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

## II. THE EFFECT OF VARIATIONS IN OSMOTIC PRESSURE AND SODIUM AND POTASSIUM CONCENTRATIONS ON FLUID PRODUCTION

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### INTRODUCTION

Ion movements across the Malpighian tubule have been studied extensively in the past (Berridge, 1968, 1969; Coast, 1969; Irvine, 1969; Maddrell, 1969; Pilcher, 1970; Ramsay, 1953, 1954, 1955, 1956). At present, the most popularly held belief is that fluid transport across Malpighian tubules is linked to solute transport. The work of Ramsay (1953, 1955) suggested that potassium was the 'prime mover' in generating the flow of urine, and this was substantiated by the results obtained by Berridge (1968) working with the tubules of *Calliphora*. However, it was found subsequently by Maddrell (1969) that tubules of *Rhodnius* could function normally in a potassium-free medium. This result led to the conclusion that although there may be a preferential handling of potassium ions, sodium ions move through the tubule wall only slightly more slowly than do potassium ions. Therefore, it was suggested that the differences between the ion-transporting characteristics of the tubules of *Rhodnius* and those of other insects may be associated with the ability of *Rhodnius* tubules to secrete fluid very much faster. It has been shown already that the Malpighian tubules of *Glomeris* provide a suitable tissue for the study of fluid transport (Farquharson, 1973*a*). The present paper will show that there are many basic differences between these tubules and those of insects with respect to ion movements. Furthermore, these differences are not readily explicable using the models of fluid transport proposed for insect Malpighian tubules.

### MATERIALS AND METHODS

Techniques for the maintenance of specimens, and the dissection and isolation of the tubules were identical to those described previously (Farquharson, 1973*a*).

The effect on the rate of fluid production by the tubules of *Glomeris* of variations in the osmotic pressure of their medium was measured using tubules isolated in serum. The osmotic pressure of serum was decreased by the addition of distilled water, and increased by the addition of 200 mM/l sucrose or 6 × Ringer solution. These solutions were added in varying quantities to a 5 $\mu$l bathing drop after which the osmotic pressure of medium and tubule fluid was measured. The effect of altered sodium and

Table 1. *The concentrations of ions and the osmotic pressure of fluid produced by Malpighian tubules of* Glomeris *when isolated in serum*

(Also shown are the concentrations of calcium in tubule fluid and medium when tubules were isolated in Ringer. Ion concentrations are expressed as mmoles/l. Osmotic pressures are expressed as the concentration of NaCl having the same freezing-point depression. All concentrations are expressed as the mean, ±S.E. of the mean.)

| | Medium | Tubule fluid |
|---|---|---|
| Na (serum) | 53·25 (±1·27) | 54·35 (±1·10) |
| K (serum) | 5·38 (±0·98) | 5·50 (±0·89) |
| Mg (serum) | 2·74 (±0·09) | 2·63 (±0·11) |
| Ca (serum) | 17·02 (±1·92) | 6·07 (±0·68) |
| Cl (serum) | 51·67 (±1·50) | 50·16 (±1·20) |
| O.P. (serum) | 84·78 (±3·06) | 89·59 (±4·06) |
| Ca (Ringer) | 9·24 (±0·50) | 8·82 (±0·64) |

potassium concentrations on fluid production was measured using tubules bathed in a Ringer solution. Aliquots of normal Ringer were mixed with aliquots of sodium-free and/or potassium-free Ringer in order to obtain all the necessary variations. The effects of experimental procedures have been expressed in terms of the percentage change in rate of fluid production compared with the rate of fluid production before the medium was altered.

The ion concentrations and osmotic pressures of serum, Ringer and tubule fluid were measured as described previously (Farquharson, 1973*a*).

