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ACTIVE TRANSPORT BY INSECT MALPIGHIAN TUBULES OF ACIDIC DYES AND OF ACYLAMIDES

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SUMMARY

Insect Malpighian tubules carry out active transport of two types of organic anion: acylamides (such as *p*-aminohippuric acid) and sulphonates (such as indigo carmine and amaranth). There are separate mechanisms for the transport of these two classes of compounds.

The degree to which these compounds are concentrated depends critically on the passive permeability of the tubule wall. In the permeable Malpighian tubules of *Calliphora*, small transported molecules readily escape from the tubule lumen. At low rates of fluid secretion the net rate of dye transport is thereby very much reduced. As a result the rate of dye transport in this insect depends on the rate of fluid secretion, although the processes are not rigidly linked. In the less permeable tubules of *Rhodnius* and *Carausius*, dye secretion is not affected by the rate of fluid secretion.

The active transport of these two types of compounds is a means of clearing from the haemolymph the conjugated compounds which are the products of detoxication of potentially toxic products of metabolism.

INTRODUCTION

Insect Malpighian tubules have long been known to concentrate acidic dyes (Lison, 1937; Palm, 1952). The present paper examines this specialized form of excretion which accompanies the diffusive filtration/resorption mechanism now so well established as the fundamental basis of the insect excretory system. An active excretion of *p*-aminohippuric acid and related compounds, by a mechanism separable from the excretion of acidic dyes has also been demonstrated.

Dye secretion by Malpighian tubules has been used by several workers as an indication of the state of activity of the Malpighian tubules. Clearly it would be interesting to know whether such an equation can, in fact, be made; in particular, are dye secretion and fluid secretion separate processes or, for example, is fast fluid secretion always accompanied by fast dye secretion?

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MATERIALS AND METHODS

In vitro preparations of Malpighian tubules were isolated in appropriate physiological saline solutions (for details see Maddrell & Gardiner, 1974). Most experiments were performed with 5th stage larvae of *Rhodnius prolixus* and adult *Calliphora erythrocephala*, but Malpighian tubules of *Carausius morosus*, *Schistocerca gregaria*, *Manduca sexta*, *Triatoma phyllosoma* and *Pieris brassicae* were also used.

Excretion of dyes

Indigo carmine and amaranth were used because they are excreted rapidly by Malpighian tubules and can be separated spectrophotometrically. To measure the dye concentration in a small (*c.* 1 μl) sample of fluid, it was diluted and mixed in 150 μl of distilled water in a small plastic centrifuge tube. The dye concentration was measured with a Beckman 151 spectrocolorimeter. In the range of concentrations used the response of the spectrocolorimeter varied linearly with the dye concentration in the sample.

Excretion of PAH and related compounds

p-Aminohippuric acid (PAH) labelled with tritium in the 2-position of the glycine moiety and with a specific activity of 150 mCi.mmol^{-1} and benzyl penicillin K labelled with ^{14}C in the 1-position of the phenylacetate side chain at a specific activity of 25 mCi.mmol^{-1} were supplied by the Radiochemical Centre, Amersham. Chromatographic analysis established that neither of these compounds were metabolized by the Malpighian tubules. The chromatograms of the secreted fluid were run in three different solvent systems (*n*-butanol:acetic acid:water (12:3:5); ethanol:ammonia:water (20:1:4) and *n*-butanol:pyridine:water (1:1:1) for PAH, and ether saturated with potassium phthalate buffer at pH 6·0; isopropanol:methanol (30:70) and acetone:acetic acid (95:5) for benzylpenicillin). The chromatograms were scanned radiometrically using a Berthold LB 2722 scanner. Thereafter concentrations of these substances were measured by conventional scintillation counting techniques using an Intertechnique ABAC SL40 scintillation counter.

RESULTS

Dye excretion

Amaranth and indigo carmine were rapidly secreted by Malpighian tubules of all the insects tested. As will be described, the extent to which the dyes are concentrated in the fluid secreted by tubules depends on several factors such as the passive permeability of the tubule wall and the rate of fluid secretion. Nonetheless it is possible to give an indication of the overall secretory capacity of the tubule by plotting the concentration of the dye in the secreted fluid against that in the bathing fluid (Fig. 1). Clearly the tubules of *Calliphora* can concentrate indigo carmine by a factor of more than 30.

Unstimulated *Rhodnius* tubules concentrated amaranth in the lumen at a level which was up to 300 times higher than in the bathing fluid. This apparently higher rate of dye secretion can be correlated with the larger size of the amaranth molecule and with the lower permeability of the tubule wall in *Rhodnius* (see p. 375). In *Rhodnius* the

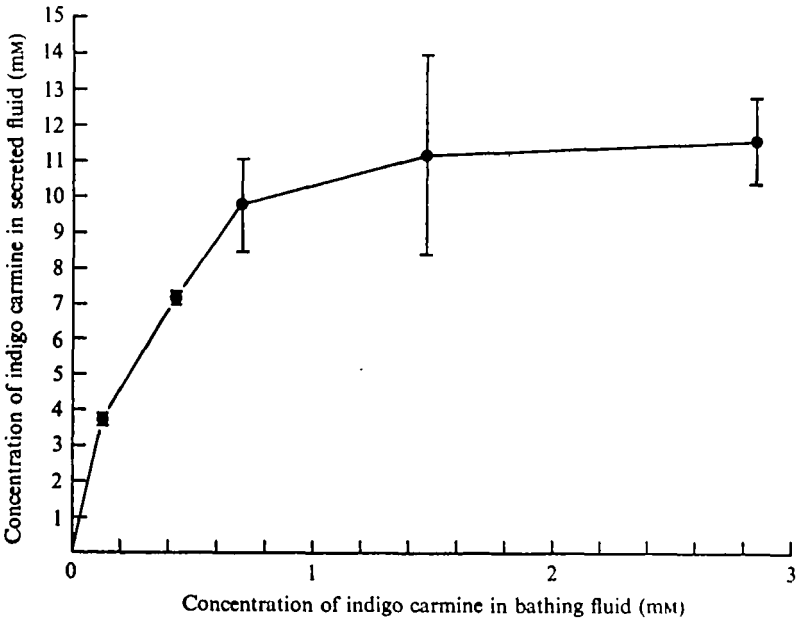


Fig. 1. The dependence of the concentration of indigo carmine in the fluid secreted by Malpighian tubules of *Calliphora* on the concentration of dye in the bathing medium. The vertical lines attached to the mean values represent ±S.E. of the mean.

Table 1. *The effects of various substances on dye transport by isolated Malpighian tubules*

Insect	Dye and concentration used (mM)		Presumptive competitor and concentration used (mM)		Effect on dye transport	No. of tubules tested
Calliphora	Indigo carmine	0·28	*p*-aminohippuric acid	3·43	No effect	12
Calliphora	Indigo carmine (Range, 0·1–2·5)		Amaranth	20	85% inhibition of indigo carmine transport	12
Calliphora	Indigo carmine	2·0	Benzoic acid	5	No effect	12
Calliphora	Indigo carmine	0·25	Uric acid	3	No effect	8
Rhodnius	Indigo carmine	1·19	Uric acid	3	No effect	12
Rhodnius	Amaranth	0·33	*p*-aminohippuric acid	10 30	No effect	12
Calliphora	Indigo carmine	2·0	Arsenate	10	70% inhibition of indigo carmine transport	12

transport of dye is manifestly uphill, for the dyes (depending on their state of dissociation) are neutral or negatively charged and the potential of the lumen of the tubule is negative to the bathing solution (Maddrell, 1971). For the tubules of *Calliphora* the situation is not quite so clear, for the tubule lumen has a potential which is positive with respect to the bathing solution. However, other anions, such as urate and benzoate, penetrate the tubule wall very much more slowly (Maddrell and Gardiner, 1974) and do not reach a concentration in the lumen which is as high as that in

