

## SECRETION OF HYPO-OSMOTIC FLUID BY THE LOWER MALPIGHIAN TUBULES OF *RHODNIUS PROLIXUS*

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### SUMMARY

1. The ability of *Rhodnius* to excrete an hypo-osmotic fluid at high rates can be attributed to the activity of the lower lengths of the Malpighian tubules.
2. In the presence of a stimulant (such as  $10^{-5}$  M 5-HT) they can absorb KCl from fluid in the lumen at rates as high as  $0.5 \mu\text{mol} \cdot \text{min}^{-1} \text{cm}^{-2}$ .
3. The solution absorbed is strongly hyperosmotic (900 mM KCl) so that the fluid in the lumen becomes hypo-osmotic (as low as 75 mOsm) and its flow is not much slowed.
4. The rate of absorption of KCl from the lumen depends on the potassium content of fluid there; the highest rates are achieved when K-rich fluid is run through the tubule at high speed from a cannula.
5. Rubidium ions are partly effective substitutes for potassium ions in the system, but sodium ions are not absorbed at measurable rates.
6. The wall of the lower tubule has a much lower permeability to *p*-amino-hippuric acid, xylose, sucrose and inulin than has the upper tubule - as expected in a system able to maintain a large osmotic concentration difference.

### INTRODUCTION

When the bloodsucking insect, *Rhodnius prolixus*, takes blood from a mammalian host it ingests up to twelve times its own weight of a fluid containing blood cells suspended in sodium-rich plasma which is distinctly hypo-osmotic to its own haemolymph. To reduce the bulk and concentrate the nutritious part of the meal and, in addition, prevent dilution of its haemolymph, *Rhodnius* rapidly excretes a markedly hypo-osmotic fluid of high-sodium content. The essential elements in this process are, first, the transport across the gut wall of a sodium chloride-based solution which is iso-osmotic with the blood meal (Farmer, 1975). Next, the upper, secretory parts of the Malpighian tubules, under the stimulus of a diuretic hormone, rapidly secrete a fluid which is iso-osmotic to the haemolymph but has a much higher potassium content and a somewhat lower sodium content (Maddrell, 1963, 1969). The fluid actually excreted by the insect, however, is considerably hypo-osmotic to the haemolymph, and has a potassium content similar to it (Maddrell, 1964). The situation is summarized in Fig. 1.

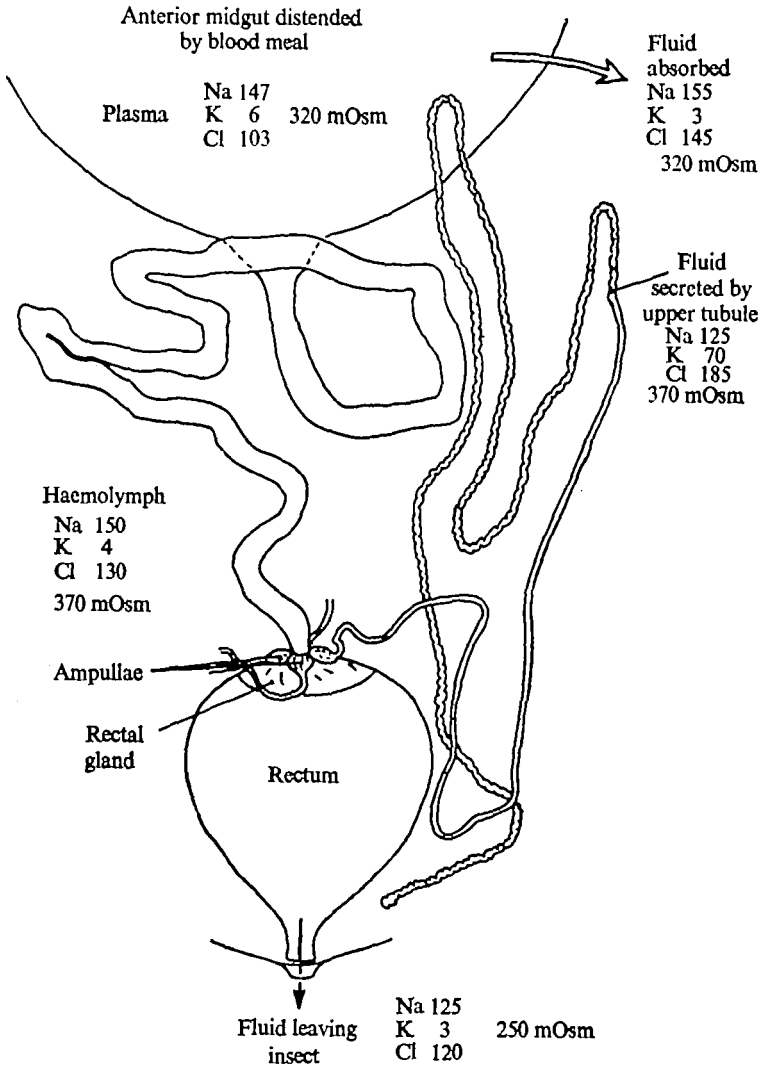


Fig. 1. The midgut, hindgut and one of the Malpighian tubules of *Rhodnius*. The figures show the composition of the fluids involved in the movement of water and ions from the lumen of the anterior midgut via the haemolymph and excretory system to the exterior.

