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A STUDY OF THE MALPIGHIAN TUBULES OF THE PILL MILLIPEDE, *GLOMERIS MARGINATA* (VILLERS).

I. THE ISOLATION OF THE TUBULES IN A RINGER SOLUTION

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INTRODUCTION

The Malpighian tubules of insects have long held the attention of physiologists so that study of non-insect Malpighian tubules has been neglected. This study represents an attempt to correct this imbalance.

Myriapod Malpighian tubules were first described by Ramdhor (1811), and an excretory function was attributed to them by Muller (1829). Studies of their function has been carried out by the injection of dyes (Kowalevsky, 1892; Duboscq, 1896; Palm, 1953), but these studies have produced results from which it is not possible to draw definitive conclusions about tubule function. Analysis of uric acid levels in chilopods have been made by Wang & Wu (1948) and by Horne (1969). Increased concentrations of uric acid in the lower tubule and hindgut observed in both cases led to the conclusion that the Malpighian tubules eliminate the nitrogenous wastes.

The main objective of the present research was to initiate the study of myriapod Malpighian tubules in isolation. (It was also of some interest to determine the function of the Malpighian tubules in a group of animals which possess alternative excretory organs, i.e. the maxillary glands.) Where possible, attempts will be made to draw correlations between insect and myriapod tubule in respect of function and structure. In subsequent papers the nature of fluid formation by the tubules of *Glomeris* will be discussed with reference to models of fluid transport proposed for insect Malpighian tubules.

MATERIALS AND METHODS

Millipedes are essentially animals of the forest floor, and *Glomeris marginata* is particularly common in climax oak and ash woodland (Blower, 1955). However, the species tends also to be plentiful on calcareous soil. Specimens were collected from rough, sloping pastureland at Whiteleaf Cross, near Princes Risborough, Buckinghamshire. The soil in this locality was well drained, overlying chalk. Collections were made predominantly during spring and summer. Specimens were kept in covered glass jars containing moist tissue paper and pieces of carrot. *Glomeris* was found to be particularly suitable for the present research. Large numbers could be collected locally and the dimensions of the body made it convenient for dissection purposes.

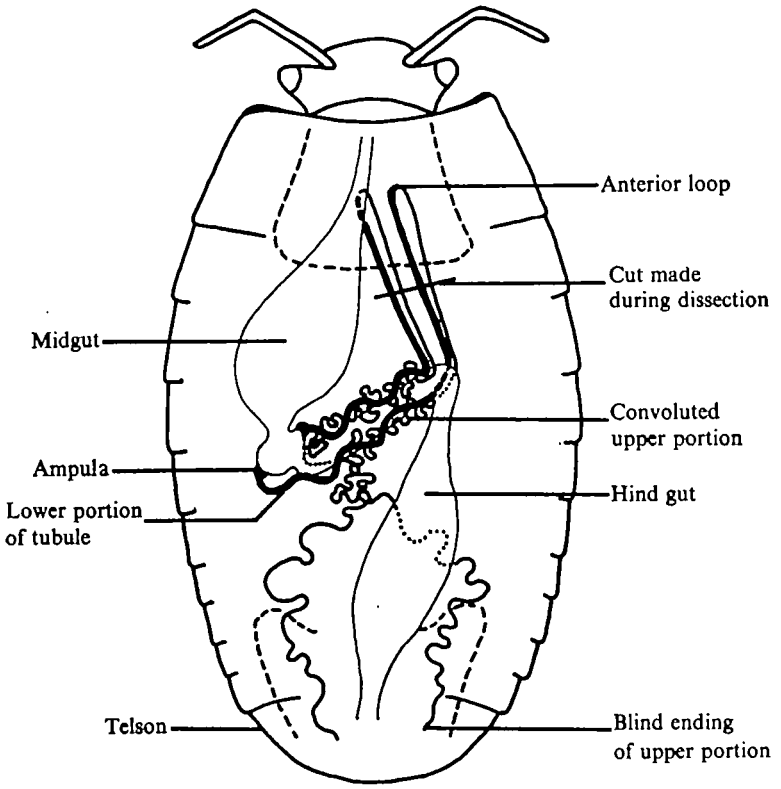

Fig. 1. The Malpighian tubules of *Glomeris marginata*, viewed from the dorsal aspect. The dashed line indicates the position of connective tissue surrounding the tubules.

Plateau (1876) described the single pair of Malpighian tubules in *Glomeris marginata* and observed the attachment of the tubules at the midgut–hindgut junction. Hubert (1970) suggested that the tubules of *Glomeris* may be differentiated into three regions in a manner similar to that found in iulids; however, the present study has shown that there are only two regions. The two long Malpighian tubules are divided into the translucent upper region (distal portion) and the wider, opaque, lower region (proximal portion). This differentiation was observed after injecting Janus Green B or neutral red (1 g/100 ml; Palm, 1953) into the animals. The arrangement of the tubules within the body is illustrated in Fig. 1. The length of a whole tubule was measured and found to be 100 mm or 7·5 times the length of the body.

For isolated preparations the convoluted upper portion lying alongside the gut was used. This region was easily accessible and could be removed by severing the gut and tracheal supply. The tubule was ligatured at its distal end, and in some preparations both tubules were left attached to a piece of the hindgut. The tubules were isolated in serum or Ringer in a manner similar to that described by Ramsay (1954). The rate of fluid production was estimated by measuring the volume of spherical drops of secreted fluid (Ramsay, 1958).

