*J. Exp. Biol.* (1970), 53, 465–484
*With* 1 *plate and* 10 *text-figures*
*Printed in Great Britain*



# THE INFLUENCE OF THE DIURETIC HORMONE ON THE PROCESS OF URINE SECRETION BY THE MALPIGHIAN TUBULES OF *CARAUSIUS MOROSUS*

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(*Received* 14 *May* 1970)

## INTRODUCTION

In his work on the Malpighian tubules of *Carausius*, Ramsay (1953–8) measured the rates of urine secretion of tubules bathed by media of differing composition. He also examined the effect which these different media had on the ionic composition of the secreted fluid. On the basis of this work he suggested that the 'prime mover' in urine formation is the transport of potassium against an electro-chemical potential gradient. Sodium can also be transported actively, but the distribution of all other ions, except possibly phosphates, can probably be accounted for by passive movements. The rate of urine secretion increases with increasing external potassium concentration over a wide range, and is also influenced to a lesser extent by the sodium concentration. Ramsay, however, used an external medium containing serum—that is, containing the diuretic hormone (Pilcher, 1970)— so it is of interest to see whether the rate of urine secretion depends upon the potassium concentration in the same way in the absence of the hormone. Moreover, using a wholly artificial medium, it is possible to investigate urine secretion in a medium from which an ion normally present in serum is absent. The performance of tubules in wholly artificial media of varying composition has therefore been examined.

The rate of urine secretion may be increased by several means, including increasing the external potassium concentration (Ramsay, 1955*b*) and adding diuretic hormone (Pilcher, 1970). An attempt has therefore been made to discover how the hormone acts upon the secretory processes involved in urine formation. The mechanism of urine secretion under the influence of the diuretic hormone has been investigated in *Rhodnius* (Maddrell, 1969), but the effect of the diuretic hormone in *Carausius* is of special interest because this species, unlike *Rhodnius*, secretes a considerable volume of urine in the absence of the hormone. The mechanism of urine secretion in the presence and absence of diuretic hormone can therefore be compared.

## MATERIALS AND METHODS

Isolated Malpighian tubules were set up and the rate of urine secretion was determined as described previously (Pilcher, 1970). The normal saline used for dissection

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and as a bathing medium for tubules is based on that used by Wood (1957). Its composition is shown in Table 1. Salines were also prepared having variations in their cation concentrations, as shown in Table 1 (A–G). In saline G the concentrations of magnesium and calcium chlorides were reduced in order to maintain a constant ionic strength, and in saline D sucrose was added to maintain the osmotic pressure. By mixing these salines in various proportions a wide variety of concentrations of sodium and potassium was produced.

Table 1. *The composition of the various salines (mM/l)*

| | A | B | C | D | E | F | G | H | J | K |
|---|---|---|---|---|---|---|---|---|---|---|
| KCl | 15·0 | . | 29·0 | . | . | 15·0 | 76·3 | . | 18·0 | . |
| $K_2HPO_4$ | . | . | . | . | . | . | . | . | . | 7·5 |
| $KH_2PO_4$ | 3·0 | . | 3·0 | 3·0 | . | 3·0 | 3·0 | . | . | 3·0 |
| KOH | . | . | 0·7 | . | . | . | 0·7 | . | . | . |
| NaCl | 12·5 | 27·5 | . | . | . | 2·0 | . | . | 13·8 | . |
| $Na_2HPO_4$ | . | 3·0 | . | . | . | . | . | . | . | 7·5 |
| NaOH* | 2·5 | 2·5 | . | . | . | 2·5 | . | . | . | . |
| $NH_4Cl$ | . | . | . | . | 27·5 | 10·5 | . | . | . | . |
| $(NH_4)_2HPO_4$ | . | . | . | . | 3·0 | . | . | . | . | . |
| $NH_4OH$ | . | . | . | . | 3·0 | . | . | . | . | . |
| $MgCl_2$ | 50·0 | 50·0 | 50·0 | 50·0 | 50·0 | 50·0 | 27·0 | . | . | . |
| $CaCl_2$ | 7·5 | 7·5 | 7·5 | 7·5 | 7·5 | 7·5 | 7·5 | . | . | . |
| $K_2SO_4$ | . | . | . | . | . | . | . | 9·0 | . | . |
| $Na_2SO_4$ | . | . | . | . | . | . | . | 8·0 | . | . |
| $MgSO_4$ | . | . | . | . | . | . | . | 33·0 | 40·0 | 35·0 |
| $CaSO_4$ | . | . | . | . | . | . | . | 2·0 | 2·0 | 2·0 |
| Glucose | 185·5 | 185·5 | 185·5 | 185·5 | 185·5 | 185·5 | 185·5 | 185·5 | 185·5 | 185·5 |
| Sucrose | . | . | . | 60·0 | . | . | . | . | . | . |

* Approximately 2·5 mM/l added until pH 6·6. Saline A is normal saline.

Table 2. *The ionic composition of serum, urine and artificial urine*

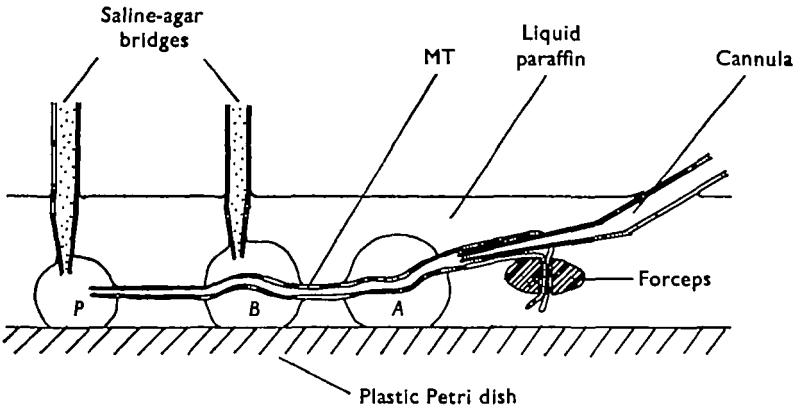
| | Concentration of ion (m-equiv/l) | | |
|---|---|---|---|
| | (1) Serum | (2) Urine | (3) Artificial urine |
| K | 18 | 145 | 150 |
| Na | 11 | 5 | 6 |
| Ca | 7 | 2 | 4 |
| Mg | 108 | 18 | 20 |
| Cl | 87 | 65 | 180 |
| $PO_4^{3-}$ | 39 | 51 | — |

(1) and (2) from Ramsay (1955*a*).

Salines containing different anions were also prepared. It was not possible to prepare a saline having phosphate as the only anion, because of the low solubility product of calcium phosphate. Preliminary investigation showed, however, that sulphate present as the only anion could support urine formation at only 4% of the rate in normal saline, and salines were therefore made up containing magnesium and calcium sulphates, with variations in the remaining anions (Table 1, salines H–K). In salines H and J, containing no phosphate, a little citrate buffer was added.

Serum was prepared as described by Ramsay (1955*a*) and was stored at −10 °C

until needed. The concentrations of ions in artificial urine (Table 2) are based on those given for urine by Ramsay (1955*a*). Breis were made by grinding the tissue with a few microlitres of distilled water. When part of this brei is added to a 50 $\mu$l drop of saline containing a Malpighian tubule, the changes in ionic concentrations produced in the large drop are negligible. After a tubule had been placed in a drop of saline a period of about 30 min was allowed for the tubule to equilibrate with the external medium before the rate of urine secretion was measured or urine collected for analysis.




Text-fig. 1. Diagram to illustrate the method of measuring the trans-wall potential in isolated, perfused tubules. The tubule (MT) is perfused by means of a fine glass cannula connected to a micrometer syringe driven by a small motor. *A* and *B* are drops of saline bathing the tubule, and *P* is a drop of perfusate which has passed through the tubule. Drop *P* is in electrical continuity with the lumen, and thus the potential difference, measured by means of saline-agar bridges connected to calomel half-cells leading to an electrometer, between drop *A* or *B* and drop *P* represents the potential difference between the external bathing solution and the lumen, across the tubule wall.

