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TRANSPORT OF URIC ACID BY THE MALPIGHIAN TUBULES OF *RHODNIUS PROLIXUS* AND OTHER INSECTS

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SUMMARY

Urate is transported against an electrochemical gradient into the lumen of the lower Malpighian tubule of *Rhodnius*. *In vivo*, higher urate transport rates are induced by the increase in haemolymph urate concentration that follows feeding. The induced rate of transport is sufficient to account for the amount of urate eliminated. *In vitro* experiments with the tsetse fly *Glossina* suggest a possible induction of transport as a direct response of the tubule cells to an increase in urate concentration in the surrounding fluid.

Urate transport has been shown for Malpighian tubules of insects from several different orders, suggesting that the mechanism is widespread. Insect urate transport differs from that of vertebrates; it is ouabain-insensitive and results in the precipitation of free uric acid instead of urate salts.

INTRODUCTION

Uric acid has long been recognized as the major nitrogenous excretory product of insects (Wigglesworth, 1965; Bursell, 1967). Two properties suit it for this purpose: it contains 33 % nitrogen and, under the acid conditions which prevail in the terminal section of the excretory system (Phillips, 1977), its solubility is quite low (1·1 mM at pH 5·5). Its elimination thus leads to the excretion of large amounts of nitrogen without incurring much loss of water.

In several insects, however, uric acid is retained internally where it may serve a variety of functions (Corbet & Rotheram, 1965; Mullins & Cochran, 1974; Berridge, 1965; Harmsen, 1966; Tucker, 1977). For insects with a low nitrogen diet, the usefulness of such retained uric acid, its low toxicity, and the expense of its synthesis (see Gilmour, 1961) may have reduced selection pressure for its elimination.

There are limits to the amounts of uric acid or urate that can be accommodated within the body, and insects with a protein-rich diet have mechanisms for uric acid elimination. The tsetse fly, *Glossina morsitans* Westw., needs to excrete nearly 50 % of the dry weight of the ingested blood in the form of nitrogen-containing compounds (mainly uric acid, but also arginine and histidine) merely to dispose of surplus nitrogen (Bursell, 1965).

Key words: Uric acid transport, insects, Malpighian tubules.

The blood-sucking bug, *Rhodnius prolixus* Stål, also rapidly eliminates uric acid after one of its large blood meals (Wigglesworth, 1931*a*). Wigglesworth (1931*b*) proposed a widely accepted model (Chapman, 1971; Roeder, 1953) for excretion of uric acid by the Malpighian tubules of adult *Rhodnius*, in which urate is first transported into the lumen of the upper fluid secreting region of the tubule. It is then carried in the fluid flow into the lower tubule, where, by reabsorption of water and acidification, uric acid crystals precipitate.

However, at least in 5th instar larvae of *Rhodnius*, no evidence has been found that urate crosses the wall of the upper tubule by any other process than passive diffusion (Maddrell & Gardiner, 1974). The present experiments were undertaken to resolve this anomaly, to characterize more fully the mechanisms involved in uric acid elimination by *Rhodnius*, and to survey the Malpighian tubules of a range of other insects to see if similar processes occur in them.

MATERIALS AND METHODS

Fifth instar and adult *Rhodnius prolixus* were taken from a laboratory culture maintained on rabbits and sheep and kept at 28 °C. Pupae of *Glossina morsitans* were obtained from the Tsetse Research Laboratory, Langford, Bristol and adults for use were allowed to emerge at 28 °C. Other insects, *Pieris brassicae* L., *Schistocerca gregaria* Forsk., *Tenodora sinensis* Saussure, and *Calliphora erythrocephala* Meigen were taken from laboratory cultures.

Malpighian tubules were isolated in drops of saline under liquid paraffin as previously described (Maddrell, 1969) and were stimulated to secrete fluid using 10^{-6} M 5-hydroxytryptamine (5-HT) (Maddrell, Pilcher & Gardiner, 1969). To perfuse the lumina of tubules, they were cannulated with fine glass pipettes and fluid was driven through them from a motor driven syringe (Maddrell, Gardiner, Pilcher & Reynolds, 1974). Radioactive [^{14}C]-uric acid was obtained from Amersham International (formerly the Radiochemical Centre, Amersham) and samples were counted with conventional scintillation techniques using a Packard 3255 liquid scintillation spectrometer.

The standard saline used for *Rhodnius* and the mantid *Tenodora sinensis* contained (mM): NaCl, 129; KCl, 8·6; $CaCl_2$, 2; $MgCl_2$, 8·5; $NaHCO_3$, 10·2; NaH_2PO_4, 4·3; and glucose, 34; giving a solution of pH 6·8 and 342 mosmol l^{-1}. A K-free and Na-free saline was prepared by substituting choline chloride for NaCl and KCl and buffering with Tris. Other salines were according to Nicolson (1976) for *Pieris brassicae*; Gee (1975) for *Glossina morsitans*; and Maddrell & Klunsuwan (1973) for *Schistocerca gregaria*.

Chromatography of uric acid samples was carried out using the three systems suggested by Amersham International:

(a) paper chromatography using t-butanol: ethyl methyl ketone: formic acid: water (40: 30: 15: 15), $R_F = 0{\cdot}20$,

(b) thin layer chromatography on cellulose in n-butanol: pyridine: water (1: 1: 1), $R_F = 0{\cdot}39$, and

(c) thin layer chromatography on PE1 cellulose using 0·15 M-sodium chloride: 95 % ethanol (4: 1), $R_F = 0{\cdot}28$.

The nature of uric acid/urate in physiological solutions

The pK of uric acid is variously given in the literature as lying within the range 3·89 (Weast, 1979) to 5·7 (Spector, 1956). However, even taking the higher value, it follows from the Henderson-Hasselbalch relationship, $pH = pK + \log_{10}$ urate/uric acid, that under the conditions of our experiments, pH 6·8, more than 90 % of uric acid in solution is present as its anionic form. We therefore refer to the substance in solution as urate in the description that follows.