Prior to this study no data existed as to the composition of the haemolymph of *Glomeris*. Therefore, measurements of the ionic composition, osmotic pressure, pH and amino acid concentrations were made with a view to the preparation of a Ringer

in which isolated tissues could remain viable. Sodium and potassium concentrations were estimated with a Unicam SP 900 emission spectrophotometer. Fluid samples of 500 nl or more were deposited in 5 ml of triple-distilled water. Calcium and magnesium concentrations were measured using a Unicam SP 90 atomic absorption flame spectrophotometer. Samples of at least 4 μl diluted in no more than 2 ml of triple-distilled water were necessary for these analyses. Chloride concentrations were estimated by the electrometric method of Ramsay, Brown & Croghan (1955), and bicarbonate concentrations were measured by the method of Little & Ruston (1970). Osmotic pressure was determined by the cryoscopic method of Ramsay & Brown (1955). Osmotic pressures are expressed as the concentration of NaCl which has the same freezing-point depression as that observed. Samples were compared with a standard (1 % NaCl) which has a freezing-point depression of 0·605 °C. Haemolymph samples were centrifuged prior to analysis employing the holders described by Riegel (1968).

The hydrogen ion concentration was estimated by a method devised by Riegel (see Farquharson, 1972). Cresol Red was added to droplets of haemolymph kept under liquid paraffin. The colour reaction obtained with haemolymph was compared with a series of buffer droplets. This technique could detect differences of 0·1 of a pH unit within the range used. Quantitative analysis of haemolymph amino acids was made using an amino-acid analyser. These analyses were carried out by Dr P. Evans (Zoology Dept. Cambridge) upon two samples of haemolymph representing volumes pooled from several millipedes. The amino acids in the haemolymph samples were isolated by ion-exchange chromatography.

Simple saline proved inadequate to support fluid production by isolated Malpighian tubules. Therefore, before the results of the amino-acid analyses became available, recourse was made to a 'serum' prepared from the haemolymph as follows. A puncture was made between the fourth and fifth 'thoracic' tergites; haemolymph was withdrawn into a pipette and accumulated under liquid paraffin. The accumulated haemolymph was taken up into a microcentrifuge tube between layers of liquid paraffin (Riegel, 1968) and the protein was precipitated by immersing the tube in boiling water. After centrifuging, the supernatant was reserved as 'serum' (Ramsay, 1955). It has been found that this serum could be stored for several months at −4 °C with no apparent deleterious effects on its ability to promote fluid production.

RESULTS

(1) *Composition of the haemolymph*

The results of the ion and amino-acid analyses and the determinations of osmotic pressure made upon the haemolymph of *Glomeris* are presented in Table 1. Glutamine and serine overlap one another on the chromatogram and therefore separate quantities of each were not determined. The results of the initial ion analyses (Table 1, column A) seemed to indicate that a cation/anion imbalance (107·02/72·96 mM/l) exists in the haemolymph. However, at the pH of the haemolymph (8·3), it is likely that most of the amino acids present would be negatively charged and could make up part of the anion deficit (107·02/97·68 mM/l). Also, subsequent analyses have shown that a large proportion of the calcium in the haemolymph is bound to protein.

The composition of the dissecting saline and the Ringer solution is also shown in

Table 1. *The ion and amino-acid concentrations of the haemolymph (A) and the composition of the experimental media (B, C)*

(Concentrations are given in mM/l. The osmotic pressure is expressed as the concentration of NaCl which has the same freezing-point depression as that observed. Values have been expressed as the mean, plus or minus the standard error of the mean.)

	Haemolymph (A)	Saline (B)		Ringer (C)
Sodium	58·50 (±3·19)	NaCl	50	50
Potassium	4·92 (±0·45)	KCl	4	4
Magnesium	2·94 (±0·58)	$MgCl_2$	3	3
Calcium	40·66 (±3·33)	$CaCl_2$	2	2
Bicarbonate	20·00 (±0·68)	$NaHCO_3$	2	2
Chloride	52·96 (±2·10)		—	—
Phosphate	?	NaH_2PO_4	5	1
Glucose	?		20	20
Tris	—		25	20
Alanine	2·91		—	2·91
Arginine	2·13		—	2·13
Aspartic acid	Trace		—	—
Glutamic acid	1·15		—	1·15
Glutamine	Present but not estimated		—	—
Glycine	3·65		—	3·65
Histidine	1·23		—	1·23
Isoleucine	1·50		—	1·50
Leucine	3·15		—	3·15
Lysine	2·85		—	2·85
Phenylalanine	1·90		—	1·90
Serine	Present but not estimated		—	—
Tyrosine	1·54		—	1·54
Valine	2·71		—	2·71
Osmotic pressure	86·99 (±2·09)		—	—
pH	8·34 (±0·05)		8·3	8·3
Penicillin	—		0·05 g/l	0·05 g/l
Streptomycin	—		0·1 g/l	0·1 g/l
		+ N HCl to adjust pH		

Table 1 (columns B, C). It may be noted that the calcium and bicarbonate levels in these media have been reduced from the haemolymph levels due to heavy precipitation of calcium bicarbonate. Also, the sodium concentration has been slightly reduced in order to maintain the normal osmotic pressure.

(2) *Studies of control tubules*

Tubules were isolated in serum and the rate and length of time of fluid production were measured. Malpighian tubules treated in this way are called 'control' tubules. Fluid secretion rates of control tubules showed considerable variation, although the tubules appeared to be in a healthy condition in both serum and the dissecting saline. The initial rate (i.e. within the first hour) was usually high, generally varying between 5–50 nl/min. The rate of secretion declined rapidly during 2–3 h following isolation (see Fig. 2), after which there was a more gradual decline. The tubules were still capable of producing fluid for 8–26 h.

