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JEB CLASSICS

ACTIVE TRANSPORT OF WATER BY INSECT MALPIGHIAN TUBULES



Simon Maddrell writes about J. A. Ramsay's 1954 publication 'Active transport of water by the Malpighian tubules of the stick insect *Dixippus morosus* (Orthoptera; Phasmidae).' A pdf file of Ramsay's paper can be accessed as supplemental data at jeb.biologists.org

Through most of his career, Arthur Ramsay was fascinated by matters osmotic. In the period after the Second World War, he worked on osmotic relations of the earthworm (Ramsay, 1949a), developing typically novel methods for measuring the melting-point and sodium content of minute quantities of fluids (Ramsay, 1949b, 1950). However, in the early 1950s, Ramsay started working on insect Malpighian tubules. He soon found that the tubules secreted potassium ions into the lumen against an electrochemical gradient, showing that this transport was an active one. This led Ramsay to the attractive idea that "the secretion of potassium (together with some anion) into the tubule will set up an osmotic pressure, which in its turn will promote a passive inward diffusion of water" (Ramsay, 1954). According to this idea "the secretion of potassium is the prime mover in generating the flow of urine; and if the theory is true it follows that the osmotic pressure of the urine should be equal to or greater than, but never less than, the osmotic pressure of the haemolymph" (Ramsay, 1954). However, in the classic paper that is the subject of the present comments, he provided evidence that he thought destroyed this simple idea.

Although we now believe him to have been wrong about this, his paper (Ramsay, 1954) is nonetheless held as a classic publication, as it describes his most novel and powerful technique with which he will always be associated.

In the paper, Ramsay tested whether the establishment of hyper-osmotic conditions in the lumen of Malpighian tubules might cause osmotic entry of water. He found that the osmotic pressure of the fluid secreted by isolated Malpighian tubules of the stick insect *Dixippus* (now *Carausius*) *morosus* was, if anything, slightly but significantly **hypo**-osmotic to the experimental bathing fluid. He believed that this made his hypothesis untenable. He did, however, point out that his results could be explained if hyperosmotic fluid were to be transported into the tubule in one region and solutes reabsorbed in another, which we now know to be the case. He observed that this argument could not be refuted on the evidence then available, but argued that active transport of water was the simplest explanation. "The onus of disproof rests upon the opponents of this view", he concluded, rather characteristically!

In fact, 50 years on, we are confident that his earlier theory was entirely correct and that fluid secretion does indeed depend on potassium transport, albeit achieved by a complex of membrane proteins in which proton transport by the ubiquitous V-ATPase is coupled with an antiporter that exchanges H^+ for K^+ (Maddrell and O'Donnell, 1992; Beyenbach, 2003). As Ramsay supposed, this potassium transport leads to anions flowing down their electrochemical potential gradient with water movements being secondary to this transport of ions. The lowered osmotic concentration of the fluid secreted by the tubules of *Dixippus* (*Carausius*), which caused Ramsay to conclude that water movement driven by potassium transport could not be correct, we now suppose to be explained by active reabsorption of solutes, probably potassium plus anion. Just such a system is found in the production of hypo-osmotic fluid by tubules from the blood-sucking insect, *Rhodnius prolixus* (Maddrell and Phillips, 1975) and tubules from several other insects behave similarly (for example, see Spring and Hazelton, 1987; Marshall et al., 1993; O'Donnell and Maddrell, 1995).

So how is it that Ramsay's paper is still so widely quoted, probably more often now even than in the first few years after it was written, even though its main conclusion is almost certainly wrong?

The answer is that the impact of the paper has been, not in its attempt to find out how water movement is achieved, but in the technique Ramsay developed to allow him to isolate the Malpighian tubules from the stick insect, keep them alive for some hours, and observe them secreting fluid. This technique, modified over the years but in essence the same as he devised, has been and still is very widely used to study not only Malpighian tubules but other fluid-secreting tubules, such as fly salivary glands (Berridge and Patel, 1968). The technique in its essentials is shown in Fig. 1, taken from his classic paper. The key points are the use of a container, a watch-glass in Ramsay's original version, with a hydrophilic surface; he used 'varnish'. On this was poured a layer of liquid paraffin (mineral oil) deep enough to cover a drop of fluid, originally haemolymph (blood) from the insect, in which a Malpighian tubule could be placed. The cut end of the tubule could then be pulled out and held outside the drop, in Ramsay's hands by a fine silk thread tied round the cut end. He then would cut the tubule near the ligature (very likely with a pair of ultrafine scissors that he used to make from tiny electrolytically sharpened tungsten wires brazed to two steel plates, joined at their farther ends) so that fluid secreted by the tubule would emerge from the cut and be held there with no tendency to run back into the drop of fluid bathing the main part of the tubule.

