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# ION AND FLUID SECRETION BY DIFFERENT SEGMENTS OF THE MALPIGHIAN TUBULES OF THE BLACK FIELD CRICKET *TELEOGRYLLUS OCEANICUS*

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## Summary

Cricket Malpighian tubules have two morphologically distinct segments, a thin distal segment, which occupies approximately 10% of the total tubule length, and a main segment. The two segments differ in secretion rates and response to corpora cardiaca extract. The secreted fluids differ in osmotic concentration and elemental composition.

The distal segment secretes fluid at a rate (per mm length) which is approximately twice that of the main segment under control conditions. After stimulation by corpora cardiaca extract (Cc) the rate from the main segment approximately doubles whilst the distal segment rate remains unchanged.

Fluid from the main segment and the whole tubule is slightly hypo-osmotic to the medium (5–11mosmol$kg^{-1}$) under control conditions, whereas that from the distal segment is slightly hyperosmotic (12mosmol$kg^{-1}$). On stimulation with Cc, the whole tubule fluid becomes slightly hyperosmotic (12mosmol$kg^{-1}$), that from the main segment remains slightly hypo-osmotic (3mosmol$kg^{-1}$) but fluid from the distal segment becomes very hyperosmotic (55mosmol$kg^{-1}$). Differences between the tubule fluid and the medium osmolality are indicated in parentheses.

Fluid from the main segment has high concentrations of K (166mmol$l^{-1}$), Cl (111mmol$l^{-1}$), Na (41mmol$l^{-1}$) and P (83mmol$l^{-1}$), whereas that from the distal segment has high concentrations of K (101mmol$l^{-1}$) and Cl (137mmol$l^{-1}$). On stimulation with Cc, the elemental concentrations in fluids from the main segments and whole tubules do not change significantly but the K and Cl concentrations in distal segment fluid increase (182 and 188mmol$l^{-1}$ respectively ). The Mg present in whole tubule fluid is derived largely from the distal segment. The ionic composition accounts for the observed osmotic concentrations in fluid from whole tubules, main segments and stimulated distal segments, but not for the concentrations in fluid from unstimulated distal segments. The fluid from unstimulated distal segments contains an unidentified organic solute accounting for approximately 90mosmol$kg^{-1}$ of the osmotic concentration.

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The distal segment contributes 22% and 11% of the fluid volume, 26% Cl, 14% K and 12% Cl, 11% K in control and Cc-stimulated tubules respectively. Considerably higher values are observed in individual tubules. The distal segment makes a significant contribution to the total ion output of the tubule.

The cyclic AMP content of tubule segments treated with corpora cardiaca extract was found to increase in both main and distal segments. When expressed in terms of protein content there was no difference between segments. However, in terms of total cell volume, the cells of the distal segment had a tenfold greater cyclic AMP content than those of the main segment. This is consistent with a 10- to 20-fold higher secretion rate of K by the distal segment.

It is suggested that the distal segment, whilst having a higher length-specific fluid secretion rate than the main segment, is, nevertheless, concerned primarily with ion and solute secretion since it is unresponsive to diuretic hormone. The prime role of the main segment, which does respond to diuretic hormone, is fluid secretion. There appear to be major differences in hydraulic conductivity between the two segments.

## Introduction

The basic principles of secretion of ions and fluid by insect Malpighian tubules are now well known (see reviews by Maddrell, 1971*a*,*b*, 1977; Phillips, 1981; Bradley 1985). It is generally agreed that the driving force for fluid secretion is the active transport of $K^+$ or, in some haematophagous insects, $Na^+$. The transport of water is said to be iso-osmotic and thought to be closely coupled to cation transport, but the exact nature of this coupling is not understood. Whilst fluid transport is approximately iso-osmotic, there is evidence that the secreted fluids may be slightly hyperosmotic, iso-osmotic or slightly to very hypo-osmotic (Taylor, 1974). Deviations from iso-osmoticity tend to be regarded as being due to resorptive processes occurring in the tubules subsequent to secretion.

Whilst many Malpighian tubules are obviously segmented (e.g. Marshall, 1973, 1974), many have a more subtle segmentation (see Bradley 1985). In the crickets *Acheta domesticus* (Hazelton *et al.* 1988) and *Teleogryllus oceanicus* (A. T. Marshall, unpublished observations) there is an obvious morphological separation into a short terminal distal segment and a main segment. These segments have marked ultrastructural differences. Much less obvious differences distinguish a further two very short more proximal regions prior to the joining of the tubules to a common ampulla (A. T. Marshall, unpublished observations). Recent investigations of the hormonal control of secretion in *Acheta* have not dealt with the possibility of significant and different contributions to the final secretion from these different segments. Studies of hormonal control of secretion from *Acheta* Malpighian tubules have been made on the whole hindgut, ureter, ampulla and tubule complex (Spring and Hazelton, 1987; Spring *et al.* 1988) and on isolated tubules (Coast, 1988, 1989; Coast *et al.* 1990, 1991; Coast and Wheeler, 1990).

On the basis of a relationship between secretion rate and length in *Acheta* Malpighian tubules, Coast (1988) considers that the terminal distal segment probably has no secretory role. Very recently, however, Kim and Spring (1992) have shown that the distal segment in *Acheta* has a higher secretion rate than the main segment under control conditions. The present paper shows that there are marked differences in the secretory behaviour of the

distal and main segments in *Teleogryllus* and also in the composition of the secretions. Furthermore, it is shown that the secretion of the morphologically insignificant distal segment is a significant contribution to total fluid and solute production. The possibility of hypo-osmotic fluid transport is also discussed.

## Materials and methods

### *Experimental animals*

Animals were adult male black field crickets (*Teleogryllus oceanicus*) that had ecdysed 2–3 weeks prior to the experiment. The colony was reared at 27˚C under a 12h/12 h reverse light cycle in the Zoology Department at La Trobe University. They were fed *ad libitum* on fresh lettuce and Barastoc mouse breeder ration and had access to water.

Experiments were started during the animals' dark phase, which is their active period.

### *Malpighian tubule saline*

Saline for Malpighian tubule preparations consisted of 115mmol $l^{-1}$ sodium chloride, 12mmol $1^{-1}$ potassium chloride, 4mmol $1^{-1}$ magnesium chloride, 4mmol $1^{-1}$ sodium dihydrogen phosphate, 10mmol $1^{-1}$ glucose, 78mmol $1^{-1}$ sucrose, 2mmol $1^{-1}$ glutamine, 2mmol $1^{-1}$ glycine, 1mmol $1^{-1}$ DL-alanine, 2mmol $1^{-1}$ L-valine, 1mmol $1^{-1}$ L-leucine, 2mmol $1^{-1}$ L-lysine, 1mmol $1^{-1}$ L-histidine, 5mmol $1^{-1}$ L-proline and 10mmol $1^{-1}$ sodium bicarbonate. After dissolving this mixture, 3.5mmol $1^{-1}$ calcium chloride was added. The osmotic pressure was checked with a vapour pressure osmometer (Wescor 5100) and adjusted if necessary to about 380mosmol$kg^{-1}$ with sucrose and to pH7.1 with sodium hydroxide. All chemicals were supplied by Sigma. The saline was used at room temperature (25˚C). Carbogen (95% oxygen/5% carbon dioxide) was bubbled through before use.

### *Preparation of corpora cardiaca extract*

A pair of corpora cardiaca was dissected from healthy adult crickets during the dark phase of the light/dark cycle and put in 100 µl of distilled water. The preparation was frozen on dry ice (carbon dioxide), thawed and homogenized by hand. The homogenate was frozen and thawed twice more, then sonicated on ice for 10min in total, taking care not to allow the homogenate to become hotter than about 30˚C. It was then centrifuged at 3000 ***g*** in a Sorvall SS1 angle centrifuge for 30min and the supernatant collected. This gives a strength of 1 gland pair/100 µl which can be used fresh or frozen in liquid nitrogen and stored at −20˚C. Immediately before use, the extract was diluted to 1 gland pair in 2500 µl of Malpighian tubule saline.