The development of a Ringer solution which incorporated a variety of amino acids (see Table 1 C) permitted the Malpighian tubules to function without any apparent

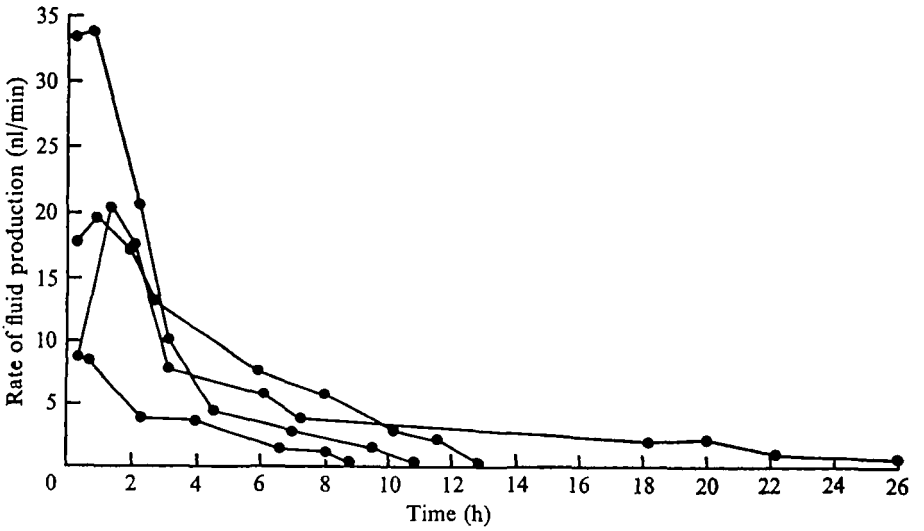

Fig. 2. Rates of fluid production by the isolated tubules of *Glomeris*.

morphological degeneration. Moreover, when serum bathing the tubules was replaced by Ringer, the rate of fluid secretion slowed, initially, by about 25 %. However, the rate soon picked up and subsequently the function of the tubules in Ringer could not be distinguished from their function in serum.

The rate of fluid secretion (average = *ca.* 15 nl/min) seemed to vary considerably from tubule to tubule. That this was due in part to variations in the lengths of tubule in different experiments is shown in Fig. 3. The effect of tubule length on the rate of secretion was measured using tubules isolated in a Ringer solution. Ligatured portions of the upper tubule were isolated in the normal manner; shorter than normal lengths were prepared simply by altering the level at which the ligature was tied. The length of the tubule was estimated at the end of the experiment, at which time the tubule was removed from the Ringer and unravelled.

The part of the upper tubule used in isolated preparations varied in length from *ca.* 10–*ca.* 50 mm; the rate of fluid production was directly related to the length of the tubule. Although there was a considerable scatter in the data, the truth of this proposition may be seen by the fact that a reduction in secretion rate was always associated with a reduction in length in cases where the tubules had been removed from the same animal (see Fig. 3). Since Ringer was their medium, the variation in secretion rates of tubules of equal length probably was linked to the condition of the animals prior to dissection. This could be expected as there have been no attempts to control the age or activity of the specimens used. Therefore, differing physiological condition of the animals as well as differing tubule lengths undoubtedly both contributed to the wide variation in secretion rates between tubules. This made it imperative that during experimental procedures each tubule must act as its own control.

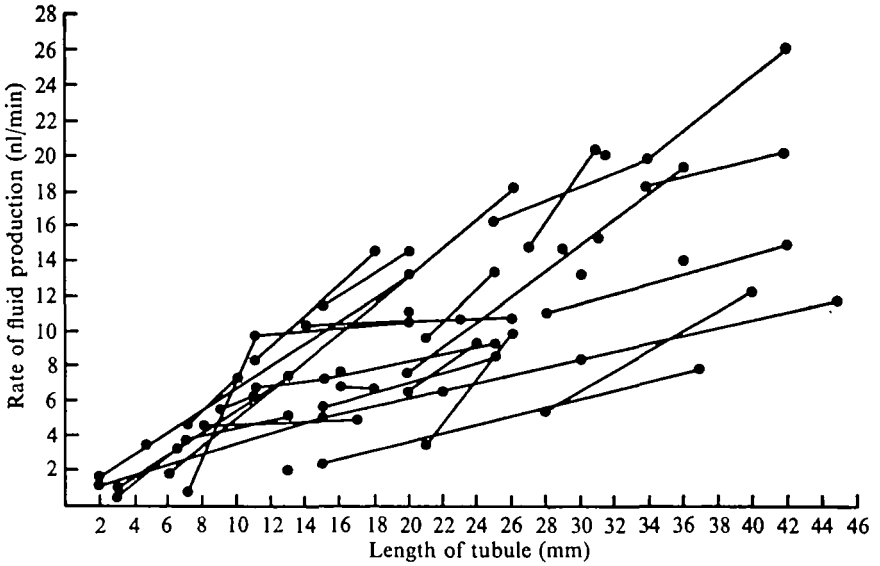

Fig. 3. The relationship between the length of the tubule and the rate of fluid production. The lines join values measured on tubules taken from the same animal.