Measurements of the potential difference across the wall of the tubule were made by the method devised by Maddrell (1970), which is illustrated in Text-fig. 1. The osmotic pressure of the urine and bathing medium were determined by the cryoscopic method of Ramsay & Brown (1955). Potassium concentrations were measured with a Unicam SP 900 flame spectrophotometer. For determinations of the concentrations of sodium, chloride or phosphate in the urine, salines were prepared containing $^{22}Na$ $^{36}Cl$ or $^{32}P$. By comparing the activity/$\mu$l of the saline (whose concentration of the ion is known) with that of the urine, the concentration of the ion in the urine can be calculated. The radioactive isotopes were counted using a Dekatron end-window counter.

Results are expressed as $\pm$ 1 S.E.

## RESULTS

### 1. *The effect on the rate of urine secretion of varying the ionic composition of the bathing medium in the presence and absence of diuretic hormone*

#### (*a*) *The ability of a single monovalent cation to support urine secretion in the absence of diuretic hormone*

Tubules were set up in media containing 33 m-equiv/l of a single monovalent cation, and the rate of urine secretion during 1 h was noted (Table 3). The rate in the potassium-only saline (C) was not significantly different from that in normal saline, but

sodium-only saline (B) is able to support urine secretion at a rate only 17·8% and ammonium-only saline (E) at only 3·7 % of the rate which potassium can support. To test whether the low rate of urine secretion in the presence of ammonium ions is due to their toxicity, part of the sodium of normal saline was replaced by ammonium the potassium remaining constant at 18 m-equiv/l. Ammonium ions at a concentration of 10·5 m-equiv/l are not toxic to the tubule cells, for the rate of urine secretion in this

Table 3. *The effect upon the rate of urine secretion of variations in the cations in the external medium*

| Saline | Concentration of ion in medium (m-equiv/l) K | Na | $NH_4$ | No. of tubules | Rate of urine secretion (nl/min) | Rate as % rate in saline A |
|---|---|---|---|---|---|---|
| A | 18 | 15 | 0 | 17 | 2·14 ± 0·17 | 100·0 |
| C | 33 | 0 | 0 | 31 | 2·05 ± 0·21 | 95·8 |
| B | 0 | 33 | 0 | 39 | 0·38 ± 0·05 | 17·8 |
| E | 0 | 0 | 33 | 15 | 0·08 ± 0·02 | 3·7 |
| A | 18 | 15 | 0 | 9 | 2·46 ± 0·17 | 100·0 |
| F | 18 | 4·5 | 10·5 | 7 | 2·43 ± 0·07 | 98·8 |

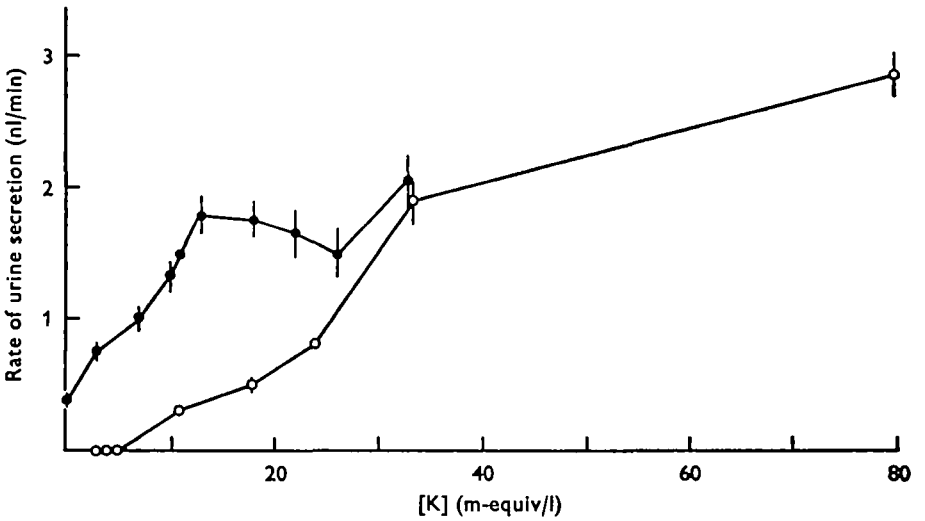
medium is not significantly different from that in the control normal saline (Table 3). Thus it seems that the tubules are able to function in a medium containing a single monovalent cation maintaining a high rate of secretion if this ion is potassium, but a lower rate if it is sodium. Ammonium ions are able to support very little urine secretion.

(*b*) *The effect of variations of sodium and potassium concentrations on the rate of urine secretion in the absence of diuretic hormone*

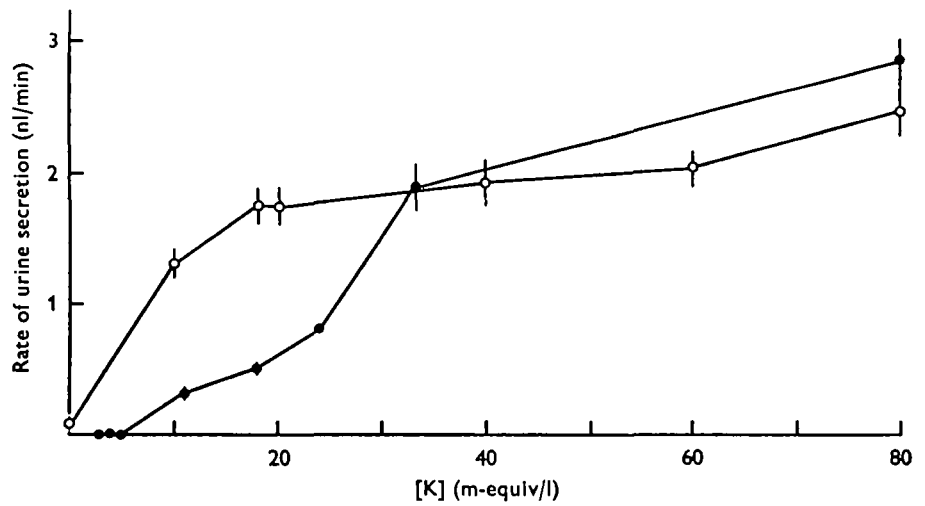
Ramsay (1955*b*) showed that the rate of urine secretion increases more or less linearly with increasing potassium concentration up to about 90 m-equiv/l potassium in the presence of 17 m-equiv/l sodium in a medium containing serum. The experiments described in the previous section show, however, that tubules in a high-potassium artificial medium in the absence of sodium do not secrete significantly faster than tubules in normal saline, which has a lower potassium concentration. The relationship between the rate of urine secretion and the concentration of sodium and potassium in wholly artificial media has therefore been investigated.

The rate of urine secretion by tubules set up in a series of media in which the 33 m-equiv/l potassium is gradually replaced by sodium, or in the absence of sodium, by sucrose (in order to maintain a constant osmotic pressure), is shown in Text-fig. 2. In the absence of sodium the rate of secretion is too low to be measured at potassium concentrations of less than 5 m-equiv/l, but above this concentration the rate of urine, secretion rises with increasing potassium concentration up to 80 m-equiv/l. When sodium is used to replace the potassium, the relationship is different. The rate of urine secretion rises steeply with increasing potassium concentration, reaching a maximum at approximately those concentrations found in the haemolymph (K = 18 m-equiv/l; Na = 15 m-equiv/l). Above this point the rate of secretion rises little as the potassium concentration is increased and the sodium concentration decreased. This constant

rate at higher potassium concentration is not due to lack of sodium, for a similar maximum is reached when the potassium concentration is varied in the presence of a constant sodium concentration of 15 m-equiv/l (Text-fig. 3). Under these conditions



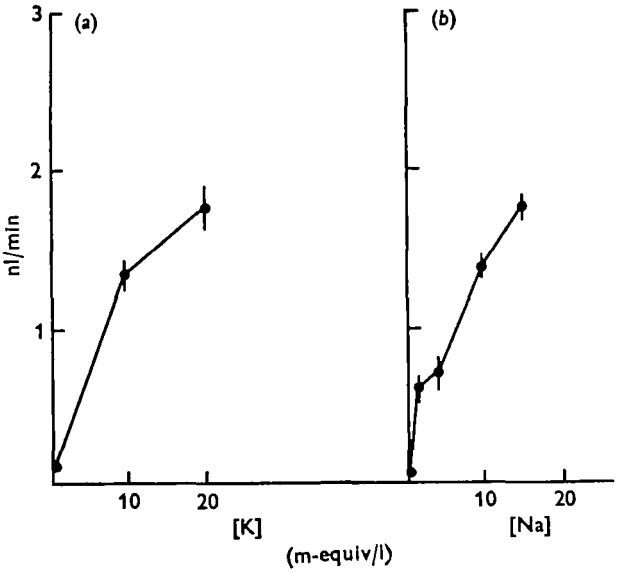

Text-fig. 2. The relationship between the rate of urine secretion and the concentration of K in the external medium, when K is gradually replaced by Na (●——●) or by sucrose (○——○).