Wigglesworth (1931*a, b*) in his work on excretion in *Rhodnius* found the urine of fed adults to be more or less iso-osmotic with the ingested blood (i.e. somewhat hypo-osmotic to the haemolymph) but although he looked at fluid movements in the Malpighian tubules this was in insects taken after the period of diuresis. Ramsay (1952) also showed that the urine of fed adults had a lowered osmotic concentration (the average value was 250 mOsm) but since he made no collections of fluid from the tubules of fed insect during diuresis he was not able to offer an explanation for the process.

This investigation was, thus, undertaken to answer two unresolved questions. First, where in its course from the upper parts of the Malpighian tubule is the K-rich

iso-osmotic fluid changed to a hypo-osmotic fluid of low K-content? Secondly, what mechanisms are involved in the modification of the fluid leaving the upper Malpighian tubules?

#### MATERIALS AND METHODS

##### *Insect material*

5th-stage larvae of *Rhodnius prolixus* from a laboratory culture maintained at 27 °C were used in all the experiments. The insects were used 1–3 weeks after the moult from 4th stage larvae.

##### *Experimental methods*

For critical determinations, osmotic concentrations of fluid samples were determined cryoscopically using a Kalber Nanolitre Osmometer (Clifton Technical Physics, New York). Where more rapid measurements of concentration were required samples of known volume (at least 50 nl.) of fluid were each diluted in 1 ml of glass distilled water in small polythene vials. From measurements of the conductivity of the water before and after addition of a sample of fluid and from the volume of the sample added the conductivity increase/ $\mu\text{l}$  of fluid added was ascertained. Conductivity was measured with a Beckman conductivity meter fitted with a CDC 114 NM probe. Since the fluids sampled were composed almost entirely of ions, conductivity measurements provide a reasonable measure of osmotic concentration. One factor affecting the results of such measurements is that K-rich samples give slightly higher increases in conductivity than do Na-rich samples so that where the most accurate measurements were required Na and K levels were also determined by conventional flame spectrophotometry.

To alter the rate at which fluid passed along the lumen of a length of Malpighian tubule, the tubule was perfused with fluid from a cannula inserted upstream of the length whose behaviour was being studied. Details of this technique are given in Maddrell, Gardiner, Pilcher & Reynolds (1974).

Measurements of the potential difference across the wall of a Malpighian tubule were made with two Agar-3 M KCl bridges (in P.E. 10 tubing) brought into contact one with the bathing medium and the other with secreted fluid emerging from the cut end of the tubule. The bridges were connected through calomel half electrodes to a Keithley 602 electrometer used to measure the potential difference. The asymmetry potential recorded with both bridges in the bathing medium was subtracted from transepithelial readings.

The basic Ringer solution used had the following composition: NaCl, 133 mM; KCl, 5 mM; MgCl<sub>2</sub>, 8.5 mM; CaCl<sub>2</sub>, 2 mM; NaH<sub>2</sub>PO<sub>4</sub>, 4.3 mM; NaHCO<sub>3</sub>, 10.2 mM and glucose, 34 mM. This solution has a pH of 6.7 and an osmotic concentration of 340 mOsm.

The technique for isolating Malpighian tubules into drops of Ringer solution under liquid paraffin so that their secretory behaviour can be observed is described in Maddrell (1969).

All experiments were done at temperatures in the range 23–25 °C.

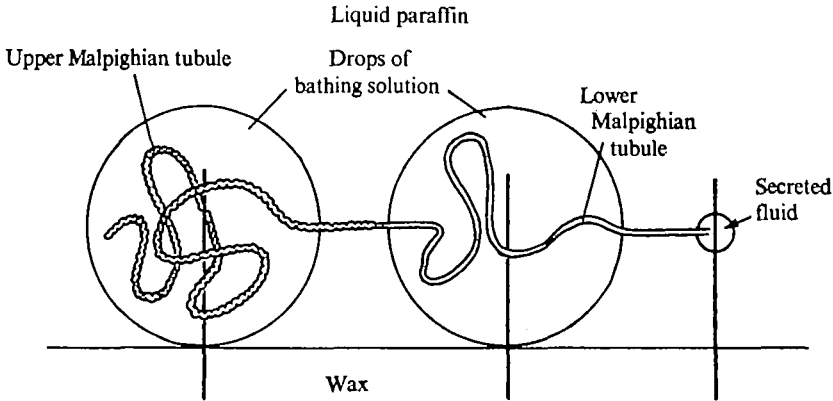


Fig. 2. The experimental arrangement involved in setting up a whole Malpighian tubule so that its upper and lower parts can separately be subjected to different treatments.