Composition of urate in lower Malpighian tubules

The levels of sodium, potassium, and urate in the large accumulations of white granules in the lower tubules were measured to determine if uric acid was present as the free acid or if it was complexed with a conjugate base. Selected lower tubules containing large amounts of such granules were dissected in normal saline, rinsed in a large volume of sodium-free and potassium-free choline saline and then placed in 2 ml of distilled water. One ml of this was analysed for its content of sodium and potassium by flame photometry using a Unicam SP90A Atomic Absorption Spectrophotometer. The remainder was added to 2 ml of saturated Li_2CO_3 and its urate content determined by measuring absorbance at 292 nm using a Unicam SP500 spectrophotometer.

Concentration of urate in haemolymph and urine

Haemolymph and urine urate concentrations were also determined spectrophotometrically as described above. However, because there is significant absorbance at 292 nm by substances other than urate, samples were incubated at 20 °C for 15 min with 0·25 units ml^{-1} uricase (Type IV, Sigma Chemical Co.) in a borate buffer (0·02 M, pH 8·5), and any remaining absorbance subtracted.

Measurement of intracellular potential differences

Glass microelectrodes filled with 3 M KCl were used in conjunction with a unity-gain, high-impedance operational amplifier to measure the intracellular/bath potential difference in lower Malpighian tubules stimulated with 5-HT.

Where a set of measurements are averaged they are shown as mean ± S.E.M. (N).

RESULTS

Permeability of upper Malpighian tubules

The dense precipitates containing uric acid, which occur along virtually the whole length of the lower Malpighian tubules of *Rhodnius*, could, in principle, arise either from a process of concentration of urate-containing fluid passing down from the upper tubule or by inward transport of urate across the walls of the lower tubule leading to saturation of the luminal fluid. This was investigated by measuring urate levels in the fluid secreted by the upper tubules. Ten adult tubules were isolated into standard saline containing 100 μM radioactive uric acid and were stimulated to secrete by the addition of 10^{-4} M 5-HT. The fluid collected from them contained uric acid at a

concentration of only 19±2 μM ($N = 10$), i.e. 17 % of that in the bathing fluid. The rate of fluid secretion was 68·3±6·7 nl min^{-1} from which it can be calculated* that the average apparent permeability to urate of the walls of the upper Malpighian tubules of adult *Rhodnius* is $1{\cdot}94 \times 10^{-6}$ cm s^{-1} (1·16 nl min^{-1} mm^{-2}); this compares with an average value of $1{\cdot}82 \times 10^{-6}$ cm s^{-1} (1·09 nl min^{-1} mm^{-2}) from similar experiments using six upper tubules from 5th instar *Rhodnius*.

It follows from these results that the uric acid deposits in the lumina of the lower tubules are very unlikely to be derived from urate present in the fluid secreted by the upper tubules. Even if the haemolymph were saturated with uric acid (presumably no more than 2 mM at the pH of the haemolymph) it can be calculated* that urate would only enter each upper tubule at a rate of about 5 pmol min^{-1} (assuming each tubule to secrete fluid at 3 nl min^{-1}). The calculated rate of uric acid transport is affected by what value is taken as the rate of fluid secretion by the tubules*. Even if this were as high as 10 nl min^{-1} $tubule^{-1}$ (which is unlikely as this would sweep uric acid crystals well downstream from the junction of upper and lower tubules where they can be seen to lie *in vivo*), urate would enter each upper tubule at a rate of only 11·8 pmol min^{-1}. It follows that the four upper tubules together might then eliminate a maximum of 68·2 nmol day^{-1} of uric acid. This, however, is still only 2·3 % of the 3 μmol (0·5 mg) which Wigglesworth (1931*a*) found to be the actual amount eliminated each day. It is clear, therefore, that passive movements of urate into the upper tubules are insufficient to meet the insect's needs.

Urate transport in the lower Malpighian tubule

To test whether the lower tubules can transport urate into the lumen, lower tubules were cannulated and standard saline perfused through them at rates of up to 50 nl min^{-1}, while they were bathed in saline containing radioactive urate at concentrations ranging from 25–150 μM. The fluid emerging from the lumen of such tubules was found in every case to contain a high concentration of tracer, usually 2–10 times that of the bathing medium. Samples of such fluid were subjected to the three chromatographic separation techniques described in Materials and Methods. This showed that virtually all the radioactivity in the samples could be attributed to unchanged urate; no other measurable spots were observed. It is clear that the lower tubules can transport urate from the bathing fluid into the lumen at high rates.

Characteristics of urate transport by the lower Malpighian tubules

To determine the capacity of lower tubules to transport urate, rates of urate transport were measured with different concentrations of urate in the bathing medium. The highest concentration used was 2·3 mM to ensure that all the urate was in

* The basis for these calculations is the formula, derived by Ramsay (1958), $S/M = b/a+b$, where S/M is the ratio of the concentration in the secreted fluid to that in the bathing fluid, b is the permeability of the tubule wall in nl min^{-1} mm^{-2} and a is the rate of fluid secretion in nl min^{-1} mm^2 of tubule wall. The areas of upper tubule wall are 7 mm^2 and 12·5 mm^2 for 5th instar larvae and adults respectively. 1 nl min^{-1} mm^{-2} is $1{\cdot}67 \times 10^{-6}$ cm s^{-1}.

For example, in the case described of an adult insect with its tubules secreting fluid at 3 nl min^{-1}, bathed in haemolymph containing 2 mM-urate, and each having a permeability to urate of 1·16 nl min^{-1} mm^{-2}, then $S/2 = 1{\cdot}16/[(3/12{\cdot}5) + 1{\cdot}16]$, whence S = 1·66 mM, and the rate of urate elimination by each tubule is 3 nl min^{-1} × 1·66 mM = 5 pmol min^{-1}.

lution. The insects were taken not more than 7 days since their previous moults.

In experiments with urate made up in standard salines, it was found that the transport rate fell away rapidly with time (Fig. 1). The urate content of the bathing drops was in all cases large enough to ensure that this was not due to depletion of urate from the bathing medium. Transport rate was better maintained in the saline of Maddrell & Gardiner (1980), which contains amino acids. Urate transport rates in this saline declined relatively little over the course of the experiment (Fig. 2), but significantly higher rates of transport occur with tubules within the first few minutes of isolation.