## RESULTS

### *Analysis of serum and tubule fluid*

Shown in Table 1 are the results of determinations of the concentrations of ions and the osmotic pressures of tubule fluid and medium when tubules were isolated in serum. With the exception of calcium, the concentrations of ions in the tubule fluid are essentially the same as the concentrations of the same ions in the serum. The concentration of calcium in the serum (17·02 mM/l) is considerably lower than that in the haemolymph (40·5 mM/l, Farquharson, 1973*a*) which suggests that a large proportion of calcium is bound to protein. Similarly, the concentration of calcium in fluid produced by isolated tubules is less than the concentration of calcium in the medium when the medium is serum (Table 1). However, when the medium is Ringer, the concentration of calcium in the tubule fluid was identical to the concentration of calcium in the medium. Therefore, it seems likely that, even in the serum, a significant proportion of the calcium remains bound to a large, heat-stable molecule.

### *The effect of altered sodium:potassium ratios*

The analyses described above revealed that neither sodium nor potassium became concentrated in fluid produced by tubules bathed in serum or Ringer in which the relative concentrations of these ions were 'normal'. Further series of experiments were carried out to determine whether this relationship holds true when the relative

Table 2. *Summary of the experiments to test the effect on fluid production of alterations in sodium and potassium concentrations of the medium*

| Experimental condition | No. tubules | Effect on fluid production* |
|---|---|---|
| K-free, choline-Ringer | 14 | Very slight reduction |
| K-free, Na-Ringer | 12 | Slight increase |
| Na-free, choline-Ringer | 10 | Rapid cessation |
| Na-free, K-Ringer | 10 | Immediate cessation |
| Reduced-Na, K-Ringer | 22 | See Fig. 1 |
| Reduced-Na, choline-Ringer | 18 | See Fig. 2 |
| Reduced-Na, choline-K-Ringer | 18 | See Fig. 2 |
| Increased-Na Ringer | 11 | See Fig. 1 |
| Na, K-free, choline-Ringer | 8 | Immediate cessation. |

* Except as noted, effect 30 min after altering the bathing medium.

concentrations of sodium and potassium are other than 'normal'. The conditions of the various experiments are summarized in Table 2.

Because the concentration of potassium in the haemolymph is normally low (*ca.* 5 mM/l), very little increment in the sodium concentration could be achieved by replacement of potassium with sodium. Furthermore, all components of the Ringer were essential for fluid production. Therefore, the only way that the sodium concentration could be increased, whilst maintaining the normal osmotic pressure, was by adding isosmotic sodium chloride solution.

When tubules were isolated in Ringer in which the potassium had been replaced by choline chloride, the rate of fluid production after 30 min was 90·7% (±3·22). Furthermore, secretion of fluid was continuous. Similarly, when the tubules were placed in Ringer in which the potassium was entirely replaced by sodium, they appeared to function normally and for several hours. The rate of fluid production was found to be 119% (±11·7) of the normal rate; this slight increase may have been due to the increase in the sodium concentration (see Fig. 1 and discussion below).

In Na-free choline Ringer the rate of fluid production diminished to 9·83% (±3·83) of normal within 30 min and soon thereafter the tubules ceased to function. In Na-free potassium Ringer fluid production ceased immediately. Addition of sufficient NaCl solution to increase the sodium concentration by 5–10 mM/l had no effect on such tubules. Further, addition of normal Ringer at 30-min intervals until the final sodium concentration was about 30 mM/l Na (6 tubules) failed to induce fluid production. Tubules appeared to recover only when their medium was replaced by normal Ringer.

As shown in Fig. 1, when sodium in the Ringer is replaced by potassium there is a reduction in the rate of fluid production. In these circumstances the sodium concentration could be reduced only to about 26 mM/l (K = 30 mM/l) before fluid production ceased. However, in a reduced-Na choline Ringer (see Fig. 2) the Ringer sodium concentration could be reduced to 10 mM/l with continued fluid production. As shown in Fig. 2, the rate of fluid production and the sodium concentration in the medium appear to be linearly related between 0 and 35–40 mM/l Na. The large scatter in the data makes it impossible to determine whether differences exist between rates of fluid production measured in the presence or absence of potassium (Fig. 2). The data summarized in Figs. 1, 2 make it apparent that fluid production by isolated Malpighian