### *Isolated tubule preparations*

Adult male crickets were killed by decapitation. The Malpighian tubule complex was removed under saline. Single tubules were dissected and placed in a small well filled with saline and covered with hydrated paraffin oil. The cut (proximal) end of the tubule was looped under paraffin around a glass peg. Wells were made from silicone elastomer (Dow

Corning Sylgard 184) to a volume of 0.75–1ml. Glass pegs were made from drawn out capillary tubes. All experiments were performed at room temperature (25˚C).

All tubules for an experiment were set up within 30min of dissection from the cricket. The saline was changed 30min after the last tubule in the well had been set up, and after this every 30min or hourly. Experiments were started up to 30min after the first change of saline.

The tubules consist of three segments: distal, main and the most proximal. Three types of preparations were used: (1) whole tubule, where both main and distal segments were in saline; (2) main segment preparation, where the distal segment was lifted out of the saline into paraffin, i.e. the distal segment was not secreting; (3) distal segment preparation, where only the distal segment was in saline and only sufficient main segment was retained to attach the tubule to a peg.

Fluid accumulated in hydrated paraffin as a droplet at the cut end of the tubule. For whole and main preparations, the urine droplets were removed every half hour, and every hour for distal preparations, by stroking the tubule with a glass probe. The collected fluid droplet was removed from the probe by lifting the probe out of the paraffin oil. The diameter of the drop was measured whilst the droplet was suspended in paraffin oil using an eyepiece graticule in a dissecting microscope. The volume of the drop was calculated assuming it was spherical.

At the end of the experiment, the length of the tubule segment(s) in saline was measured using an eyepiece graticule by gently pulling the proximal base of the tubule, which was anchored around the glass peg, away from the well until the segment in saline was fully extended. The total length of the tubule was measured by placing the tubule in 50% glycerine on a glass slide. A digitizer interfaced to a computer was used to measure the length of the tubule from an enlarged positive photographic print taken of the slide.

### *Secretion rates*

The secretion rate of an *in vitro* Malpighian tubule preparation decreased slightly with time. Secretion rates of control and stimulated tubules were obtained from the same tubule in the following way.

Fluid was collected from a control tubule over three time periods starting usually 1 h after dissection (time zero). The tubule was then incubated with corpora cardiaca extract and its secretion rate was measured three times. The relationship of secretion rate with time was determined for the control tubule and it was assumed that the secretion rate of the tubule after treatment would decay at the same rate (this assumption was checked by measuring the secretion rate after treatment at three time intervals). This relationship was then used to scale the control and treatment secretion rates to time zero. Secretion rates were expressed in $\mathrm{nl\,mm^{-1}\,h^{-1}}$.

### *Osmotic concentrations*

The osmotic concentrations of fluid droplets and the incubating saline in each well were measured simultaneously with a set of standards using a nanolitre osmometer (Clifton Technical Physics, New York). The data were converted from freezing point

depression to mosmol$kg^{-1}$. Saline osmotic concentrations were also checked with a Wescor 5100 vapour pressure osmometer. For statistical analysis, fluid osmotic concentrations were expressed as a percentage of that of the incubation saline.

### *X-ray microanalysis*

Microdroplets of secreted fluid were prepared for X-ray microanalysis by a modification of the method of Hyatt and Marshall (1985). The main difference was that rubidium nitrate was used as a standard in place of cobalt nitrate.

Analysis of microdroplets of secreted fluid was carried out in a JEOL JEM 1200EX scanning transmission electron microscope fitted with a Link ultrathin window energy dispersive X-ray detector interfaced to an AN10000 analyser. Spectra were collected at 120kV for 200s livetime with an electron dose of 58 $e^{-}$ $nm^{-2}$.

Spectra were processed using the ratio standard method for thin films (RTS-2/FLS), with rubidium as the reference element. Elemental concentrations were calculated using the formula from Cliff and Lorimer (1975), in which the concentrations of all elements in the fluid sample were scaled to that of rubidium. Thus, results are obtained in mmol$l^{-1}$. Sensitivity values were obtained from binary standards after Shuman *et al.* (1976).

### *Cyclic AMP determinations*

Twelve male crickets were fed on lettuce and ground rat chow with water *ad libitum* for 2 days, then held without food or water for 1 day before being used in the experiment. The Malpighian tubules were dissected out in saline and divided into two equal portions per insect. The distal segments from each portion were removed and the two main segment samples and the two distal segment samples were placed in tared microcentrifuge tubes (0.1mg) containing 90 μl of saline and 0.25mmol$l^{-1}$ 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor). The microcentrifuge tubes were reweighed, and the wet mass of the tubule segment recorded. Unfortunately, the mass of the distal segments was typically less than the ability of the scale to discriminate (i.e. less than 0.1mg).

The assay was initiated by adding 10 μl of a saline extract of corpora cardiaca (1 gland pair/200 μl) to the microcentrifuge tube or 10 μl of saline for control tissues. For each pair of tissues (distal or middle segments), one acted as control and the other received the corpora cardiaca extract. After 5min of incubation at room temperature (24°C), the reaction was stopped by adding 5 μl of 2mol$l^{-1}$ HCl. The incubation fluid and tissue were sonicated for 30s to release the cyclic AMP, followed by placing the tubes in boiling water for 10min. The tubes were then centrifuged at 13000revs$min^{-1}$ for 3min and frozen at −20°C until assayed.

The concentration of cyclic AMP was determined by radioimmunoassay using the method of Steiner *et al.* (1972) as modified by Hunt *et al.* (1980). The antisera for cyclic AMP were raised in rabbits using keyhole limpet haemocyanin as the conjugating protein. The tyrosine methyl ester derivatives of succinyl cyclic AMP (Sigma) were labelled with $^{125}I$ using the chloramine T reaction (Steiner *et al.* 1972).

Total cell volume was estimated from the product of tubule cross-sectional area and

segment length. Cross-sectional areas for each segment were obtained by digitising a number of segment profiles in light micrographs of 1.0 μm sections of freeze-substituted Malpighian tubules. Cross-sectional area was calculated from the difference between the area of tubules digitised around the basement membrane and the area of the lumen digitised around the apical side of the brush border. Tubules were frozen by plunging into propane, freeze-substituted in an ether/acrolein mixture and embedded in a methacrylate resin.

### *Data analysis*

Secretion rates and fluid osmotic concentrations were analysed with the *t*-test and least significant difference test (after one-way analysis of variance) using the computer statistics package Statcalc. Linear regressions were performed using the computer program Statview. The amiloride and cyclic AMP experiments were analysed using the least significance difference test after one-way analysis of variance using Statview. Fluid ionic concentrations were analysed with the least significant difference test after one-way analysis of variance using the computer package SPSS-X. Values are presented as mean±S.E.

## Results

### *Rate of fluid secretion*

#### *Control rates*

The tubules of *Teleogryllus* vary in size (mean length for 18 measurements of 7.04±0.05mm) with a distal segment of about 10–12% of the total length. Secretion rates, measured as a function of length from approximately 130 tubules of different lengths with some 80% of each tubule in the medium, showed a non-linear relationship with length (Fig. 1). The highest rates were obtained from the shortest tubules.

This relationship was also observed in data from a series of tubules in which specific rates were measured for different lengths of tubules immersed in the physiological saline, thereby eliminating the possibility that shorter tubules were physiologically different from longer ones. This was investigated further by cutting tubules short and plotting specific rates of secretion against the length of the distal segment as a percentage of the total length of the tubule. Thus, in intact tubules, the percentage would be approximately 10% and in tubules in which only the distal segment remained this would, of course, be 100%. The results are shown in Fig. 2.

