sulphate-transporting ability induced in the Malpighian tubules (Fig. 4) is such that with 35 mM in the haemolymph they each eliminate sulphate at a rate of about 100 pmol min^{-1}, which means the full complement of five tubules can excrete 30 nmol h^{-1} of sulphate ions, i.e. easily sufficient to match the rate at which sulphate ions are taken up. In fact one might wonder why the level of sulphate in the haemolymph is not kept at lower levels. Two factors may be involved. The first is that the rate of fluid secretion affects sulphate elimination (Fig. 11 and p. 197); if the tubules are not maximally hormonally stimulated they secrete fluid more slowly and sulphate elimination is reduced. As discussed on p. 198, this seems most likely to be a consequence of the relatively high passive permeability of the tubule wall; at low rates of fluid secretion the concentration of sulphate ions in the tubule lumen rises and so the ions diffuse faster back through the tubule wall into the haemolymph and so reduce the effective rate at which they are removed from the haemolymph. A second factor which might reduce sulphate elimination from the insect is the possibility that not all the fluid running into the alimentary canal from the Malpighian tubules may pass backwards to the hindgut. In larvae of other mosquitoes, Ramsay (1950) and Stobbart (1971) have shown that some tubule fluid passes forward to the midgut. If this also happens in *A. taeniorhyncus*, then sulphate ions in the fluid would be reabsorbed into the haemolymph. In view of these two factors, what appears at first sight to be unnecessarily fast transport of sulphate ions by the Malpighian tubules becomes more understandable.

Our findings provide evidence that *Aedes taeniorhynchus* larvae are able to release into their haemolymph a diuretic hormone capable of increasing the rate of fluid secretion of the Malpighian tubules by up to 20 times. What role might this ability play in the normal physiology of the insect?

Water uptake by larvae living and feeding in 100% sea water occurs solely by drinking and at rates which have variously been measured at between 100–300 nl h^{-1} per larva (Bradley & Phillips, 1975; Bradley & Phillips, 1977*b*; this paper). Water loss occurs osmotically through the cuticle and by fluid secretion by the Malpighian tubules and by the rectum. Osmotic loss through the cuticle has not been measured for *A. taeniorhynchus* but has been shown to be significant in another saline-water mosquito, *Opifex fuscus* (Nicolson & Leader, 1974). Larvae of *A. campestris* living in very concentrated fluid are known to suffer decreases in body volume of around 5–10% and they greatly increase the rates at which they drink, presumably in attempts to replace the water loss (Kiceniuk & Phillips, 1974). This also suggests that osmotic loss can be a significant feature in the water balance of these insects.

Based on these sorts of findings, Bradley & Phillips (1975) have suggested that osmotic loss from a larva might amount to around 40 nl h^{-1} in feeding larvae of *A. taeniorhynchus* living in 100% sea water. If we take (from Fig. 2) values of water uptake per larva of 150 nl h^{-1} and 65 nl h^{-1} for feeding and non-feeding larvae respectively and assume that osmotic loss might be of the order of 40 nl h^{-1} per larva

this would leave 110 nl h^{-1} and 25 nl h^{-1} to be removed by the excretory system in the two cases. Bradley & Phillips (1975) estimate the mean rate of rectal secretion of fluid as about 20 nl h^{-1} per larva. From this it follows that the rate of fluid secretion by the Malpighian tubules might need to vary from only 5 nl h^{-1} in unfed insects to 90 nl h^{-1} in fed larvae. Plainly the accuracy of these estimates is debatable, but in qualitative terms it is clear that relatively small variations in the rate of water intake will require much larger changes in the rate of water elimination by the Malpighian tubules. If, as in other mosquitoes, fluid produced by the Malpighian tubules can either pass back into the hindgut or forward into the midgut (Stobbart, 1971) this would seem to be an alternative way in which the amount of water eliminated by the excretory system could be regulated. However, such a system could not eliminate fluid any faster than the Malpighian tubules secrete it, so that high rates of fluid elimination still demand that the Malpighian tubules secrete fluid at rates considerably higher than they achieve in the unstimulated state. The highest rates of fluid elimination would be necessary in feeding larvae living in media hypo-osmotic to the haemolymph, where osmotic influx would increase the rate at which water has to be eliminated. From our present results (Fig. 7) the full complement of five tubules when fully stimulated could secrete fluid at a combined rate of the order of 900 nl h^{-1}. Even if a significant fraction of this were to be reabsorbed (as is known to occur during diuresis in another dipteran, *Calliphora vomitoria* (Knowles, 1976)), it seems that the capacity of the Malpighian tubules to secrete fluid is sufficient to meet the needs of the insect even under extreme conditions.

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