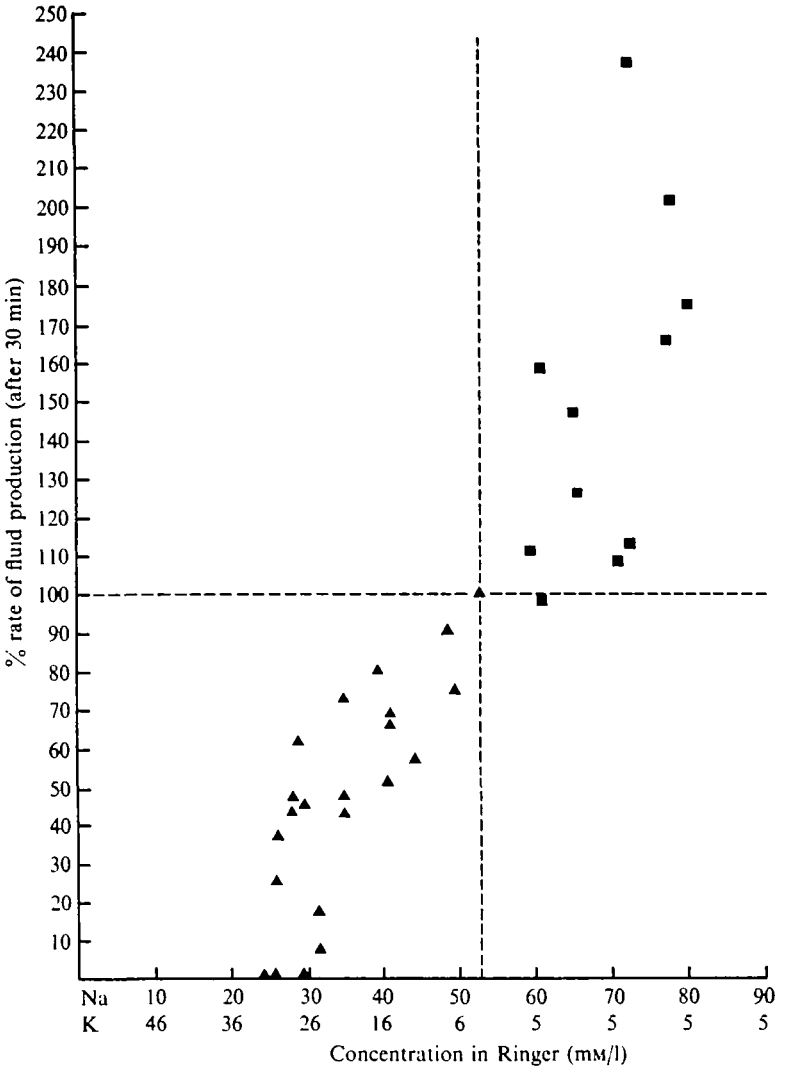


Fig. 1. The effect of variations in sodium and potassium concentrations on the rate of fluid production by the Malphigian tubules of *Glomeris*. Sodium ions in the medium have been replaced by potassium ions (▲), or their concentration in the medium increased by the addition of 90 mM/l NaCl (■).

tubules of *Glomeris* is dependent upon sodium concentration. An increase in the sodium concentration of the medium above the normal level always resulted in a corresponding increase in the rate of fluid production (Fig. 1). It appears also from the results that relatively high concentrations of potassium inhibit fluid production. Finally, tubules were unable to function in a Ringer in which both sodium and potassium were replaced by choline chloride (Table 2).

Measurement of the concentrations of sodium and potassium has shown that throughout the range of concentrations of the medium (Na = 0–80 mM/l, K = 30–0 mM/l), the tubule fluid was isomolar with the medium in respect of both ions (Fig. 3). It is clear from the data in Fig. 3 that elevated concentrations of neithe