In all three of the foregoing experiments the shortest tubules (in some cases virtually distal segments only) had length-specific secretion rates up to four times the rates of long intact tubules.

#### *Stimulated rates*

Stimulation by corpora cardiaca extract at a dose of 1 pair/2500 μl produced an increase in secretion rates in whole tubules. The mean increase for 25 tubules from 5 insects was from 11.16±0.63–22.17±1.47nl$mm^{-1}h^{-1}$, i.e. an increase of almost twice the control rate. Individual increases ranged from 1.37 to 3.1 times the control value.

When tubules of varying lengths were stimulated, the greatest percentage increase in

secretion rate was observed in tubules in which the distal segment accounted for 10–20 % of the total tubule length. No increase was observed when the distal segment accounted for 100% of the total length (Fig. 3).

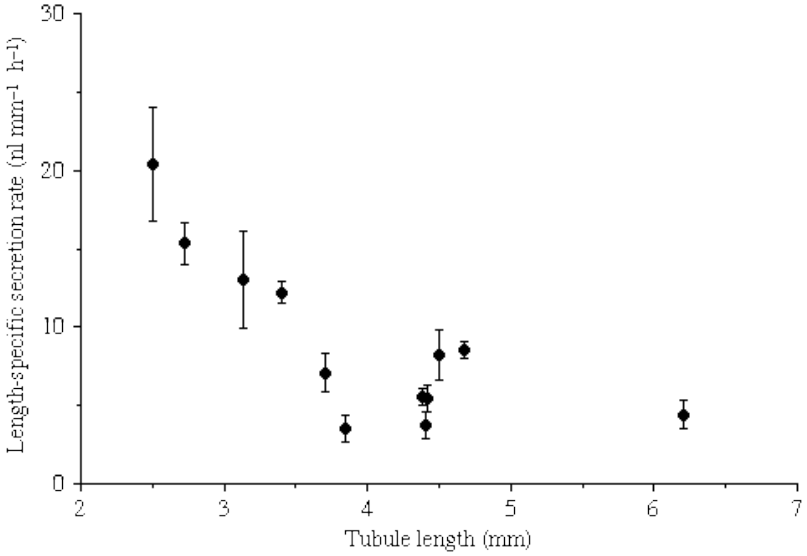


Fig. 1. Relationship between *in vitro* length-specific secretion rate of single Malpighian tubules and total tubule length. Each point represents the mean with standard error bar of 4–11 tubules from a single cricket. About 80% of the tubule was in the bathing medium.

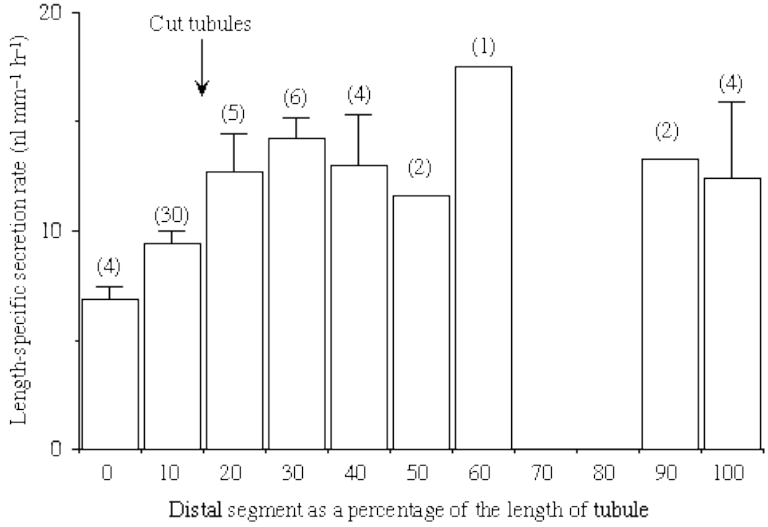


Fig. 2. Relationship between *in vitro* length-specific secretion rate of single Malpighian tubules and the proportion of the distal segment to total tubule length. Values are mean and standard error with sample sizes in parentheses. Intact tubules correspond to a proportion of about 10%; at greater proportions, more of the proximal end of the tubule was removed; at 100% only the distal segment remained.

*Secretion rates from different segments*

The foregoing experiments suggested that the distal segment may have higher control secretion rates than the main segment but that these do not increase on treatment with corpora cardiaca extract. This possibility was therefore further investigated by comparison of length-specific secretion rates from preparations from the same tubules of the distal segment, main segment and whole or intact tubule. The results (Fig. 4) clearly indicate that control secretion rates in whole and main segment preparations are significantly lower than in the distal segment ($P$=0.002). The control and stimulated rates are significantly different for the whole and main segment preparations ($P$<0.0005) but not for the distal segment preparations.

*Volume contribution of distal segment*

Measurements of the absolute secretion rate from both whole preparations and distal segment preparations were made on 13 tubules from the same insect under control and stimulated conditions (Table 1). The fluid flow from the distal segment is 30% of the flow from the whole tubule under control conditions and 18% after stimulation with corpora cardiaca extract.

*Dependence of secretion rate on osmotic concentration of the medium*

As has been found for other species, the rate of secretion of whole tubules decreased with increasing osmotic concentration of the bathing medium (Fig. 5). The relationship is more precisely demonstrated in Fig. 6, which shows changes in specific rate for six tubules all measured at three different osmotic concentrations of bathing medium.

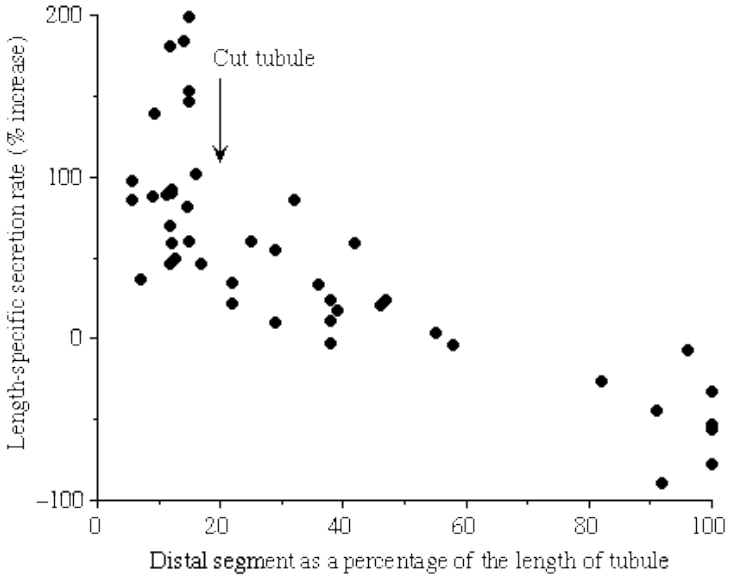


Fig. 3. The increase in *in vitro* length-specific secretion rate of single Malpighian tubules stimulated with corpora cardiaca extract (1 gland pair/2500 μl) plotted against the proportion of the distal segment to total tubule length.

*Dependence of secretion rate on potassium*

Specific secretion rates of whole tubules under control conditions increased as potassium concentration in the bathing medium increased, whilst cationic balance was maintained by reducing sodium and substituting potassium salts for sodium dihydrogen phosphate and sodium bicarbonate (Fig. 7). At the highest concentration of potassium, sodium was nominally absent.