(3) *Factors affecting the rate of fluid production*

(*a*) *Measuring a constant rate*

The continuous deterioration in the rate of fluid production described previously occurred when the tubules were isolated in serum or Ringer (see Fig. 4(1)). However, if the fluid produced by tubules were placed back in the bathing drop after each volume measurement, the deterioration of the fluid production rate was arrested, and it became possible to measure a constant rate of fluid production (Fig. 4(2)). (Hereafter, this procedure will be referred to as 'measuring a constant rate'.) Furthermore, if the medium (serum or Ringer) drops were replaced entirely with a fresh drop of either, an increase in the rate of fluid production would result (Fig. 4(3)). However, if the medium drop was renewed after 'measuring a constant rate', there was no effect (Fig. 4(4)). It may be noted that the ion concentrations and osmotic pressures of fluid produced by isolated Malpighian tubules of *Glomeris* are identical to those of the serum or Ringer which bathes them (Farquharson, 1973*a*). These results therefore imply that the deterioration in rate of fluid secretion results from loss of metabolites from the medium; presumably these metabolites are not destroyed during fluid formation.

(*b*) *Amino acids*

The important role of amino acids in fluid production could be demonstrated with the following experiments. When Ringer of the medium drop was replaced by saline a significant reduction in rate resulted and eventually fluid production stopped (Fig. 5*a*). However, when tubules were isolated in Ringer which lacked glucose but contained amino acids there was no apparent deleterious effect on secretion rate. In similar experiments replacement of the bathing Ringer with tubule fluid after a 'constant rate' had been measured resulted in a substantial increase in the rate of fluid production (see Fig. 5*b*).

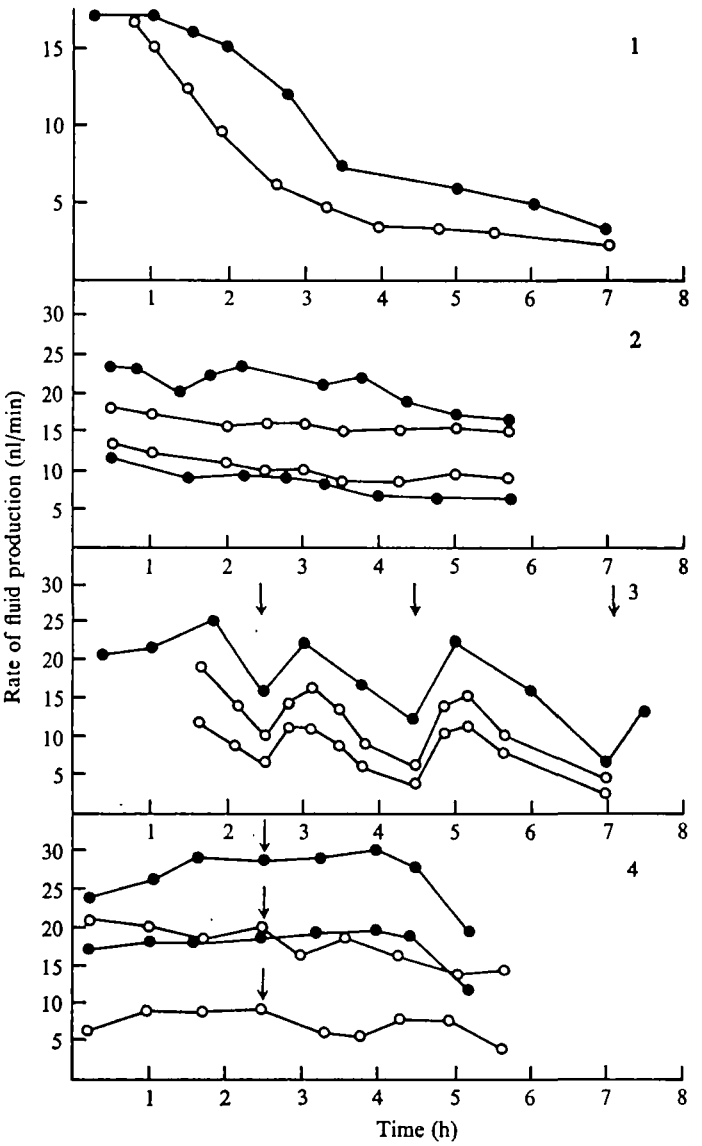

Fig. 4. 'Measuring a constant rate' and the effect of fresh serum and Ringer on fluid production by isolated tubules. (1) Control tubules; (2) fluid production rates when the tubule fluid is put back into the bathing drop after each volume measurement; (3) the effect of adding fresh Ringer or serum on the rate of fluid production of control tubules. (4) the effect of adding fresh Ringer or serum to tubules after 'measuring a constant rate' (i.e. as in (2)). Tubules were bathed in Ringer (○) or serum (●).

Preliminary attempts to identify the amino acid(s) most directly involved in fluid production were made using a saline solution containing 20 mM/l glutamic acid or alanine. These amino acids were chosen because of their ability to support urine production by isolated tubules of *Calliphora* (Berridge, 1966*b*); furthermore they appeared in high concentration in the fluid produced by Malpighian tubules of