The concentration/transport curves for urate transport at various times after isolation (Fig. 3) show that, at least for a short while, *Rhodnius* lower Malpighian tubules *in vitro* can eliminate urate at a rate of 350 pmol min^{-1} when the bathing medium contains about 2 mM-urate. The rate of secretion varies linearly with urate concentration; there is no evidence that the pumping mechanism becomes saturated at the higher concentrations.

Some tubules transported urate at such high rates that the apparently clear secreted fluid contained very high concentrations of urate, often around 10–15 mM and, exceptionally, as high as 19 mM. The solutions were apparently true solutions. Four 0·2 μl samples of fluid secreted by Malpighian tubules in saline containing 2 mM urate plus radioactive urate as tracer were examined under dark field illumination at 400×. None showed any traces of crystalline deposits though their urate concentrations were 8·5, 7·6, 8·9, and 9·7 mM.

pH in the lower tubule

To investigate whether high urate concentrations could be explained by the pH of the lumen of the lower tubule, 5th stage insects fed 24 h previously were injected with 10 μl of saline containing 0·38 mM-chlorophenol red (3′,3″-dichlorophenol sulphonphthalein, an indicator which is yellow at pH 5·0 and red at pH 6·6). After 2–3 h the dye had appeared in the lumina of the lower tubules and showed there as an orange-yellow

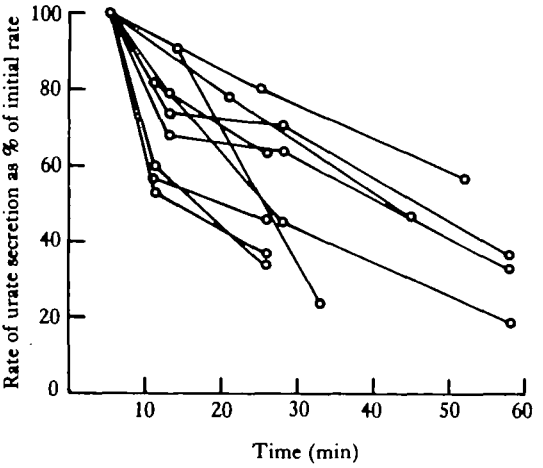

Fig. 1. Declining rates of transport of urate by Malpighian tubules isolated from 5th stage *Rhodnius* into standard saline.

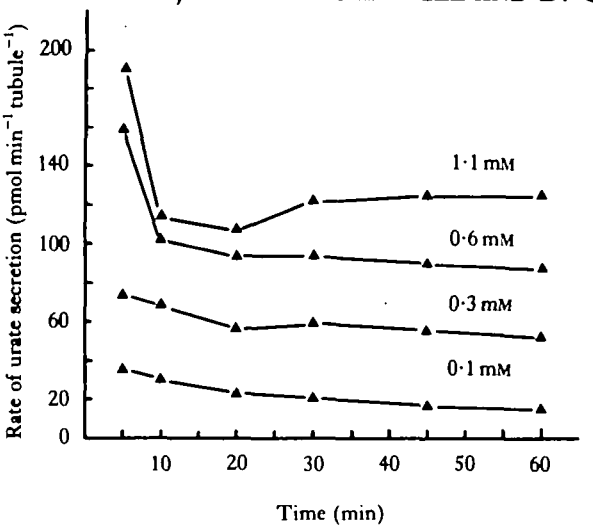

Fig. 2. Rates of urate transport by individual Malpighian tubules of 5th instar *Rhodnius* isolated into an amino acid-containing saline. The figures by each line indicate the urate concentration in the bath.

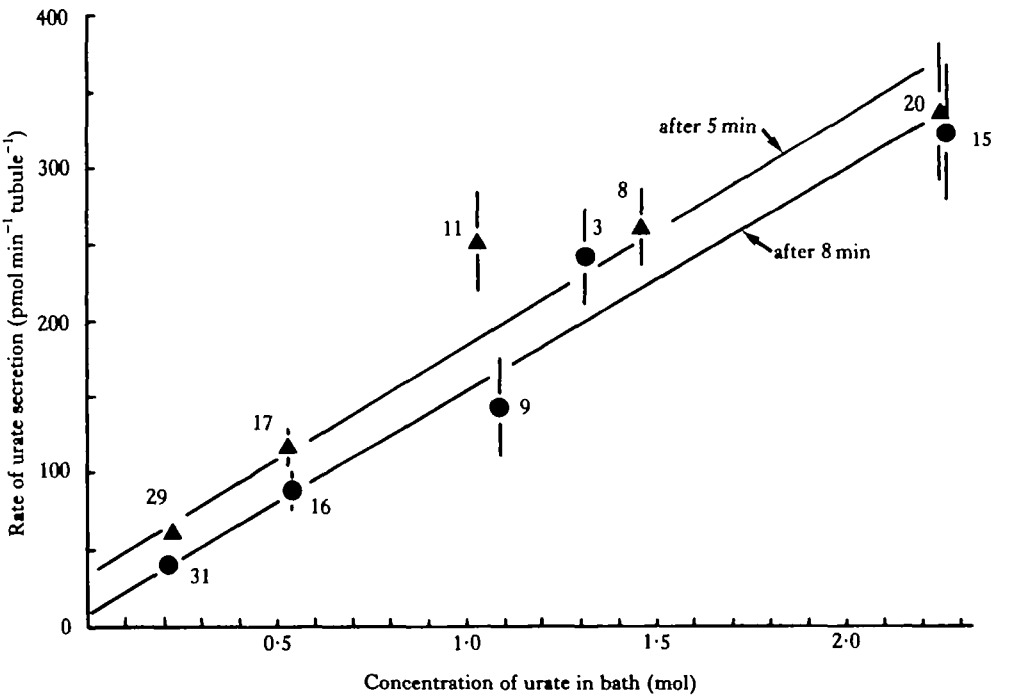

Fig. 3. Rates of urate transport by isolated Malpighian tubules of *Rhodnius* at various concentrations of urate in the bath. The determinations were made either after 5 min (upper line, solid triangles) or after 8 min (lower line, solid circles). The vertical lines attached to the points represent ±S.E.

plour, indicating a pH of 6·2 as judged by comparison with indicator in solutions of known pH.