## RESULTS

### *The site of reduction in osmotic concentration*

Iso-osmotic fluid from the upper lengths of the Malpighian tubules passes next through the lower tubules which expose an extensive surface area to the fluid in the lumen; they are clearly well suited to produce the rapid reduction in osmotic concentration observed *in vivo*.

To test the possibility that these parts of the Malpighian tubules are involved, whole tubules (consisting of both upper and lower lengths) were isolated into Ringer solution to which was added 5-hydroxytryptamine to stimulate fluid secretion by the upper lengths of the tubules. Under such conditions the fluid emerging from the cut end of the Malpighian tubules had an osmotic concentration of  $251 \pm 5$  mOsm (mean  $\pm$  s.e. of observations made on 12 tubules). For comparison, the bathing Ringer's solution had an osmotic concentration of 340 mOsm as did fluid secreted by the upper lengths of Malpighian tubule bathed in the same solution. This reduction in osmotic concentration is very similar to that achieved *in vivo* indicating that the lower Malpighian tubule is capable, *on its own*, of bringing about the required lowering of osmotic concentration. One need not suppose, therefore, that the ampullae situated at the base of each tubule nor the rectum plays a large part in altering the osmotic concentration of the urine produced – at least during the diuretic period.

### *Stimulation of action of the lower Malpighian tubule*

To discover whether the lowering of osmotic concentration described above requires the presence of a stimulant, whole Malpighian tubules were isolated and arranged to run through two separate drops. One drop contained the upper length and the other the lower part of the tubule (Fig. 2). In this way the two different parts of the tubule could separately be treated with potential stimulants. When the upper length of the tubule was treated with its diuretic hormone (Maddrell, 1963) and the lower length bathed only by Ringer's solution, the fluid emerging from the tubule was still iso-osmotic. By contrast, if 5-HT was included in the medium bathing the lower tubule, the osmotic concentration of fluid passing through it rapidly fell. In over one

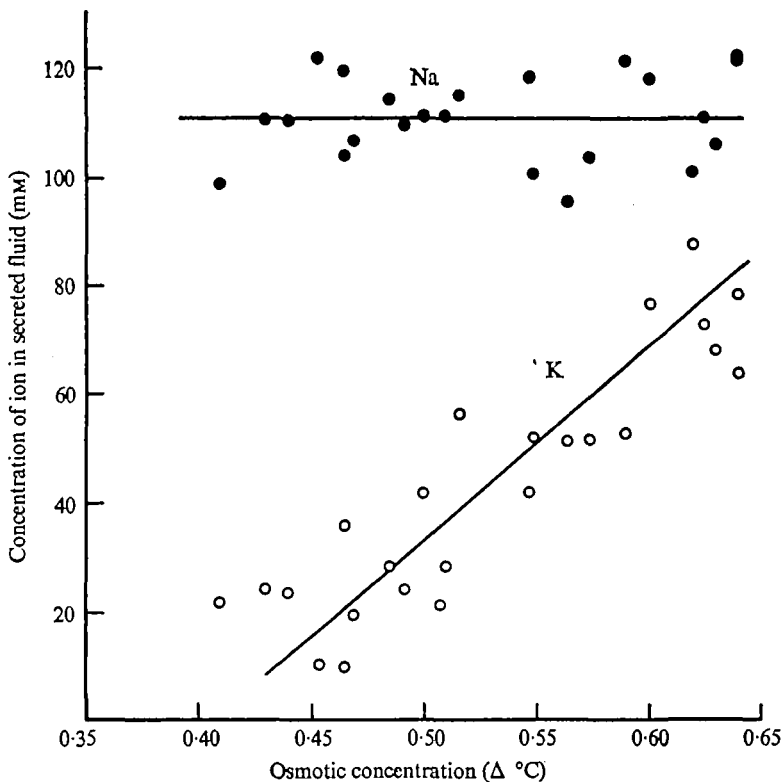


Fig. 3. The relation between the osmotic concentration and the Na and K levels in fluid secreted by whole Malpighian tubules. The lines through the points are linear regression lines fitted by the least squares method.

hundred trials the lower Malpighian tubule has not been observed to change the osmotic concentration of fluid passing through it except when it has been bathed in a medium containing 5-HT, or some other stimulant – (see Maddrell & Phillips, 1975). In all subsequent experiments, therefore, the lower tubule was routinely stimulated with  $5 \times 10^{-5}$  M-5-HT.