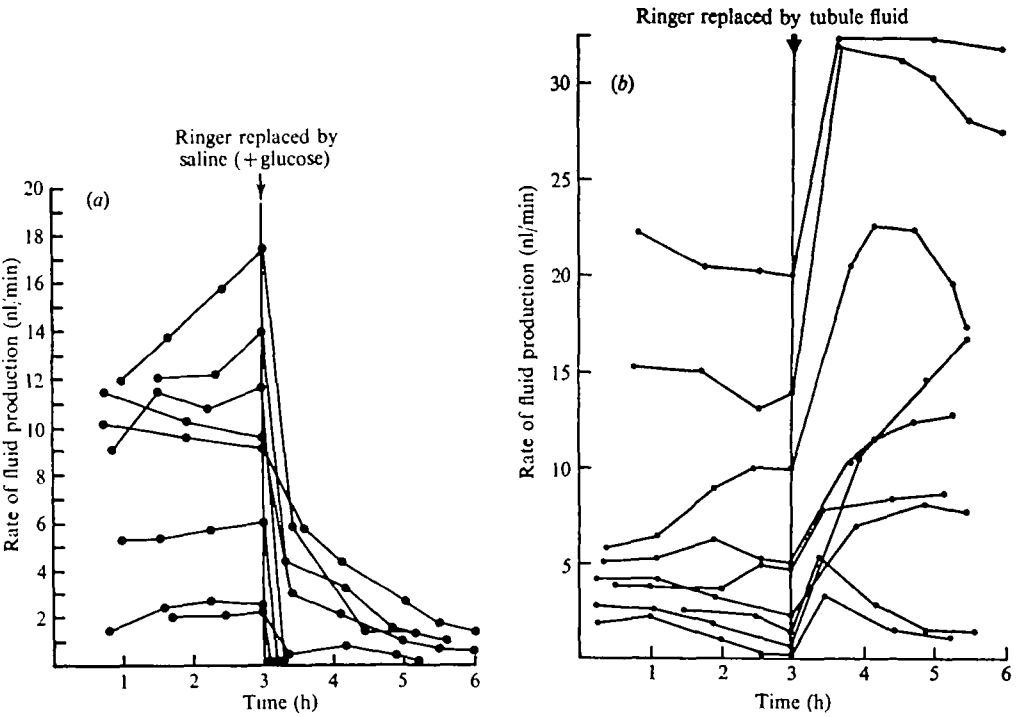

Fig. 5. The effect of saline solution (Ringer minus amino acids) (*a*) and tubule fluid (*b*) on the rate of fluid production of tubules bathed initially in Ringer.

Glomeris (as shown by two-way chromatography). In saline containing glutamic acid or alanine the tubules produced fluid at rates characteristic of normal saline (i.e. 13–14% of the control). However, it should be noted that glutamic acid reduced the pH of the saline and it is possible that any effect of the substrate was masked by a pH effect.

(4) *The effect of metabolic inhibitors on fluid production*

A measured volume of a solution containing a known concentration of inhibitor was added to tubules bathed in Ringer solution. Solutions were either made up fresh each day or stored at 4 °C.

2,4-dinitrophenol is thought to inhibit cell metabolism by preventing oxidative phosphorylation. This compound was found to be effective in slowing the rate of fluid production at a concentration of 1×10^{-4} M/l (Fig. 6). Cyanide and azide are known to inhibit cytochrome oxidase. These compounds were added to isolated tubules in the following concentrations:

Cyanide 10^{-3} 2×10^{-3} 3×10^{-3} 5×10^{-3} 10^{-2} M/l
Azide 10^{-3} 2×10^{-3} 3×10^{-3} 5×10^{-3} 6×10^{-3} 8×10^{-3} 10^{-2} M/l.

The tubules of *Glomeris* are relatively insensitive to cyanide and azide. Inhibition occurs at concentrations of 8×10^{-3} M/l (azide) and 3×10^{-3} M/l (cyanide) (Fig. 6).

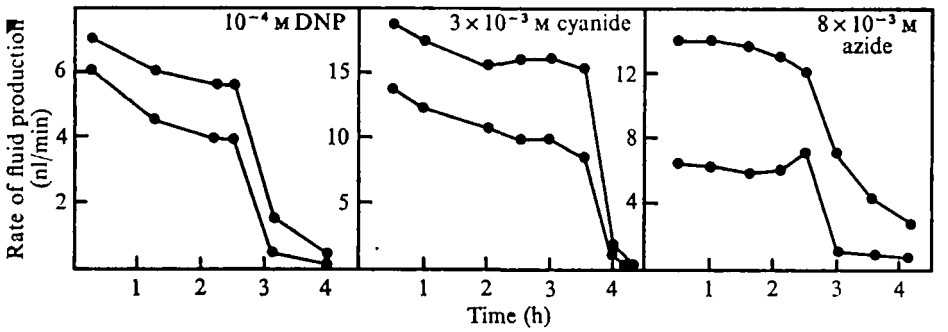

Fig. 6. The effect of 2,4-dinitrophenol, cyanide and azide on the rate of fluid production by isolated tubules.

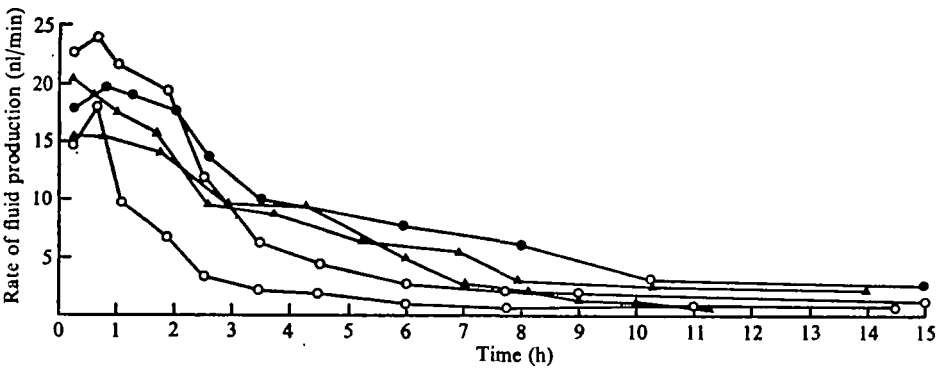

Fig. 7. The effect of diluting serum with saline on the rate of fluid production. Tubules were isolated in serum (●), serum:saline 1:1 (○), or serum:saline 1:3 (▲).

