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EXCRETION IN THE HOUSE CRICKET (*ACHETA DOMESTICUS*): STIMULATION OF DIURESIS BY TISSUE HOMOGENATES

BY JEFFREY H. SPRING AND SHELIA R. HAZELTON

Department of Biology, University of Southwestern Louisiana, Lafayette, LA 70504, USA

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SUMMARY

1. A new method is described for maintaining cricket Malpighian tubules *in vitro*. Warmed, oxygenated saline is circulated rapidly past the tubules, while the secreted urine is collected under oil for analysis. This technique allows the cricket tubules to be observed and manipulated for extended periods (6 h), in contrast to their short life (<1 h) using conventional methods.

2. Cricket tubules show extreme sensitivity to oxygen deprivation, such that 15 min of anoxia represents the median lethal dose (LD_{50}) for *in vitro* preparations.

3. Homogenates of corpus cardiacum (CC) cause the rate of fluid secretion by the tubules to double. The maximum stimulation is dose-dependent over the range 0·01 to 1·0 CC. Homogenates of brain and other ganglia show much smaller stimulatory effects (0·01–0·02 CC-equivalents). Cyclic AMP mimics the increase in secretion rate, but has an inhibitory effect on the smooth muscle of the ureter.

4. Control preparations maintain a urine osmotic pressure (OP) that is hyperosmotic to the bath by 5–10 mosmol l^{-1}. CC homogenate produces a decrease in urine OP to 10–12 mosmol l^{-1} hypo-osmotic to the bath. This suggests that active solute reabsorption is occurring in the lower tubule or ampulla.

5. Stimulation by CC homogenate increases the urine potassium concentration slightly less than two-fold, whereas the sodium concentration increases by a maximum of five-fold and remains at a higher concentration than potassium throughout the experiment. Tubule secretion rate is drastically inhibited in nominally sodium-free saline.

INTRODUCTION

The hormonal control of diuresis has been under investigation for more than three decades with results as diverse as the insects studied. Much of our knowledge of this control comes from studies on blood-feeding insects (*Rhodnius*, tsetse fly, mosquito) that periodically consume a blood meal of several times their body mass, excreting the ingested fluid within a few hours after feeding (reviewed by Raabe, 1982; Phillips, 1983). During the non-feeding intervals urine production is greatly reduced, to the extent that Maddrell & Nordmann (1979) have proposed that a single

Key words: cricket, diuresis, Malpighian tubule, corpus cardiacum.

diuretic hormone (DH) is sufficient to control excretion in *Rhodnius*. Conversely, the bulk of our knowledge of the reabsorptive processes has been obtained using the xeric insect, *Schistocerca*, in which the reabsorptive tissue, the rectum, is very highly developed (reviewed by Phillips, 1983).

Obviously, the majority of insect species fall somewhere between these extremes, neither facing the massive periodic saline loading of the blood-feeders nor tolerating extreme desiccating conditions as do the xeric insects. For this study, the easily reared house cricket, *Acheta domesticus*, was selected to represent the generality of insects. In addition, the morphology of the cricket excretory system is well-suited to studies of diuresis. In *Acheta*, as in other Gryllidae, the anterior hindgut extends nearly to the level of the gastric caeca and the midgut is greatly reduced. The Malpighian tubules have become completely dissociated from the gut, and fuse to form a common, bladder-like ampulla which lies free in the haemocoel. The ampulla empties *via* a single muscular ureter into the hindgut slightly anterior to the rectum. The morphology and ultrastructure of this system have been described in detail elsewhere (Martoja, 1966; Parker & Spring, 1985).

The development of a method for maintaining *Acheta* Malpighian tubules for prolonged periods *in vitro* allowed us to address two broad areas of investigation. First, we characterized the Malpighian tubule preparation as fully as possible, in terms of oxygen and temperature dependence, rate of urine formation, transported ions, etc. Second, after baselines for the above variables had been established, we determined whether they would be measurably affected by homogenates of tissues reported to contain diuretic (DH) and antidiuretic (ADH) hormones.

MATERIALS AND METHODS

Experimental animals

Virgin adult female *Acheta domesticus* were obtained by courtesy of Dr J. P. Woodring, Louisiana State University at Baton Rouge. Crickets were maintained in our laboratory at 30 ± 1 °C on a 14L:10D photoperiod and were provided with Purina cricket chow and fresh water *ad libitum*. Experimental animals were kept in groups of 20 in 4-l containers and used when they were 15–30 days past the final moult.