## *Composition of secreted fluid*

*Osmotic concentration*

Whole tubules secreted a fluid which was close to iso-osmoticity with bathing medium at an osmotic concentration of 300mosmolkg$^{-1}$. However, as the osmolality of the bathing medium increased, the secreted fluid became increasingly hypo-osmotic (Fig. 8). The slope of the regression line is significantly different from zero ($P$=0.0001) as well as being different from the slope of the iso-osmotic line (=1) ($P$=0.0001).

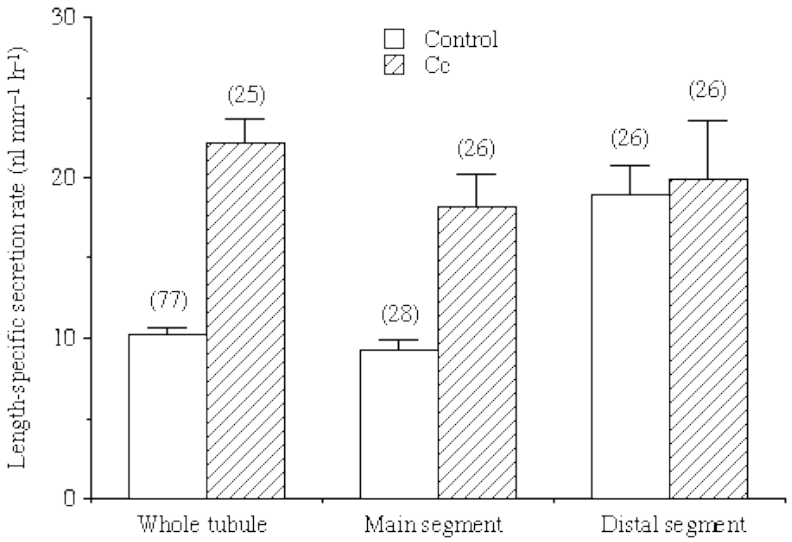


Fig. 4. *In vitro* length-specific secretion rate of whole Malpighian tubules and tubule segment preparations under control conditions and when stimulated with corpora cardiaca extract (1 gland pair/2500 μl). Values are mean and standard error with sample sizes in parentheses.

Table 1. *Control and corpora-cardiaca-stimulated secretion rates from whole tubule and distal segment preparations*

| | Secretion rate (nl h$^{-1}$) | |
|---|---|---|
| | Control | Stimulated |
| Whole tubule | 50.1±3.1 | 98.6±8.7 |
| Distal segment | 15.1±2.5 | 17.5±3.7 |

Mean ± S.E., $N$ = 13.

Considerable variability was observed with individual tubules placed in media at particular osmotic concentrations (Fig. 8). Six tubules were therefore allowed to secrete sequentially in three bathing solutions of increasing osmotic concentration. The pattern of

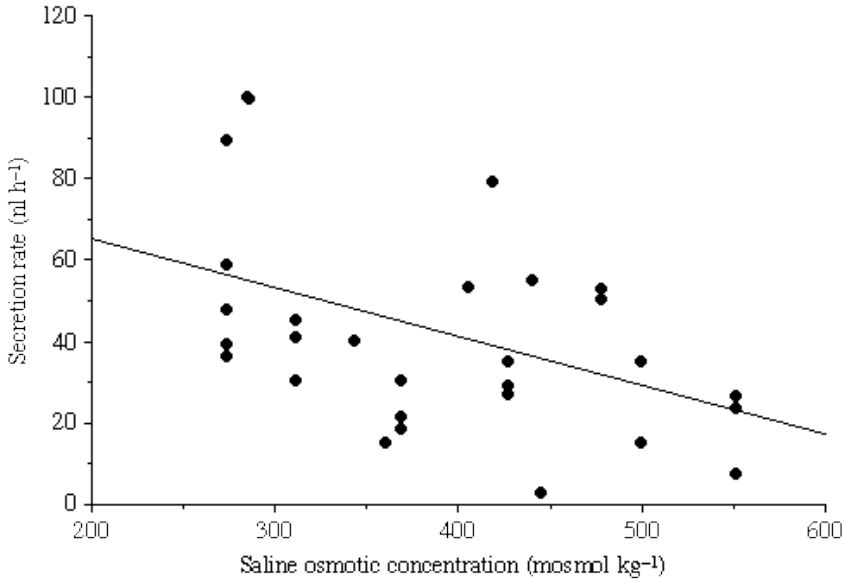


Fig. 5. Relationship between *in vitro* secretion rate of single Malpighian tubules and the osmotic concentration of the bathing medium. The relationship is described by the linear regression $y=89.3-0.12x$ ($P=0.009$) with a correlation coefficient $r=0.45$.

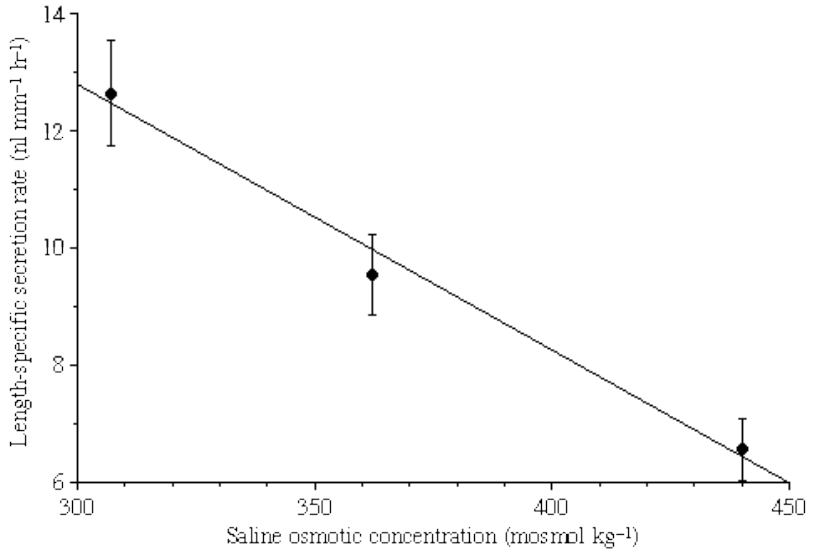


Fig. 6. *In vitro* length-specific secretion rate of single Malpighian tubules incubated sequentially in bathing media of increasing osmotic strength. Values are means with standard error bars for six tubules. The relationship is described by the linear regression $y=26.25-0.05x$ ($P=0.0001$) with a correlation coefficient $r=0.81$.

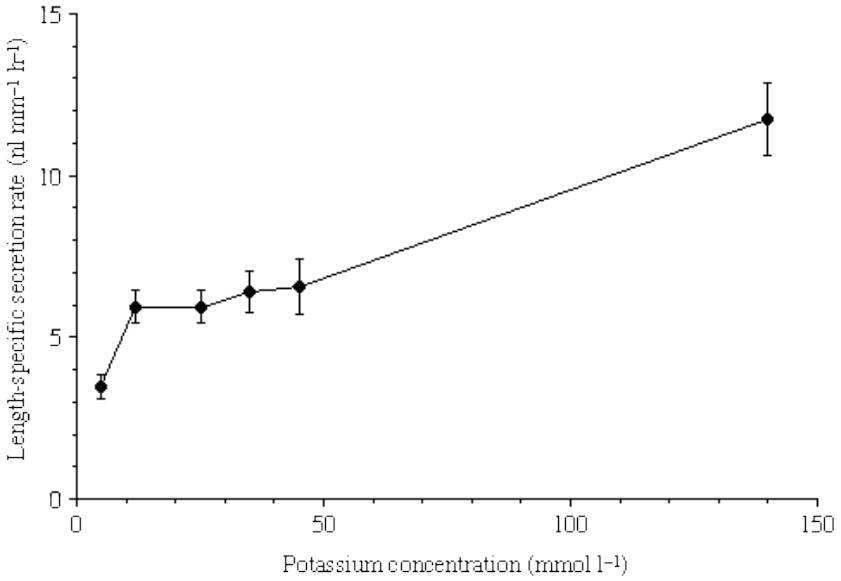


Fig. 7. Effect of varying potassium concentration in the bathing medium on *in vitro* length-specific secretion rate of single Malpighian tubules. Values are means and standard errors for 9–20 tubules. Cationic balance was maintained by adjusting the concentration of sodium chloride and substituting potassium salts for sodium dihydrogen phosphate and sodium bicarbonate in the bathing medium. At the highest potassium concentration, the medium was nominally sodium-free.