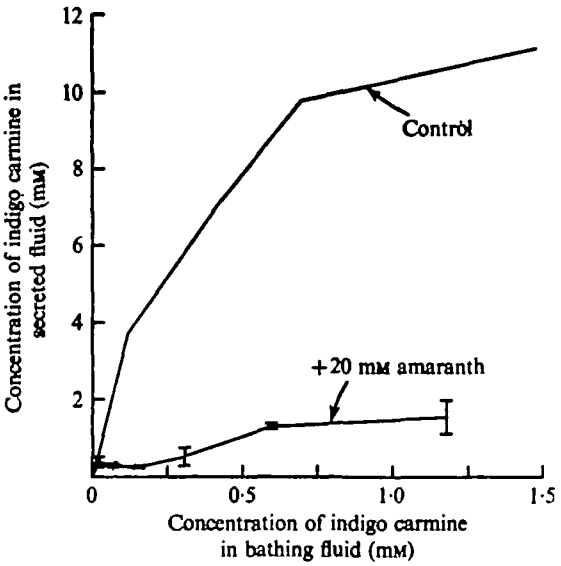


Fig. 2. The effect of amaranth on the concentration of indigo carmine in the fluid secreted by Malpighian tubules of *Calliphora*. Upper curve, without amaranth, figures from Fig. 1; lower curve, with 20 mM amaranth.

the bathing solution even though they are of lower molecular weight than the dye molecules. Moreover, the steep concentration gradient for dyes across the tubule wall is unlikely to be passively maintained by the small positive potential. It can, therefore, be concluded that dye transport by these two insect Malpighian tubules occurs actively.

Competition and inhibition studies

p-Aminohippuric acid does not affect dye transport (Table 1) although in vertebrate systems it has pronounced inhibitory effects, for example, on dye transport by nephrons of the flounder (Forster & Taggart, 1950). As might be expected, the addition of a second transported dye markedly reduces the secretion of the first dye; Fig. 2 shows the effect of added amaranth on indigo carmine secretion by *Calliphora* tubules. Benzoic and uric acids, which are not transported, have no effect on dye transport. It is interesting that arsenate ions should have such a strong inhibitory effect on dye secretion, for these ions are known competitively to inhibit phosphate transport in other organisms (such as yeast (Rothstein, 1963)) as well as inhibiting phosphate transport in Malpighian tubules of *Calliphora* (Berridge, 1969). This point is discussed on p. 374.

The relationship between fluid secretion and dye transport

Rhodnius

Malpighian tubules of *Rhodnius* were treated with small doses of 5-hydroxytryptamine so as to produce initially high rates of fluid secretion which were followed by a steady decline (Maddrell, Pilcher & Gardiner, 1971). By collecting a series of drops so produced we could measure dye transport at various rates of fluid secretion. Fig. 3 shows that dye transport was very little affected by the rate of fluid secretion. Only in

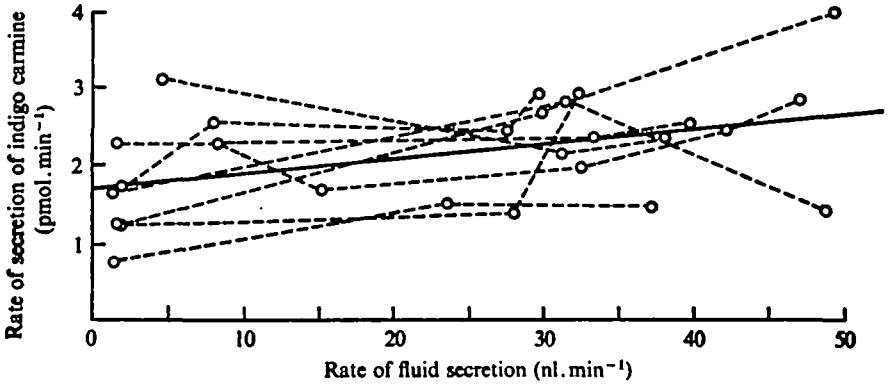


Fig. 3. The effect on the rate of secretion of indigo carmine by Malpighian tubules of *Rhodnius* of changes in the rate of fluid secretion. Dotted lines join determinations made on one tubule. The continuous line is the linear regression line computed by the least squares method.

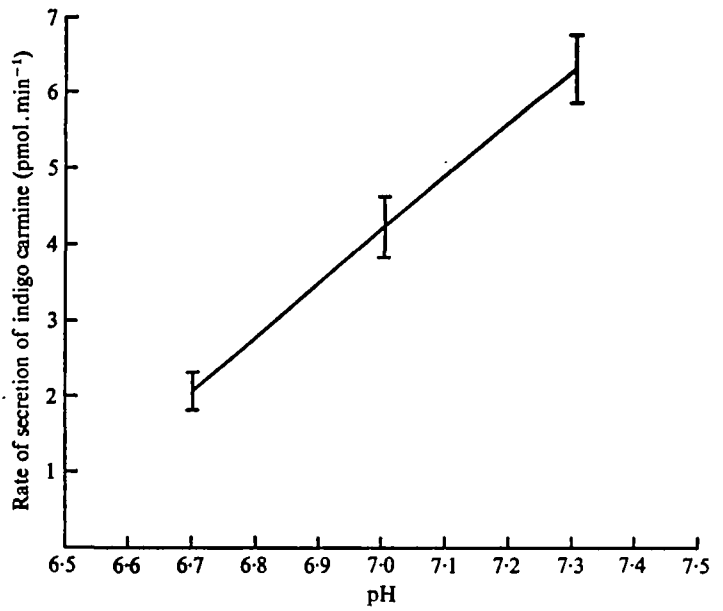


Fig. 4. The effect of pH on the rate of secretion of indigo carmine by Malpighian tubules of *Rhodnius* in a bathing solution containing 0·1 mM of the dye. The vertical lines attached to the mean values represent ±S.E. of the mean.

tubules secreting fluid at a slow rate did the secreted fluid contain dye at a higher concentration than in the bathing fluid.

Effects of pH

The rate of indigo carmine secretion by *Rhodnius* tubules was measured at three different pHs: 6·7, 7·0 and 7·3. It was found that dye secretion occurred more rapidly at the higher pHs (Fig. 4). It seems probable then that it is the concentration of the anionic form of this dye which determines the rate at which it is transported.

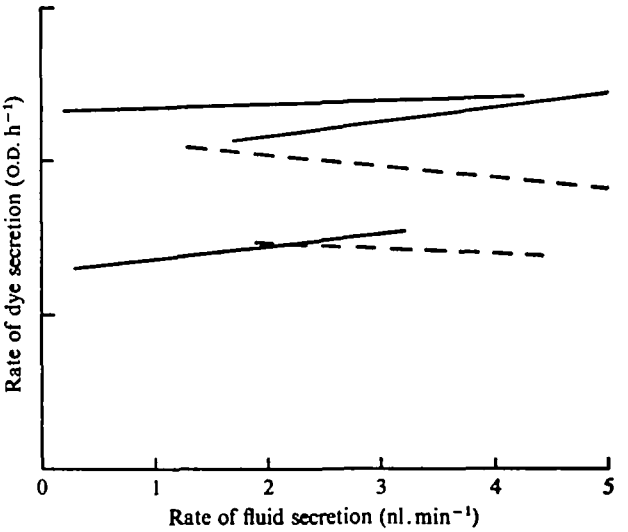


Fig. 5. The rate of secretion of dyes by isolated Malpighian tubules of *Carausius* as a function of the rate of fluid secretion. Each line represents the linear regression calculated for the tubules from a single insect (an average of ten tubules for each insect). The continuous lines represent results from experiments with indigo carmine and the dashed lines the results of experiments using neutral red.