Solubility of urate/uric acid in solutions of different pH

To gain some insight into the solubility of uric acid/urate, the following procedure was carried out. Two mmol of either uric acid or its sodium salt was dissolved in 15 ml of N NaOH (giving a clear solution containing 133 mM urate at pH 12·75). 1·5 mmol of Tris was added as buffer, a trace of phenol red as indicator, and 70 ml of distilled water. A little N HCl was then slowly run in while the mixture was stirred continuously and its pH monitored (with a Pye PW 9409 digital pH meter). 12 ml aliquots were removed when the pH reached 7·50, 7·00, 6·50 and 6·00 respectively. 5 μl samples of the clear supernatant were taken at intervals and the urate concentration determined spectrophotometrically. These results (Fig. 4) clearly show that concentrated urate solutions in the pH range 6·0 to 7·5 are not stable but precipitate slowly and somewhat erratically over a long period. However, at times less than 1 h, concentrations of urate in solution up to at least 20 mM can readily be obtained.

To check that the urate was in solution and not a colloidal suspension of uric acid, we centrifuged portions of the supernatant from those samples of a solution initially containing 20 mM urate (plus radioactive urate as a marker) at pH 12·5, but brought to pH 7, 6·5 and 6 in the three different samples. In each case considerable amounts of uric acid had precipitated before the samples were taken and the supernatants from the three contained 3·5, 6·8 and 5·6 mM urate respectively before being centrifuged. Each sample was then subjected to 100 000 **g** for 1 h when the supernatant was assayed for its urate content. The three samples now contained 2·7, 5·5 and 5·3 mM urate. After leaving them to stand for a further 18 h aliquots taken from the surface contained 1·8, 3·6 and 2·0 mM urate. These results suggest that centrifugation even at 100 000 **g**

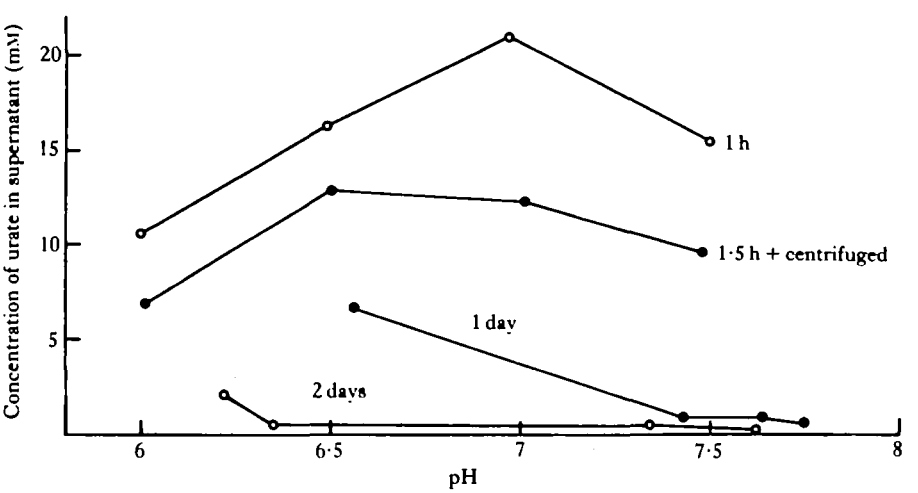

Fig. 4. Concentrations of urate and pH of solutions, initially with 20 mM urate and pH 7·5, 7·0, 6·5 and 6·0. The upper lines indicate the results of two experiments in which the solutions were left 1 h (open circles) or 1·5 h and were centrifuged (solid circles). The solutions from the first experiment were left for 2 days and gave the results shown by the bottom line (open circles); the solutions from the second experiment were left overnight and gave the results shown (solid circles, lower line).

did not have a marked effect on the rate of precipitation of uric acid from these solutions.

As a further test, we examined under dark field illumination at 400×, samples of fluid made up so as to contain 10 mM urate at pH 7·0. Five such samples showed no evidence of light scattering, suggesting that there were no suspended small crystals of uric acid in the fluid.

The results of both these sets of experiments suggest that uric acid can exist in apparently true solution at concentrations of up to 10 mM. The above results indicate that fluid produced by stimulated whole Malpighian tubules when they are bathed in a saline containing 1–2 mM urate could well contain as much as 10–20 mM in solution and that this would only relatively slowly precipitate out at the slightly alkaline pH involved.

The significance of changes in fluid secretion rate

In the *in vivo* situation, it is likely that high rates of urate transport (such as occur after feeding – see below) into the lumen, containing slowly moving fluid at pH of about 6, would lead to a relatively much more rapid precipitation of uric acid. In an insect eliminating 7 μmol of uric acid day^{-1} (see below) it follows that each tubule transports urate at a rate sufficient to deposit 0·3 mg of uric acid day^{-1} in the lumen. The specific gravity of uric acid is 1·86, so this would occupy a volume of 160 nl, more than twice the volume of the lumen of the lower tubule which is 76 nl. In unfed insects the upper tubule may secrete fluid at only 0·1 nl min^{-1} or 145 nl day^{-1}, which might not be sufficient to flush the precipitated uric acid out of the lower tubule into the rectum. However, the rate of fluid secretion increases after feeding and reaches 2–3 nl min^{-1} in tubules a few days after the meal (S. H. P. Maddrell, unpublished results). Part of the function of this increase may thus be to prevent the choking of the lower tubules with precipitated uric acid.