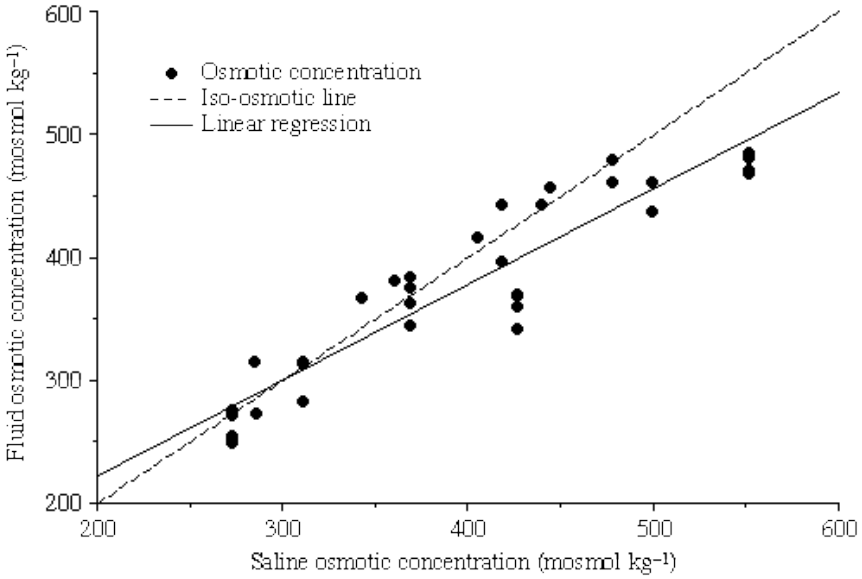


Fig. 8. The effect of osmotic concentration of the bathing medium on osmotic concentration of fluid collected from single Malpighian tubules. The relationship is described by the linear regression $y=67.8+0.78x$ ($P=0.0001$) with a correlation coefficient $r=0.93$

secretion was essentially the same as previously, with the secretion becoming hypo-osmotic at bath concentrations above 300mosmol$kg^{-1}$ (Fig. 9).

*Osmotic concentration of fluids from different segments*

Fluid was collected from whole, main and distal segment preparations. Because the osmotic concentrations of the bathing media differed slightly, the fluid osmotic concentrations were expressed as percentages of bath concentrations (Fig. 10). Comparison of the transformed data by *t*-tests indicates that the fluid from the control whole tubule is hypo-osmotic to the bath ($P<0.001$) and that after stimulation the secreted fluid becomes hyperosmotic ($P<0.02$). The fluid from the main control segment is slightly hypo-osmotic ($P<0.027$) and becomes iso-osmotic upon stimulation. Fluid from the control distal segment is not significantly different from the bath osmotic concentration, although the data suggest slight hyperosmoticity. However, fluid from the stimulated distal segment is significantly hyperosmotic ($P<0.005$). One-way analysis of variance shows that the fluids from control and stimulated distal segments are significantly higher in osmotic concentration than fluids from both of the respective whole tubule and main segment preparations ($P<0.0001$).

*Ion concentrations*

Fluids secreted from whole tubule, main and distal segment preparations under control and corpora-cardiaca-stimulated conditions were analysed by X-ray microanalysis.

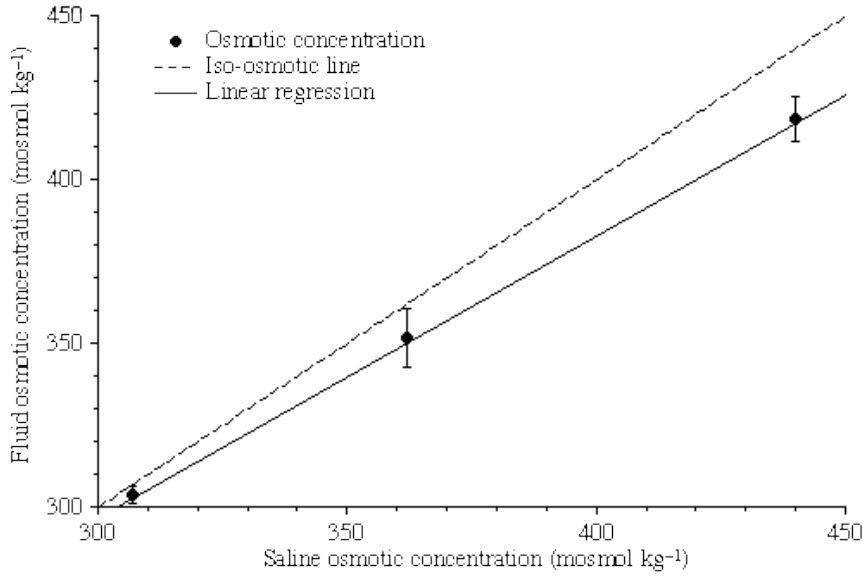


Fig. 9. Osmotic concentration of fluid collected from single Malpighian tubules incubated sequentially in media of increasing osmotic strength. Values are means with standard error bars for six tubules. The relationship is described by the linear regression $y=39.3+0.86x$ ($P=0.0001$) with a correlation coefficient $r=0.95$.

Fluid from the whole tubule was rich in K (175±13mmol $l^{-1}$) and Cl (119±11mmol $l^{-1}$). High concentrations of Na (49±8mmol $l^{-1}$) and P (95±6mmol $l^{-1}$) were also present, with Mg (13±1mmol $l^{-1}$), S (6±1mmol $l^{-1}$) and Ca (4±0mmol $l^{-1}$) in relatively low concentrations. (Values are means±S.E., $N$=26.)

A comparison of whole tubule, main and distal segment preparations (Fig. 11) shows that the patterns of ion concentrations in fluids from the whole tubule and main segment are very similar. There are no significant differences in ion concentrations in fluids from control and corpora-cardiaca-stimulated preparations.

The distal segment, however, produced a fluid in which the pattern of ion concentration is both different from the patterns in the other two preparations and differs between control and stimulated preparations. Sodium was lower in concentration ($P$<0.05) than in fluid from whole tubule and main segment preparations, Mg was higher ($P$<0.05) and P was lower in the control. Whereas K concentrations were higher than Cl concentrations in the whole tubule and main segment fluids, the reverse was true for fluid from control distal segments and the concentrations were equal in fluid from stimulated preparations. The K concentration in fluid from the stimulated distal segment is significantly higher ($P$<0.05) than that in fluid from the control distal segment.