Carausius

The rate of fluid secretion by Malpighian tubules of *Carausius* cannot be varied over such a large range as with *Rhodnius*. However, as is clear from Fig. 5, the results plainly show that, as in *Rhodnius*, dye secretion is virtually unaffected by changes in the rate of fluid secretion.

Calliphora

In these experiments the rate of fluid secretion was altered by changing the potassium or the osmotic concentration of the bathing fluid. Unlike the situation in *Rhodnius* and *Carausius*, dye secretion is very greatly affected by changes in the rate of fluid secretion, being approximately linearly related (Fig. 6).

This finding could have two possible explanations. First, it is conceivable that dye transport is linked with the active processes driving fluid secretion, being different in this respect from both *Rhodnius* and *Carausius* in which the two processes are independent. Secondly, it is possible that because *Calliphora* tubules are very permeable and transport dye at high rates, the transported dye leaks back into the bathing medium (from high concentrations in the tubule cells or lumen) nearly as fast as its active transport into the tubules.

Three lines of evidence have convinced us that the second explanation is the correct one.

There is good evidence that fluid secretion in insect Malpighian tubules is achieved by a primary active transport of ions which entrains a passive movement of water in osmotically compensating amounts (Maddrell, 1972). In bathing solutions of differing osmotic concentrations the rate of ion transport is rather little affected, the movement

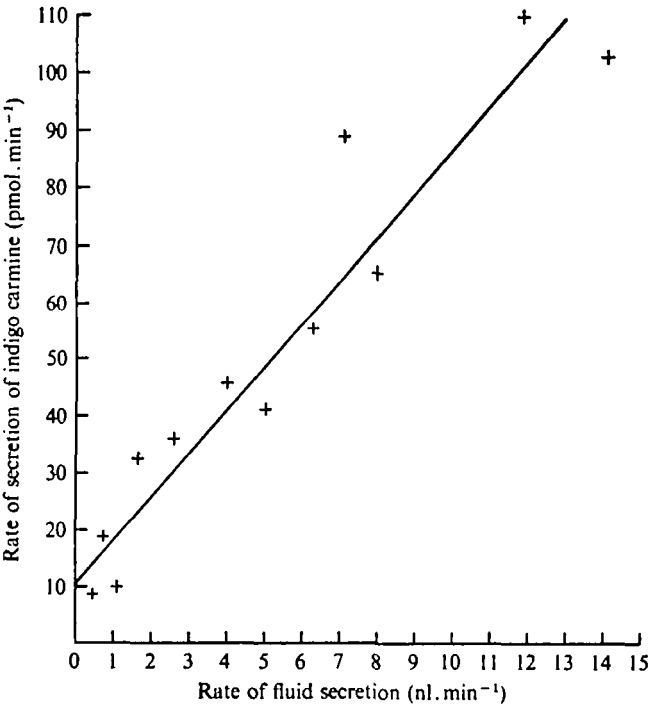


Fig. 6. The dependence of the rate of secretion of indigo carmine by Malpighian tubules of *Calliphora* on the rate of secretion of fluid. The line is the linear regression line fitted to the points by the least squares method.

of water being markedly affected (Maddrell, 1972). In *Calliphora* Malpighian tubules, the rate of ion transport can be altered by variations in the potassium concentration of the bathing medium (Berridge, 1968). By combining changes in potassium concentrations with appropriate changes in total osmotic concentration of the bathing media we could independently alter the rates of ion and fluid secretion. For example, by increasing the potassium concentration and increasing the osmotic concentration we could increase the rate of ion transport and yet slow fluid transport. The opposite effect (an increase in the rate of fluid transport and a slowing of ion transport) could be achieved by appropriate decreases in the potassium and osmotic concentrations of the bathing medium. When dye secretion was followed under these conditions it was clear that the rate of fluid transport was the factor that affected dye transport. For example, an increase in the rate of fluid transport was always accompanied by an increase in the rate of dye secretion even though ion secretion might be slowed (Figs. 7 and 8). Since dye transport is not affected by changes in ion transport, but only by changes in the secondary process of water transport, it seems unlikely that dye transport and fluid secretion are linked.

This conclusion is supported by observations on the rates of dye transport with different concentrations of dye in the bathing medium, experiments which provide a measure of the apparent capacity of the dye transporting mechanism. Tubules secreting fluid at low rates appear to have only a limited capability for dye transport, the mechanism apparently being saturated at a low level. If, however, the tubules are

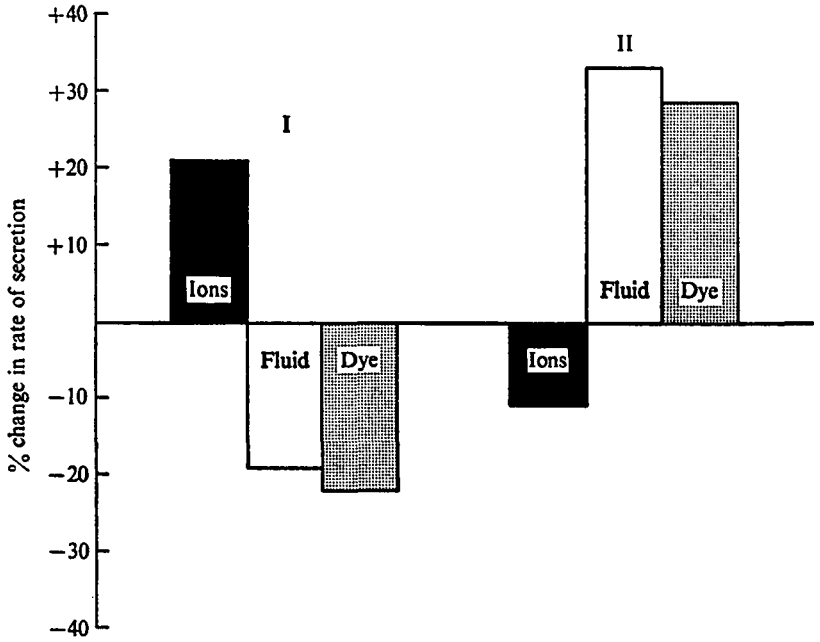


Fig. 7. The results of two experiments to show the independence of dye and ion transport and the dependence of dye movements on fluid movements in Malpighian tubules of *Calliphora*. In I, the osmotic concentration of the bathing solution was increased by raising the potassium concentration. This decreased the rate of fluid transport while allowing an increase in the rate of ion transport. In II, the osmotic concentration of the bathing solution was lowered by a decrease in its potassium content. This increased the rate of fluid transport but caused a drop in the rate of ion transport.

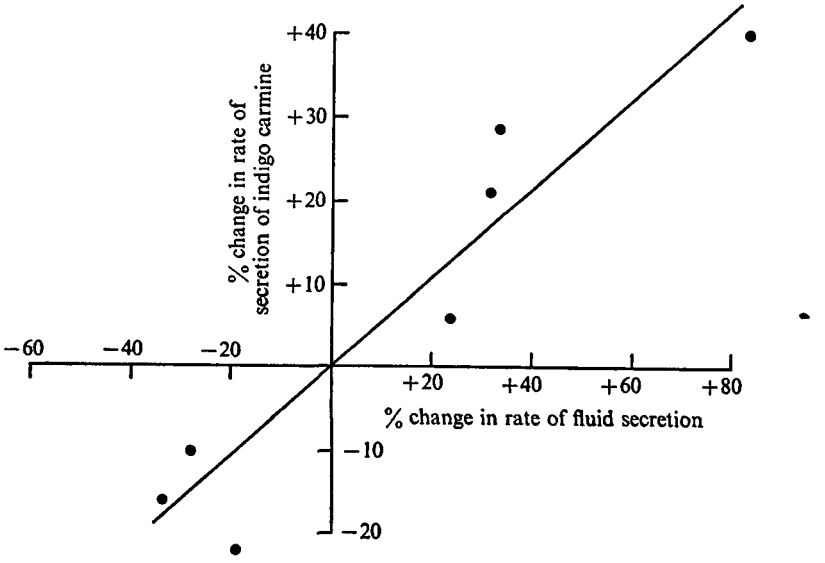


Fig. 8. The dependence of changes in the rate of secretion of indigo carmine by Malpighian tubules of *Calliphora* on changes in the rate of fluid secretion. The line is the linear regression line fitted to the points by the least squares method.