Effect of feeding upon transport rate

Malpighian tubules from 5th instar *Rhodnius* showed a dramatic increase in *in vitro* urate secretion within 2–3 days of feeding (Fig. 5). Peak levels of secretion in 0·5 mM urate, 170–200 pmol min^{-1} $tubule^{-1}$, were seven times those of unfed control animals of the same age. In 2 mM urate, peak rates of secretion 5–6 days after feeding were 814±70 pmol min^{-1} $tubule^{-1}$ ($N = 17$) after 5 min *in vitro* and 726 ± 77 pmol min^{-1} $tubule^{-1}$ after 10 min. Urate transport by all four tubules at these rates would be sufficient to account for close to 5 μmol day^{-1}.

To determine how far the urate transport rate measured *in vitro* could account for *in vivo* urate excretion the rate at which 5th instar *Rhodnius* eliminate uric acid and the concentration of urate in the haemolymph were measured. The rate of elimination of uric acid was measured in 5th instar *Rhodnius* fed about a month after their previous moult. After diuresis (5 h), each insect was placed in an individual 60 ml cylindrical plastic specimen container. At intervals, when the insects were seen to have excreted, they were moved to fresh containers. The used containers were washed out with saturated Li_2CO_3 solution and the fluid assayed spectrophotometrically to determine its content of urate. From the urate content of an insect's excreta and a knowledge of the time at which its previous excretion had taken place, the daily rate of its uric ac

elimination during the intervening period was calculated. The rate of urate elimination increased considerably during the first few days after a meal to reach a peak of about 6–7 μmol day^{-1} (Fig. 6). Thereafter the rate declined steadily until a few days after ecdysis when the rate reached a near-negligible value. The fall in uric acid elimination that occurs after the first 2 days probably does not represent a fall in the rate of urate transport. Water elimination after the meal goes on at a slow rate for a day or so then virtually ceases. The rectum then fills with a thick suspension of uric acid crystals rather than a more watery urine and there is consequently a longer delay before urate transported through the Malpighian tubules is actually eliminated from the body.

The area under the curve shown in Fig. 6 represents the total amount of uric acid eliminated after the meal. Evaluation of this showed that, on average, each fed insect eliminated 83 μmol of uric acid by 25 days after its meal.

To interpret the rates of elimination in terms of active transport, the urate concentration in the haemolymph was determined after feeding. The spectrophotometric absorbance of haemolymph samples from fed animals at 292 nm was measured before and after addition of uricase to the cuvettes. Only $38 \pm 5\%$ ($N = 12$) of the initial absorbance was due to uric acid, whose concentration in the haemolymph was $2{\cdot}0 \pm 0{\cdot}3$ mM ($N = 12$). Haemolymph urate concentrations were also measured at intervals after feeding. The concentration was $1{\cdot}2 \pm 0{\cdot}2$ mM ($N = 14$) within the first

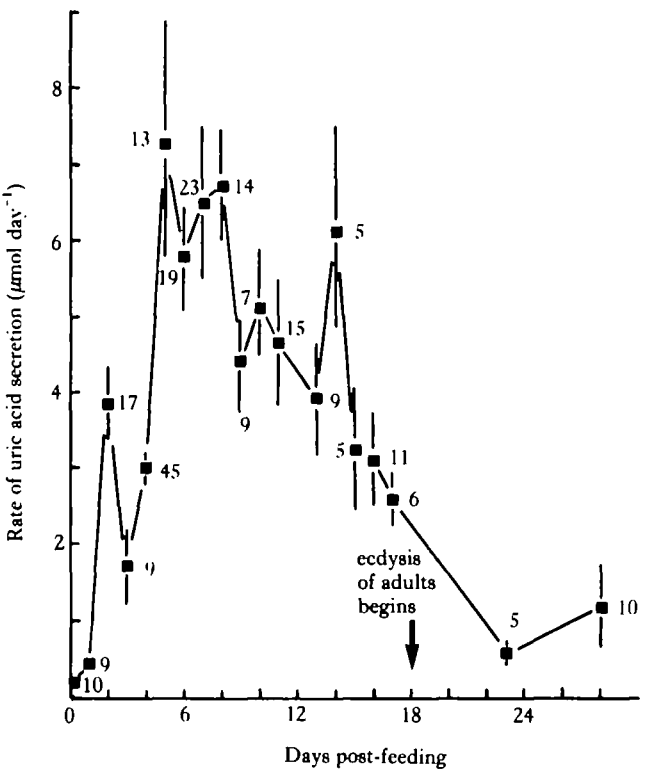

Fig. 5. Daily rates (mean ± S.E.) of excretion of uric acid by 5th instar *Rhodnius* after feeding. Number of animals indicated adjacent to each point.

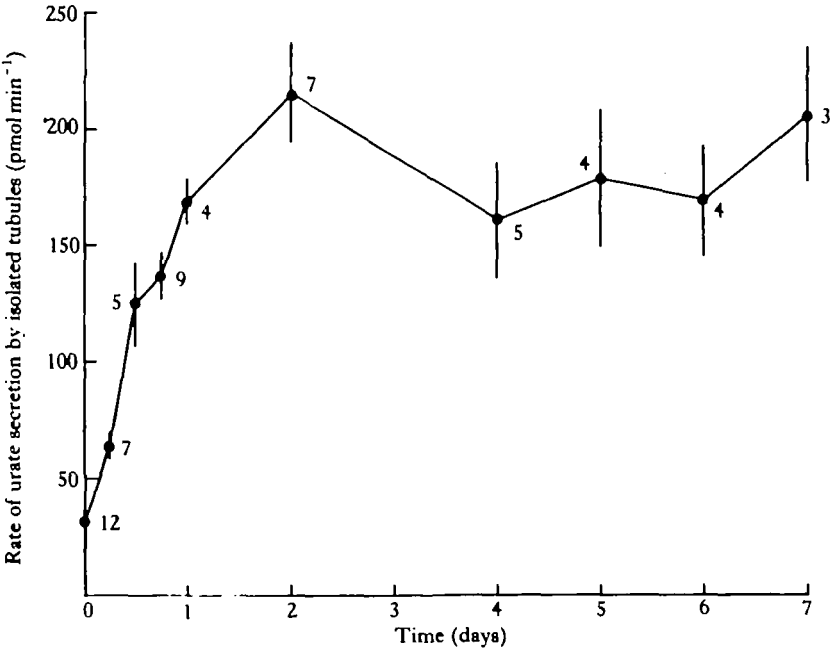

Fig. 6. Induction of urate secretion in 5th instar *Rhodnius* Malpighian tubules in response to feeding. Urate secretion rates were measured *in vitro* in enriched saline containing 0·5 mM urate.