#### *Ionic changes involved in the reduction of osmotic concentration*

The fluid secreted by the upper Malpighian tubules differs markedly in ionic composition, especially in potassium concentration, from the urine voided by the insect during diuresis. When bathed in fluid of similar composition to the haemolymph the upper Malpighian tubules secrete a fluid containing around 70–80 mM K (Maddrell, 1969) as compared with the final urine concentration of about 4–5 mM K (Ramsay, 1952; Farmer, 1975). Accordingly, a series of measurements was made of the osmotic concentration and K and Na levels in fluid secreted by whole Malpighian tubules. By varying the length of the lower part of a tubule included in a preparation it was possible to vary the extent of the reduction in final osmotic concentration achieved. While the Na content seemed not to vary there was an obvious correlation between the osmotic concentration and the K content of the secreted fluid (Fig. 3), suggesting that the change in K concentration caused the osmotic pressure change. While it is

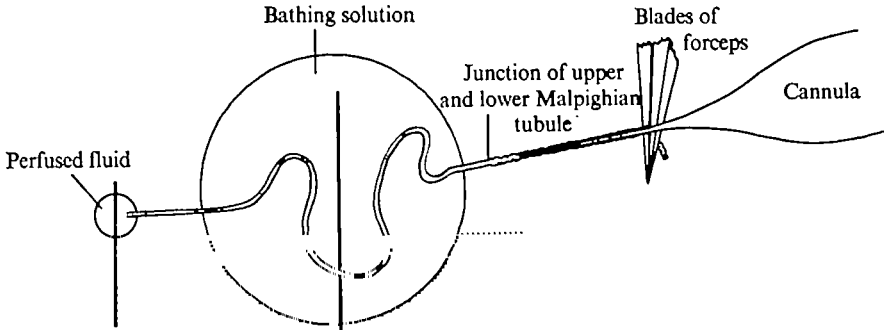


Fig. 4. The experimental arrangement involved in perfusing fluid through a lower Malpighian tubule via a cannula inserted into a short length of attached upper tubule.

likely that the reduction in K concentration resulted from reabsorption it could also have been achieved by movement of water and some sodium chloride into the fluid. The following experiments were designed to test this possibility.

*Water movements occurring during the production of hypo-osmotic fluid*

In each of these experiments a whole Malpighian tubule was taken and most of the upper part cut away. The remainder of the upper part was grasped in forceps held in a micromanipulator, and a cannula was thrust down its lumen. Potassium-rich fluid\* was perfused through the cannula at a constant rate from a syringe operated by a clock motor (Maddrell *et al.* 1974). The attached lower length of the tubule was then set up in a drop of normal Ringer's solution, the upper length being used for access to the lumen so as to achieve maximal exposure of the lower tubule to Ringer's solution (Fig. 4).

In each experiment, fluid was perfused for a while through the lower tubule which was bathed in Ringer's solution alone, drops of perfused fluid being collected from the end of the tubule at suitable intervals. The volume of fluid emerging from the tubule per minute was calculated from the diameter of the drops. 5-HT was then added to the bathing fluid and the new rate of fluid emergence determined. The tubule was then washed in stimulant-free solution and the rate of fluid emergence again calculated. Finally, the cannula was pulled out of the captive tubule and the rate of perfusion ascertained. The conductivity of all the drops was measured so that changes in osmotic concentration could be monitored. The results of one such experiment are summarized in Fig. 5, nine other experiments yielding essentially similar results, and show that in the absence of a stimulant there are no net movements of fluid across the tubule wall. However, while the lower tubule reduces the osmotic concentration of fluid passing along its lumen in response to 5-HT stimulation, there is a net movement of water from the lumen into the bathing solution. Clearly then the fall in osmotic concentration is caused by the removal of potassium (chloride) from the lumen and *not* by inward movements of water and sodium chloride.

\* This fluid contained 138 mM KCl; 8.5 mM MgCl<sub>2</sub>; 2 mM CaCl<sub>2</sub>; 10.2 mM NaHCO<sub>3</sub>; 4.3 mM NaH<sub>2</sub>PO<sub>4</sub> and 3.4 mM glucose.