Ramsay began the Summary of his paper by stating "1) *Single Malpighian tubules of the stick insect have been studied as preparations isolated in drops of haemolymph under liquid paraffin.*" It is this part of the paper that has so effectively

survived and had such a major effect on the world of insect fluid-secreting tubules. Indeed a very recent paper (Beyenbach, 2003) contains as part of its first figure a representation of technique instantly recognizable from Ramsay's original. The technique is engagingly simple and elegant and, using it, one can often see within seconds whether an experiment, say with a suspected stimulant, has worked.

The technique has now been modified so as to allow easier observation and handling of the secreted fluid. So the floor of the container is now usually paraffin wax or silicone with a series of depressions in it so that many tubules can be studied at once without the tendency of the droplets of a bathing fluid to coalesce. For example up to 20 tubules from adult *Drosophila* can be conveniently studied at once. The transparent floor allows the tubules to be observed by transmitted light. The cut ends of the tubules are today usually looped over small metal or glass pins stuck in the wax or silicone to which they adhere. Secreted drops are either dislodged with very fine glass rods or sucked off with a Gilson pipette and deposited on the container floor. Their volumes are calculated just as Ramsay did by observing their diameters with a micrometer eyepiece in the viewing binocular microscope. He assumed the droplets resting on the floor of the container to be spherical, as we do now, but he thought this was manifestly untrue. However, tests of this, using radioactive counting of droplets of known radioactive content, have in fact showed that the droplets are indeed almost perfectly spherical, provided they are smaller than about 1 μl . This may be due to the use of relatively high-density liquid

paraffin, which means that the droplets are more nearly neutrally buoyant and so made spherical by surface tension, a powerful force when the droplets are small. Ramsay felt it necessary to hold a small bubble of oxygen against the bathing drop so as to supply the tubule with this gas. No-one now does this. It seems that the surface area/volume ratio of a Malpighian tubule is so large that oxygen dissolved in the bathing drop has easy access to the tubule and supports secretion relatively unchecked. However, rather larger drops of fluid are used to bathe an isolated tubule so that the oxygen demands of the tubule do not exhaust the oxygen content of the drop. In any case, oxygen diffuses through the liquid paraffin surrounding the bathing drop and the tubule in it. Ramsay was impeded in his research by the need to include some haemolymph in the fluid that bathed the isolated tubules. Other tubules, it turned out, required no such special treatment; the tubules of *Rhodnius* would secrete for hours in a simple saline containing only glucose as an energy supply, indeed they would secrete at 35% of the normal rate in a solution of ammonium nitrate plus glucose, containing no potassium, sodium or chloride (Maddrell, 1969). Ironically, it has emerged that many Malpighian tubules will only secrete normally when bathed in a fluid containing amino acids, particularly glutamine, glutamate or aspartate, possibly because they function as compatible intracellular osmolytes that are necessary for sustained secretion at high rates by the Malpighian tubules (Hazel et al., 2003). The irony derives from Ramsay's development of a dissecting fluid which indeed contained glutamate, histidine and glycine, any of which, it is now known, will support rapid fluid secretion when added to a simple salt-based saline (Hazel et al., 2003). He supposed that the composition of his "dissecting fluid should be put on record, though it has no special merits to recommend it"!

The major effect of Ramsay's paper is that it has allowed a whole field of investigation to be opened up, in which fluid-secreting tubules can be studied in isolation. In early days, studies were made of the effects of stimulants, such as hormones, on the rates of fluid secretion and the effects of salines with different concentrations of the various physiologically important ions. Later, it was possible to measure *trans*-epithelial potential differences merely by placing electrodes in the bathing drop and the droplet of secreted fluid (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001), though this method cannot be used