(5) *Hormonal control of secretion*

Preliminary serum dilution experiments were performed in order to determine whether the serum contained an essential diuretic factor. If a diuretic hormone were playing an effective role, then the dilution of serum with a saline solution should result in a decrease in secretion rate or length of time of secretion (Ramsay, 1955).

Tubules were isolated in serum:saline in the ratios 1:1 and 1:3 (20 tubules; see Fig. 7). It was found that the tubules could function for up to 14 h in serum:saline mixtures with no obvious deleterious effects. Saline was also added to tubules isolated in serum after 'measuring a constant rate'. Using a 1:1 serum:saline mixture (8 tubules) the rate of fluid production after 30 min was about 102% of the original rate with little deterioration after 3 h. It would therefore seem unlikely that a diuretic hormone is involved in the control of normal fluid secretion by the tubules of *Glomeris*.

Previous studies have shown that fluid secretion by Malpighian tubules (Maddrell, Pilcher & Gardiner, 1969, 1971) and salivary glands of insects (Berridge, 1970; Berridge & Patel, 1968; Berridge & Prince, 1972; Oschman & Berridge, 1970) is stimulated by 5-hydroxytryptamine, 3′,5′-cyclic adenosine monophosphate (AMP) and theophylline. These compounds were added to the Ringer bathing the isolated Malpighian tubules of *Glomeris*. However, in no instance was it possible to observe an increase in the rate of fluid production due to this procedure.

Table 2. *The effect of various pharmaceuticals on the fluid production rate of isolated Malpighian tubules of* Glomeris

Rates are expressed as the mean plus or minus the standard error of the mean.

Compound	Concentration (M/l)	Rate of secretion 30 min after administration of drug (as % of original rate) (%)	Number of tubules
5-HT	10^{-4}	70·74 (±6·22)*	6
	10^{-5}	80·77 (±8·26)*	8
	10^{-6}	86·51 (±5·57)*	6
	10^{-7}	94·52 (±3·53)	6
	10^{-8}	96·42 (±4·61)	4
Dibutyryl cyclic AMP	10^{-2}	27·41 (±2·85) (*R*)	16
	10^{-2}	28·69 (±4·17) (*S*)	8
	10^{-3}	66·20 (±3·83) (*R*)*	8
Theophylline	10^{-3}	50·57 (±2·56) (*R*)	10
	10^{-2}	81·10 (±6·93) (*R*)*	6
Imidazole	10^{-2}	96·47 (±6·33)	10

* Tubules showed a recovery to a normal rate of secretion.
(*S*) experiment carried out with tubules isolated in serum.
(*R*) experiments carried out with tubules isolated in a Ringer.

As shown in Table 2, when 5-HT was added to the medium at concentrations effective in stimulating other tissues (10^{-6}, 10^{-7} M/l), there was generally no effect on fluid production. However, when 5-HT was administered at higher concentrations (10^{-6}, 10^{-5}, 10^{-4} M/l), the secretion rate decreased initially and then returned to normal within approximately 1½ hours. Addition of theophylline (10^{-3} M/l) to Ringer bathing the tubules resulted in only a slight decrease in secretion rate (81·10%). At a higher concentration (10^{-2} M/l) the fluid secretion rate was reduced by about one half. Theophylline, a methyl xanthine, is an inhibitor of the phosphodiesterase which degrades cyclic AMP (Butcher & Sutherland, 1962). Therefore, administration of theophylline can lead indirectly to an increase in cyclic AMP concentration within the cell. Thus, if theophylline does not stimulate fluid production, it is only to be expected that cyclic AMP would have no stimulatory effect on fluid production.

In the present study the dibutyryl derivative of cyclic AMP was used, which, it is believed (Henion, Sutherland & Pasternak, 1967) penetrates cells more easily and is less susceptible to hydrolysis by phosphodiesterase than cyclic AMP. When this compound was added to the bathing Ringer there was a rapid decrease in rate of fluid production. As was the case with theophylline, there was no recovery period at concentrations higher than 10^{-2} M/l.

Finally, imidazole had no effect on fluid production by Malpighian tubules of *Glomeris*. This result is consistent with the results of experiments with theophylline and cyclic AMP. Imidazole has been shown to reduce the net yield of cyclic AMP by increasing the activity of cyclic nucleotide phosphodiesterase (Butcher & Sutherland, 1962).

(6) *Seasonal variation in the functional capacity of the Malpighian tubules of* Glomeris

For a considerable portion of the year the Malpighian tubules of *Glomeris* are incapable of producing fluid *in vitro*. The annual secretory cycle of Malpighian tubules, based on observations made during the present study, is as follows.

The isolated Malphigian tubules stop functioning at the end of June/early July. This period coincides with the release of eggs from the body. During the following month the tubules appeared to be in poor condition and had a tendency to tear easily during dissection. At the end of July and beginning of August the animals moulted. The fragile nature of the tubules persisted until mid-October at which time they again appeared to be capable of producing fluid. Serum prepared from haemolymph of animals with functional Malpighian tubules and stored frozen would not stimulate fluid production during July, August and September. However, such serum could stimulate isolated tubules in October, whilst freshly prepared serum remained ineffective in promoting fluid production. At the end of March and beginning of April the tubules showed a similar temporary halt in fluid production. During this period they appeared healthy, although the lumen might be filled with crystals which are probably uric acid.