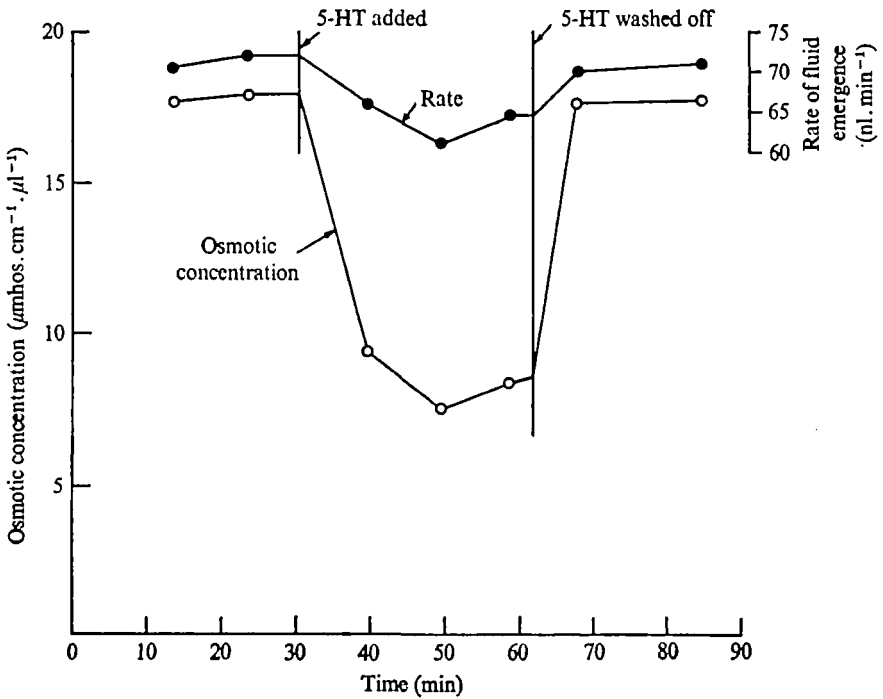


Fig. 5. 5-HT-induced changes in the rate of flow and osmotic concentration of fluid emerging from the cut end of a lower Malpighian tubule being perfused with K-rich fluid at a constant rate of 71 nl.min<sup>-1</sup>.

From the reduction in osmotic concentration and the water movement accompanying it one can calculate the osmotic concentration of the fluid crossing the tubule wall. Ten determinations carried out at fluid perfusion rates in the range 60–75 nl min<sup>-1</sup> gave an average figure for the osmotic concentration of this fluid of  $1760 \pm 140$  mOsm. If, as seems to be the case, this fluid comprises mainly potassium chloride ions in solution it must contain about 900 mM KCl. The average rate of fluid absorption was 6.13 nl.min<sup>-1</sup> which corresponds to a rate of KCl absorption of 5.38 nmol.min<sup>-1</sup>.

#### *Effects of changes in the ionic composition of the luminal fluid*

If, as seems likely from the results so far described, potassium reabsorption accounts for the lowering of osmotic concentration in fluid passing through a stimulated length of lower Malpighian tubule, then the extent of the lowering in osmotic concentration should be dictated by and be dependent on the potassium content of the fluid entering the tubule. To test this, K-free fluid was run through the lower tubule either from a cannula or from an attached length of upper tubule bathed in a K-free solution. A total of eight experiments all showed that the lower tubule did not alter the osmotic concentration of such a fluid. As a test of the other extreme, K-rich fluid was run through lower tubules either through a cannula or from their own upper tubules bathed this time in a Na-free solution. Under these conditions very extensive K reabsorption occurred, the osmotic concentration of the fluid falling to the lowest values recorded: as low as 75 mOsm in some experiments. This compares with the

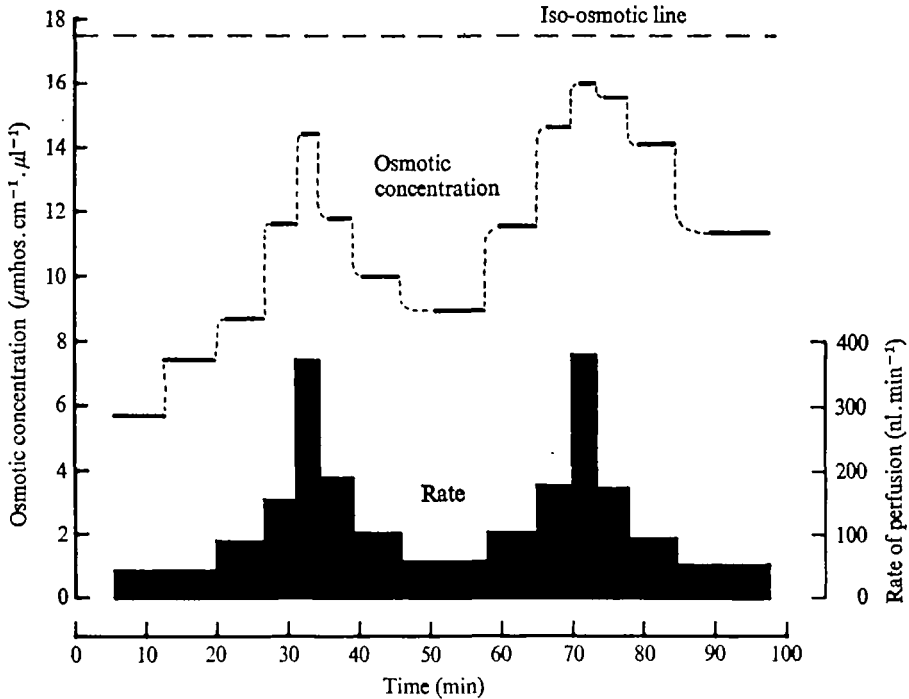


Fig. 6. Changes in the osmotic concentration of fluid emerging from the cut end of a lower Malpighian tubule in response to changes in the rate at which it was perfused with K-rich fluid.

figure of 250 mOsm for fluid containing 60–70 mM K as it enters the lower tubule (p. 674).