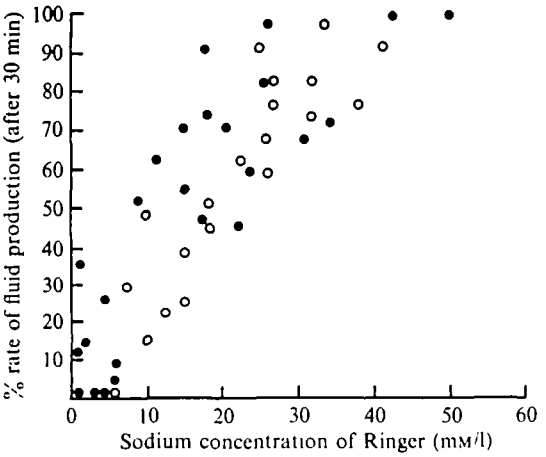


Fig. 2. The effect of reducing the sodium concentration of the medium on the rate of fluid production. Sodium was replaced by choline chloride in the absence of potassium (○) or in the presence of 5 mM/l potassium (●).

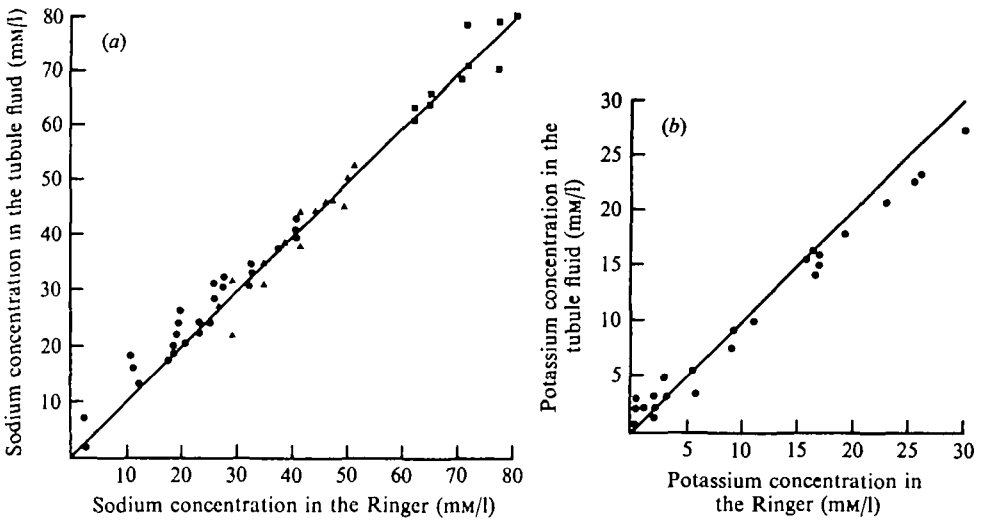


Fig. 3. The correlation between sodium and potassium concentrations in the tubule fluid and the bathing Ringer solution. (*a*) Sodium ions were either replaced by potassium ions (▲) or by choline chloride (●). Increased sodium concentrations were obtained by the addition of isosmotic NaCl solution to the bathing medium (■). (*b*) High concentrations of potassium in the medium were obtained by replacement of sodium ions with potassium ions. In both figures the straight line from the origin represents equal concentrations in the tubule fluid and Ringer.

potassium nor sodium were found in fluid produced by tubules of *Glomeris*, which makes them unique amongst Malpighian tubules. As shown in Fig. 3*b*, when the potassium concentration in the medium was greater than about 15 mM/l the potassium concentration of the tubule fluid tended to be depressed (by *ca.* 10%). Whether or not this represents a significant trend was not explored.