DISCUSSION

It is of interest to compare the haemolymph composition of *Glomeris* with that of insects. An earlier comparison was made by Sutcliffe (1963) who suggested that evolutionary trends were shown between myriapods and insects and within the insect groups themselves with respect to ion ratios and the amino-acid composition of the haemolymph. It was found that the contribution of sodium and chloride ions to the total osmolar concentration of the haemolymph of *Iulus* (diplopod) had been reduced in comparison with isopods, arachnids and chilopods and was therefore more similar to some of the exopterygote insects. Further analyses of the amino-acid composition of the haemolymph of a diplopod and a chilopod (Sundara Rajulu, 1970) have shown that the amino-acid content is much higher than that reported for crustaceans and arachnids and is therefore also more comparable to the primitive insect condition. Such attributes are shown by the haemolymph of *Glomeris*. Sodium and chloride ions can account for the major proportion of the total osmotic concentration, but there is also a fairly high concentration of amino acids (*ca.* 13 % of total O.P.). Thirteen of the seventeen amino acids described by Sundara Rajulu and found in both diplopod and chilopod haemolymph are also present in the haemolymph of *Glomeris*. However, cystine was absent. It had been suggested that this amino acid was characteristic of the haemolymph of myriapods and has also been reported to be present in the haemolymph of the millipede, *Jonespeltis* (Krishnan Nair & Prabhu, 1971).

The high calcium level of the haemolymph of *Glomeris* represents a condition which has not previously been described in insects or myriapods. This may either be a result of the specimens living on, and ingesting, a chalky soil; or could be associated with the requirement of calcium for the highly calcified exoskeleton (Reichle, Shanks & Crossley, 1969). Such aspects need further investigation, although it can be noted that a large proportion of the calcium in the haemolymph is bound to protein. This was made evident by analyses of serum and tubule fluid (Farquharson, 1973*b*).

Average rates of fluid production by isolated tubules (15 nl/min) are similar to those found in *Calpodes* (Irvine, 1969) and are considerably higher than those of *Carausius* (*ca.* 2 nl/min; Ramsay, 1954), *Calliphora* (*ca.* 4 nl/min; Berridge, 1966*b*), and *Dysdercus* (*ca.* 1 nl/min; Berridge, 1966*a*). However, they are not as high as rates of fluid production of tubules of *Rhodnius* during diuresis (*ca.* 40 nl/min; Maddrell, 1969). The wide variation in the secretion rates of control tubules is probably a result of differing lengths of tubule being used in isolated preparations. Berridge & Oschman (1969) and Maddrell (1969) have shown the direct relationship between the rate of fluid production and the length of the Malpighian tubule whilst bathed by an artificial medium. As expected, a similar relationship is evident for the tubules of *Glomeris*. The variation in fluid production rates for isolated tubules of equal length could be associated with differing physiological conditions of the animals.

Although the tubules can potentially secrete at a high rate, there is a fast decline from the initial rate measured in tubules *in vitro*. Berridge (1966*b*), whilst investigating the oviposition cycle in *Calliphora*, also found that the secretion rate of isolated tubules declined over a 32 h period. The decline was more marked when the tubules were secreting at high rates. A similar effect has been shown by Irvine (1969) who noted that the addition of fresh medium to the bathing droplet stimulated the tubules of *Calpodes*. It is unlikely that the deterioration in secretion rate observed in studies of tubules of *Glomeris* was due to a change in pH, since the bathing Ringer was buffered (Tris/HCl). In preliminary experiments where the pH of the Ringer was varied on either side of the optimum (pH = 8·3), the rate of fluid production did decrease (cf. *Carausius*; Ramsay, 1956). However, if this were the cause of the general deterioration in rate then it would be necessary to postulate that addition of small volumes of Ringer or tubule fluid to the bathing drop would have a buffering effect tending to return the pH of the Ringer to optimum (as this procedure stabilizes the rate). The possibility of the decline in secretion rate being associated with an insufficient supply of oxygen should also be considered. Other transport systems are very sensitive to lowered oxygen tensions (Haskell, Clemons & Harvey, 1965). Therefore, it would not be surprising if a similar effect were observed in this case. A decrease in the partial pressure of oxygen in the bathing drop could easily account for the decline in secretion rate; however, in view of the stimulatory action of tubule fluid it becomes essential to assume that the oxygen tension is much higher in the tubule fluid than in the bathing media. Such a proposition seems unlikely, although obviously further investigations are required as the effect of variations in oxygen tension on fluid production.

As the tubules are unable to function in a Ringer which lacks amino acids, stress can be put on the role of amino acids in fluid production. The stabilizing effect on the rate of secretion of returning the tubule fluid to the bathing drop would seem to suggest that the deterioration in rate could be a result of a loss of amino acids across the tubule wall, rather than a depletion in source solely due to metabolization. This proposition is supported by the fact that the tubules can function whilst bathed by tubule fluid; however, this procedure also results in a consistent increase in the rate of fluid production. As the tubule fluid is known to possess the same osmotic pressure and ion concentrations as the bathing media (Farquharson, 1973*b*), this effect could be the result of an increased concentration of metabolite(s) necessary for tubule function.

Permeability studies carried out on tubules of *Glomeris* (Farquharson, 1973*c*) would suggest that there is little resistance to the movement of these substances across the tubule wall. It may also be significant that whereas only four amino acids could be detected in two-way chromatograms of haemolymph, 10 amino acids were obviously present in tubule fluid (Farquharson, 1972). Bennett (1971) has also found that a number of amino acids can be identified in the excreta eliminated by the diplopod, *Cylindroiulus*. Measurement of TF/M and metabolization studies of amino acids involved may reveal the significance of their role in fluid production.