Text-fig. 3. The relationship between the rate of urine secretion and the concentration of K in the external medium, in the presence of Na (15 m-equiv/l, ○——○) and in its absence (sucrose present, ●——●).

the magnesium concentration is reduced to maintain constant osmotic pressure. It seems that sodium only enhances urine secretion at lower potassium concentrations, but at higher potassium concentrations a maximum rate of secretion is reached. This enhancement by sodium at lower potassium concentrations depends upon the concentration of sodium present. Text-fig. 4(*b*) shows the effect of different concentrations

of sodium when the potassium concentration remains at 18 m-equiv/l. Comparison with Text-fig. 4(*a*), which is taken from Text-fig. 3, reveals that variation in either potassium or sodium, while the other ion remains at its normal concentration, has a similar effect upon the rate of urine secretion.




Text-fig. 4. The relationship between the rate of urine secretion and the concentration in the external medium of (*a*) K, with Na remaining constant at the concentration present in normal saline (15 m-equiv/l). (*b*) Na, with K remaining constant at the concentration present in normal saline (18 m-equiv/l).

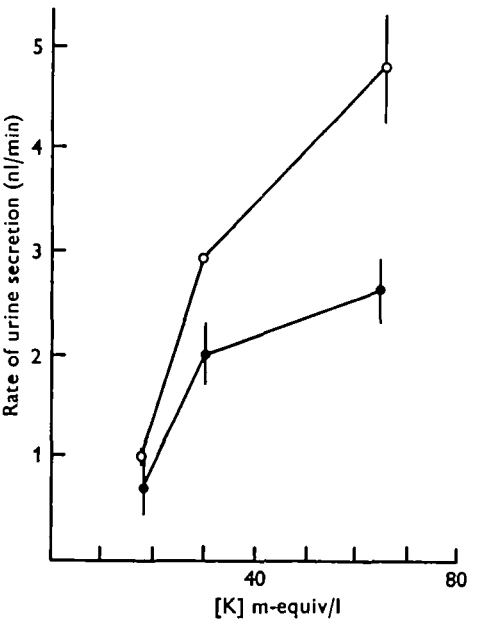
(*c*) *The effect of the diuretic hormone on the rate of urine secretion by tubules in media of differing composition*

Tubules bathed by media with various concentrations of sodium and potassium were treated with breis of brain and corpora cardiaca. Diuresis was found to occur in tubules in a medium containing potassium as the only monovalent cation (saline C), but not when sodium was the only monovalent cation (saline B). Diuresis was possible when the potassium concentration was as low as 3·3 m-equiv/l (Na = 29·7 m-equiv/l). It seems that the diuretic hormone cannot stimulate the urine secretion which occurs in the absence of potassium, but that urine secretion in the absence of sodium can be stimulated by the hormone.

Rates of urine secretion in artificial media and in a mixture of 1 part serum:3 parts saline of various potassium concentrations were determined, and are shown in Text-fig. 5. The concentrations of sodium and potassium in media containing serum were not measured, but were estimated from the figures given for the concentration of these ions in serum by Ramsay (1955*a*) (Table 2). In these experiments, as in those performed by Ramsay (1955*b*), the range of ionic concentrations which could be used was limited by the presence of serum. The sodium concentration of these media is low because in order to obtain high potassium concentrations, serum or normal saline was mixed with saline G (K = 80 m-equiv/l; Na = 0 m-equiv/l). Therefore in order to

make the results comparable, low sodium concentrations were also used at the low potassium concentrations.

Whereas in totally artificial media the rate of urine secretion rises little at potassium concentrations above about 18 m-equiv/l, in the presence of serum the rate continues to rise at potassium concentrations above this value, and the appearance of the graph is rather similar to that obtained by Ramsay (1955*b*, fig. 2).




Text-fig. 5. The rate of urine secretion by tubules in saline (●——●) or in a mixture of 1 part serum : 3 parts saline (○——○), at various concentrations of K in the external medium (Na concentration, 5 m-equiv/l).

### 2. *The role of anions in urine secretion*

#### (*a*) *The effect of variations of the anions in the external medium upon the rate of urine secretion*

In order to study the ability of various anions to accompany cations, rates of urine secretion by tubules in normal saline were compared with rates of secretion by tubules from the same insect set up in saline containing different anions (Table 4). The rate of urine secretion in sulphate-only saline (H) is only 4·8% of that in normal saline, but the replacement of part of the sulphate with 31 m-equiv/l chloride (saline J) allows a rate of 39%, and with 33 m-equiv/l phosphates (saline K, $HPO_4''$ and $H_2PO_4'$ to give pH 6·6), a rate of 57% of that in normal saline is obtained. The limited ability of sulphate ions to accompany cations in secretion probably reflects the low permeability of the tissue to these ions. This has been demonstrated in many other tissues, and is thought to be due to the large hydrated radius of the sulphate ion. The rate of secretion in phosphate saline is significantly higher than in chloride saline at the 1% level (*t* test).

Table 4. *The rate of urine secretion in media containing various anions in the presence and absence of* $Cu^{2+}$

| Saline | Anions present | No. of tubules | Rate of urine secretion (expressed as % of rate during first hour in normal saline) Before $Cu^{2+}$ | After $Cu^{2+}$ |
|---|---|---|---|---|
| A | $Cl'$, $HPO_4''$, $H_2PO_4'$ | 42 | 100·0 ± 6·9 | 13·0 ± 1·4 |
| H | $SO_4''$ | 11 | 4·8 ± 1·8 | 4·3 ± 1·1 |
| J | $SO_4''$, $Cl'$ | 24 | 39·1 ± 3·1 | 11·5 ± 1·7 |
| K | $SO_4''$, $HPO_4''$, $H_2PO_4'$ | 15 | 56·8 ± 5·8 | 11·7 ± 2·5 |
| A | Control, no $Cu^{2+}$ added | 41 | . | 92·6 ± 3·8 |

(*b*) *The effect of copper ions on the rate of urine secretion*

Copper is thought to render the membranes of frog skin impermeable to chloride (Koefoed-Johnsen & Ussing, 1958), and Berridge (1969) has suggested that copper inhibits the passage of anions through pores in the membranes of the Malpighian tubules of *Calliphora*. He finds that phosphate movement is unaffected by copper and postulates a carrier system for phosphate transport.

*Carausius* tubules were set up in saline containing different anions, and were treated with $10^{-4}$ M cupric sulphate, made up in saline immediately beforehand to avoid the precipitation of copper hydroxide or phosphates which occurs on standing. The rates of urine secretion in the different media before and after treatment with cupric ions are shown in Table 4. It can be seen that the presence of cupric ions does not cause a significant change in the rate of secretion in the presence of sulphate only ($P > 50\%$, *t* test), but in salines containing sulphate and phosphate or sulphate and chloride, and in normal saline (phosphate and chloride) there is a large fall in rate. Since the performance of tubules in sulphate-only saline is unimpaired, it seems that the effect on the tubules in other media is not due to the toxicity of the cupric ion.