The very highest rates of lowering of osmotic concentration were shown in tubules perfused at high rates with K-rich fluid (Fig. 9). Under these conditions fluid passed so rapidly down the lumen that its K content, although subject to intense reabsorption, remained higher than in tubules which were perfused more slowly.

All these results lead to the same conclusion that the ability of the lower Malpighian tubule to reduce the osmotic concentration of fluid passing along its lumen depends on the potassium concentration of the fluid. One may conclude that it is the reabsorption of potassium (chloride) which causes the reduction in osmotic concentration.

#### *Effects of changes in rate of flow of fluid through the lower Malpighian tubule*

To measure the capacity of the lower Malpighian tubule to change the osmotic concentration of fluid going through it, long lengths of lower tubule were perfused with K-rich fluid (containing 138 mM K and 14 mM Na) at rates which were changed every few minutes. After each change in rate enough fluid was allowed to flow through the tubule to wash out all that which had entered the tubule at the previous rate of flow. A sample was then collected during a measured time interval so that the rate of perfusion could be calculated, the conductivity of the sample being also measured to find out the reduction in its osmotic concentration. Such experiments showed that the lower Malpighian tubule is capable of causing a significant change in the osmotic concentration of fluid passing through it even at rates of flow close to 400 nl. min<sup>-1</sup> (Fig. 6). I



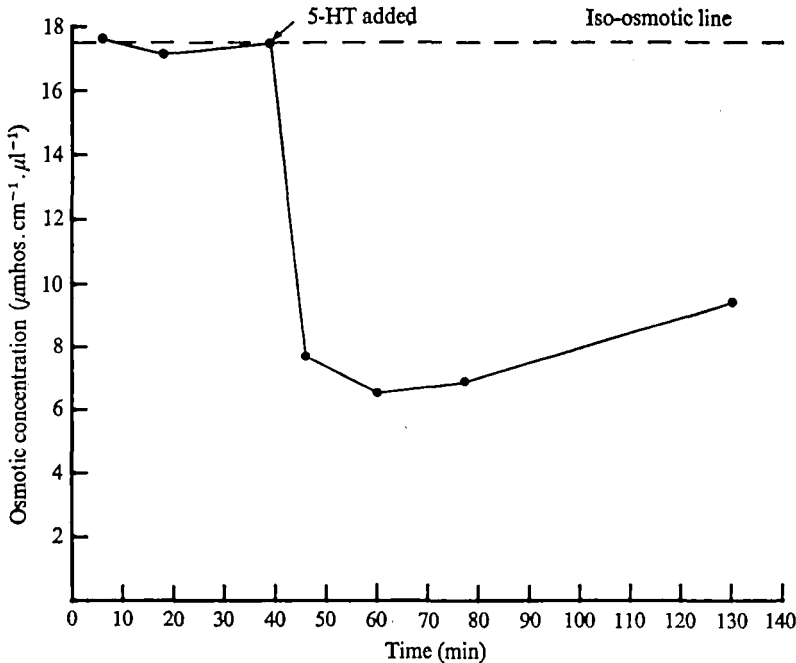


Fig. 7. The osmotic concentration of fluid emerging from the cut end of a lower Malpighian tubule while being perfused with K-rich fluid at  $60 \text{ nl. min}^{-1}$ .

*in vivo* the rate of flow through the lower tubule is only about  $150 \text{ nl. min}^{-1}$  (Maddrell, 1964).

The activity of the lower tubule appears to decline fairly rapidly with time (Fig. 6). This change is partly attributable to the effects of very high rates of perfusion, as tubules perfused at more normal rates are better able to maintain their activity (Fig. 7).

Several such experiments gave essentially similar results to those shown in Fig. 6. The effect that changes in perfusion rate have on the osmotic concentration of the emergent fluid are summarized in Fig. 8. The rate of KCl absorption can be calculated from the rate of perfusion and the change in osmotic concentration. Fig. 9 shows how this rate is related to the osmotic concentration of the emerging fluid, the highest values being attained at the highest rates of perfusion, when the osmotic concentration of the perfused fluid is not much reduced by the time it emerges.

#### *Substitution of K by Rb*

In many potassium-transporting systems rubidium ions are an effective substitute for  $\text{K}^+$  ions (cf. Harvey & Wood, 1973). To discover whether this also applies to the lower Malpighian tubules of *Rhodnius* a series was perfused either with a K-based solution, containing  $138 \text{ mM K}$  and  $14 \text{ mM Na}$ , or with a Rb-based solution, containing  $138 \text{ mM Rb}$  and  $14 \text{ mM Na}$ . The osmotic concentration of the fluid leaving the tubule was  $236 \pm 13 \text{ mOsm}$  during perfusion with Rb-rich fluid and  $136 \pm 6 \text{ mOsm}$  during K perfusion. So although the lower tubule still reduced the osmotic concentration of an Rb-rich solution this cation acted as only a partial substitute for K in this system.