Fluid production was inhibited by 2,4-dinitrophenol at a concentration comparable to those which inhibit fluid production by the Malpighian tubules of *Calliphora* (Berridge, 1966*b*) and *Rhodnius* (Maddrell, 1969). This effect suggests that fluid production requires a continuous formation of high-energy phosphate compounds. In comparison to insect Malpighian tubules (Berridge, 1966; Maddrell, 1969) the tubules of *Glomeris* are relatively insensitive to cyanide and azide. As these compounds inhibit cytochrome oxidase, this insensitivity could be attributed to an excess of that enzyme. However, work carried out by Hall, Hollingworth & Shankland (1971) on the millipedes *Euryurus leachii* and *Pleuroloma flavipes butleri* suggests that cyanide tolerance in millipedes could be due to a resistant terminal oxidase. Presumably cyanide resistance would be necessary to enable millipedes to tolerate their own defensive secretions which consist largely of hydrogen cyanide (see also Dwarakanath, Berlin & Thilager, 1971).

The results of studies in which the serum was diluted with saline solution were similar to those of Ramsay (1955) in that there was no effect on secretion rate. However in this work, unlike Ramsay's, there was no obvious effect on secretion time. This observation, taken together with the ability of the tubules of *Glomeris* to secrete at a normal rate in a Ringer solution, suggests that a diuretic factor is probably not involved in the control of the normal secretory mechanism. However, the period of inactivity associated with a time of oviposition and active moulting may be under hormonal control. Were the seasonal inactivity of the tubules due to the lack of a diuretic factor it would be expected that 'active' serum could induce fluid production. This would not appear to be the case, except during late October. Effective antidiuretic factors have been found to control secretion by insect Malpighian tubules (Wall & Ralph, 1964; Cazal & Girardie, 1968; Vietinghoff, 1967); however, at present, there is no direct evidence to suggest similar activity in *Glomeris*. It is possible that the maxillary glands could assume the major excretory role during the antidiuretic phase of the tubules. It would be expected that the animal would need to conserve water when undergoing ecdysis.

It has been found that the stimulatory effect of insect diuretic hormone can be simulated by 5-HT (Maddrell *et al.* 1969, 1971; Pilcher, 1970*b*). No response was noted when threshold concentrations of 5-HT were added to Ringer bathing the tubules of *Glomeris*, suggesting that this compound is not involved in any diuretic activity; its inhibitory effect at high concentrations is probably an unspecific effect. Investigations by Berridge (1970) and Berridge & Patel (1968) on the salivary glands of *Calliphora* indicated that the action of 5-HT was probably mediated by cyclic AMP which stimulated a potassium pump on the apical membrane. Extensive studies have been carried out on the role of cyclic AMP in the mediation of hormone action in a

large variety of tissues (review by Major & Kilpatrick, 1972). Maddrell *et al.* (1971) have found that the tubules of *Rhodnius* and *Carausius* were stimulated by cyclic AMP, but there was a lack of response of tubules of *Rhodnius* to aminophylline and theophylline, the latter reducing the rate of secretion of the tubules at high concentrations. It was felt that this was due possibly to a low unstimulated rate of cyclic AMP production so that the partial inhibition of phosphodiesterase activity may have had little effect on the cyclic AMP level. It was also believed that any response to theophylline was masked by the adverse reaction of the cells to high concentrations. Such a phenomenon is not universal as concentrations of 10^{-3}, 10^{-2} M/l theophylline and cyclic AMP are frequently used on transporting tissues with no adverse effects. However, the high concentrations of cyclic AMP and theophylline added to the medium bathing the tubules of *Glomeris* resulted in a considerable reduction in the rate of secretion. This effect was minimized by reducing the concentration, but no stimulatory effect could be observed. This response could similarly be interpreted as an unspecific action on the cells at high concentrations. However, it would be expected that some stimulatory activity of cyclic AMP could be measured at lower concentrations, and that imidazole, which stimulates phosphodiesterase activity, would slow down the secretion rate. Assuming that the concentration of cyclic AMP within the cells had been affected by the presence of theophylline or imidazole, the results could signify either (1) that the intracellular level of cyclic AMP is very low and has little or no effect in stimulating fluid production, inhibiting unspecifically at high concentrations, or (2) that it is a mediator of an antidiuretic hormone and affects the permeability of the tubule wall. Orloff & Handler (1964, 1967) have shown that the antidiuretic effect of vasopressin on toad bladder is mediated by cyclic AMP. However, for such a mechanism to operate in this case would necessitate a reduction in permeability to water, whereas in toad bladder vasopressin stimulates sodium transport and increases permeability to water. There seem to have been no previous reports describing an inhibitory effect of cyclic AMP on fluid transport.

SUMMARY

1. The Malphigian tubules of the pill millipede, *Glomeris marginata* are described.

2. After the necessary analyses of the haemolymph had been carried out a Ringer solution was prepared which would support the secretion of tubule fluid *in vitro*.

3. The reasons for the variation and deterioration in the secretion rates of control tubules are discussed. The presence of various amino acids in the bathing medium appears to be essential for fluid production.

4. There would not appear to be a diuretic hormone involved in the control of secretion; and 5-HT, cylic AMP and theophylline will not stimulate fluid production.

5. Secretion is inhibited by the metabolic inhibitors 2,4-DNP, azide and cyanide. The relative insensitivity shown to the latter two compounds is probably associated with a resistance mechanism.

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