12 h, 1·8 ± 0·2 mM ($N = 29$) after 12–24 h, and 1·3 ± 0·1 mM ($N = 11$) after 36–48 h. The initial decrease is probably the result of the increase in haemolymph volume which follows feeding (Maddrell & Gardiner, 1980). The change between 24 and 48 h is more likely to be due to an increase in the rate of urate elimination by the lower tubules (see Fig. 6).

In vivo, then, uric acid is eliminated at a peak rate of 6–7 μmol day^{-1}, when the haemolymph contains about 2 mM of urate. As we have seen, tubules from insects at this stage when isolated into saline containing 2 mM urate, can transport urate fast enough to account for the elimination of 5 μmol day^{-1}, suggesting that *in vivo* they could well eliminate urate at the required rate.

Induction of urate transport after feeding

The results suggest an induction of urate transport in response to feeding. To test whether there was a direct effect of urate concentration upon the tubules, we followed the response in unfed insects to an injection of 10 μl of saline containing 18 mM urate and at a pH of 7·0. After 24 h, 26 Malpighian tubules from 12 insects transported urate at a rate of 52·1 ± 2·7 pmol min^{-1}, when isolated in saline containing 0·5 mM urate. Eleven tubules from six unfed insects which had been injected with 10 μl of saline alone transported urate at a rate of 23·8 ± 1·7 pmol min^{-1}. Evidently the injection of 180 nmol of urate, although this is only equivalent to the amount eliminated in 2 h, is sufficient to induce a doubling of the urate transport rate by the Malpighian tubules. This result suggests that induction is a direct response of the tubules to an increase in haemolymph urate concentration after feeding. It is difficult to test the la[illegible]

possibility directly using *in vitro* preparations of *Rhodnius* tubules because the time required for the induction is longer than it is feasible to maintain *in vitro* tubules showing secretion of urate at significant rates. However, *in vitro* experiments with Malpighian tubules of the tsetse fly, *Glossina morsitans* (Fig. 7) showed a fairly steady rate of urate secretion for periods of more than an hour. These tubules therefore appeared suitable for direct *in vitro* measurements of the effects of an increase in bathing fluid urate concentration upon the rate of urate transport.

In vitro *induction of urate transport in Malpighian tubules of* Glossina

Tubules bathed in saline containing 0·1 mM urate showed a steady transport rate after 10 min (Fig. 8). In response to a step change in bath urate concentration from 0·1 mM urate to either 0·17 mM or 0·3 mM urate after 60 min, all tubules from unfed animals, or animals fed the previous day, increased their urate transport rates for 30–60 min after the change. For unfed animals (Fig. 8A, B) the mean increase in transport rate from t = 70 min to t = 90 min was 26 % in 0·17 mM urate and 35 % in 0·3 mM urate. For fed animals (Fig. 8C), the mean increase between t = 70 min and t = 90 min was 25 %. For both fed and unfed animals, paired *t*-tests showed that the differences were significant at the 5 % level.

These delayed increases are unlikely to reflect simply the time required for the establishment of a new steady state, because steady state transport rates in low (0·1 mM) urate concentrations are established in about 10 min (Fig. 8). This is partly because fluid secretion rates were sufficient to flush the luminal contents in less than a minute. The delayed response seems more likely to be due to an induction of urate transport following exposure of tubules to an increase in urate concentration. The apparent induction is a rapid effect; higher rates of transport are established within 30–60 min of the concentration increase. Fig. 8 also suggests that induction *in vivo* in response to feeding decays rapidly; animals fed the previous day do not show higher rates of urate transport in 0·1 mM urate.

Effects of obstructing the Malpighian tubules

Wigglesworth (1931*c*) studied the function of different segments of the Malpighian tubules by obstructing the tubules at different positions. After 12 h, uratic granules

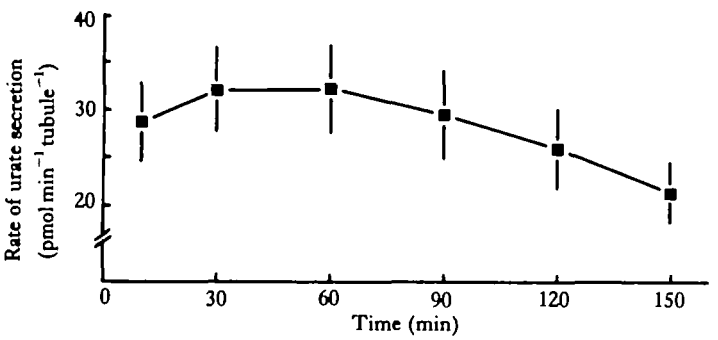

Fig. 7. *In vitro* urate secretion rates of *Glossina* Malpighian tubules, bathed in 0·55 mM urate. Secretion rates increase or are stable for longer periods of time than in *Rhodnius* tubules. $N = 9$.