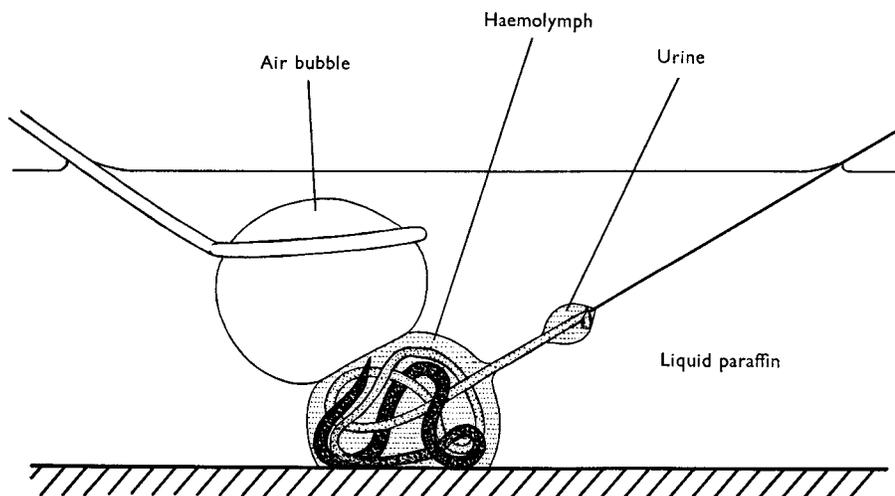


Fig. 1. Reproduced from Ramsay's 1954 paper. This figure shows the essentials of Ramsay's method, which is still in use today.

to study tubules with too narrow a lumen (Aneshansley et al., 1988). It has been used to study the effects of genetically modifying the relative proportions of the different cell types (Denholm et al., 2003). It is very pleasing that such a simple, elegant and powerful technique has survived close to 50 years essentially without change.

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ACTIVE TRANSPORT OF WATER BY THE MALPIGHIAN
TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS*
(ORTHOPTERA, PHASMIDAE)

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INTRODUCTION

In a recent study of excretion in insects (Ramsay, 1953 *b*) it has been shown that in eight different genera potassium ion is actively transported across the wall of the Malpighian tubule from haemolymph to urine against an electro-chemical gradient. It is suggested that this process is likely to be of general occurrence among insects. In two of these genera it has further been shown (Ramsay, 1952, 1953 *a*) that some of the potassium secreted into the urine is later reabsorbed into the haemolymph, so that there is a circulation of potassium within the insect's body; this again may well prove to be of general occurrence.

One is tempted to see this circulation as something which is fundamental to the process of urine formation in Malpighian tubules. It is easy to imagine that the secretion of potassium (together with some anion) into the tubule will set up an osmotic pressure which in its turn will promote a passive inward diffusion of water. According to this theory the secretion of potassium is, as it were, the prime mover in generating the flow of urine; and if the theory is true it follows that the osmotic pressure of the urine (O.P._u) should be equal to or greater than, but never less than, the osmotic pressure of the haemolymph (O.P._h).

Measurements of osmotic pressure reported in earlier work (Ramsay, 1950, 1951, 1952) show that, although the urine and the haemolymph are more or less isotonic, in certain cases $O.P._u < O.P._h$ by a small but apparently significant amount. These measurements taken at their face value are sufficient in themselves to disprove the theory; but before they are accepted it is necessary to point out that the conditions under which the fluids were collected were not entirely satisfactory where small differences of osmotic pressure are in question. In some experiments the insect was opened in a stream of moist air and some slight evaporation or condensation may have occurred. The circulation was seriously impaired as a result of the operation, and it is not impossible that haemolymph was collected from one region exposed to the air while the main part of the tubule lay in another region of the body unaffected by exposure. In other experiments haemolymph was obtained by tearing open the body of a small larva with gross damage to the tissues.

The present experiments were planned to avoid these disadvantages by making use of single Malpighian tubules removed from the body of the insect and set up in drops of haemolymph under liquid paraffin. In this way it is possible to ensure

that the sample taken is representative of the haemolymph surrounding the tubule, and differences in osmotic pressure can be interpreted with more confidence.

MATERIAL AND METHODS

The Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae), are particularly suitable for study. De Sinéty (1901) recognized three kinds of tubules. (1) 'Superior' tubules, opening at the annulus between midgut and hindgut, making a short forward loop and running back to end blindly close to the posterior region of the hindgut. The 'superior' tubules are of fairly uniform appearance throughout their length and are supplied by small branches of the tracheal system reaching them at various points. They are of relatively large diameter and the cells are transparent. (2) 'Inferior' tubules, opening at the annulus in pairs and running directly backwards. Their distal portions are dilated and filled with a milky fluid and they terminate in clumps of cells (cells of Sidorot) embedded in the fat body. Each tubule has its own trachea which accompanies it over most of its length. Over their non-dilated proximal portions the 'inferior' tubules have the same appearance as the 'superior' tubules. (3) 'Appendices of the midgut.' These appear to be very thin tubules opening separately into the midgut and running back over the hindgut.