Solutions

The composition of the physiological saline used in all experiments was as follows (in mmol l^{-1}): NaCl, 100; K_2SO_4, 10; $MgSO_4.7H_2O$, 10; $NaHCO_3$, 10; $CaCl_2.2H_2O$, 3·5; imidazole, 15; glucose, 10; glycine, 10; proline, 10; glutamine, 2; histidine, 2; leucine, 2; lysine, 4; tyrosine, 2; valine, 4; pH 7·3, OP 300 mosmol l^{-1}. The amino acid concentrations were based on the haemolymph values for virgin females as outlined by Woodring & Blakeney (1980). Unless otherwise specified, the saline was constantly oxygenated, even during dissections, with water-saturated 95 % O_2:5 % CO_2. For one set of experiments, a nominally sodium-free saline was used, which had the following composition (in mmol l^{-1}): choline chloride, 100; K_2SO_4,

5; $MgSO_4.7H_2O$, 10; $KHCO_3$, 10; $CaCl_2.2H_2O$, 3·5; imidazole, 15; glucose, 10; glycine, 10; proline, 10; glutamine, 2; histidine, 2; leucine, 2; lysine, 4; tyrosine, 2; valine, 4; pH 7·3, OP 300 mosmol l^{-1}. For both normal and sodium-free salines, 15 min of bubbling with water-saturated 95 % O_2:5 % CO_2 reduced the pH to 7·0, where it stabilized for the remainder of the experiment.

Malpighian tubule preparations

Crickets were killed by decapitation and the bodies pinned dorsal-side uppermost on a wax-filled dissecting dish. The abdomen was opened with a midline and two lateral incisions and the cuticle flaps were pinned open. The paired ovaries were removed using fine forceps and the preparation was quickly flooded with warm (30 °C), oxygenated saline. Sufficient tracheae were severed to free the hindgut and permit it to be uncoiled and pinned straight. The gut was nicked several millimetres anterior to the ureter junction and a polyethylene cannula (PE 50) was inserted into the rectum. Several millilitres of saline were flushed through the hindgut, distending it slightly and removing all particulate matter. The large cannula was removed and a smaller one (PE 10), about 3 or 4 cm long, inserted in its place. This cannula was pushed through the lumen of the gut until the flared end was located just posterior to the ureter valve. The cannula was secured with a thread ligature, and a second ligature, located anteriorly to the ureter junction, sealed the gut. All excess gut tissue was trimmed away, releasing the cannula and its attached piece of gut, and any Malpighian tubules that were attached to the body wall were gently teased free until the entire Malpighian tubule/ampulla/ureter/hindgut/cannula preparation was floating freely in the saline. The cannula was then trimmed to 5–6 mm in length and the preparation transferred to the incubation chamber illustrated in Fig. 1. The cannula fitted snugly into the notch in the Plexiglas wall separating the saline and oil baths and a seal was formed using silicone grease. The saline was circulated rapidly past the tubule preparation using a gas-lift pump which also served to saturate the saline with oxygen. A cover on the reservoir (*R*; Fig. 1) reduced evaporation and splattering of the saline and the bath temperature was maintained at 30 ± 2 °C.

Single tubule preparations

For those experiments in which it was necessary to examine the urine from a single tubule, an apparatus similar to that first described by Ramsay (1954) was used. A loop of 6 mm o.d. glass tubing was heated and shaped to fit in the bottom of a 2-cm deep glass Petri dish where it was buried within a 1-cm deep layer of paraffin wax. The dish was filled with silicone oil and heated water circulated through the tubing to maintain the oil bath temperature at 30 ± 2 °C. A large drop (300–500 μl) of oxygenated saline was set up in a depression in the wax and kept from moving with several circumferentially placed minuten pins. One further pin was placed 1–2 mm from the edge of the drop. A cricket was prepared as described above with the exception that instead of removing the entire Malpighian tubule system, a single tubule was severed 3–4 mm from the ampulla and transferred to the saline drop. Care was taken to handle the tubule only at the cut end. This end was extended from the