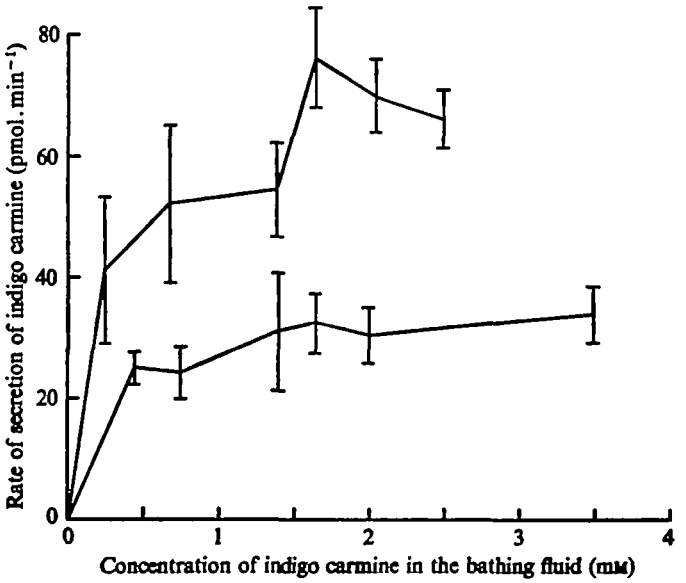


Fig. 9. The effect of the rate of fluid secretion on the apparent saturation of the transport mechanism for indigo carmine in Malpighian tubules of *Calliphora*. Upper curve, tubules made to secrete fluid at more than 8 nl.min^{-1}; lower curve, tubules made to secrete fluid at less than 4 nl.min^{-1}. The vertical lines attached to the points represent ±S.E. of the mean.

made to secrete fluid faster by, for example, a decrease in osmotic concentration of the bathing medium, the capacity for dye transport is greatly enhanced (Fig. 9). The results strongly suggest that the dye transport-system is not, in fact, saturated in tubules secreting fluid at low rates but that some other process makes it appear so. This could easily be a passive leak back into the bathing medium.

If the leakage is from concentrated dye solution in the lumen of the Malpighian tubule then it should be possible to reduce such a leakage by perfusing the lumen with a dye-free solution. To do this the apparatus shown in Fig. 10 was used. Essentially it consists of a motor-driven syringe which slowly empties its contents through a fine glass cannula *via* a thick-walled plastic tube. The experimental procedure was as follows. A Malpighian tubule was dissected from the insect and, as usual, isolated into a drop of Ringer's solution under liquid paraffin. A pair of fine forceps mounted on a micromanipulator was brought close to the bathing drop and the distal end of the tubule looped round the nearer point of the forceps, which were arranged so that their points opened and closed in the horizontal plane. The points of the forceps were held together with a small clamp. The fluid filled cannula also mounted on a micromanipulator was then brought up to the wall of the tubule close to where it is held by the forceps. The cannula was pushed through the wall of the tubule and on down the lumen until the taper of the cannula filled the lumen. Perfusion was then started.

Using this technique it was possible to measure the rate of dye secretion before, during and after perfusion.

For these experiments we used tubules made to secrete fluid at low rates, at elatively high concentrations of dye in the bathing medium, because, as shown in

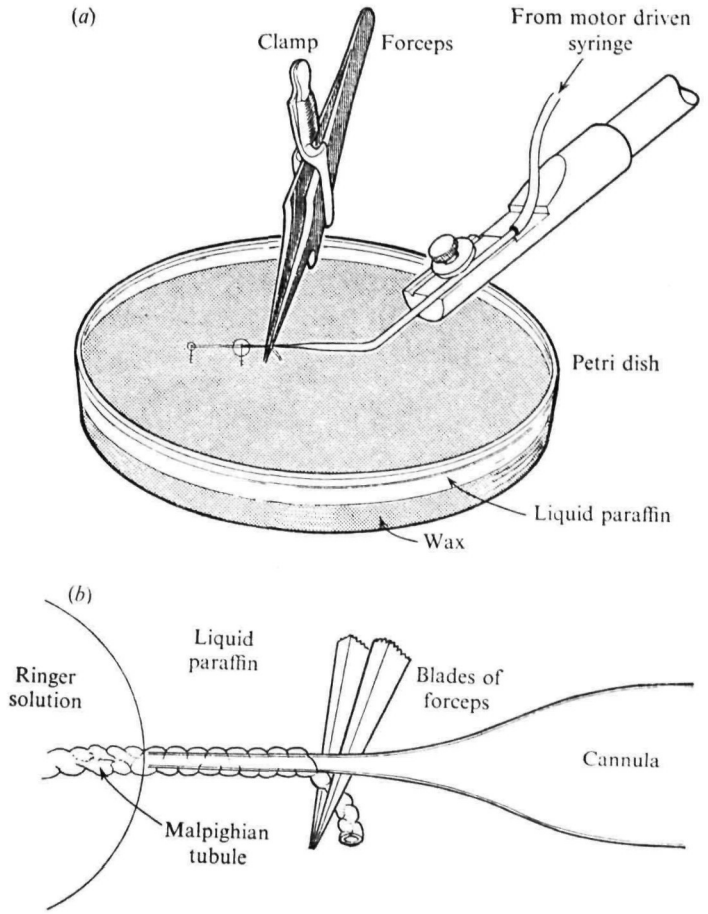


Fig. 10. Apparatus used to cannulate a Malpighian tubule from *Calliphora* so that the lumen could be perfused with fluid. (*a*) general view; (*b*) close-up view of the cannula tip entering the Malpighian tubule.

Fig. 9, it is under these conditions that the capability for dye transport is at its most limited. The results of such an experiment are shown in Fig. 11. Five further experiments gave essentially similar results. It is clear that perfusion of the lumen of a tubule during dye secretion can greatly increase the net transport of dye into the lumen. This evidence, together with that of the type shown in Figs. 7, 8 and 9, make it plain that active dye secretion is not directly linked to fluid secretion in *Calliphora*. The strong correlation between rate of fluid and dye secretion shown in Fig. 6 probably results merely from a large passive leak of secreted dye back into the bathing medium at low rates of fluid secretion, the rate of leak being reduced at higher rates of fluid secretion. The former effect would be expected if there were a higher concentration of dye in the lumen at such low rates of fluid secretion. Several questions now arise. Are there in fact higher concentrations of dye in the lumen at low rates of fluid secretion? If so, are they high enough to explain the proposed rate of leak back into the bathing solution and what must be the permeability of the wall to dye? Finally (assuming a reasonable value for the permeability of the wall) do the calculated

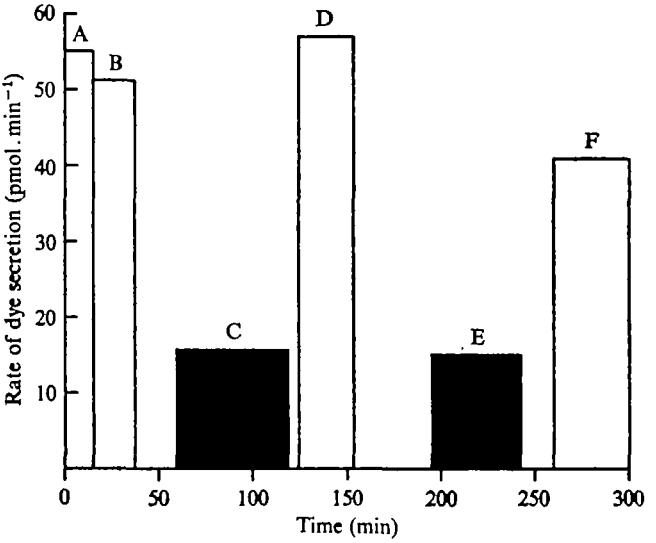


Fig. 11. The effect of perfusing with Ringer's solution the lumen of a Malpighian tubule of *Calliphora* on the net rate of transport of amaranth into the lumen from a concentration of 6·2 mM in the bathing solution. During periods A, B, D and F the tubules were perfused; during periods C and E, they were not.