De Sinéty further investigated the dilatations of the 'inferior' tubules and came to the conclusion that their contents were the carbonates of calcium and magnesium which accumulated in these dilatations throughout the insect's life. From these observations and from comparison of the superior and inferior tubules under the microscope it seems likely that the proximal portion of the 'inferior' tubule is a region where urine is produced, whereas the distal region is a 'kidney of accumulation' where alkaline earth carbonates are precipitated and fluid is reabsorbed. Some further evidence bearing upon this point was obtained during the course of the present work; it was found that potassium, secreted into the tubule in the proximal portion, passed back into the haemolymph in the distal portion. However, this circulation within the tubule is relatively slow, as is shown by the use of phenol red which is rapidly concentrated in the lumen of the proximal portion but only slowly moves into the distal portion. It seems that by far the greater part of the urine passes down the tubule and into the gut.

Both the 'superior' and 'inferior' tubules can be used in these investigations, but for purposes of removal from the body the 'inferior' tubules have the advantages of lying superficially and of being without many fine tracheal connexions. As will be pointed out in the next paragraph the speed with which dissection can be completed is an important consideration. For all the experiments reported in this paper 'inferior' tubules were used, either entire or with the distal portion ligatured off and cut away.

While it is not impossible to dissect out a Malpighian tubule from the body of a stick insect opened in air, the dissection is very much easier and quicker with the insect under saline. Unfortunately, the Malpighian tubules are very sensitive to

the composition of the medium in which they are immersed, and whereas other insect tissues survive well in physiological saline the Malpighian tubules show abnormal changes after a relatively short time (Wigglesworth, 1953). As described in the Appendix a dissecting fluid was made up in which the tubules could survive for a few hours as judged by their ability to concentrate phenol red. In practice the dissection could be completed in 20 min., after which the tubules were returned to haemolymph as described below.

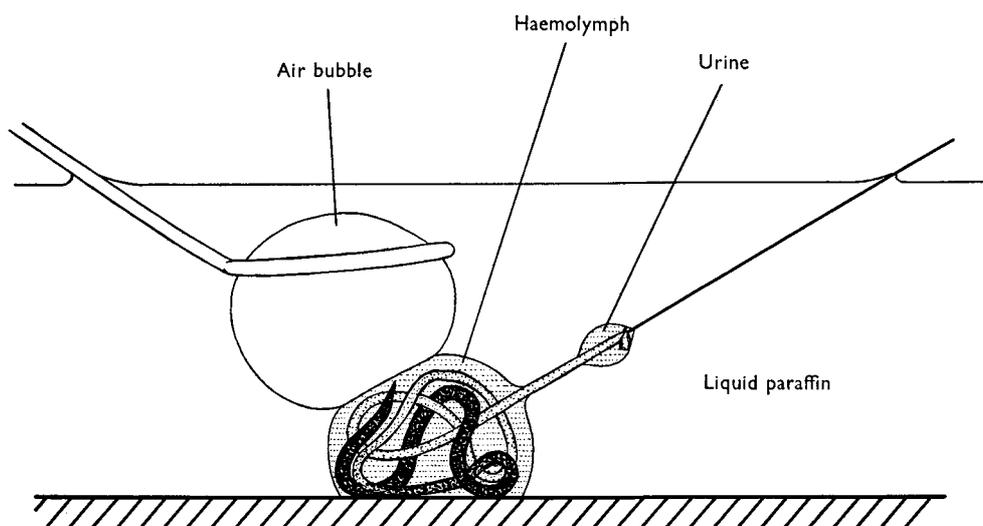


Fig. 1. For explanation see text.

The insect was pinned down ventral surface uppermost in a small dissecting dish. The abdomen was opened and as much haemolymph as possible was collected and stored under liquid paraffin in a varnished watch-glass. Dissecting fluid was then added to cover the insect and some half-dozen tubules were separated and cut free close to their openings into the gut. These tubules were removed by means of a pipette each to a separate varnished watch-glass containing liquid paraffin; here the dissecting fluid was sucked away and replaced with a drop of haemolymph. Tubules prepared in this way have remained alive for as much as 48 hr., exhibiting characteristic writhing movements and being able to concentrate phenol red.