3. *The effect of the diuretic hormone on the osmotic pressure and composition of urine secreted by tubules in normal saline*

(*a*) *Osmotic pressure*

The osmotic pressure of the urine was measured before and after the addition of a brain or corpus cardiacum brei. The results set out in Table 5 show that there is no significant change in the osmotic pressure during the hour following the addition of diuretic hormone; that is, when the diuresis is greatest (Pilcher, 1970). The urine remains more or less isosmotic with the bathing medium. The mean freezing-point depression of the urine secreted in the absence of diuretic hormone is 98·7 ± 1·1 % ($n = 10$) of that of the medium bathing the tubule. This difference between the osmotic pressure of the urine and medium is not significant ($P > 5\%$, *t* test).

Table 5. *The effect of diuretic hormone on the depression of freezing-point (Δ °C) of urine*

| Time, $t$ (min) | No. of tubules | Δ °C | $\left(\frac{\Delta_t}{\Delta_o} \times 100\right)$ % |
|---|---|---|---|
| −10 | 8 | 0·732 ± 0·012 | 100·0 |
| | | Hormone added | |
| +10 | 8 | 0·730 ± 0·009 | 101·7 |
| 20 | 10 | 0·738 ± 0·011 | 101·8 |
| 30 | 9 | 0·736 ± 0·008 | 101·1 |
| 40 | 9 | 0·747 ± 0·015 | 102·6 |
| 50 | 8 | 0·747 ± 0·010 | 102·4 |
| 60 | 6 | 0·733 ± 0·015 | 99·5 |
| Medium | 9 | 0·738 ± 0·012 | |

$\Delta_o$ = Δ °C before addition of hormone,
$\Delta_t$ = Δ °C at time $t$ after addition of hormone.

In the calculation of $\frac{\Delta_t}{\Delta_o} \times 100$ (%), $\Delta_t$ is expressed as a percentage of $\Delta_o$ for the same tubule,

Table 6. *The concentration of certain ions in the urine secreted by tubules in the presence and absence of diuretic hormone*

| | Ion | | | |
|---|---|---|---|---|
| | K | Na | Cl | Phosphate |
| 1st h | | | | |
| $n$ | 15 | 14 | . | . |
| Concn of ion, tubule in saline (m-equiv/l) | 154·4 ± 6·2 | 5·1 ± 0·4 | . | . |
| $n$ | 8 | 13 | . | . |
| Concn of tubule in 1:3 serum:saline (m-equiv/l) | 153·8 ± 7·9 | 5·1 ± 0·5 | . | . |
| 2nd h | | | | |
| +hormone | | | | |
| $n$ | 23 | 20 | 12 | 10 |
| Concn (m-equiv/l) | 150 ± 4·8 | 5·1 ± 0·4 | . | . |
| Concn as % 1st h | 97·1 ± 3·1 | 99·3 ± 7·3 | 99·3 ± 5·1 | 105·0 ± 6·9 |
| $P$ ($t$ test) for diff. from 1st h | > 25% | > 50% | > 50% | > 50% |
| No hormone (control) | | | | |
| $n$ | 4 | 15 | 13 | 9 |
| Concn (m-equiv/l) | . | 6·7 ± 0·5 | . | . |
| Concn as % 1st h | 97·3 ± 1·4 | 130·4 ± 9·9 | 93·1 ± 4·9 | 86·0 ± 8·7 |
| $P$ ($t$ test) for diff. from 1st h | > 50% | < 0·1%* | > 50% | > 10% |

* $P$ for difference between control and tubule with hormone < 2·5%.

(*b*) *Ions*

The concentrations of potassium and sodium in the urine secreted by tubules bathed in saline, in a mixture of saline and serum and in saline to which a brei of brain or corpus cardiacum had been added are shown in Table 6. The concentration of these ions is similar in the urine secreted by tubules in saline and in the saline/serum mixture. The addition of diuretic hormone caused no significant change in the potassium concentration of the urine ($P > 25\%$, $t$ test), and the four control measurements fall within the same range, indicating that changes in rates of potassium secretion and urine flow must be proportional. Addition of diuretic hormone causes no significant change in the sodium concentration of the urine; that is, increased sodium

secretion occurs with increased urine flow. In the control tubules, however, although the rate of urine flow falls, the rate of sodium secretion remains constant ($101 \cdot 9 \pm 9 \cdot 9\%$), causing a significant rise in the sodium concentration of the urine. Thus sodium secretion rises on addition of diuretic hormone, but remains constant in its absence, while potassium secretion rises on addition of diuretic hormone, but falls in its absence during the second hour.

The concentration of chloride in the urine secreted during the second hour in the presence or absence of diuretic hormone is not significantly different from that secreted during the first hour ($P > 50\%$ in both cases). The $U/P$ ratio for chloride, that is,

$$\frac{\text{cpm}/\mu\text{l in urine}}{\text{cpm}/\mu\text{l in medium}},$$

was close to, but slightly less than, 1 (0·80, 0·98), which indicates that the chloride concentration in the urine approaches that in the medium. The phosphate concentration in the urine was also measured. $Na_2HPO_4$, labelled with $^{32}P$ was added to the external medium containing $H_2PO_4'$ and $HPO_4''$. $^{32}P$ would thus become distributed between these two forms of phosphate. Ramsay (1956) found that the pH of the urine secreted in a medium of pH 6·6, as in the salines used here, was about pH 7·3.

Table 7. *The* U/P *ratio for* $^{32}P$ *before and after the addition of diuretic hormone*

| | $U/P$ ratio | | | | | |
|---|---|---|---|---|---|---|
| Before addition of hormone | 2·27 | 2·31 | 2·46 | 2·59 | 2·76 | 2·96 |
| After addition of hormone | 2·55 | 2·75 | 3·55 | · | · | · |

Thus the ratio of $H_2PO_4'$ to $HPO_4''$ will be different in the urine and medium. Ramsay (1956) pointed out the difficulty of interpreting results obtained for concentrations of phosphate by this method. It can be seen, however, from Table 7 that the concentration of $^{32}P$ is not significantly altered in the presence of a diuretic brei, in the second hour as compared with the first hour, nor does it fall significantly in the control tubules. The $U/P$ ratio remains greater than 1 on addition of a brei.

It seems therefore that the diuretic hormone does not cause changes in the composition of the urine, but causes the secretion of urine of the same composition at a faster rate.

### 4. (*a*) *The potential difference across the tubule wall*

Measurement of the potential difference across the wall of the Malpighian tubules *in situ* have been made in several species by Ramsay (1953), but it is difficult to compare these results with those obtained for single isolated tubules in the present investigation. Whilst Ramsay's measurements are a better reflexion of the situation *in vivo*, since the tubules are surrounded by and filled with their natural fluids, the limitations of this as an experimental technique are severe. It is not possible to control the composition of the internal solution, and, since the tubules remain attached to the insect, large volumes of external bathing solution are needed. Thus vast amounts of hormone are required to produce a concentration which will affect the tubules. Both the internal

and external environment of isolated, perfused tubules can be controlled, and since the rate of perfusion is large (in the order of 40 nl/min) compared with the rate of urine flow (around 2 nl/min), changes in the composition of the internal solution due to ion secretion will be small. Moreover, in Ramsay's preparation, the tubule wall is damaged when it is pierced by an electrode, whereas in the isolated preparation the potential is measured across the undamaged wall.

Table 8. *Trans-wall potentials in tubules bathed in normal saline and perfused with artificial urine*

| Insect | Tubule | Potential (mV)* |
|---|---|---|
| 1 | a | −24, −24 |
| | b | 0, −14, −14 |
| 2 | a | +64 |
| | b | +51 |
| 3 | a | −6 |
| | b | +5 |
| 4 | . | −10 |
| 5 | . | −20 |
| 6 | . | +20 |

* A negative potential indicates that the lumen is negative with respect to the bathing medium.