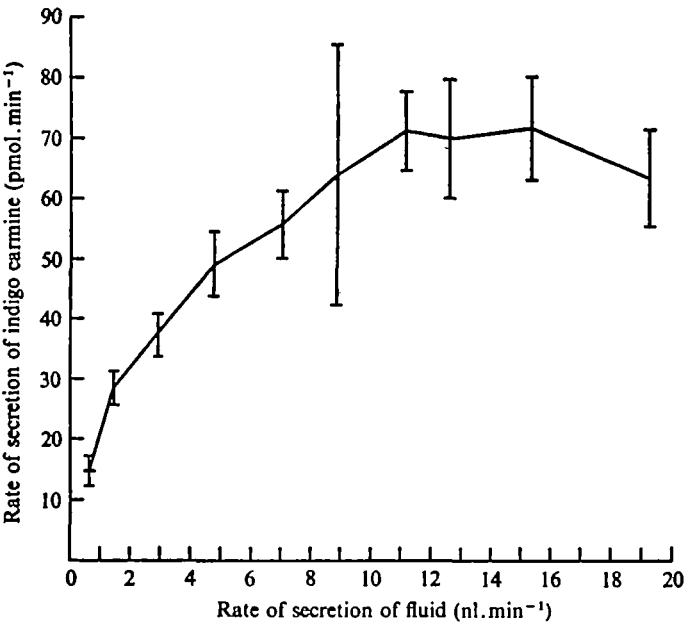


Fig. 12. The dependence of secretion of indigo carmine into the lumen of Malpighian tubules of *Calliphora* on the rate of secretion of fluid. The vertical lines attached to the points represent ±S.E. of the mean.

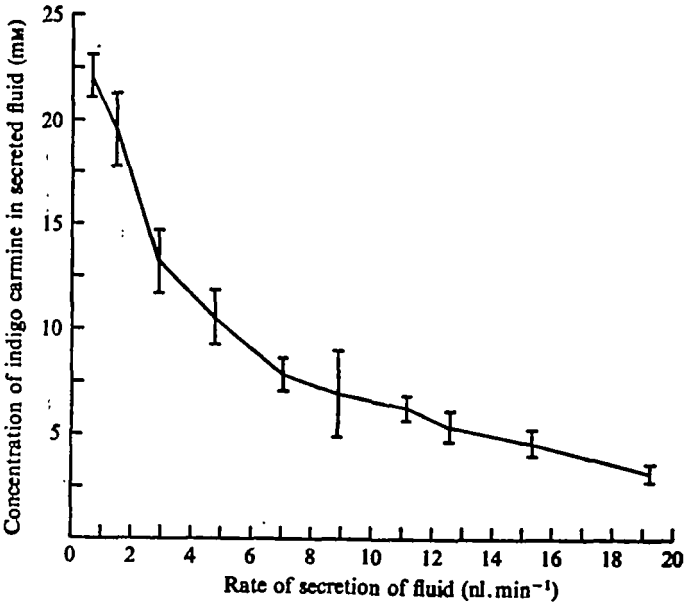


Fig. 13. The dependence of the concentration of indigo carmine in the fluid secreted by Malpighian tubules of *Calliphora* on the rate of secretion of fluid. The vertical lines attached to the points represent ±S.E. of the mean.

figures for diffusion of dye across the wall out of the tubule added to the measured net rates of dye transport, give figures for dye transport into the lumen which are independent of the rate of fluid secretion? To answer these questions in a satisfactory manner required considerable data. To this end more than 180 determinations of rate of net dye transport, rate of fluid secretion and concentration of dye in the secreted fluid were made at various rates of fluid secretion, using concentrations of indigo carmine in the bathing fluid of about 2 mM. The relationships between net transport of dye and the rate of fluid secretion and between concentrations of dye in the secreted fluid and rate of fluid secretion are shown in Figs. 12 and 13 respectively. Fig. 12 shows that net dye transport is low at low rates of fluid secretion, increases with the rate of fluid secretion below about 10 nl.min^{-1} but is not much affected by further increases in the rate of fluid secretion. Fig. 13 shows that, at low rates of fluid secretion, the concentration of dye in the secreted fluid is many times higher than both that of the bathing fluid and that of the fluid secreted at high rates. Are these concentrations high enough to explain the proposed loss from the lumen by diffusion? *Calliphora* Malpighian tubules are known to be very permeable (Maddrell & Gardiner, 1974) and for indigo carmine (whose molecular weight is 420) the permeability of the tubule wall would be predicted to be of the order of 0·35 nl.mm^{-2}.min^{-1}, although, as emphasized by Maddrell and Gardiner, the actual permeability for a particular compound might differ from that predicted by a factor of two or so in either direction. Accordingly, a permeability for indigo carmine of between 0·15 and 0·80 nl.mm^{-2}.min^{-1} would be consistent with the known permeability of *Calliphora* Malpighian tubules to other substances.

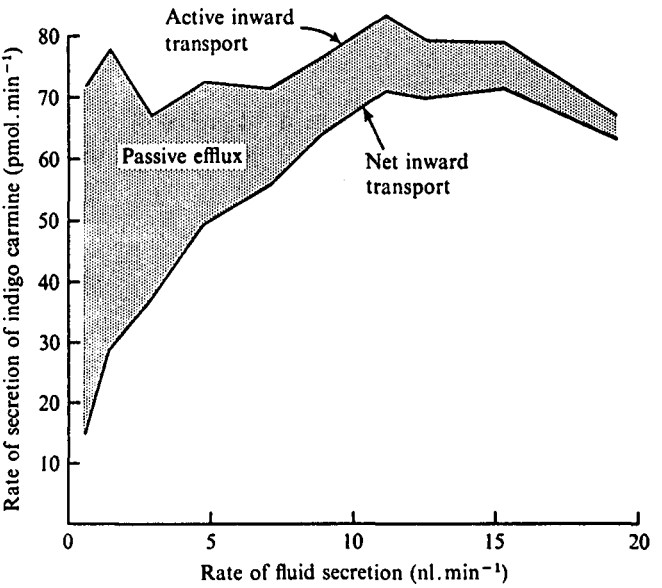


Fig. 14. Indigo carmine transport by Malpighian tubules of *Calliphora* as a function of the rate of secretion of fluid. The lower curve shows the rate of net inward dye transport actually observed (figures from Fig. 12). The upper curve shows the active inward transport and was constructed by adding the calculated rates of dye efflux to the lower curve, assuming the tubule wall to have a permeability to indigo carmine of 0·70 nl.mm^{-2}.min^{-1}. The stippled area between the two curves represents the extent of passive dye efflux.

The rate of passive efflux of dye from the lumen is given by the formula

$$\text{Dye efflux} = ba\,(S-M),$$

where b is the permeability of the wall, a the area of the wall and S and M the dye concentrations of the secreted fluid and bathing medium respectively.