### *Effect of stimulation on cyclic AMP concentrations*

Treatment of tubules with corpora cardiaca extract resulted in significant increases in cyclic AMP concentration in both main ($P$=0.016) and distal ($P$=0.012) segments (Table 2). The amount of cyclic AMP was significantly greater in the main than in the distal segment in control and stimulated preparations ($P$=0.05) (Table 2). When cyclic

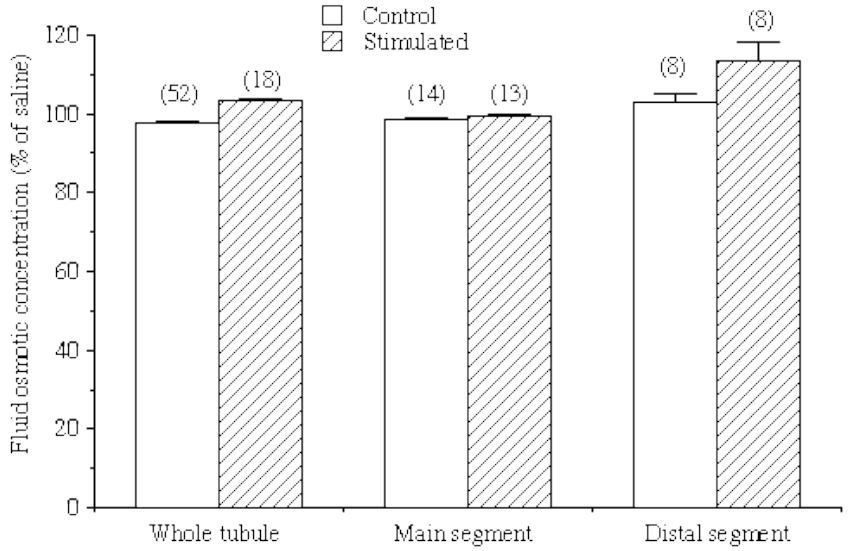


Fig. 10. Osmotic concentrations of fluid collected from whole Malpighian tubules and tubule segments expressed as a percentage of that of the bathing medium under control conditions and when stimulated with corpora cardiaca extract (1 gland pair/2500 μl). Values are mean and standard error with sample sizes in parentheses.

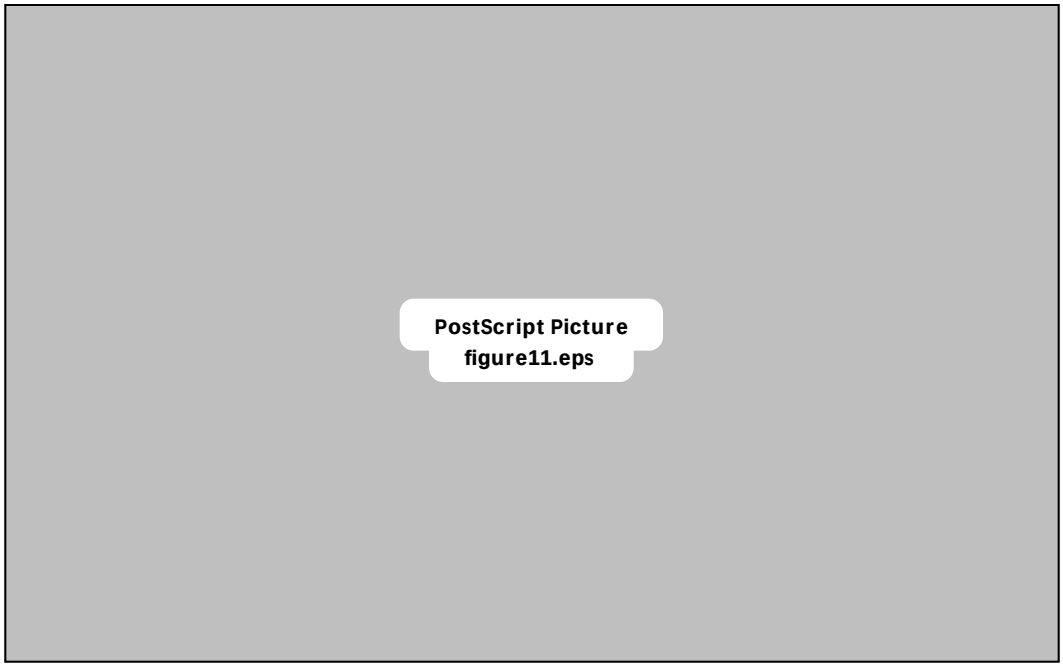


Fig. 11. X-ray microanalysis of fluid collected from whole Malpighian tubules (A) and from main (B) and distal (C) segment preparations under control conditions and when stimulated with corpora cardiaca extract (1 gland pair/2500 μl). Values are mean and standard error with sample sizes in parentheses.

AMP concentrations are expressed in terms of protein content, there is no significant difference between the two segments (Table 2). However, if cyclic AMP concentrations are expressed in terms of cell volume the concentration is ten times higher in the distal segment cells than in those of the main segment (Table 2).

## Discussion

The isolated Malpighian tubules of the cricket *Teleogryllus oceanicus* survive in a functional state in physiological saline for many hours, as shown by Coast (1988) for the Malpighian tubules of *Acheta domesticus*. Secretion rates are maintained with only a slight decrease over very long periods.

The tubules respond to a diuretic factor, or factors, in crude corpora cardiaca extracts by a doubling of secretion rates. This has also been shown in *Acheta* by Coast (1988) using isolated tubules and by Spring and Hazelton (1987) using the isolated tubules/ampulla/ureter complex (TAU).

Whilst recognising that *Acheta* Malpighian tubules have two morphologically different segments, which are easily distinguished, Coast (1988) and Spring and Hazelton (1987) have considered the short, narrow terminal distal segment to be insignificant or non-functional as far as fluid secretion is concerned. Recently, however, Kim and Spring

Table 2. *Cyclic AMP measurements in control and stimulated Malpighian tubule segments*

| | Main segment | | Distal segment | |
|---|---|---|---|---|
| | Control | Stimulated | Control | Stimulated |
| Protein content of approximately 100 segments ($\mu$g) | 4.46±0.71 (23) | | 1.44±0.31 (22)* | |
| Cyclic AMP content of approximately 100 segments (pmol) | 7.07±1.35 (11)* | 13.58±1.91 (12)* | 2.68±0.70 (10)* | 3.75±1.01 (12)* |
| Cyclic AMP concentration (pmol $\mu g^{-1}$ protein) | 1.94±0.49 (11) | 4.24±0.88 (12) | 2.22±0.50 (10) | 3.60±0.98 (12) |
| Total cell volume (nl) | 10.2 | | 0.37 | |
| Cyclic AMP concentration ($\mu$mol $l^{-1}$ cell volume) | 6.9 | 13.3 | 72.4 | 101.4 |

Values are means ± standard errors with the number of crickets in parentheses. Approximately 100 segments were pooled for each cricket.
Pooled segments were stimulated with corpora cardiaca extract (1 gland pair/2500$\mu$l).
Significant differences ($P$<0.05) between values for the two segments are indicated with an asterisk, except for cyclic AMP concentration (pmol $\mu g^{-1}$ protein), where there are no significant differences between segments, but control segments are significantly different ($P$<0.05) from stimulated segments.

Table 3. *Calculated fluid and elemental secretion rates for whole tubules and distal segments*

| | Fluid (nl $h^{-1}$) | K (nmol $h^{-1}$) | Cl (nmol $h^{-1}$) | Na (nmol $h^{-1}$) | P (nmol $h^{-1}$) |
|---|---|---|---|---|---|
| Whole tubule | | | | | |
| Control | 65.8 | 12.3 | 7.6 | 2.6 | 6.8 |
| Cc | 143.2 | 25.2 | 19.4 | 4.2 | 10.1 |
| Distal segment | | | | | |
| Control | 14.6 | 1.5 | 2.0 | 0.6 | 0.3 |
| Cc | 15.3 | 2.9 | 2.8 | 0.5 | 0.7 |

Calculation is based on secretion rates given in Fig. 4, concentrations in Fig. 11 and measurements of tubules used to obtain the rates in Fig. 4 (whole tubules 6.45mm, distal segment 0.77mm).
Cc indicates stimulation with corpora cardiaca extract (1 gland pair/2500 μl).