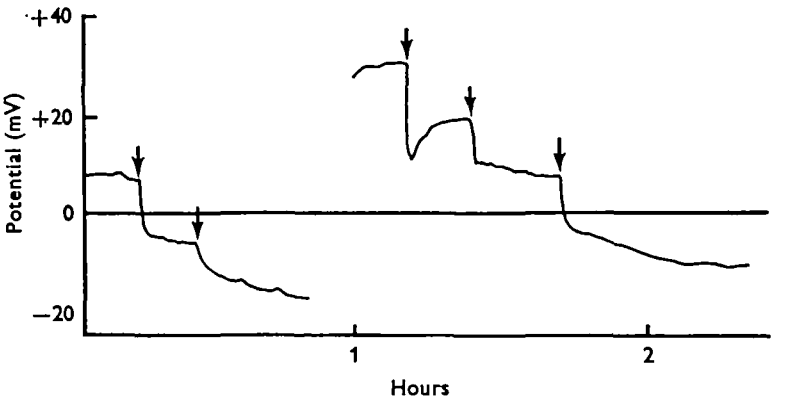
Table 9. *The trans-wall potential in a number of tubules bathed in and perfused with identical salines*

| $[K^+]_o = [K^+]_i$ (m-equiv/l) | Potential (mV) |
|---|---|
| 18 | +12, +10, +7, +5, +3, −2, −3, −5, −10, −10, −10, −12, −14 (+48, +35, +34, +26)* |
| 80 | +45, +30, +30, +13, +10 |

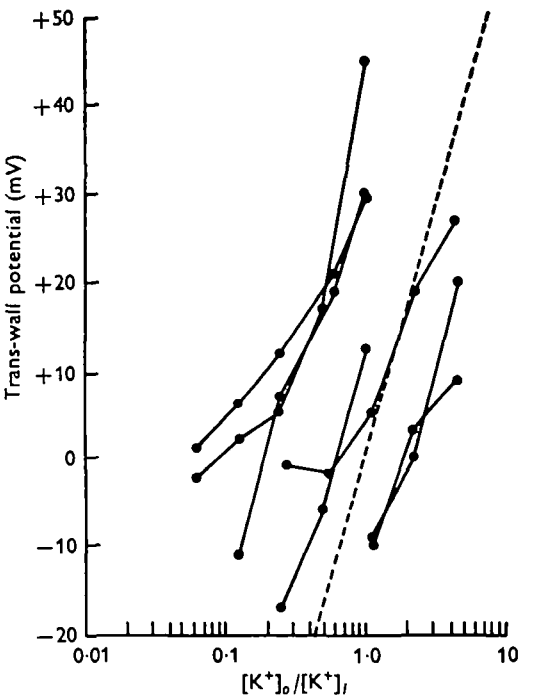
* Recorded from tubules which were poorly cannulated.

Experiments in which tubules were bathed in normal saline and perfused with artificial urine gave results for the trans-wall potential which were very variable (Table 8). The potential varies not only between tubules from one insect, but also along the length of the tubule. Some of the variation may be due to variations in the rate of perfusion and also in ambient temperature, both of which have a profound effect upon the trans-wall potential in *Rhodnius* tubules (Maddrell, 1970). Table 9 shows the trans-wall potential measured in tubules bathed in and perfused with identical salines. In tubules which were poorly cannulated, in which little fluid passed along the lumen, but instead accumulated around the point of cannulation, the trans-wall potential is consistently higher; that is, the lumen is more positive to the bathing fluid. Since the potentials recorded across the walls of the tubules surrounded by normal saline and perfused with artificial urine (Table 8) are much lower than those found by Ramsay (1953), it seems possible that the rate of perfusion may be higher than optimum, thus reducing the potential, but it seems likely that the changes in potential still reflect those which would occur *in vivo*, under optimal conditions.

If the external potassium concentration is decreased while the internal concentration remains constant, potential changes can be recorded, as shown in Text-fig. 6. If the steady values of these potentials ($E$ mV) are plotted against the logarithm of the ratio of the external to the internal potassium concentration ($\log_{10} K_o/K_i$), the slope of




Text-fig. 6. The effect on the trans-wall potential of changes in the external K concentration. In each case, the initial internal and external K concentration was 80 m-equiv/l, but at intervals, indicated by arrows, the external K concentration was halved by the addition of an equal volume of K-free saline.




Text-fig. 7. The relationship between the trans-wall potential and the ratio of external to internal K concentration, $[K^+]_o/[K^+]_i$, (log-scale). Measurements on the same part of a single tubule are joined. – – – –, Theoretical slope (58 mV change in potential for a tenfold change in K ratio), predicted by the Nernst equation.

the graph (Text-fig. 7) is similar to that predicted by the Nernst equation for a potassium diffusion potential:

$$E = \frac{RT}{zF} \log_e \frac{C_o}{C_i}$$

where $E$ = potential difference (mV), $R$ = gas constant, $z$ = valency of ion, $F$ = Faraday's constant, $C_o$ = external concentration of ion, $C_i$ = internal concentration of ion, or, by substituting for potassium at 18 °C,

$$E = 58 \log_{10} \frac{K_o}{K_i}.$$

This indicates that one barrier in the tubule, accessible from the blood side, is selectively permeable to potassium. Since it is not practicable to change the potassium concentration in the internal bathing solution, it is not possible to demonstrate conclusively whether this potassium-selective barrier is at the blood-side or lumen-side border of the cell, but it seems probable that it is at the blood-side.

Table 10. *The trans-wall potential in tubules bathed in and perfused with identical salines containing various anions*

| Concentration of ion (m-equiv/l) | | | (mM/l) | Potential | Rate of urine secretion |
|---|---|---|---|---|---|
| $Cl'$ | $HCO_3'$ | $SO_4''$ | $HPO_4'' + H_2PO_4'$ | (mv) | (%) |
| 143 | 0 | 0 | 3 | +12, +10, +7, +5, +3, −3, −5, −10, −10, −10, −12, −14 | 100·0 ± 6·9, $n$ = 42 |
| 0 | 12 | 92 | 3 | +10, +23, +26, +35 | 23·4 ± 1·6, $n$ = 24 |

The potential across the wall of tubules bathed and perfused by saline in which the chloride has been replaced by sulphate and bicarbonate is consistently higher than in tubules in normal saline perfused at a similar rate (Table 10). In a sulphate and bicarbonate saline the rate of urine secretion is lower than in normal saline.

4. (*b*) *The effect of the diuretic hormone on the trans-wall potential*

The addition of diuretic hormone as a brei or serum results in an increase in the lumen-positive potential, as shown in Text-fig. 8 and Tables 11 and 12. The time course of this change is similar to that of the change of rate of urine secretion. This change in potential occurs without any change in the concentration of potassium, sodium, chloride or phosphate in either the bathing or perfusing media, for it was shown in §3(*b*) that the addition of diuretic hormone causes no change in the concentration of these ions in the urine. Similar rises in potential are seen on addition of diuretic hormone when the tubules are bathed and perfused by differing salines, and can be seen when the potassium concentration is as low as 3·3 m-equiv/l and as high as 80 m-equiv/l (Table 13). Diuresis is also possible at both these low and high potassium concentrations.