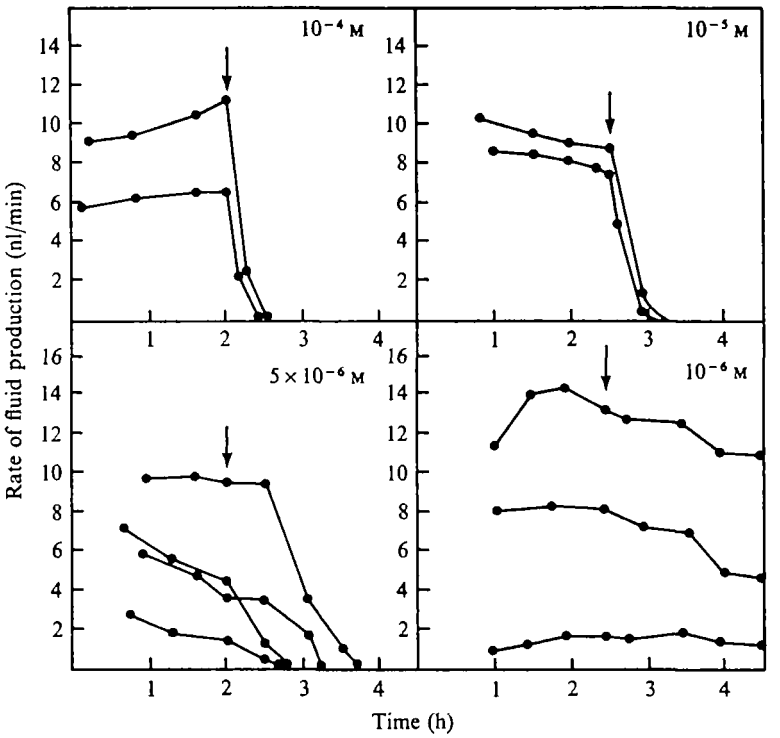


Fig. 4. The effect of various concentrations of ouabain on fluid production by isolated tubules.

*The effect of ouabain on fluid production*

Ouabain is a cardiac glycoside which inhibits a variety of transport processes involving sodium and potassium. It is thought that it exerts its effect by the inhibition of Na/K activated ATPase (Glynn, 1964). Ouabain has no effect on insect Malphigian tubules; therefore it became a matter of interest to determine whether it would inhibit the tubules of *Glomeris*. Ouabain was added to Ringer bathing isolated tubules so as to make final concentrations within the range of 1 mmol ($10^{-3}$ M) and one $\mu$mol ($10^{-6}$ M).

As shown in Fig. 4, low concentrations of ouabain ($5 \times 10^{-6}$, $10^{-5}$ M) were effective in inhibiting fluid production by isolated Malpighian tubules of *Glomeris*. However, the drug had no effect on the sodium and potassium concentrations of the secreted fluid ($5 = 10^{-6}$ M/l, 7 tubules). The threshold concentration for a response to ouabain was $5 \times 10^{-6}$ M, a concentration which is comparable to concentrations effective in the inhibition of other fluid-transporting tissues (Glynn, 1964; Skou, 1960).

*The effect of variations in osmotic pressure on fluid production*

The results of experiments to test the effect of varying the osmotic pressure of the medium on the rate of fluid production by the tubules of *Glomeris* isolated in serum are shown in Fig. 5. The rate of fluid formation varied approximately inversely with the osmotic pressure of the medium. Furthermore, as shown in Fig. 6, the tubule fluid remained nearly isosmotic to the bathing medium. It was possible to approximately double or halve the normal osmotic pressure of the medium whilst fluid production continued. However, fluid production ceased when the osmotic pressure of the mediu

