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JEB Classics

ACTIVE TRANSPORT OF WATER BY INSECT MALPIGHIAN TUBULES

[104]

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

By J. A. RAMSAY

Zoological Laboratory, University of Cambridge

(*Received* 8 *July* 1953)

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

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*"!

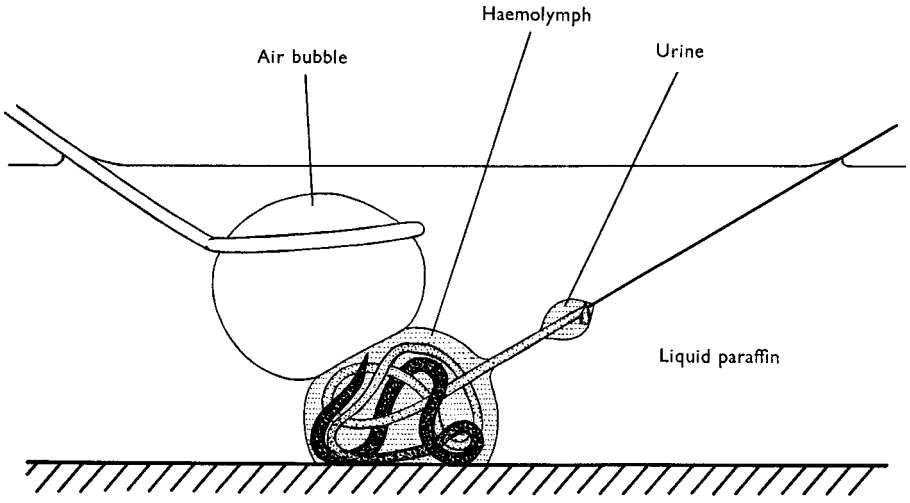

Fig. 1. Reproduced from Ramsay's 1954 paper. This figure shows the essentials of Ramsay's method, which is still in use today.

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

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.

10.1242/jeb.00846

Simon Maddrell
Cambridge University
shpm100@hermes.cam.ac.uk

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