Table 11. *The effect of diuretic hormone on the trans-wall potential in tubules bathed in and perfused with normal saline*

| Steady potential (mV) | | |
|---|---|---|
| Before hormone | After hormone | Change in potential (mV) |
| +7·5 | +11·0 | +3·5 |
| +2·5 | +17·0 | +14·5 |
| −5·0 | +20·0 | +25·0 |
| −10·0 | +7·5 | +17·5 |
| +7·0 | +18·0 | +11·0 |
| −2·5 | +12·5 | +15·0 |
| +2·5 | +12·0 | +9·5 |
| −14·0 | +10·0 | +28·0 |
| +10·0 | +27·0 | +17·0 |

Table 12. *The effect of the addition of serum on the trans-wall potential in tubules bathed in normal saline and perfused with artificial urine*

| Steady potential (mV) | | |
|---|---|---|
| Before serum | After serum | Change in potential (mV) |
| −24 | 0 | +24 |
| −22 | +6 | +28 |
| 0 | +10 | +10 |

Table 13. *The effect of addition of diuretic hormone on the trans-wall potential in tubules bathed in and perfused with salines of various composition*

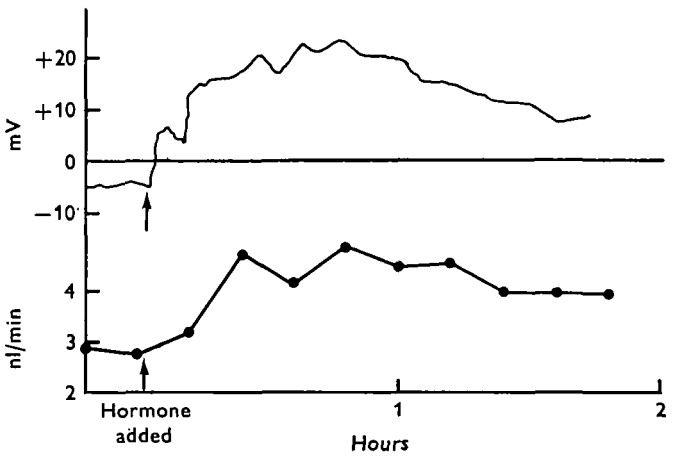
| [K+] (m-equiv/l) | [K+] (m-equiv/l) | Steady potential (mV) | | Change in potential (mV) |
|---|---|---|---|---|
| | | Before hormone | After hormone | |
| 80 | 18 | +10 | +25 | +15 |
| 33 | 18 | +50 | +85 | +35 |
| 18 | 150 | −2 | +24 | +26 |
| 18 | 80 | +26 | +40 | +14 |
| 3·3 | 18 | +10 | +30 | +20 |

### 5. *The effect of ouabain and dinitrophenol on the rate of urine secretion and trans-wall potential*

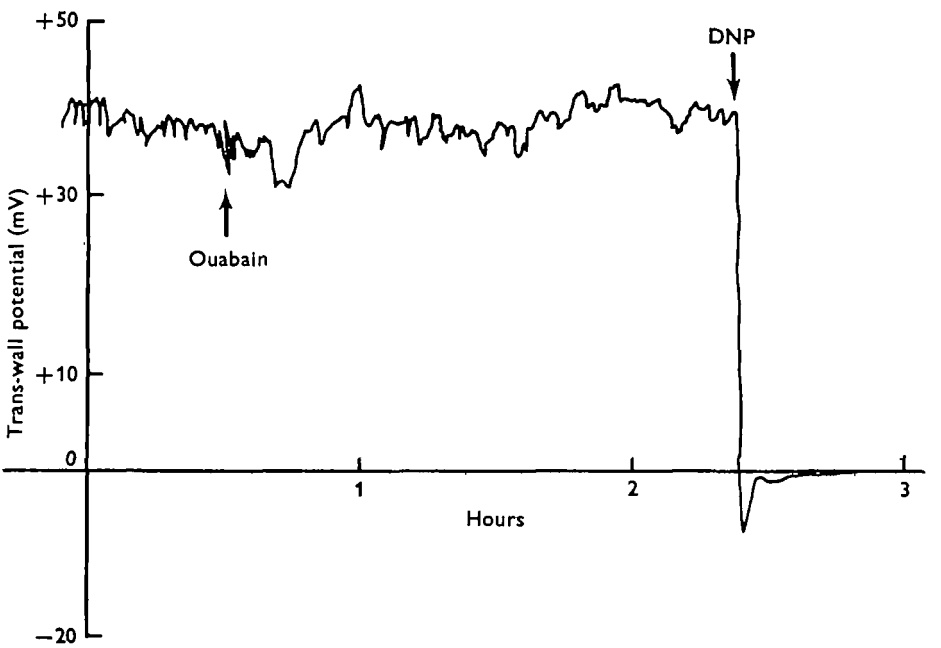
The cardiac glycoside, ouabain, has been shown to interfere with the ATPase involved in sodium/potassium forced exchange pumps in a number of tissues (see Glynn, 1964). The midgut of *Cecropia* and the Malpighian tubules of *Calliphora* and *Rhodnius*, all of which are involved in potassium transport, are, however, insensitive to ouabain (Haskell, Clemons & Harvey, 1965; Berridge, 1968; Maddrell, 1969). 2,4-Dinitrophenol (DNP) is a metabolic inhibitor which uncouples oxidative phosphorylation and thus cuts off the energy supply to the active tissues. The effect of these substances added to the external bathing solution on the rate of urine secretion and on the trans-wall potential has been tested.

Ouabain ($10^{-4}$ M) has no effect on the rate of secretion or on the trans-wall potential, whereas DNP ($10^{-4}$ M) abolishes both urine flow and, when the tubules are bathed

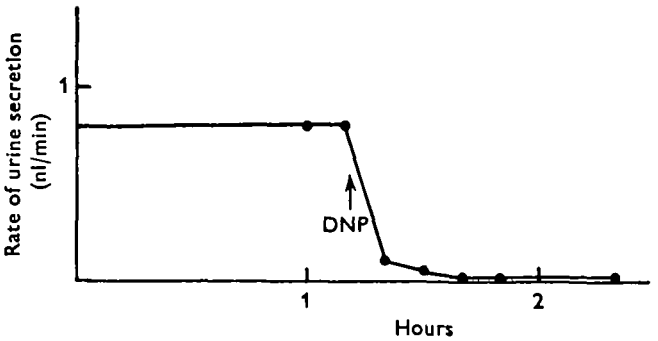
and perfused by identical salines, the potential also (Text-figs. 9, 10; Table 14). Ouabain, moreover, does not affect diuresis or the potential changes associated with it. It therefore seems that if a sodium/potassium forced exchange pump is important in urine secretion, it is not of the conventional ouabain-sensitive type. Alternatively, it may be that a sodium/potassium exchange pump is not directly involved in urine secretion, but may exist, as in many cells, and serve to maintain the intracellular ionic concentrations.




Text-fig. 8. The effect of the addition of diuretic hormone on the trans-wall potential (upper record), in a tubule bathed in and perfused with normal saline. The time course of the potential change can be compared with that of the change of rate of urine secretion (lower record).




Text-fig. 9. The effect upon the trans-wall potential of $10^{-3}$ M ouabain and $2 \times 10^{-4}$ M DNP. (Tubule bathed in and perfused with normal saline.)




Text-fig. 10. The effect upon the rate of urine secretion of $2 \times 10^{-4}$ M DNP.

Table 14. *The effect of ouabain on the rate of urine secretion*

| Concentration of ouabain | No. of tubules | Rate of urine secretion in presence of ouabain (% of rate in its absence during the previous hour) |
|---|---|---|
| $1 \times 10^{-5}$ M | 10 | $83{\cdot}3 \pm 5{\cdot}4$ |
| $2{\cdot}5 \times 10^{-4}$ M | 9 | $88{\cdot}6 \pm 5{\cdot}0$ |
| Absent (control) | 20 | $86{\cdot}6 \pm 3{\cdot}1$ |

## DISCUSSION

The results described above are in agreement with Ramsay's theory (1953) that the 'prime mover' in urine secretion is active transport of potassium. Potassium as the only monovalent cation present in a saline is able to support urine secretion at a far higher rate than sodium, while ammonium, calcium and magnesium can support only a very low rate. In the absence of potassium the tubules must be transporting sodium, but it is not possible to tell whether sodium and potassium use the same or different secretory channels. Ramsay (1955*b*) showed that *Carausius* tubules can pump sodium in the presence of potassium, and found no evidence of competition between sodium and potassium for a common secretory channel.

The ability of tubules to secrete urine is influenced profoundly by the concentration of sodium. The difference between the rates of urine secretion in the presence of 15 m-equiv/l sodium and in its absence at lower potassium concentrations is far too great to be accounted for by urine secretion resulting from sodium transport, at least of the type seen in the absence of potassium. It seems that the sodium in some way allows more potassium to be pumped at the same potassium concentration. A similar sodium effect has been demonstrated in *Calliphora* (Berridge, 1968). In *Rhodnius*, however, the rate of urine secretion remains more or less constant over the range of concentrations K = 1, Na = 149 m-equiv/l to K = 150, Na = 0 m-equiv/l, but an increase in sodium concentration from 0 to 1 m-equiv/l results in an acceleration of about $2\frac{1}{2}$ times (Maddrell, 1969).