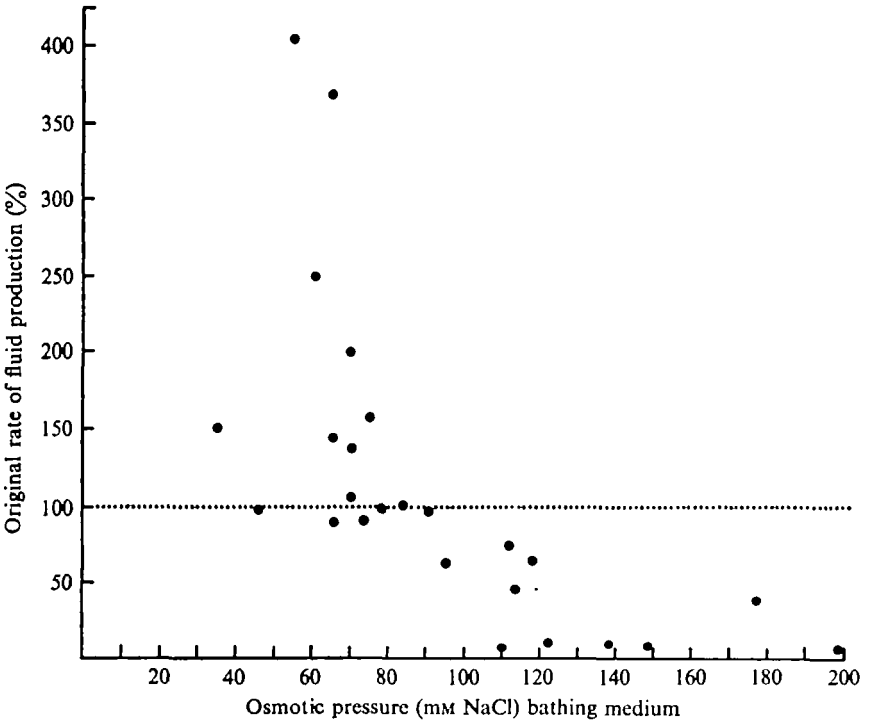


Fig. 5. Rates of fluid production by tubules bathed in media of varying osmotic pressure.

exceeded a concentration equivalent to 200 mM/l NaCl. From Fig. 6 there is some suggestion that the fluid produced by the tubules of *Glomeris* is also hyperosmotic to the bathing medium. However, statistical analysis of the data has shown that the values are not significantly different from the isosmotic line.

### *Electrical measurements*

Attempts were made to determine whether or not an electrical potential difference exists across the wall of Malphigian tubules of *Glomeris*. The method of study was that described by Pilcher (1970). The tips of glass electrodes filled with 3 mol KCl in agar were placed in the drop of medium and in the droplet of fluid being produced by isolated tubule. The electrodes were connected via agar/KCl bridges and calomel half-cells to a Dymar microvoltmeter (type 721). There was no need to perfuse the tubule lumen as the tubule fluid has essentially the same ionic composition as the bathing Ringer drop. The potential difference recorded as described above varied between 0·06 and 0·75 mV (average, 0·46 mV) lumen negative (15 tubules), and the tip potential was in the order of 0·5 mV. It is unlikely that the trans-wall potential is significantly different from zero. The accuracy of this method of measuring trans-epithelial potential differences has been investigated by Berridge & Prince (1972) employing microelectrodes placed in the lumen of the salivary gland of *Calliphora*. Furthermore, potential differences across insect Malpighian tubules measured by Pilcher (1970), Coast (1969) and Maddrell (1971) using the method described above compare favourably with potential differences determined by Ramsay (1953) who

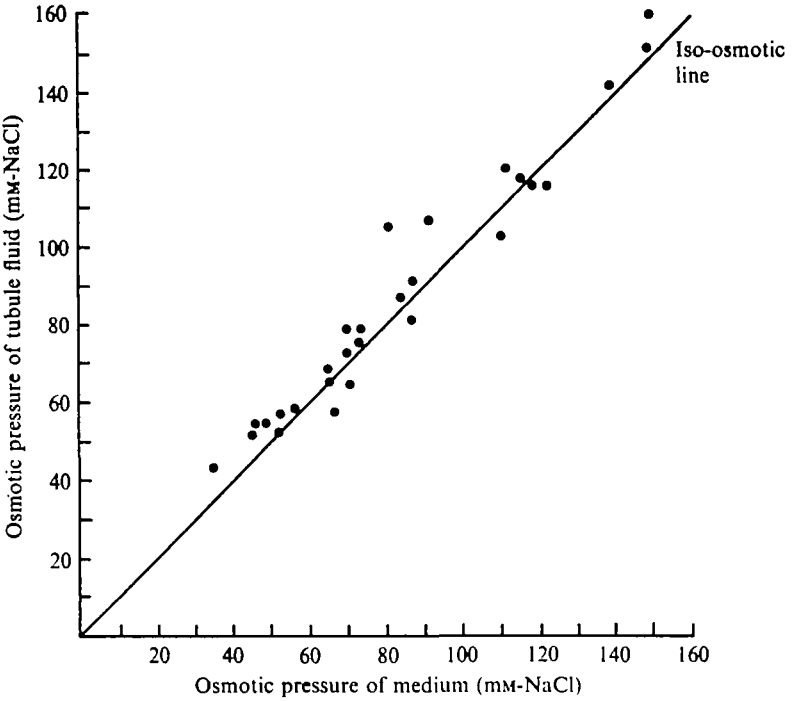


Fig. 6. The osmotic pressure of tubule fluid produced by tubules bathed in media of varying osmotic pressure.