Table 4. *Elemental concentrations (mmol $l^{-1}$) in fluid secreted by* Teleogryllus *and* Acheta *Malpighian tubules*

| Preparation | Na | Mg | P | S | Cl | K | Ca |
|---|---|---|---|---|---|---|---|
| *Teleogryllus* (high-phosphate medium) | | | | | | | |
| Control tubules (8) | 39±5 | 11±3 | 104±6 | 8±2 | 116±14 | 118±16 | 1±0 |
| Cc tubules (6) | 29±8 | 5±2 | 70±16 | 4±1 | 135±17 | 176±24 | 3±1 |
| Control TAU* (5) | 44±5 | 35±4 | 24±3 | 0 | 110±5 | 233±16 | 32±2 |
| *Acheta*† (high-sulphate medium) | | | | | | | |
| Cc tubules (5) | 50±10 | – | – | – | 95±6 | 24±5 | – |
| Control TAU (10) | 20±8 | 17±4 | – | 40 | 60-110 | 20 | 7.5 |
| Cc TAU (9) | 52±10 | 6 | – | – | 110±8 | 33±3 | – |

*Hyatt and Marshall (1985); †Spring and Hazelton (1987).
Means ± S.E. (*N*).
Cc indicates stimulation with corpora cardiaca extract.

(1992) have shown that it has a secretory role. It is shown here that this segment in *Teleogryllus* has considerable functional significance, although it is much smaller in diameter than the main segment and only some 10–12% of the total tubule length.

Few measurements of fluid secretion rates from different segments of segmented Malpighian tubules appear to have been made. The lower tubule segment of *Rhodnius prolixus* does not secrete any fluid (Maddrell and Phillips, 1975) and appears to have a primarily resorptive function. However, the different segments of the Malpighian tubules of *Cenocorixa bifida* and *Cenocorixa blaisdelli* have been shown to secrete fluid at different rates (Szibbo and Scudder, 1979; Cooper *et al.* 1989). Similarly, segmental differences in the ionic composition of fluid secreted by Malpighian tubules have been observed in relatively few insects (Stobbart and Shaw, 1974) and these appear to be largely due to the resorption of $K^+$, although in some instances $Na^+$ is secreted (Ramsay, 1955, 1976; Irvine, 1969; Maddrell and Phillips, 1975; Maddrell, 1978; Green, 1979).

### *Secretion rates*

In *Teleogryllus*, shorter whole tubules have a higher length-specific rate of secretion than longer tubules. This is due to the distal segment in short tubules occupying a higher proportion of the total length. In tubules which have not been stimulated by corpora cardiaca extract, the distal segment secretes at a length-specific rate which is up to twice that of the main segment and whole tubule. When stimulated, the specific secretion rates of the main segment and whole tubules approximately double but there is virtually no increase in rate from the distal segment.

The fluid from the distal segment accounted for some 30% and 18% of the fluid volume secreted from unstimulated and stimulated tubules, respectively, when determined by direct experiment. When volumes are calculated from mean length-specific secretion rates and mean tubule lengths, the percentage volume contributions are 22% and 11% for unstimulated and stimulated tubules respectively. Since whole tubules vary in length but distal segments are less variable, it is probable that the percentage volume contribution also varies from one tubule to another.

In *Acheta*, Kim and Spring (1992) report a fourfold higher specific secretion rate for the distal segment compared to the main segment in control tubules but a fall to zero secretion rate in distal segments stimulated with corpora cardiaca extract. The contribution to total fluid volume by the distal segment under control conditions is given as more than 55% in *Acheta*.

### *Ion secretion*

The concentrations of $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ in the fluid from the whole tubule account, with counterions, for the observed osmotic concentration of fluid from unstimulated tubules. In fluid from the main segment, the same cations account for all of the observed osmotic concentrations in both unstimulated and stimulated preparations. This is not so for fluid from the distal segment, in which $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ concentrations account for only 73% of the osmotic concentrations in fluid from unstimulated tubules. In stimulated tubules, these cations and counterions account for almost all of the observed osmotic concentration. The observed osmotic concentration is approximately $50 mosmol kg^{-1}$ higher in fluid from stimulated tubules compared to that in fluid from unstimulated tubules, but the calculated difference is approximately $140 mosmol l^{-1}$. Thus some $90 mosmol l^{-1}$ must be due to an organic component that ceases to be secreted on stimulation of the tubules with Cc extract.

The pattern of ion secretion from the main and distal segments is different. The main segment secretes $K^+$, $Cl^-$, $Na^+$ and P (probably as $PO_4^{3-}$) whereas the distal segment secretes $K^+$, $Cl^-$ and $Mg^{2+}$ as the principal ions. Furthermore, the ion concentrations from the main segment do not change on stimulation by corpora cardiaca extract, whereas $K^+$ and $Cl^-$ concentrations increase markedly in the secretion from the distal segment.

Absolute fluid and ion secretion rates can be calculated and are shown in Table 3. It can be inferred that in the main segment the effect of Cc stimulation is to double secretion rates of $K^+$ and $Na^+$, triple $Cl^-$ secretion rate and increase P secretion by half. In the distal segment, the secretion rate of $K^+$ is also doubled, as is the P rate, but the $Cl^-$ rate only

increases by one-third and the $Na^+$ rate does not change. At the same time, fluid secretion rate doubles in the main segment but is unchanged in the distal segment.

When flow rates and ion concentrations are considered, it can be shown that, in terms of $mmol\,h^{-1}$, the unstimulated distal segment contributes 26% of the $Cl^-$ secreted by the whole tubule and 12% of the $K^+$. In the stimulated state, the distal segment contributes 14% of the $Cl^-$ and 11% of the $K^+$. Values of up to 50% of total $Cl^-$ secretion and 23% of total $K^+$ secretion in unstimulated tubules, and 43% of total $Cl^-$ and 33% of total $K^+$ secretion in stimulated tubules, were recorded from a number of tubules. The distal segment, whilst making a significant contribution to the total ion output of the whole Malpighian tubule, is clearly also secreting some so far unidentified component or components. One possible candidate is proline, which has been shown to be secreted by locust Malpighian tubules (Chamberlin and Phillips, 1980).

If specific secretion rates for $K^+$ are calculated, it can be seen that they are very similar for the main segment (control $1.9 nmol mm^{-1} h^{-1}$, Cc $3.9 nmol mm^{-1} h^{-1}$) and distal segment (control $1.9 nmol mm^{-1} h^{-1}$, Cc $3.8 nmol mm^{-1} h^{-1}$). Since the cross-sectional area of the lumen in the main segment is greater than that of the distal segment it seems probable that the secretion rate of $K^+$ in the distal segment, in terms of area of apical cell surface, would be significantly higher than that of the main segment. This may well account for the fluid secretion rate from the distal segment being higher than that of the main segment under control conditions. The fluid secretion rate does not change in the distal segment after Cc stimulation, although $K^+$ secretion rate increases proportionately to that in the main segment and the secreted fluid is hyperosmotic to the bathing medium. This suggests that there is some limitation on the hydraulic conductivity of the cells of this segment.

The elemental concentrations measured in this investigation in fluid secreted by whole tubules and by the TAU preparation (Hyatt and Marshall, 1985) differ greatly from those recorded by Spring and Hazelton (1987) and Kim and Spring (1992) from single tubule preparations and from TAU preparations of *Acheta*. A comparison is given in Table 4. On the basis of their measurements, Spring and Hazelton (1987) concluded that the Malpighian tubule fluid was richer in $Na^+$ than in $K^+$ and became more so after stimulation. They also suggested that $Ca^{2+}$, $Mg^{2+}$ and $SO_4^{2-}$ were either being actively transported into the tubule lumen against their concentration gradients or that they were concentrated by fluid resorption in the 'lower tubules/ampulla' region.