To take a specific example from Figs. 12 and 13, at a rate of fluid secretion of 1·5 nl.min^{-1}, the net rate of dye transport is 28·50 *p*mol.min^{-1}, and the concentration of dye in the lumen 19·49 mM. The concentration of dye in the bathing medium was 1·85 mM and taking the surface area of the tubule as 4 mm^2 (Maddrell & Gardiner, 1974), the calculated passive efflux of dye is 70·56b *p*mol.min^{-1}. From Fig. 12, net dye transport at high rates of fluid transport, when presumably dye efflux is much smaller, is of the order of 70 *p*mol.min^{-1}. Thus at 1·5 nl.min^{-1}, dye efflux must at least account for this difference (i.e. 70–28·50 > 41·50b *p*mol.min^{-1}) whence b must have a value of at least 0·59 nl.mm^{-2}.min^{-1} and this is within the range expected for a compound of the size of indigo carmine.

In fact if similar calculations are done for each point from Figs. 12 and 13 one can construct a graph to show the rate of transport of dye into the lumen at various rates of fluid transport, in each case the calculated rate of dye efflux being added to the observed net rate of dye transport. As Fig. 14 shows, taking the permeability of the tubule wall for indigo carmine to be 0·70 nl.mm^{-2}.min^{-1} gives figures for dye transport into the lumen which are independent of the rate of fluid secretion.

In *Calliphora*, then, active dye secretion into the lumen proceeds at a high rate so

Table 2. *Concentration of PAH in fluid secreted by Malpighian tubules of various insects as ratio of concentration in the secreted fluid to concentration in the bathing medium (S/M ratio)*

Insect	*S/M* ratio achieved	Conc. of PAH in bathing medium (mM)	No. of tubules tested
Rhodnius prolixus	9·57	0·008	7
Schistocerca gregaria	23·35	0·013	13
Calliphora erythrocephala	3·46	0·050	10
Manduca sexta	2·52	0·014	10
Triatoma phyllosoma	2·20	0·014	4

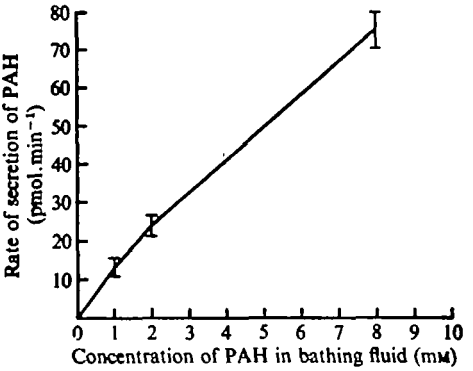


Fig. 15

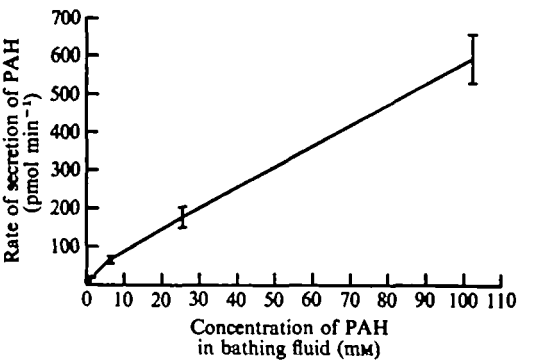


Fig. 16

Fig. 15. The rate of secretion of PAH by Malpighian tubules of *Rhodnius* as a function of the concentration of PAH in the bathing fluid. The vertical lines attached to the points represent ±S.E. of the mean.

Fig. 16. The rate of secretion of PAH by Malpighian tubules of *Calliphora* as a function of the concentration of PAH in the bathing fluid. The vertical lines attached to the points represent ±S.E. of the mean.

that a high concentration of dye is reached in the lumen at all but high rates of fluid secretion. Because the tubule wall is passively permeable to dye, a large proportion of transported dye diffuses back out of the lumen into the bathing fluid. As a result, the net amount of dye transported into the lumen depends very much on the rate of fluid secretion.

In *Rhodnius*, by contrast, where tubule walls are much less permeable (Maddrell & Gardiner, 1974) considerably higher dye concentration gradients can be maintained across the tubule wall (see p. 371). This has the result that far less of the transported dye leaks back into the bathing fluid and so the net amount transported into the lumen is very little affected by the rate of fluid secretion.

Acylamide excretion

In most experiments *p*-aminohippuric acid (PAH) was used, partly because of its availability and because it has been so widely used in investigations of other excretory systems. Table 2 shows that all Malpighian tubules tested transported PAH fast enough to concentrate it in the secreted fluid.

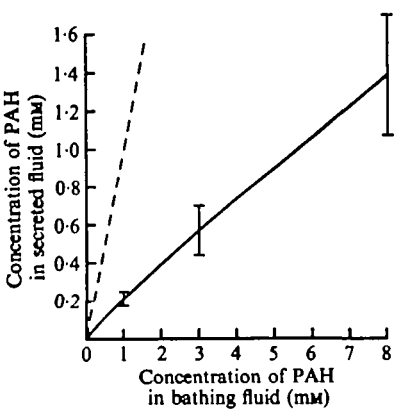


Fig. 17

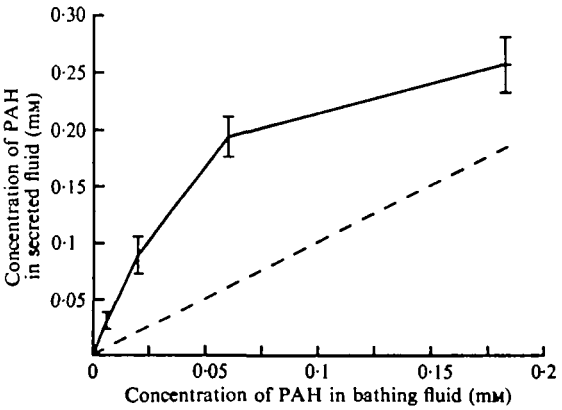


Fig. 18

Fig. 17. The concentration of PAH in the fluid secreted by Malpighian tubules of *Rhodnius* as a function of the concentration of PAH in the bathing fluid. The dotted line is that of a relationship in which the concentrations of PAH in the two fluids are the same.

Fig. 18. The concentration of PAH in the fluid secreted by Malpighian tubules of *Rhodnius* at concentrations of PAH in the bathing fluid of less than 0·2 mM. The dotted line is that of a relationship in which the concentrations of PAH in the two fluids are the same.

The rate of PAH transport into the secreted fluid was initially measured at concentrations in the bathing fluid of more than 1 mM. With *Rhodnius* and *Calliphora* it appeared that the transport was non-saturable, PAH excretion increasing directly with the concentration of PAH in the bathing fluid (Figs. 15 and 16). However, PAH is a relatively small compound (MW 194) and because Malpighian tubules are such permeable organs (Maddrell & Gardiner, 1974 and see p. 370) one might expect PAH to move across the tubule wall passively at a rate depending on the concentration gradient across it. It is clear, for *Rhodnius* tubules, that there is a steep concentration gradient which would drive PAH into the secreted fluid (Fig. 17). From figures for the permeability of the tubule wall to similarly sized compounds one can calculate that nearly all the PAH in the secreted fluid in the experiments shown in Figs. 15 and 17 could have arrived there passively. However, if similar experiments are done with concentrations of PAH in the bathing fluid of less than 0·2 mM, one can disassociate the active and passive elements of PAH transport, for the concentration of PAH in the secreted fluid is now higher than in the bathing fluid. The passive movements of PAH would, thus, be in the opposite direction (Fig. 18). Under these conditions *Rhodnius* tubules actively transport PAH into the lumen at a maximal rate of about 5 *p*mol.min^{-1} and with a *Km* of 0·05 mM.