obtained his results by perforating the tubule wall with a microelectrode. It seems a reasonable assumption that no potential difference exists across the tubule wall of *Glomeris*.

## DISCUSSION

It is clear from the results presented here that in their function, at least superficially, the Malpighian tubules of *Glomeris* do not conform to the 'typical' insect pattern. In fact, there is no evidence to suggest that the mechanisms underlying function in *Glomeris* are necessarily of the kind postulated for insect tubules. Work on insect tubules has implicated potassium as the most important ion involved in fluid transport. Its entry into the cell may be facilitated by sodium ions, with an electrogenic pump on the apical surface of the cell. *Rhodnius* tubules differ in that they are able to pump sodium ions as well, resulting in a normal rate of fluid secretion in a potassium-free medium. The tubules of *Glomeris* are similar to those of *Rhodnius* (Maddrell, 1969) in that they are able to secrete fluid at a normal rate in a potassium-free medium. However, the tubules of *Rhodnius* are able to secrete at a rate which is totally independent of the potassium concentration, whereas a higher concentration of this ion appears to have an inhibitory effect on the tubules of *Glomeris*. Furthermore, the tubule fluid produced by *Glomeris* is not characterized by elevated potassium concentrations. In potassium-secreting tubules, e.g. *Calliphora* (Berridge, 1968), and *Carausius* (Ramsay, 1955), the potassium concentration in the tubule fluid was always higher than that in the medium. Maddrell (1969) has shown also that a similar phenomenon exists in the

tubules of *Rhodnius* where the potassium concentration covers the range 0–110 mM/l. The potassium concentration of the final tubule fluid produced by the tubules of *Calpodes* (Irvine, 1969) tends to be very close to that of the medium. However, this is due to the balance of differing secretory and reabsorptive mechanisms operating along the length of the tubule which is composed of four distinct regions. The upper portion of the Malpighian tubule of *Glomeris* is composed of only a single region. Therefore, when considering fluid production by the tubules of *Glomeris*, there seems to be no evidence to suggest the involvement of potassium pumps. Also, the lack of a trans-wall electrical potential difference indicates that fluid transport does not require or generate an electrical potential and therefore does not favour the presence of an electrogenic pump.

Emphasis may be given, however, to the role of sodium in fluid secretion, whose rate is directly related to the sodium concentration in the medium, with no secretion in a sodium-free Ringer. Depriving a transporting tissue of sodium ions may also have secondary effects. Maddrell (1971) has shown that the passage of chloride ions across the wall of the tubule of *Rhodnius* is slowed in the absence of sodium. Fox *et al.* (1964) have found also that active transport of amino acids (glycine, $\alpha$-amino isobutyric acid) in slices of rat kidney cortex was abolished in a sodium-free medium and inhibited by potassium. Thier (1968) has investigated amino-acid accumulation by the toad bladder, whose cells concentrate amino acids by uptake across the serosal surface. It was suggested that the dependence of $\alpha$-amino isobutyric acid uptake on the presence of sodium in fluid bathing the serosal surface of the bladder may be explained by the coupled movement of the amino acid and sodium across the membrane. The active transport of amino acids by a variety of tissue preparations *in vitro* has been shown to be dependent on sodium (see Thier, 1968 for references). Remembering the important role of amino acids in fluid transport by tubules of *Glomeris* (Farquharson, 1973*a*), it is possible that sodium and amino-acid transport are linked in this tissue also. Therefore, fluid production which appears to be dependent upon amino acids may be inhibited in a sodium-free solution because of the interdependence of sodium and amino-acid transport.