Urine was collected in either of two ways: (1) the cut end of the tubule was seized with forceps and a pipette was inserted as described previously (Ramsay, 1953*b*), or (2) a fine silk thread was tied around the severed end of the tubule which was then drawn a short distance out of the drop of haemolymph and a cut was made close to the ligature to allow the urine to escape; the urine accumulated as a droplet around the ligature and did not tend to run back into the drop of haemolymph. Oxygen was provided from a bubble of air which was held in a loop of wire and pressed against the drop of haemolymph. The arrangement is shown in Fig. 1.

Osmotic pressure was determined by the depression of freezing-point method previously described (Ramsay, 1949), and is expressed as that concentration of

NaCl, in millimoles per litre of solution, which has the same freezing-point depression. Since the highest accuracy was called for, all (or nearly all) determinations were made in quadruplicate. The standard error of the mean of four determinations by this method is less than ± 0.7 mM/l. NaCl.

RESULTS

(a) *Relation between $O.P._h$ and $O.P._u$.* In the first series of experiments urine production was studied on single tubules each enclosed in a relatively large (4-5 cu.mm.) drop of haemolymph, it being assumed that the osmotic pressure of the haemolymph would not be significantly altered by the relatively small amount

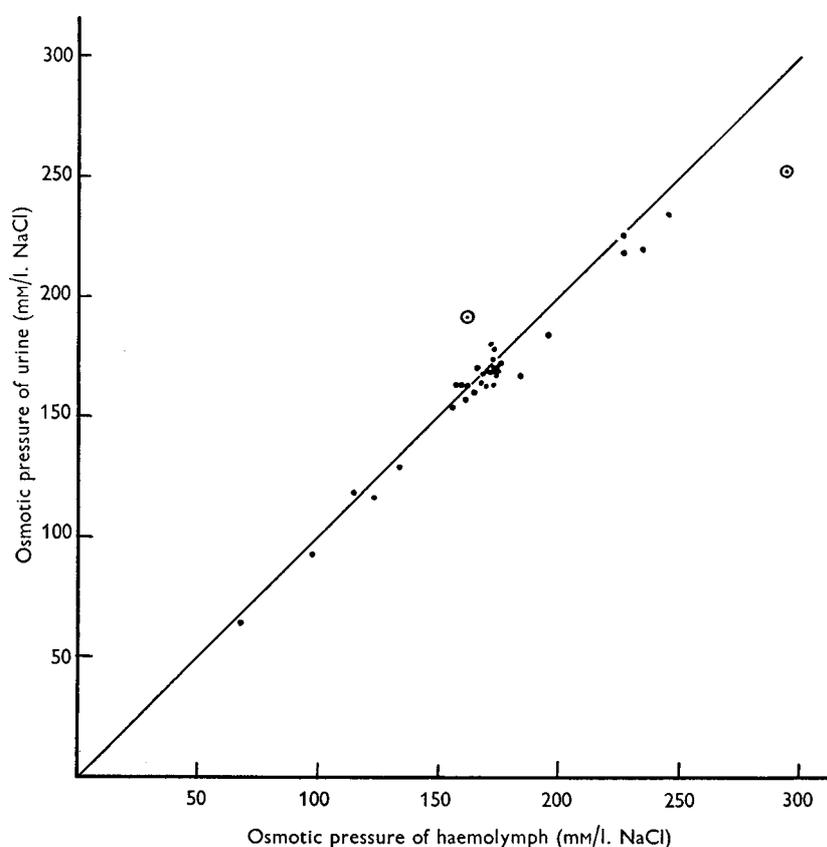


Fig. 2. For explanation see text.

of urine produced. In many of these experiments, after an initial collection of urine had been made and a sample of haemolymph taken, the remaining haemolymph was diluted with distilled water or its osmotic pressure was raised by the addition of sugar (see Appendix, component A) and further collections were made.

In Fig. 2, $O.P._u$ is plotted against $O.P._h$ for all the thirty-four observations made in this series of experiments. Apart from two cases, which are ringed in Fig. 2, the

difference $O.P._h - O.P._u$ is small; neglecting the two ringed observations the average value of the difference is 2.6 mm/l. Possibly this difference is not statistically significant in relation to the scatter of the observations, but that is an issue of secondary importance. What is important is that in a majority of cases $O.P._h$ exceeds $O.P._u$ by an amount which is significant in relation to the errors of analysis. It is impossible to doubt that the tubules can and often do produce hypotonic urine, and this is decisive in demanding the rejection of the theory of urine production which was outlined in the Introduction.