In *Rhodnius*, then, passive influx must account for any PAH transport in excess of 5 *p*mol.min^{-1}, so that the very high rates of transport, shown in Fig. 15, are largely explicable as passive movements. From the rates of passive entry of PAH and the concentration gradient one can calculate the permeability of the walls of the Malpighian tubules of *Rhodnius* and *Calliphora* to PAH to be 1·51 nl.mm^{-2}.min^{-1} and 9·50 nl.mm^{-2}.min^{-1} respectively. These values agree well with ones for other similar sized compounds (Maddrell and Gardiner, 1974).

Table 3. *The effects of various substances on acylamide transport by isolated Malpighian tubules of* Rhodnius

Acylamide and concentration used (mM)	Presumptive competitor and concentration used (mM)	Effect on transport	No. of tubules tested
Benzylpenicillin 0·33	PAH 20	65 % inhibition	8
Benzylpenicillin 0·22	Allantoin 20	None	8
Benzylpenicillin 0·40	Amaranth 1·8	None	8
PAH 0·045	Amaranth 4	None	10
PAH 0·045	Allantoin 20	None	8
PAH 0·045	Benzylpenicillin 20	> 95 % inhibition	20

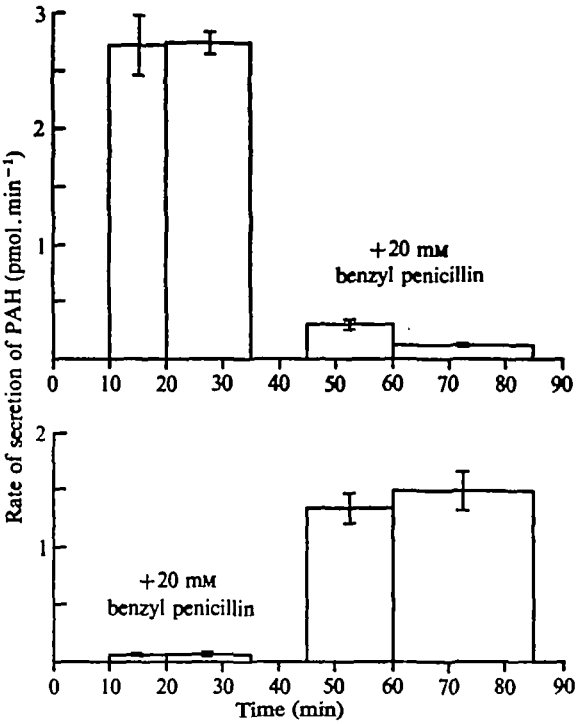


Fig. 19. Two experiments to show the effects of 20 mM benzyl penicillin on the rate of secretion of PAH by Malpighian tubules of *Rhodnius* from a bathing solution containing 0·05 mM PAH. In the upper graph, a set of tubules were allowed to secrete in a control medium lacking benzyl penicillin before treatment. The lower graph shows the recovery in the rate of PAH secretion on removal of benzyl penicillin from the bathing solution.

Competition studies

The effects on acylamide secretion by *Rhodnius* tubules of adding various compounds with similar structures to the bathing medium are summarized in Table 3. They show that the presence of benzyl penicillin strongly depresses the excretion of PAH (Fig. 19). PAH, although inhibiting the transport of benzyl penicillin, is less effective than benzyl penicillin in inhibiting PAH transport. Allantoin, an insect excretory product of somewhat similar structure to PAH, has no inhibitory effect on PAH excretion in *Rhodnius* although there is some evidence that it may be actively

concentrated by the Malpighian tubules of *Calliphora* (M. J. Berridge, personal communication).

Addition of dyes to acylamide-containing solutions do not, in general, inhibit the acylamide transport. At moderately high concentrations, however, they do have a depressing effect on such transport, but at these concentrations fluid secretion itself is also slowed. It seems likely that dyes have some general toxic effect on Malpighian tubules.

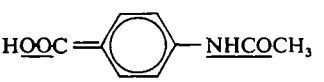
Effects of changes in concentration of potassium and calcium ions on dye and acylamide secretion

In other dye- and acylamide-transporting tissues, transport of these molecules is prevented if the bathing medium lacks either K or Ca (Puck, Wasserman & Fishman, 1952). We have, therefore, tested the ability of Malpighian tubules to secrete dyes and acylamides under such conditions. In these experiments Malpighian tubules of *Rhodnius* were used rather than those of *Calliphora*, as the former secrete fluid at unchanged rates in the absence of K or Ca. In *Rhodnius* tubules, the transport of amaranth, indigo carmine, benzyl penicillin and PAH was unaffected by the absence either of K or of Ca. One series of experiments also showed that neither amaranth nor fluid secretion was affected by the absence of both K and Ca.

DISCUSSION

The above evidence shows that insect Malpighian tubules actively excrete organic anions of two types: acylamides, such as *p*-aminohippuric acid (PAH), and sulphonates, such as indigo carmine and amaranth. Competitive inhibition between members of the two groups was not demonstrated although, within either group, the presence of one compound will interfere with the secretion of another of the same group. It seems, therefore, that insects possess two separate mechanisms for the excretion of organic anions.

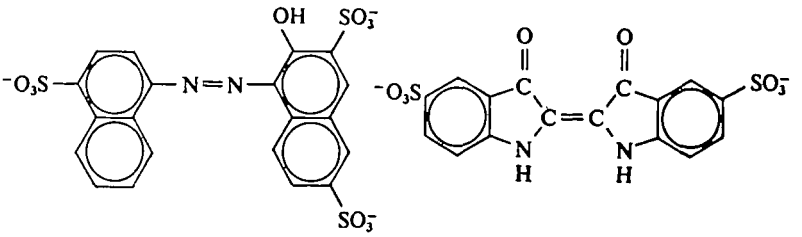
From a survey of the excretion of organic anions by vertebrate kidney tubules, Despopoulos (1965) has suggested that a transported anion must make a three-point contact with a receptor molecule on the kidney cell. Two oxygen atoms must make reinforced ionic bonds and a further oxygen or a nitrogen must make a supporting hydrogen bond with the receptor for transport to occur. He concludes, however, that two types of substrate fulfil these conditions: one in which the three reactive atoms are very close together as in sulphonates (R-SO_2OH) and sulphonamides (R-SO_2—NH—) and one in which the reactive groups are a good deal more widely separated as, for example, in 4-acetamidobenzoate,



It is perhaps surprising, therefore, that, in vertebrates, both types of compound seem to participate in a single discrete renal transport system, for they are excreted similarly and mutually interfere during transport (Smith, 1951).

In insects, members of these two classes of compounds are carried by separate

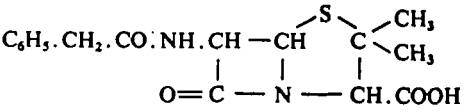
mechanisms, although it must be admitted that they are excreted in rather a similar fashion. For example, *Rhodnius* tubules will secrete PAH at a maximal rate of about 5 *p*mol.min^{-1} (with a Km of about 0·05 mM) and will secrete amaranth at a maximal rate of about 3–4 *p*mol.min^{-1} (with a Km of about 0·01 mM). The structural formulae of amaranth, indigo carmine, benzyl penicillin and PAH are set out below.



Amaranth

Indigo carmine

PAH



Benzyl penicillin

As Despopoulos (1965) points out, the sidechain of PAH which contains the reactive groups can exist in a folded or an extended state and, in the vertebrate kidney, it might make satisfactory interactions with the receptor molecule in either state. The failure of PAH to compete with dye molecules for transport by Malpighian tubules suggests that, at least in insects, the sidechain of PAH interacts with the transport sites only in its extended state.

The inhibiting effect of arsenate ions on dye transport (p. 360) may reflect the ability of these anions to interact transiently with the receptor so as to form an ionic bond. As a result arsenate ions could interfere competitively with dye transport without necessarily being themselves transported.