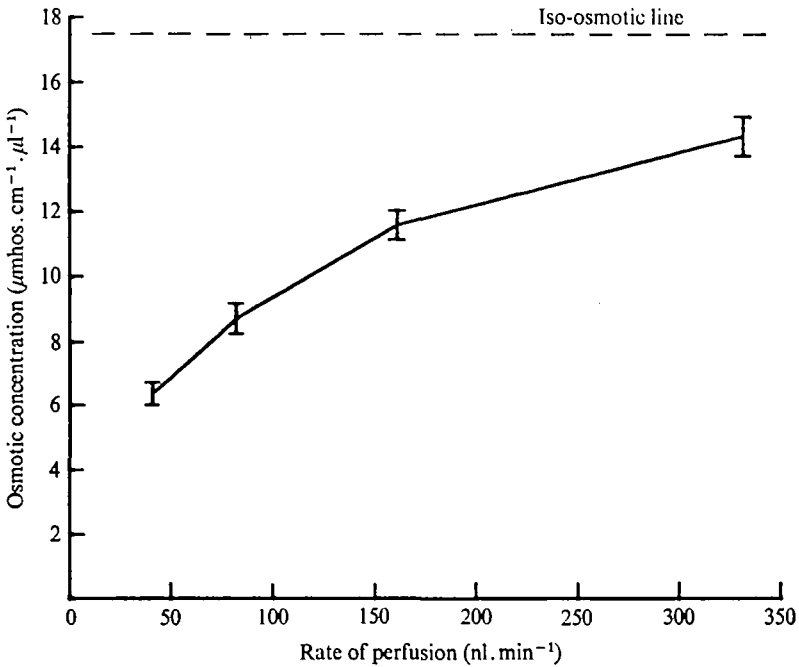


Fig. 8. The osmotic concentration of initially K-rich fluid after perfusion through a lower Malpighian tubule, as a function of the rate of perfusion. The vertical lines attached to the points represent the extent of the standard error of the mean of several determinations.

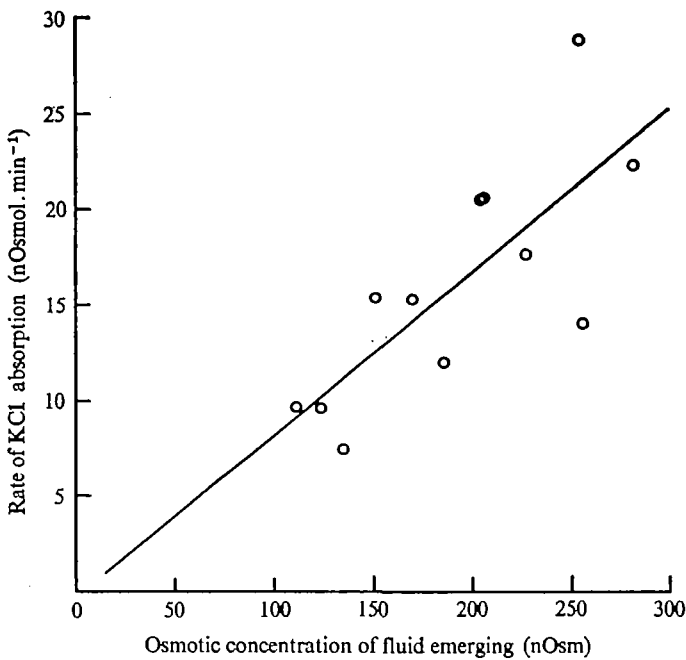


Fig. 9. The relationship between the rate of KCl absorption by lower Malpighian tubules and the emergent osmotic concentration of initially K-rich fluid perfused through it. The line drawn through the points is the linear regression line fitted by the least squares method.