A second series of experiments was undertaken in which the distal portion of the tubule was ligatured off and cut away and the proximal portion only was enclosed in a relatively small (0.25–0.4 cu.mm.) drop of haemolymph. The main purpose of these experiments was to study the changes in the ionic composition of the haemolymph which were brought about by the activity of the tubule; it so happened that they provided confirmation of the conclusions drawn from the first series of experiments.

Table 1

t, time in minutes since start of experiment.
 $O.P._h$, osmotic pressure of haemolymph in mm/l. NaCl.
 $O.P._u$, osmotic pressure of urine in mm/l. NaCl.

Tubule no.	<i>t</i>	$O.P._h$	$O.P._u$	$O.P._h - O.P._u$
1	30	192	193	-1
	105	195	177	18
	195	194	176	18
	380	201	187	14
2	40	187	181	6
	115	181	164	17
	200	181	162	19
	385	188	162	26
3	55	193	181	12
	120	196	177	19
	205	196	180	16
	390	195	156	39

Urine was collected and a sample of haemolymph was taken at various times after the start of the experiment. The osmotic pressures of these samples are recorded in Table 1. The variations in $O.P._h$ during these experiments call for comment. During the first period the tubule is recovering in haemolymph from its previous immersion in dissecting fluid; its activities during this period and their effects upon the composition of the haemolymph may well be unrepresentative. During the second and third periods the tubule is probably working normally and $O.P._u$ is constant. By the end of the fourth period urine production has come to a standstill, and the tubule is no longer able to concentrate phenol red; this is quite probably the result of the changes (e.g. reduction of potassium) in the haemolymph which have been brought about by the activity of the tubule.

The figures in Table 1 show that apart from the first period of the experiment the urine is consistently hypotonic to the haemolymph and that $O.P._h - O.P._u$ is

many times greater than the average difference recorded in the first series of experiments in which large drops were used.

(b) *Rate of urine production.* Although it would appear to be a relatively simple matter to collect the urine which accumulates in a given time and measure its volume, this will not necessarily be an accurate measure of the rate of urine production. If one collects by means of a pipette the urine rises rapidly into the pipette, and the walls of the tubule collapse around the orifice. The pipette is withdrawn, the urine is ejected and the pipette must then be reinserted. It is scarcely practicable to insert a pipette into a collapsed tubule without its orifice becoming blocked; on the other hand, if one waits until the tubule is distended there is liable to be some loss during the process of penetration. If one collects the urine by the second method, described on p. 106, there is an immediate rush of urine after the tubule is cut, but the edges of the cut then come together and the pressure subsequently developed is not always sufficient to force them apart again. There is also the further difficulty that if the free outflow of urine is restricted and the tubule becomes over-distended there may be some inhibition of urine production (see below under Secretion Pressure). Measurements of the rate of urine production are therefore liable to considerable error.

Rough estimates of volume were made on the urine samples by measuring the diameter of the droplets as they rested under liquid paraffin upon the bottom of a watch-glass. The droplets were assumed to be spherical, an assumption which was manifestly untrue, but the errors arising from this approximation are small compared with the errors discussed in the previous paragraph.

The volumes of the urine samples whose osmotic pressures are recorded in Fig. 2 were measured in this way, and the times of collection being also recorded it was possible to calculate the corresponding rates of urine production. In Fig. 3 the rate of urine production is plotted against O.P._h and shows an obvious tendency to increase with increasing dilution of the haemolymph; but the scatter is so great that no more accurate description of the relationship can usefully be given.

The length of a tubule is about 20 mm., of which the proximal portion (where it is assumed that the urine is produced) represents about 15 mm. The outer diameter of this region may be of the order of 120 μ . From Fig. 3, 0.4×10^{-3} cu.mm./min. could be taken as a normal rate of urine production. From this data the rate of urine production per unit area of tubule surface works out as of the order of 0.07×10^{-3} cu.mm./sq.mm./min. or 0.42×10^{-3} c.c./sq.cm./hr.

(c) *Secretion pressure of urine.* It is commonly observed (e.g. Wigglesworth, 1931, 1933) that ligatured Malpighian tubules become distended but do not swell up so much as to burst, from which it may be assumed that urine production ceases after a certain degree of distension has been suffered. This in its turn implies that there is a maximum hydrostatic pressure against which urine can be secreted by the tubule. This matter was investigated in the following way.