The addition of the diuretic hormone results in faster secretion of urine without any change in the concentrations of potassium, sodium, chloride or total phosphate. Since potassium secretion, which is thought to be the 'prime mover' in urine secretion

(Ramsay, 1953), is accelerated, it seems likely that the action of the diuretic hormone is primary to stimulate potassium transport.

During the second hour, in the absence of diuretic hormone, the rate of urine secretion falls but the rate of sodium secretion is maintained, leading to a rise in the sodium concentration of the urine. Since it is not known to what extent the sodium in the urine can be accounted for by active transport and to what extent by passive movements, it is difficult to explain this observation. It does, however, indicate a degree of independence between the rates of potassium and sodium secretion.

The trans-wall potential is dependent for its maintenance upon metabolic energy, and thus might be due either to an electrogenic ion pump or to diffusion resulting from an electrically neutral pump working together with a selectively permeable membrane. The increase in potential without changes in concentration of ions in either the medium or urine on the addition of diuretic hormone suggests that it might be the former, but there is insufficient evidence to decide this point.

The reduction in the rate of urine secretion when chloride or phosphate is replaced by sulphate suggests that cation transport (mainly potassium) requires an accompanying anion, and that sulphate ions are not able to pass through the cell fast enough to accompany the transported potassium, so that the rate of potassium transport is slowed. The rise in potential seen in sulphate/bicarbonate saline is consistent with the idea that there might be an electrogenic potassium pump, with chloride and other permeant anions following passively, but it is also possible that the same situation might arise with other types of pumps.

The significantly higher rate of urine secretion in phosphate saline than in chloride saline is in accordance with Ramsay's (1956) finding that the replacement of chloride by phosphate in a medium containing serum results in a higher rate of urine secretion. The hydrated ionic radii of $H_2PO_4'$ and $HPO_4''$ are 4·04 and 5·11 Å respectively, and both of these are considerably greater than those of chloride (1·90 Å) and sulphate (3·64 Å) (calculated from Araki, Ito & Oscarsson, 1961; Solomon, 1960). If the inability of sulphate ions to accompany secreted cations is due to their large hydrated radius, as was suggested earlier, then it seems probable that the passage of the larger phosphate ions into the urine is not by simple diffusion alone. Berridge (1969) has shown that the tubules of *Calliphora*, like those of *Carausius* (Ramsay, 1956) are able to concentrate phosphate in the urine, and that the rate of urine secretion in a saline containing these anions is higher than in saline containing other anions of a similar size. Since the secretion of urine by *Calliphora* tubules in phosphate saline is not inhibited by cupric ions, it is suggested that a carrier mechanism is involved in phosphate secretion (Berridge, 1969). Ramsay (1956) has pointed out that there are insufficient grounds for assuming that phosphate is actively transported in *Carausius* tubules, and the demonstration that urine secretion by tubules in phosphate saline is inhibited by cupric ions suggests that the mechanism of phosphate secretion is different in the two species.

If potassium transport occurs by an intracellular route, potassium ions must cross both a basal and an apical barrier. It is possible that there are active steps at both barriers. Indeed, it is difficult to visualize purely passive fluxes of this size occurring at either barrier. Ultrastructural studies (Plate 1) of *Carausius* Malpighian tubules reveal basal infoldings and apical microvilli, each with abundant mitochondria which

would be necessary if there were both a basal and apical active step. Urine secretion occurs when tubules are surrounded by as little as 3·3 m-equiv/l potassium in the absence of sodium, so the tubule cells must be able to take up potassium from this low external concentration into the cell. In other tissues, the intracellular potassium concentration has been found to lie within the range 50–100 m-equiv/l (e.g. Steinbach & Spiegelman, 1943; Keynes & Lewis, 1951; Shaw, 1955; Robertson, 1957; Harvey & Zerahn, 1969). The concentration gradient may be reduced by, for example, binding of intracellular potassium, which may occur in frog muscle (Ling & Cope, 1969) and Harvey & Zerahn (1969) have shown that the transported potassium does not mix with all the intracellular potassium in the midgut of *Cecropia*. Nevertheless, there must still be a substantial concentration gradient against which potassium must enter the cell. Whilst potassium could enter the cell against such a concentration gradient if there were a potential across the barrier, it is probable that such a potential would be actively maintained by some sort of pump.

There are many possible models which might help to explain the process of urine secretion in *Carausius* (Pilcher, 1969), and while none of these is wholly satisfactory, it is worth considering briefly some of the possibilities. It is possible that there is an apical potassium pump and two different basal mechanisms for entry of potassium into the cell. In the absence of sodium, potassium would enter slowly at a rate dependent upon the external potassium concentration. In the presence of sodium, however, entry would be enhanced, at least at lower potassium concentrations, by an additional sodium-dependent mechanism. This is similar to the model proposed by Berridge (1968) for *Calliphora* tubules. At lower potassium concentrations the rate of urine secretion appears to be limited by the rate at which potassium can enter the cell, since the rate of urine secretion in the absence of sodium is increased by the addition of sodium, that is, when the sodium-dependent entry mechanism is activated. At higher external potassium concentrations, however, the presence of sodium does not enhance urine secretion, that is, an increase in rate of entry of potassium into the cell does not result in an increase in rate of urine secretion. One possible explanation is that at higher external concentrations of potassium the rate of urine secretion is limited not by the rate of potassium entry, but by the apical potassium transport step.

The action of the diuretic hormone must now be considered. Diuresis can occur both at lower potassium concentrations (K = 3·3; Na = 29·7 m-equiv/l) when potassium entry, according to the model proposed above, may be limiting, and at higher concentrations (K = 33, Na = 0 m-equiv/l) when the apical pump may be saturated. It therefore seems possible that the diuretic hormone is able both to increase the rate of entry of potassium into the cell, and to relieve the saturation of the apical pump, perhaps by accelerating it. This would permit faster pumping of potassium without alteration of the composition of the urine.

It is possible to speculate about the nature of the pumps involved (Pilcher, 1969), but not to reach any satisfactory conclusion. An investigation of the potential profile of the cell might help in this respect. It is possible that the apical pump might be an electrogenic potassium pump or an electrically neutral potassium chloride pump. It was suggested that there may be two basal entry mechanisms for potassium. The sodium-independent mechanism might conceivably involve diffusion down an electrical

gradient. The sodium-dependent entry mechanism is unknown, but it appears that an ouabain-sensitive sodium/potassium exchange pump is not involved.

SUMMARY

1. The rate of urine secretion and the trans-wall potential have been measured in single, isolated Malpighian tubules in media of varying ionic composition.

2. Potassium, present as the only monovalent cation in a saline, is able to support urine secretion at a higher rate than sodium. In the absence of sodium, the rate of urine secretion depends upon the concentration of potassium, but is enhanced in the presence of sodium, especially at low potassium concentrations.

3. The replacement of part or all of the anions normally present in saline by sulphate results in a reduction in rate of urine secretion and a rise in trans-wall potential.

4. Neither the rate of urine secretion nor the trans-wall potential is affected by ouabain, but both are reduced by 2,4-dinitrophenol.

5. The ionic composition of the urine remains unchanged by the addition of diuretic hormone to the medium surrounding the tubule, but the rate of urine secretion and the trans-wall potential are increased.

6. It is suggested that the diuretic hormone acts by stimulating active potassium transport.

This paper represents part of a thesis submitted to the University of Cambridge for the degree of Ph.D. I am indebted to Dr S. H. P. Maddrell for his advice and supervision, and for much helpful discussion. I am grateful to Dr M. J. Berridge for his valuable criticism of the manuscript, and to Mr D. Chapman for the electron micrograph (Plate 1). I wish to thank Girton College, Cambridge, and the Science Research Council for financial support during tenure of Studentships.

REFERENCES

ARAKI, T., ITO, M. & OSCARSSON, O. (1961). Anion permeability of the synaptic and non-synaptic motoneurone membrane. *J. Physiol., Lond.* **159**, 410–35.