When the ion concentrations for *Teleogryllus* and *Acheta* are compared, a large difference is obvious. *Teleogryllus* fluid is $K^+$-rich and changes very little in composition on stimulation with corpora cardiaca extract, whereas *Acheta* fluid is $Na^+$-rich and becomes even more so on stimulation. In *Teleogryllus*, as in *Acheta*, $Mg^{2+}$ and S (presumably as $SO_4^{2-}$) are concentrated against their chemical gradients but S is in very low concentration in *Teleogryllus* fluid (with a bath nominally free of $SO_4^{2-}$) whereas P (presumably as $PO_4^{3-}$) is present in very high concentrations, although only at very low concentrations in the bath. Calcium is concentrated in the fluid from TAU preparations in both *Teleogryllus* and *Acheta*, but is at the concentration of the bathing medium in fluid from *Teleogryllus* tubules. Phosphate concentration is lower in the TAU fluid than in the tubule fluid in *Teleogryllus*, which may indicate resorption in the ampulla. It is difficult to

account for the major differences in $Na^+$, $Cl^-$ and $K^+$ concentrations between *Teleogryllus* and *Acheta*. These may be species differences but, if so, they are surprising since the feeding habit and anatomy of the excretory systems are essentially the same and the species are closely related.

### *Cellular regulation of secretion*

The distal segment appears to be predominantly engaged in ion secretion and the removal of unidentified solutes, whereas a major role of the main segment appears to be fluid secretion. Although the specific rate of fluid secretion by the distal segment is higher than that of the main segment, the absolute rate is much lower and the rate does not change under the influence of diuretic hormone. It might be anticipated, therefore, that some differences in control would be observable between the two segments.

In insects, most crude extracts of diuretic hormone exert their effects *via* cyclic AMP (Rafaeli, 1990). It is known, however, that in some insects some diuretic hormones produce no increase in cyclic AMP level, e.g. in *Manduca sexta* (Kataoka *et al.* 1989) and in *Locusta migratoria* stimulation with crude extract produces increases in cyclic AMP and inositol trisphosphate concentrations (Fogg *et al.* 1990). Morgan and Mordue (1985) have also shown in *Locusta* that two diuretic peptides act separately, one *via* cyclic AMP and the other *via* a $Ca^{2+}$-dependent mechanism. Coast *et al.* (1991) discuss and cite the evidence for the concept that cyclic AMP mediates increases in $Na^+$ and $K^+$ transport whereas $Ca^{2+}$ mediates increases in $Cl^-$ conductance. Consequently, it was considered that relative changes in cyclic AMP concentration may indicate whether there are major differences in the cellular control of secretion in the two segments of cricket Malpighian tubules.

At first inspection, there appear to be no indications of differences in cyclic AMP response to hormonal stimulation in the main and distal segments. There is an approximate doubling of cyclic AMP per unit mass of protein in both segments on stimulation and there are no significant differences in concentration between segments. However, if concentration is expressed in terms of cyclic AMP per unit volume of cell, then the concentration in the distal segment is approximately ten times that in the main segment. This is consistent with the observation that the calculated secretion rates of $K^+$ (from measured fluid secretion rates, ion concentrations and estimated segmental cell volumes) by control and stimulated distal segments are $0.86\times10^{-5}$ and $2.16\times10^{-5}$ nmol $h^{-1}$ $m^{-3}$, respectively, and by control and stimulated main segments are $0.07\times10^{-5}$ and $0.11\times10^{-5}$ nmol $h^{-1}$ $m^{-3}$, i.e. a 10- to 20-fold higher rate in the distal segments. The concentrations of cyclic AMP in the whole tubules (distal and main segments) are 9.5 μmol $l^{-1}$ cellvolume for control and 16.8 μmol $l^{-1}$ cellvolume for stimulated tubules. These are consistent with those of control (0.9–5.7 μmol $l^{-1}$) and stimulated (3.3–70 μmol $l^{-1}$) tubules from *Acheta* reported by Coast *et al.* (1991).

These calculations suggest that the two segments may have the same control mechanism for regulating $K^+$ secretion. There must, however, be some other mechanism for regulating hydraulic conductivity, since the distal segment did not respond to stimulation with corpora cardiaca extract by increasing fluid secretion although the osmotic concentration of the secreted fluid increased by approximately 50mosmolkg$^{-1}$.

The similarity in cyclic AMP per unit mass of protein in main and distal segments and the large difference in cyclic AMP per unit volume implies a considerable difference in protein density between the two segments. This may be due in part to the different cellular structure of the two segments. The cells of the main segment have a complex and extensive network of endoplasmic reticulum whilst the cells of the distal segment have a comparatively reduced endoplasmic reticulum (A. T. Marshall, unpublished data).

*Osmotic concentrations and hypo-osmotic secretion*

The osmotic concentration of fluid secreted from unstimulated whole tubule preparations appears to be iso-osmotic when the osmotic concentration of the bath is approximately $300 mosmol kg^{-1}$ but becomes increasingly hypo-osmotic as the osmotic concentration increases. On stimulation with corpora cardiaca extract, the secreted fluid becomes hyperosmotic. Fluid from the main segment is also hypo-osmotic but appears to remain hypo-osmotic on stimulation. Since the osmotic concentration of the haemolymph of hydrated crickets is about $390 mosmol kg^{-1}$ (Hyatt and Marshall, 1978) secretion may be expected to be normally hypo-osmotic. Fluid from the distal segment is probably slightly hyperosmotic in unstimulated tubules but becomes very hyperosmotic (increases by approximately $50 mosmol kg^{-1}$) on stimulation. Hyperosmotic secretion by Malpighian tubules may not be uncommon, as pointed out by Taylor (1971), and presents no conceptual difficulties. However, hypo-osmotic secretion is difficult to explain.

It is generally accepted that fluid secretion in insect Malpighian tubules is iso-osmotic or slightly hyperosmotic and that the production of hypo-osmotic fluid is a consequence of reabsorptive processes (Bradley, 1985). This may not always be the case. It has been shown that the secreted fluid from the inferior Malpighian tubules of *Carausius morosus* can be hypo-osmotic when functioning in an artificial saline (Taylor, 1974). This has also been observed by Ramsay (1954) when tubules were bathed in haemolymph. Taylor (1974) has argued on morphological grounds that resorption is unlikely to be the cause of hypo-osmotic fluid secretion in *Carausius*. In *Teleogryllus*, it was observed that different lengths of the main segment were all capable of producing hypo-osmotic fluid. This suggests that there is no specialised ‘downstream’ region of the main segment where ion resorption takes place. It does not exclude the possibility that specialised resorptive cells may be dispersed throughout the epithelium. Morphologically distinct cells have been observed only very occasionally and must have an extremely low frequency of occurrence (A. T. Marshall, unpublished results). However, it has been shown by Maddrell (1978, 1980) that similarity of cell structure in Malpighian tubules does not necessarily imply physiological identity. A point that may be of significance to future considerations of the mechanism of hypo-osmotic fluid secretion is that the structure of the principal (type 1) cells of the inferior tubules of *Carausius* (Taylor, 1971) appears to be identical to that of the cells of the main segment in *Teleogryllus* (A. T. Marshall, unpublished results).

Experiments described here on whole tubule preparations show that the Malpighian tubule physiology has no strikingly unusual features which might indicate why the secretion is hypo-osmotic. Secretion rate falls as bath osmotic concentration increases and increases as $K^+$ concentration in the bathing medium increases. These are commonly

observed physiological characteristics of Malpighian tubules (Maddrell, 1971*b*). Preliminary X-ray microanalytical investigations of the main segment cells in *Teleogryllus* have shown in unstimulated preparations that a marked increase in $K^+$ and $Cl^-$ concentrations occurs in the brush border (Marshall, 1981, 1982). This observation suggests that some form of local osmotic gradient is involved in the osmotic coupling process.

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