As stated above, when a tubule is ligatured it swells to a certain limited extent. The diameter of the partially distended tubule is first measured, the tubule is cut so that the urine escapes with a rush and the tubule shrinks to its 'resting' diameter

which is then measured again. It is also possible artificially to distend the tubule by inflation. For this purpose a steeply tapered pipette is used, partially filled with a solution of 30 mM/l. NaCl + 150 mM/l. KCl which approximates to urine in composition at least as far as the cations are concerned. The pipette is connected to a water manometer and is thrust well into the tubule so that its taper seals the wound. The hydrostatic pressure necessary to produce varying degrees of distension of the tubule can then be measured, allowance being made for the capillary forces at the meniscus in the pipette.

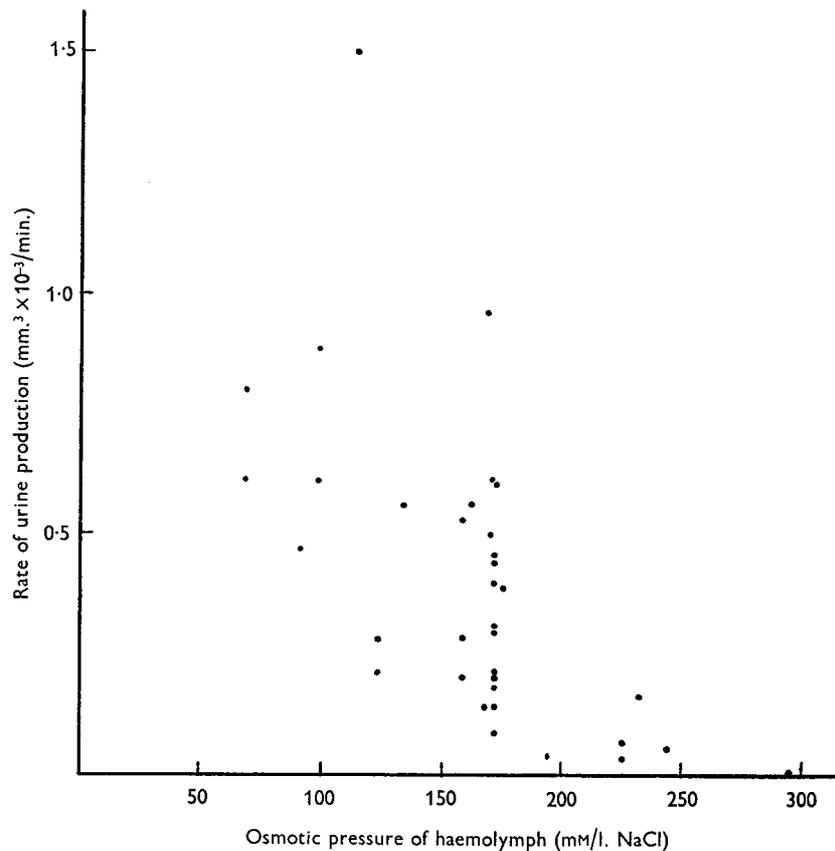


Fig. 3. For explanation see text.

Several tubules were investigated in this way. A pressure of 10–15 cm. H₂O was required to produce any visible distension of the tubule beyond its 'resting' diameter. With pressures increasing from 15 to 40 cm. H₂O the diameter of the tubule increased to about double the 'resting' diameter, and the contractile elements in the wall seemed to be unable to produce the writhing movements against this degree of turgor. Beyond a pressure of 40 cm. H₂O there was no further increase in diameter up to the pressure at which the seal gave way.

An increase in diameter to double the 'resting' diameter has only once been observed in a ligatured tubule; for the most part the distended diameter of a ligatured tubule is about 120–130% of the resting diameter, which would indicate a secretion pressure of the order of 20 cm. H₂O. Now it has been shown (Table 1) that urine can be produced against an osmotic gradient of some 20 mM/l. NaCl or more. In terms of hydrostatic pressure this is very many times greater than 20 cm. H₂O. There is therefore no question of the secretory mechanism being unable to overcome the hydrostatic pressure developed inside the ligatured tubule; if secretion fails this is more likely to be the result of the distortion suffered by the secretory cells as the tubule becomes distended.

It was interesting to observe that the fully distended tubule was unable to concentrate phenol red, a dye which the normal tubule concentrates very rapidly. This failure might have been due to the distension of the tubule *per se*, as suggested above, but it might also have been due to some metabolic disturbance resulting from the presence of the distending fluid in the lumen, since this fluid only roughly approximates to urine in composition. To put this to the test an experiment was carried out as follows. Phenol red was added to the haemolymph surrounding the fully distended tubule; the dye was not concentrated in the lumen. The tip of the tubule was then cut so that all the previous contents of the tubule were flushed out and only the distending fluid remained in the lumen. When the pressure was released and the walls collapsed the tubule began to concentrate the phenol red with its usual efficiency. It therefore appears likely that the active transport not only of water but of dissolved substances is adversely affected by distension.