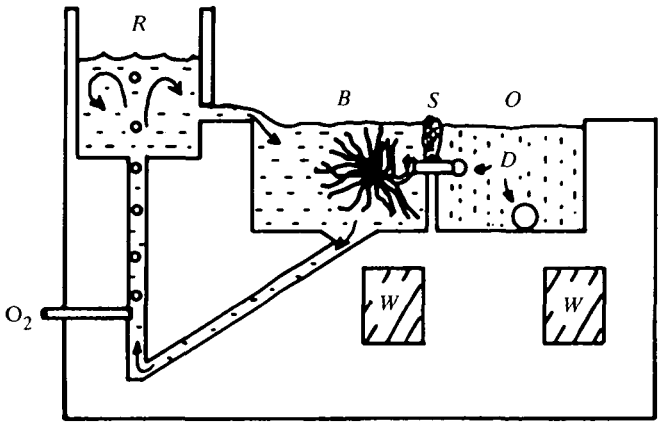

Fig. 1. Schematic diagram of apparatus used to maintain *Acheta* Malpighian tubules *in vitro*. *R*, saline reservoir; *B*, saline bath; O_2, inlet for gas-lift pump. Saline circulation is in the direction of the arrows. *S*, silicone grease; *D*, urine droplets, forming at the end of the cannula and resting on the floor of the oil bath (*O*). *W*, water jacket for heating or cooling the preparation. For complete explanation, see text.

main drop to the outlying pin and looped around it. The droplet of urine that formed at the cut end was usually small (30–60 nl) but sufficient for the determination of elemental concentrations as described below.

Volume and rate determinations

During the normal course of secretion, a droplet of urine would form continuously at the tip of the cannula under the silicone oil. At timed intervals, droplets would be removed from the cannula using the tip of an insect pin, and the diameter of the droplet measured with a graticule ocular. The volume of each droplet and the rate of secretion could then be determined as described by Maddrell (1980). Droplets were kept in separate depressions in the oil bath for further analysis.

Preparation of homogenates and drugs

To expose the corpora cardiaca (CC), a freshly-severed head was placed posterior-side down on a wax dissecting surface. A single-edged razor blade was then used to cut off the upper portion of the head capsule, the incision being made at the level of the lateral ocelli. The lower portion was placed in the normal vertical position and secured with insect pins. The bluish-white CC were then readily visible near the pars intercerebralis (PI) and could be dissected free with fine forceps and iris scissors. The CC were immediately macerated in distilled water in a Potter–Elvehjem ground glass homogenizer at a concentration of 1·0 pair/100 μl. Homogenates were stored on ice until used.

In *A. domesticus*, the CC receive the bulk of their neurosecretory material from the roughly 900 medial neurosecretory cells (MNC) *via* the axons forming nervus corporis cardiaci I (NCCI; Geldiay & Edwards, 1973). The two groups of MNC lie near the surface of the PI and are also bluish-white in appearance. For some

experiments a head capsule was prepared as above and a sufficiently large volume of the PI was removed to ensure that it contained all of the MNC. This tissue was homogenized as above and designated PI. Entire suboesophageal ganglia (SOG) and terminal abdominal ganglia (TAG) were dissected free and homogenized as above.

Dibutyryl adenosine 3′,5′-cyclic monophosphate (cyclic AMP) was prepared as a $0{\cdot}1\ \mathrm{mol\,l^{-1}}$ stock solution in physiological saline and stored at 4°C until used. The phosphodiesterase inhibitor 3-isobutyl 1-methylxanthine (IBMX) was prepared as a $1\ \mathrm{mmol\,l^{-1}}$ stock solution in physiological saline and stored at 4°C. Synthetic proctolin was prepared as a $1\ \mu\mathrm{mol\,l^{-1}}$ stock solution in distilled water and stored frozen. All three drugs were obtained from the Sigma Chemical Company (St Louis, MO, USA).

Assay procedures

Malpighian tubule preparations were set up in physiological saline and allowed to stabilize for 45–90 min. This period was used to determine the viability of the preparation and to establish the control secretion rate. For those experiments involving physical changes (i.e. temperature, oxygen saturation), the variable was altered at the time the final control droplet was removed from the cannula and the experimental period timed from that point. Although such changes were not instantaneous, they were treated as step functions for the purposes of these experiments as the sample interval (15 min) was greater than the expected equilibration time.

Concentrated stock solutions of tissue homogenates and drugs required the addition of 5–100 μl of stock to reach the final concentration desired. When the volume of stock to be added exceeded 40 μl, an equivalent volume of bath saline was first removed to minimize dilution changes. For IBMX, which was not sufficiently soluble to be prepared as a concentrated stock, the bath saline was replaced with three changes of warm, oxygenated saline containing the drug at its final concentration. For all preparations, at the end of the experimental period, the chambers were flushed with seven changes of fresh, warmed, oxygenated saline. Test preparations containing dyed saline indicated that this removed all of the experimental saline. Preparations were allowed to recover for a further 45–60 min to re-establish the control secretion rate.