The ability of insect Malpighian tubules to secrete acidic dyes is widespread (Lison, 1937; Palm, 1952). It is clear from the results presented in this paper that the Malpighian tubules of several insects can also concentrate compounds containing an acylamide configuration. The question arises as to what is the function of such an ability. From the intensive studies of recent years on the metabolic fate of insecticides and related compounds (see, for example, Smith, 1962; O'Brien, 1967), it is clear that many potentially toxic molecules (both those produced by metabolism within the insect and foreign compounds) are altered biochemically to form a series of compounds which are much less toxic. The compounds formed are hippuric acids, ethereal sulphates, β-glucosides and β-glucuronides, acetamido derivatives and methylated compounds (Smith, 1962). It is now clear from the results of the present work that it is just these sorts of compounds for which Malpighian tubules have active transporting systems. Compounds of the size of the products of detoxifying systems listed above would be able to penetrate the permeable walls of Malpighian tubules but only at a

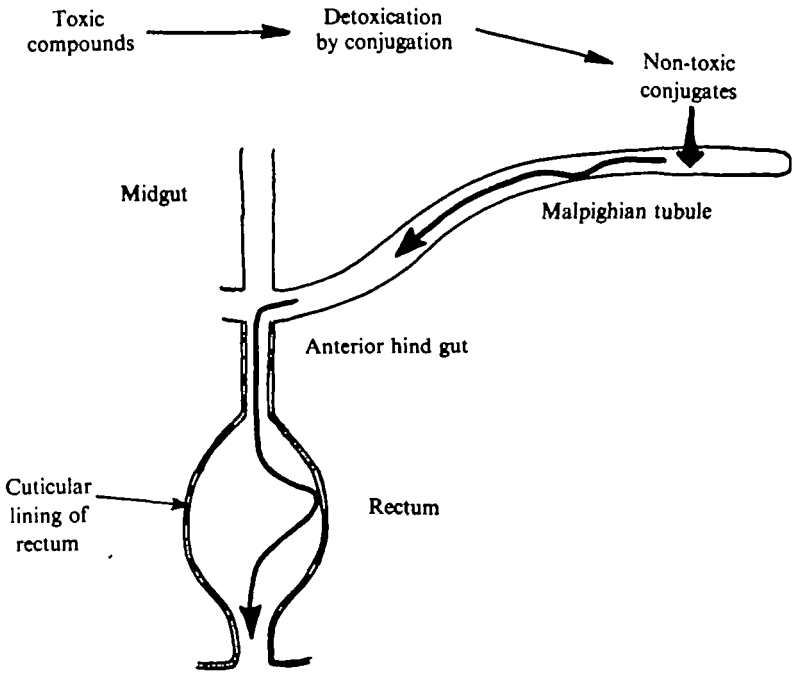


Fig. 20. The handling by the insect excretory system of the conjugated products of detoxification. After transport into the Malpighian tubules, these compounds are too large readily to escape from the tubule lumen still less to be able to diffuse through the cuticular lining of the hindgut.

relatively slow rate (Maddrell & Gardiner, 1974). The existence of a transporting system for these compounds thus enormously speeds up their excretion.

Detoxification by conjugation not only fits toxic molecules for active excretion by Malpighian tubules, but also, of course, increases their size. This in turn increases the effectiveness with which they are excreted. First, because such large compounds can less easily diffuse passively back into the haemolymph across the walls of the Malpighian tubules. The transport of smaller compounds would, on the other hand, lead to a continual erosion of the concentrative effects of the active transport due to the high passive permeability of the tubule wall. Secondly, when the excretory products are concentrated by water reabsorption in the rectum (Maddrell, 1971), such conjugated compounds are too large to penetrate the cuticular lining of the rectum and so cannot be passively returned to the haemolymph. These ideas are illustrated in Fig. 20.

At first sight, the ability of insect Malpighian tubules to transport dyes is less pronounced than it is in vertebrate nephrons. For example, kidney tubules of the flounder can concentrate phenol red by a factor of up to 4000 times (Puck, Wasserman & Fishman, 1952). The Malpighian tubules of *Calliphora* can concentrate indigo carmine only by a factor of about 30 times (Fig. 1). However, it is very probable that this reflects no more than a difference in passive permeability, it being important for the function of Malpighian tubules that they be permeable (Maddrell & Gardiner, 1974). *Rhodnius* tubules which are less permeable than those of *Calliphora* can concentrate amaranth by a factor of up to 300 times (see p. 358).

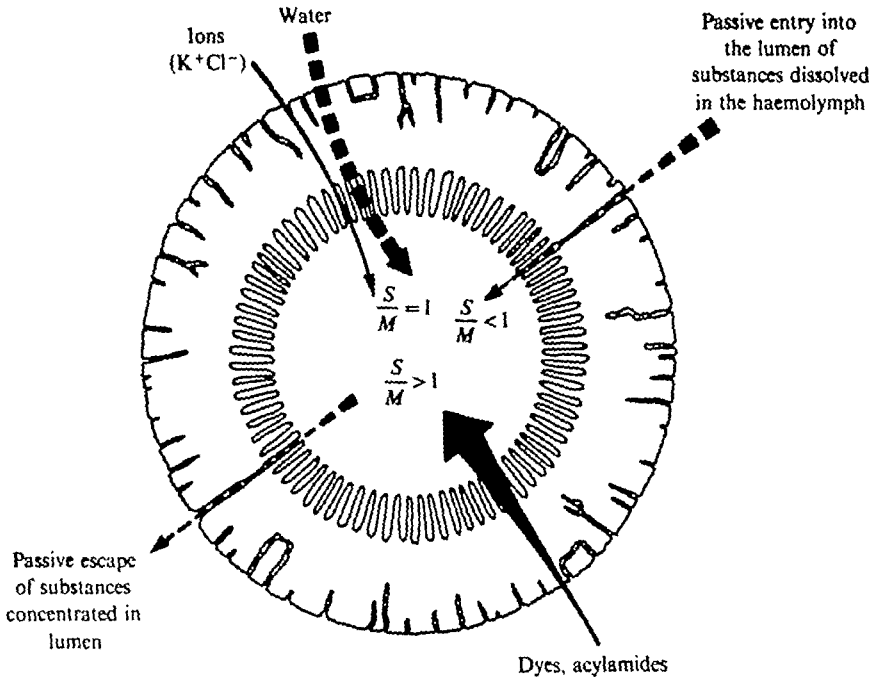


Fig. 21. Schematic section of the wall of a Malpighian tubule to show the routes of transport of ions, water, dyes and acylamides and diffusion of organic substances through the tubule wall. Active processes are represented by the continuous lines, passive processes by the broken lines.

The rate of dye secretion by insect Malpighian tubules has been used by some workers as a measure of the rate of fluid secretion (p. 357). It is now clear that the transport of dye and of fluid are only indirectly linked. The rate of dye secretion is only a guide to fluid secretion in Malpighian tubules, such as those of *Calliphora*, which transport dyes very rapidly and fluid relatively slowly and which have highly permeable walls. In other insects, such as *Rhodnius* and *Carausius*, the rate of dye secretion is almost unaffected by the rate of fluid secretion. One needs, therefore, to establish that dye secretion and fluid secretion are apparently related before dye transport can be used as an indicator of fluid transport.

Our conception of the operation of Malpighian tubules must be modified in the light of the results presented in this paper. Active transport of ions through the cells entrains an accompanying flow of water in osmotically compensating amounts (Maddrell, 1971). Compounds in solution in the haemolymph may reach the lumen by passive diffusion through the relatively leaky intercellular junctions (Maddrell & Gardiner, 1974), or, one can now add, by active transport, presumably through the tubule cells. Fig. 21 summarizes these ideas.

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