*The permeability of the lower Malpighian tubule wall*

Any epithelium which produces or maintains an osmotic gradient across itself must be relatively impermeable or osmotic equilibration would rapidly occur. The lower Malpighian tubule in *Rhodnius* can, in extreme cases, reduce the osmotic concentration of fluid passing along it by a factor of 5 and might reasonably be presumed to be relatively impermeable. To test this possibility lengths of lower Malpighian tubule were bathed in normal Ringer's solution containing 5-HT and one of a series of labelled radioactive organic substances. We determined the rate of appearance of label in the fluid passing through the lower tubule, from a cannula or from its own upper tubule bathed in a separate, unlabelled, drop of solution. From the length of the lower tubule and its rate of perfusion, the permeability of the tubule wall was calculated and compared with values for the wall of the upper tubule (Maddrell & Gardiner, 1974; Maddrell *et al.* 1974). In these experiments the permeability of the lower tubule to *p*-aminohippuric acid (PAH), xylose, sucrose and inulin was as follows: for PAH ( $n = 30$ ),  $0.049 \pm 0.008$  nl.mm<sup>-2</sup>.min<sup>-1</sup> (upper tubule  $1.51$  nl.mm<sup>-2</sup>.min<sup>-1</sup>); for xylose ( $n = 52$ ),  $0.152 \pm 0.015$  nl.mm<sup>-2</sup>.min<sup>-1</sup> (upper tubule  $2.8$  nl.mm<sup>-2</sup>.min<sup>-1</sup>); for sucrose, ( $n = 27$ ),  $0.036 \pm 0.006$  nl.mm<sup>-2</sup>.min<sup>-1</sup> (upper tubule  $0.39$  nl.mm<sup>-2</sup>.min<sup>-1</sup>) and for inulin ( $n = 8$ ),  $0.0068 \pm 0.0006$  nl.mm<sup>-2</sup>.min<sup>-1</sup> (upper tubule  $0.050$  nl.mm<sup>-2</sup>.min<sup>-1</sup>). These results show that the lower tubule is up to thirty times less permeable to the test substances than is the upper tubule.

## DISCUSSION

The results show that, in diuresis, *Rhodnius* excretes an hypo-osmotic fluid by a process which involves cycling of potassium and chloride ions. In the upper Malpighian tubule, sodium, potassium and chloride ions move into the lumen each accompanied by about 150 water molecules; that is the fluid secreted is iso-osmotic. In the lower tubule, potassium and chloride ions move out of the lumen each accompanied by only about 30 water molecules, so the fluid left in the lumen of the tubule becomes hypo-osmotic and its content of potassium is greatly reduced.

It is, perhaps, surprising that potassium ions should play such a key role in this process. The ingested plasma contains little potassium, and the haemolymph very little more, so that the commitment of potassium ions to the flow of excretory fluid would seem to be inappropriate. Such an apparently paradoxical use of potassium ions also occurs in other insects (Ramsay, 1953). A possible explanation is that insects may primitively have been plant-feeders and as such have long been faced with the problem of excess potassium in their diet. The excretory system may thus retain a dependence on potassium secretion even when it is no longer so appropriate. An interesting exception is found in the tsetse fly, *Glossina austeni*, which faces much the same problems as *Rhodnius*, yet has Malpighian tubules which secrete sodium-rich fluid with potassium ions being very little involved (Gee, 1974).

The production of an hypo-osmotic fluid by first secreting an iso-osmotic one from which ions are recovered without osmotically compensating amounts of water closely parallels the mechanism of absorption of water by insect recta, where ion cycling is also a key element in the process (Wall, Oschman & Schmidt-Nielsen, 1970; Maddrell, 1971).

That selective resorption, of potassium chloride in this case, should occur in the Malpighian tubules and not, as is more often the case, in the hindgut requires explanation. One important factor may be that only the lower Malpighian tubules have a surface area/volume ratio large enough to allow the rapid movements of potassium and chloride out of the fluid secreted by the upper tubules. None of this is to deny the ampullae and rectal gland (Fig. 1) any function; they are clearly important in the more leisurely activities of the system after diuresis, when the osmotic and potassium concentrations of the excreted fluid rises steeply (Ramsay, 1952).

Epithelia which secrete iso-osmotic fluids are characterized by extensive close packed microvilli or foldings (Diamond, 1971; Berridge & Oschman, 1972) and the upper Malpighian tubules of *Rhodnius* conform to this stereotype. By contrast, the lower Malpighian tubules of *Rhodnius* which are now shown to transport a hyperosmotic fluid have a luminal surface characterized by relatively short microvilli which are more widely separated from one another (Wigglesworth & Salpeter, 1962). Such an arrangement would be expected to minimize the standing osmotic gradients which encourage iso-osmotic flow (Diamond, 1971) and allow ions to move with relatively fewer water molecules accompanying them. In short, the ultrastructure of the lower Malpighian tubule is suited to its function.

Since the function of the lower Malpighian tubule during diuresis results in a difference in osmotic concentration across its wall, which may reach 270 mOsm *in vitro*, the wall must have a relatively low permeability. That this is indeed the case is shown by the lower rates of penetration through the wall of several organic substances.

Finally, the speed of operation of the system should be emphasized. *In vivo*, fluid flows through the lower tubule at 150 nl.min<sup>-1</sup>, and this is subject to reabsorption for only 30 sec and yet in this time its osmotic concentration falls from 360 mOsm to 240 mOsm, a rate of change of 4 mOsm.sec<sup>-1</sup>. Its potassium concentration falls from 70 mM to around 4 mM at a rate of 2.2 mM.sec<sup>-1</sup>. *In vitro*, when perfused with a K-rich fluid, absorption of KCl can cause a drop in osmotic concentration at a rate as high as 8 mOsm.sec<sup>-1</sup> with the potassium concentration falling at about 4.5 mM.sec<sup>-1</sup>.

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