Osmotic concentration determinations

During all experiments, the osmotic concentration of the bathing saline was measured at roughly 15-min intervals using a Wescor 5100B vapour pressure osmometer. Due to the relatively small volume of the bath (1·5 ml) and its elevated temperature (10°C above ambient), the osmotic concentration would rise 5–15 $\mathrm{mosmol\,l^{-1}}$ between measurements. Therefore, distilled water was added and the osmotic concentration remeasured at each interval in order to maintain the bath osmotic concentration as close to $300\ \mathrm{mosmol\,l^{-1}}$ as possible.

During some experiments, the osmotic concentration of the urine was also determined. For these and the ionic concentration determinations (see below), the

delay in the formation of the droplet due to the dead volume of the cannula was significant. At the end of these experiments, the cannula was dissected free of tissue and the length determined to the nearest 0·25 mm. The dead volume was then calculated from the known internal diameter (0·28 mm). Using the secretion rate measured for each interval, the actual time interval represented by each droplet was determined and its position on the x-axis altered accordingly (see Figs 6, 7). To minimize any changes in the concentration of the droplets due to evaporative or diffusive losses, osmotic concentrations were determined within 15 min of the time the droplet was collected. A 10–20 nl sample of each droplet was taken and the osmotic concentration measured (as freezing point depression) with a Clifton Nanolitre Osmometer.

Elemental concentration determinations

Elemental concentrations of sodium, magnesium, phosphorus, sulphur, chlorine, potassium and calcium were measured with energy dispersive X-ray microanalysis (EDS), using a modification of the method of Hyatt & Marshall (1985). Briefly, urine droplets were mixed under oil with measured volumes of cobalt standard to give final cobalt concentrations of approximately 25 mmol l^{-1}. Picolitre samples of the mixed droplets were transferred to nickel finder grids, which had previously been coated with Formvar and carbon, and allowed to air dry. Full grids were glued to aluminium sample stubs, dried *in vacuo* for 12 h, and carbon-coated to prevent rehydration. Droplets were examined with a Hitachi S-450 SEM and the elemental concentrations determined using a Kevex 7700 series X-ray analyser. EDS spectra were measured using an accelerating voltage of 20 kV, a specimen current of 2×10^{-10} A and a collection time of 100 s. A minimum of three samples from each original droplet was analysed and the mean value for each element used in further calculations. The ratios of the elements to the internal cobalt standard were determined and the millimolar concentrations determined from standard curves for each element and the calculated cobalt concentration in the original mixed droplets.

RESULTS

Control secretion rates

Secretion rates in control preparations were followed for a 6-h period (Fig. 2). Initial secretion rates were nearly 4 μl h^{-1}, declining to approximately 1 μl h^{-1} at 6 h. Although these preparations never reached a true steady-state condition, the decline in rate was linear and this pseudo-steady-state provided an acceptable baseline for further experiments. All experiments in which the preparations were manipulated were completed within the 6-h period for which tubules have been shown to remain viable. Since every preparation was allowed to stabilize for 45–90 min prior to experimentation, it would be possible to extrapolate the basal secretion rate and measure experimental changes against the expected next value. However, there was always some variability within individual preparations, so experimental rates were compared with the last rate observed during the stabilization period. This treatment

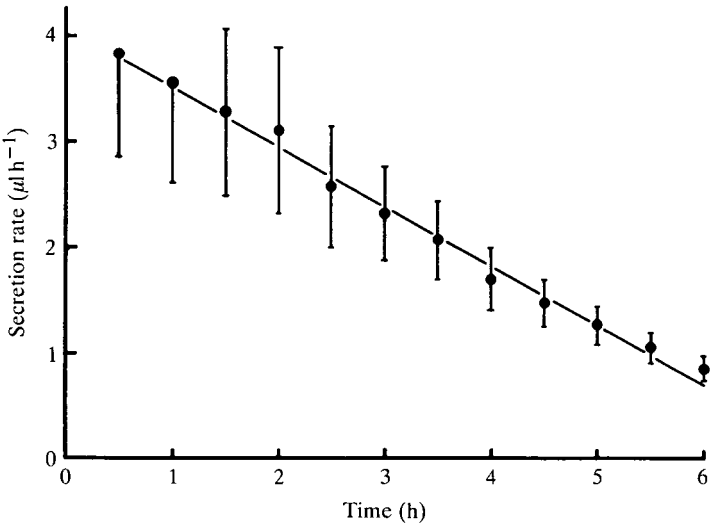

Fig. 2. Control secretion rate. Each point represents the mean (±1 S.E.) for 10 preparations. The slope ($\mu l\ h^{-1}$) = −0·56x+4·08. The correlation coefficient (r) is 0·996.

of the data would consistently underestimate the effects of diuretics while overestimating the effects of antidiuretics.