BERRIDGE, M. J. (1968). Urine formation by the Malpighian tubules of *Calliphora*. I. Cations. *J. exp. Biol.* **48**, 159–74.

BERRIDGE, M. J. (1969). Urine formation by the Malpighian tubules of *Calliphora*. II. Anions. *J. exp. Biol.* **50**, 15–28.

GLYNN, I. M. (1964). The action of cardiac glycosides on ion movements. *Pharmac. Rev.* **16**, 381–407.

HARVEY, W. R. & ZERAHN, K. (1969). Kinetics and route of active K-transport in the isolated midgut of *Hyalophora cecropia*. *J. exp. Biol.* **50**, 297–306.

HASKELL, J. A., CLEMONS, R. D. & HARVEY, W. R. (1965). Active transport by the Cecropia midgut. I. Inhibitors, stimulants and potassium transport. *J. cell. comp. Physiol.* **65**, 45–56.

KEYNES, R. D. & LEWIS, P. R. (1951). The sodium and potassium content of Cephalopod nerve fibres. *J. Physiol., Lond.* **114**, 151–82.

KOEFOED-JOHNSON, V. & USSING, H. H. (1958). The nature of the frog skin potential. *Acta physiol. scand.* **42**, 298–308.

LING, G. N. & COPE, F. W. (1969). Potassium ion: is the bulk of intracellular $K^+$ adsorbed? *Science, N.Y.* **163**, 1335–6.

MADDRELL, S. H. P. (1969). Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. *J. exp. Biol.* **51**, 71–97.

MADDRELL, S. H. P. (1970). Secretion by the Malpighian tubules of *Rhodnius*. Electrical events. (In preparation.)

PILCHER, D. E. M. (1969). Hormonal control of the Malpighian tubules of the stick insect, *Carausius morosus*. Ph.D. thesis, University of Cambridge.

PILCHER, D. E. M. (1970). Hormonal control of the Malpighian tubules of the stick insect, *Carausius morosus*. *J. exp. Biol.* **52**, 653–665.

RAMSAY, J. A. (1953). Active transport of potassium by the Malpighian tubules of Insects. *J. exp. Biol.* **30**, 358–69.

RAMSAY, J. A. (1954). Active transport of water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. exp. Biol.* **31**, 104–13.

RAMSAY, J. A. (1955*a*). The excretory system of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. exp. Biol.* **32**, 183–99.

RAMSAY, J. A. (1955*b*). The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae). *J. exp. Biol.* **32**, 200–16.

RAMSAY, J. A. (1956). Excretion by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae): calcium, magnesium, chloride, phosphate and hydrogen ions. *J. exp. Biol.* **33**, 697–708.

RAMSAY, J. A. (1958). Excretion by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae): amino acids, sugars and urea. *J. exp. Biol.* **35**, 871–91.

RAMSAY, J. A. & BROWN, R. H. J. (1955). Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid. *J. scient. Instrum.* **32**, 372–5.

ROBERTSON, J. D. (1957). Osmotic and ionic regulation in aquatic invertebrates. In *Recent Advances in Invertebrate Physiology* (ed. B. T. Scheer), pp. 229–46. Eugene, Oregon: University of Oregon Publications.

SHAW, J. (1955). Ionic regulation in the muscle fibres of *Carcinus maenas*. *J. exp. Biol.* **32**, 383–96.

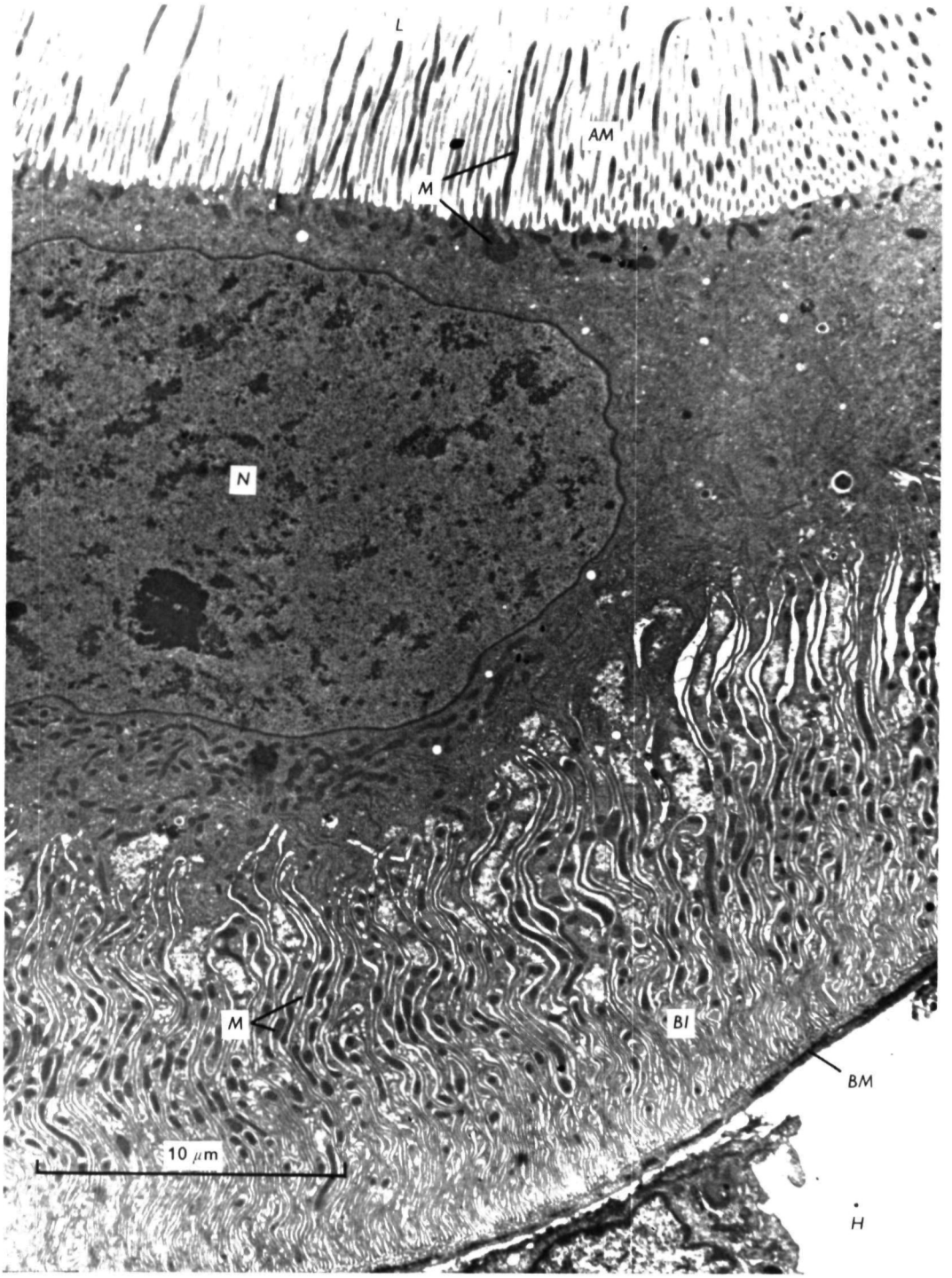
SOLOMON, A. K. (1960). Red cell membrane structure and ion transport. *J. gen. Physiol.* **43** (Suppl.), 1–15.

STEINBACH, H. B. & SPIEGELMAN, S. (1943). The sodium and potassium balance in squid nerve axoplasm. *J. cell. comp. Physiol.* **22**, 187–96.

WOOD, D. W. (1957). The effect of ions upon neuromuscular transmission in a herbivorous insect. *J. Physiol., Lond.* **138**, 119–39.

## EXPLANATION OF PLATE

Text-fig. 11. Low power electron micrograph of tubule cell. The section is at right angles to the long axis of the tubule. Basal infoldings (*BI*) and apical microvilli (*AM*) are visible, both containing mitochondria (*M*). *L*, lumen; *N*, nucleus; *BM*, basement membrane; *H*, haemocoele.




DIANA E. M. PILCHER

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