It is not possible at this time to do more than speculate about the mechanism responsible for causing sodium to move across the tubule wall. A neutral NaCl pump in which the transport of one Na ion is coupled directly to the transport of one Cl ion could be operating. This kind of mechanism has been postulated by Diamond (1962) to be present in the gallbladder. Significantly, fluid transport by that organ also is inhibited by ouabain. Evidence to support this proposition arises from the observation that no electrical potential difference could be recorded across the tubule wall.

In contrast to insect Malpighian tubules, the tubules of *Glomeris* are very sensitive to low concentrations of ouabain. Berridge (1968), Maddrell (1969) and Pilcher (1970) failed to show an inhibitory effect of ouabain on fluid production by the tubules of *Calliphora*, *Rhodnius* and *Carausius* at concentrations of $10^{-3}$ and $2{\cdot}5 \times 10^{-4}$ M. It is known that the active movement of sodium and potassium across cell membranes involves the hydrolysis of ATP and that the system is inhibited by cardiac glycosides. The nature of the bonds formed between the glycoside and receptor molecules in tissues, and the changes which lead to the inhibition of ATPase activity and cation transport, remain obscure. Although most investigations have been related to the

active, linked transport of sodium and potassium across cell membranes, Diamond (1962) has shown also that ouabain will inhibit the transport of both Na and Cl ions across gallbladder epithelium. The transport of amino acids across intestine (Csaky, 1961) and toad bladder (Thier, 1968) is also sensitive to cardiac glycosides, although this may not be entirely independent of the movements of sodium and potassium. The sensitivity of the tubules of *Glomeris* to ouabain lends further support to the idea that an active sodium pump is involved in fluid transport. However, the ability of the tubules to function normally in a potassium-free Ringer suggests that an active Na/K exchange pump is not an integral part of the secretory mechanism. If it were, then it would be expected that a potassium-free solution would inhibit fluid production in similar manner to ouabain.

In an attempt to account for fluid transport across the Malpighian tubule where there is no obvious hydrostatic pressure gradient, it has been necessary to postulate that water movement is osmotically linked to the movement of ions. Previous studies (Ramsay, 1954; Berridge, 1966*a*, 1968; Maddrell, 1969; Coast, 1969) and the effects of osmotic pressure on fluid production by the tubules of *Glomeris* are consistent with such a proposal. Hence, models of fluid transport have been suggested (Diamond & Bossert, 1967, 1968; Berridge & Oschman, 1969; Maddrell, 1971) which envisage the formation of substantial osmotic gradients within the tissue, and which can account for the production of an isosmotic fluid. Ultrastructural and permeability studies of the tubules of *Glomeris* (Farquharson, 1973*b*) have shown that it is unlikely that localized areas of standing osmotic gradients can be maintained within this tissue. One must, therefore, question the validity of postulating ion pumps before determining how such pumps may be involved in the mechanism of fluid transport. Perhaps it is sufficient to say that fluid transport by the tubules of *Glomeris* is dependent on the availability of sodium ions and that there may be some form of active sodium pump, although how this is involved in the mechanism of fluid transport is not immediately obvious.

SUMMARY

1. Isolated Malpighian tubules of *Glomeris* produce fluid which is identical to the bathing media with respect to ion concentrations and osmotic pressure.

2. The tubules secrete normally in a potassium-free Ringer, but they cease to function in a sodium-free Ringer.

3. The rate of fluid production is dependent on the availability of sodium ions, but it is also inhibited by higher concentrations (25 mM/l) of potassium ions.

4. Throughout a wide variation in concentrations of sodium and potassium ions in the bathing Ringer, the concentrations of these ions in the tubule fluid are the same as their concentrations in the Ringer.

5. Fluid production by the tubules of *Glomeris* is inhibited by ouabain at a concentration of $5 \times 10^{-6}$ M/l or greater.

6. The rate of fluid production varies inversely with the osmotic pressure, and the secreted fluid remains approximately isosmotic to the bathing media.

7. No electrical potential difference could be recorded across the tubule wall.

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