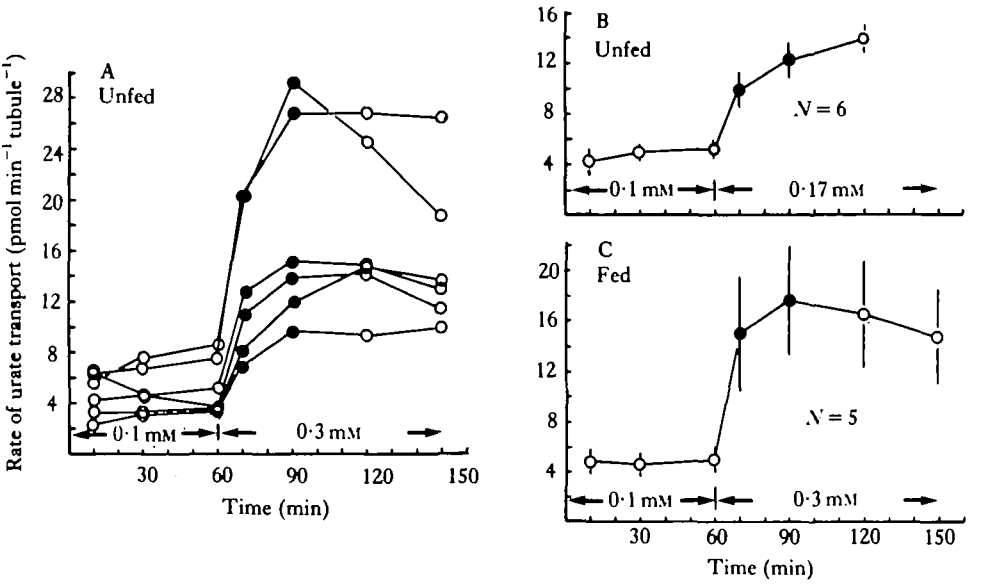

Fig. 8. *In vitro* rates of urate transport by *Glossina* Malpighian tubules in response to a change in bath urate concentration at t = 60 min. Bath urate concentrations are indicated above the time axis. In (A) values for individual tubules are presented. In (B) and (C), mean values (± S.E.) are given. Animals in (C) were fed the previous day.

were formed below a single ligature on the lower tubule, but not between two ligatures.

The latter result is incompatible with secretion of urate by the lower tubules; the following experiments were therefore done to determine the cause of this discrepancy. Within 3·5 h of feeding lower tubules were ligated with silk threads at either one or two positions along their length. The opening in the body cavity was covered with a thin sheet of polyethylene, which was held in place by the surface tension of the haemolymph between the sheet and the cuticle. The animals were dissected and examined 24–28 h later.

For tubules with one ligature, the results were similar to those of Wigglesworth (1931*c*). For 12 tubules from eight animals, uratic granules were present between the ligature and the hindgut in 11 cases; granules were present above the ligature in four cases, but in relatively small amounts.

For tubules ligated at two positions along their lower segment, a precipitate was found between the ligatures in 12 out of 18 animals. This result could only occur if the cells of the lower tubule secreted urate into the lumen, and differs from the results of Wigglesworth's (1931*c*) study, in which no precipitate was found between the ligatures. It is unlikely that disruption of tracheal supplies during dissection could prevent precipitation of urate between the ligatures. For four animals in which the posterior three spiracles on one side were blocked with wax 1–2 h after feeding, precipitate was observed in normal quantities in all tubules when dissected 22–23 h after feeding. A further possibility is that the ligatures in Wigglesworth's experiments may have been too close together. In our experiments, uric acid precipitation was found between ligatures which were, on average, 7·7 mm apart (range 2·5–12 mm

but such precipitation was not found between ligatures which were only 3·2 mm apart (range 1·6–8 mm).

Effects of varying pH on urate transport

The transport of an acidic species such as uric acid might be affected by the pH of the surrounding medium. In fact, urate secretion was not markedly dependent upon pH. When bathed in 0·033 mM urate at pH 6·3, tubules secreted $14{\cdot}2 \pm 1{\cdot}4$ ($N = 9$) pmol min^{-1} $tubule^{-1}$. At pH 7, and in the same concentration of urate, 9 pmol min^{-1} $tubule^{-1}$ was secreted. At a pH of 8·3, $7{\cdot}9 \pm 1{\cdot}4$ ($N = 9$) pmol min^{-1} $tubule^{-1}$ was secreted from a bath containing 0·023 mM urate; the corresponding rate at pH 7 was 6·3 pmol min^{-1} $tubule^{-1}$.

Comparisons with vertebrate urate transport

Composition of uratic granules in lower Malpighian tubules

The mean sodium/urate ratio was $0{\cdot}054 \pm 0{\cdot}015$ ($N = 10$), and the mean potassium/urate ratio was $0{\cdot}158 \pm 0{\cdot}030$ ($N = 10$). These values suggest that the majority of the urate is present as uric acid, because each molecule of a urate salt would contain two cations, in which case the sodium/urate ratio and/or potassium/urate ratio would be much higher.

Effects of ouabain

Mean secretion rate was $41{\cdot}5 \pm 4{\cdot}6$ pmol min^{-1} $tubule^{-1}$ ($N = 6$), when the tubules were bathed in enriched saline containing 0·11 mM urate and 10^{-4} M ouabain. For six control tubules from the same animals, the corresponding rate was $37{\cdot}9 \pm 4{\cdot}9$ pmol min^{-1} $tubule^{-1}$. A *t*-test indicated that the differences were not significant, and that ouabain did not, therefore, inhibit urate transport.

Site of the urate pump

If urate is actively transported across the basal membrane of the lower Malpighian tubule, and subsequently diffuses passively into the lumen, intracellular urate concentrations should be higher than expected from the electrochemical potential between the cell and the bathing medium. Estimation of intracellular urate concentration required a measurement of the volume of the cells of the lower Malpighian tubules. For this purpose, the lower tubules were bathed in saline containing a penetrant solute, [^{14}C]-labelled thiourea, and were also cannulated so that they could be perfused with the same solution. After 10 min, the solution in the cannula was replaced with thiourea-free saline, which was used to flush the luminal contents for 1–2 s. The tubule was then quickly pulled through three washes of complex saline (Maddrell & Gardiner, 1980) to remove any thiourea adhering to the external surfaces, and placed in a drop of distilled water. Radioactivity in the drop was determined by liquid scintillation spectrometry. By assuming equilibration of intracellular thiourea concentration with the bathing solution, lower tubule volume was calculated as $34{\cdot}2 \pm 5{\cdot}1$ nl ($N = 6$).