DISCUSSION

Before proceeding further it is as well to be clear as to how far the evidence justifies the conclusion that water is actively transported. Most fresh-water animals produce urine which is hypotonic to the blood, and at first sight it would be natural to suppose that this involved the active transport of water. We know, however, that certainly in the Amphibia and probably also in most of the higher invertebrates there is a primary process of ultrafiltration followed by the reabsorption of salts, so that hypotonic urine can be produced without active transport of water. It could be argued that an ultrafiltrate was transported into the Malpighian tubule in one region and solutes reabsorbed in another. This argument cannot be refuted on the evidence so far obtained; one can but point out that there is no direct or circumstantial evidence in favour of the argument, and that since active transport of water is the simpler explanation the onus of disproof rests upon the opponents of this view.

It was an attractive feature of what might have been called the 'potassium theory' of urine formation that it accounted for the otherwise meaningless circulation of potassium as being necessary to maintain a current of water through the excretory system. This theory is now shown to be untenable. Water does not have to wait upon potassium but can be actively transported. It is true that the mechanism of

uric acid excretion proposed by Wigglesworth (1931) involves a circulation of potassium, but in fact the essential feature of the mechanism is the circulation of base, and Wigglesworth chose potassium rather than sodium to represent the base for no other reason than that the urate of potassium is the more soluble (personal communication). In any case it seems *prima facie* most unlikely that the amount of uric acid excreted bears any relationship to the very much greater amount of potassium circulated in the same time. The significance of the circulation of potassium remains obscure. It seems probable, however, that the movements of water are normally adjusted to the movements of solutes so as to produce urine which is more or less isotonic with the haemolymph. How this adjustment is brought about is not easy to see at present; the observed fact that urine production increases with dilution might have several explanations. It will presumably be necessary to study all the major constituents of the urine and the way in which their excretion is influenced by their concentrations in the haemolymph and in the urine before one can hope to see any clear picture, and it will be some time before this ground can be adequately covered.

SUMMARY

1. Single Malpighian tubules of the stick insect have been studied as preparations isolated in drops of haemolymph under liquid paraffin. Measurements of osmotic pressure have been made on haemolymph and urine.
2. The urine is more or less isotonic with the haemolymph over a wide (45-145%) range of osmotic pressure. But in a majority of cases the urine is slightly but significantly hypotonic to the haemolymph.
3. From these and other observations it is concluded that water must be actively transported across the wall of the tubule. The implications of this conclusion are discussed.
4. The normal rate of urine production is of the order of 0.07×10^{-3} cu.mm./sq.mm./min. The rate increases with increasing dilution of the haemolymph and vice versa.
5. Urine production normally ceases, probably as a result of distension of the tubule, when the pressure in the lumen rises to about 20 cm. H_2O .

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APPENDIX

After some consideration I have come to the view that the composition of the dissecting fluid used in these experiments should be put on record, although it has no special merits to recommend it. No complete analysis of stick insect haemolymph has been published. Figures for sodium and potassium have been given by Boné (1944), and more recently Duchâteau, Sarlet & Florkin (1952) have given a comprehensive list of the amino-acids and their concentrations. Based upon these observations and upon the general background of knowledge summarized by Wigglesworth (1953), a dissecting fluid was prepared as described below. Since micro-organisms very soon multiply in this fluid it was found

convenient to make it up fresh every few days from component solutions, each of which was stored in a number of sealed and sterilized bottles.

Component A (sugars)	Glucose	9 g./l.
	Sucrose	342 g./l.
Component B (amino-acids)	Glycine	1.25 g./l.
	Glutamic acid	2.8 g./l.
	Histidine	3.0 g./l.
	Lysine	1.6 g./l.
	Threonine	1.6 g./l.
	Valine	1.55 g./l.
Component C (alkaline earths)	CaCl ₂	2.7 g./l.
	MgCl ₂ ·6H ₂ O	8.1 g./l.
Component D (alkalis)	NaCl	11.9 g./l.
	NaOH	4.0 g./l.
	KCl	14.9 g./l.
	KOH	5.6 g./l.

Take 20 c.c. of A, 25 c.c. each of B and C, 5 c.c. of D. Make up to 100 c.c. Add more D drop by drop to adjust pH to 7.

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