Using this volume estimate, the ratio of urate concentration in the lower tubule to that the bathing medium was determined. Cannulated tubules secreting [^{14}C]-urate

were flushed with urate-free saline and washed, as for thiourea. Concentrations intracellular urate were $0{\cdot}88 \pm 0{\cdot}18$ ($N = 9$) times those of the bath, which contained 0·094 mM-urate. The S/M ratio for tubules which had been secreting urate for 7–12 min in a comparable concentration was $5{\cdot}4 \pm 1{\cdot}2$ ($N = 8$).

These results indicated that intracellular urate concentrations were below those in the secretion, but not much less than the bath concentration. However, microelectrode potential measurements showed that the interior of the cells was negative with respect to the bathing medium, so that entry of the negatively charged urate ion into the cells is against an electrical gradient. In the lowermost one-third of the lower tubule, the intracellular potential was -14 ± 1 mV ($N = 9$). Potentials varied with position along the upper two-thirds of the lower tubule, so that the results were more variable; here the intracellular potential averaged 32 ± 9 mV negative, with respect to the bath. If the electrochemical potential difference between the bath and the cell interior were zero, the intracellular urate concentration should be in the range 30–60 % of that in the surrounding medium. Since the intracellular concentration is higher than this, it seems that urate ions must be transported actively into the cell with pumping sites on the basal cell membranes of the lower Malpighian tubules. Passive movement of urate from within the cell into the lumen is down an electrical gradient. Measurements of the transepithelial potential difference showed that the lumen was negative with respect to the bathing medium by an average of $17{\cdot}2 \pm 3{\cdot}4$ mV ($N = 10$). The potential step at the luminal face is thus between 0 and 15 mV, cell negative, which could account for a doubling of the luminal concentration above that of the cell. However, since luminal urate concentrations are 2–10 times those of the bath, there are likely to be additional pumping sites for urate on the luminal surfaces.

Urate secretion by other insects

To determine if active urate secretion is a common physiological mechanism in insects, S/M ratios for several other species were determined in bathing fluids containing 30–70 μmol l^{-1} radiolabelled urate. The mean values were: for larvae of a lepidopteran, the large cabbage white butterfly, *Pieris brassicae*, $1{\cdot}33 \pm 0{\cdot}13$ ($N = 6$); for an orthopteran, the desert locust, *Schistocerca gregaria*, $2{\cdot}8 \pm 0{\cdot}5$ ($N = 10$); for a dipteran, a tsetse fly, *Glossina morsitans*, $6{\cdot}4 \pm 2{\cdot}6$ ($N = 6$); and for a carnivorous dictyopteran, a Chinese praying mantis, *Tenodora sinensis*, $15{\cdot}8 \pm 0{\cdot}8$ ($N = 14$). Thin layer chromatography indicated that the secretion contained unchanged uric acid in all these animals. These results show that the Malpighian tubules of these insects can transport urate into the lumen against a concentration gradient. However, since in most insect Malpighian tubules the lumen is at a potential positive to the bathing medium (Ramsay, 1953; Gee, 1976), such transport is not necessarily active. However, the passive permeability of Malpighian tubule to substances of similar molecular size to uric acid is not high (Maddrell & Gardiner, 1974). It is likely, therefore, that urate transport in these insects is active as it is in *Rhodnius*.

For another dipteran, the blowfly, *Calliphora erythrocephala*, thin layer chromatography indicated that uric acid was converted to allantoin (as earlier discovered by Dr M. J. Berridge; personal communication). The ratio of the concentration

allantoin in the secreted fluid to the bath concentration of urate was 28·2 ± 3·8 (N = 22).

DISCUSSION

This study has shown active urate secretion by the lower Malpighian tubules of *Rhodnius*. In recently fed animals, *in vitro*, the rates of secretion are sufficient to account for the observed levels of urate excretion *in vivo*. Experiments with insects from other orders suggest that active secretion of urate by Malpighian tubules is a widespread phenomenon. In *Calliphora*, Malpighian tubules convert uric acid to a related nitrogenous compound, allantoin, which is then excreted.

Previous proposals for the mechanism of urate excretion in *Rhodnius* (Wigglesworth, 1931*a*,*b*,*c*) involved transport into the upper tubule, and subsequent precipitation in the lower tubule as a result of acidification and water removal. However, our results indicate that transport through the upper tubules is sufficient to account for, at most, only 1·5 % of the total urate excretion. Wigglesworth's proposal was based partly on the results of ligation experiments, which showed that uratic granules did not appear between two ligatures on a lower tubule. The discrepancy between these results and those described above may partly be accounted for by differences in the time between ligation and dissection; Wigglesworth dissected the tubules 12 h after ligation, whereas our results suggest that precipitation does not begin until 5 h after feeding, and therefore we allowed at least 20 h to elapse before dissection. As discussed above, a further possibility is that the ligatures in Wigglesworth's experiments may have been too close together.

An important advantage of active urate pumping by the lower tubules may be that urate elimination can be increased when necessary with little effect on the movements of other metabolites. If excretion involved passive movement into the tubule and subsequent precipitation, an increase in excretion would demand an increase in fluid secretion rate and/or an increase in the permeability of the tubule to urate. However, such an increase would produce accelerated movement of other molecules of similar size and charge; the animal would either lose these or be forced to reabsorb them actively.

In *Rhodnius*, the rate of urate transport increases after feeding, presumably because the early stages of digestion of the blood meal produce large amounts of nitrogenous waste. In *Glossina*, there appears to be a direct induction of the tubules in response to an increase in ambient urate concentration. The rapid onset and decay of induction in *Glossina* may be necessary in an animal which feeds at frequent intervals. Similar induction effects for the secretion of *p*-aminohippuric acid have been observed in *Rhodnius* (Maddrell & Gardiner, 1975).

Whilst both vertebrates (Dantzler & Braun, 1980) and insects actively secrete urate, the observed lack of effect of ouabain suggests that there are differences in the mechanism of urate transport in the two groups. Urate secretion by mammalian renal tubules is irreversibly inhibited by ouabain (Chonko, 1980). Another difference is that large quantities of sodium and potassium are present in the urate precipitate excreted by reptiles, whereas the precipitate in *Rhodnius* lower tubules appears to be free uric acid.

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