Effects of temperature and oxygen

When the temperature of the bathing saline was lowered from 30° to 20°C over a 10-min period, the secretion rate declined to about one-half the control value. Restoring the bath to 30°C restored the secretion rate (Fig. 3A). These observations are consistent with the tissue having a Q_{10} of approximately 2, as expected for most biological systems, and confirm the necessity of maintaining an elevated bath temperature to obtain optimal secretion rates.

In some experiments, the gas mixture (95 % O_2: 5 % CO_2) in the gas-lift pump was replaced with nitrogen at identical flow rates to maintain a constant bath circulation (Fig. 3B). Fifteen minutes of oxygen deprivation permanently stopped the secretion of four out of ten preparations. One further preparation failed to recover after normal oxygen levels were restored. The five surviving preparations partially recovered, but secretion rates did not return to pre-anoxic levels.

Stimulation of corpora cardiaca

The addition of 1·0 pair of homogenized CC to the bath caused the rate of secretion nearly to double over the next 30 min (Fig. 4). The rate remained elevated for a further 30 min, following which it began to decline increasingly quickly. Rinsing the tissue with fresh saline caused the secretion rate to return to control levels and recovery appeared to be complete (i.e. the slope of the baseline is identical before and after stimulation). It should be noted also that if the increased velocity with which the secretion rate was declining between 1 and 2 h after stimulation were

maintained, the preparations would have returned to the control rate 3 h after stimulation. This is suggestive of breakdown of the active CC factor, either through metabolic degradation by the tubules or simple biochemical breakdown in the saline.

The peak increase in secretion rate with varying doses of CC homogenates is shown in Fig. 5. The maximum stimulatory effect occurred with 1·0 pair of CC and the minimum dose which could be detected was 0·01 pair. Although variability was high, the response appeared to increase with the logarithm of the CC dose.

Effects of cyclic AMP and other drugs

When cyclic AMP was added to the bath at a final concentration of 1·0 mmol l^{-1}, the preparation immediately stopped moving. The individual tubules ceased their usual constant writhing and the ureter stopped its peristaltic pumping and became greatly distended. In spite of this, urine secretion increased by 45% over the first 30 min following stimulation (Table 1), but the preparations were not viable for

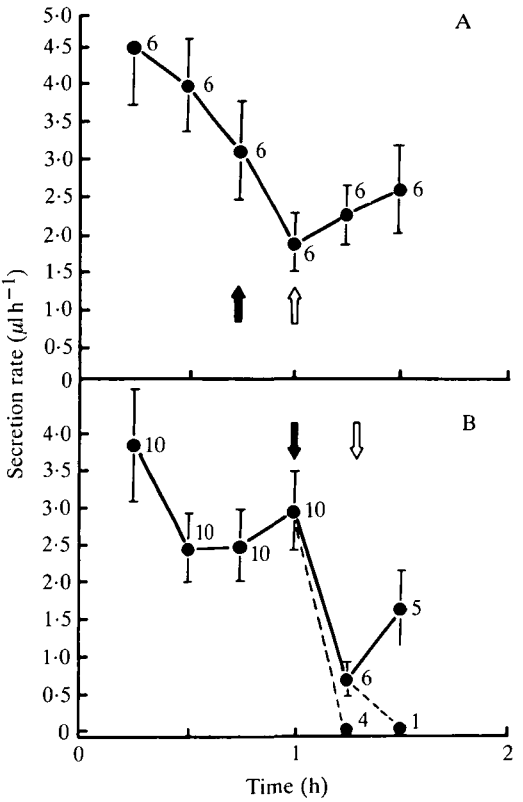

Fig. 3. (A) Effect of temperature on urine secretion rate. Bath temperature lowered from 30° to 20°C at filled arrow. Bath restored to 30°C at open arrow. (B) Effect of oxygen deprivation on urine secretion rate. Nitrogen subsituted for usual gas mixture (95% O_2:5% CO_2) in gas-lift pumps at filled arrow. Carbogen circulation restored at open arrow. Dotted lines follow the secretion rates for those preparations which did not survive the anoxic period. For both figures, vertical bars represent ±1 S.E. and the number of trials for each point is given.

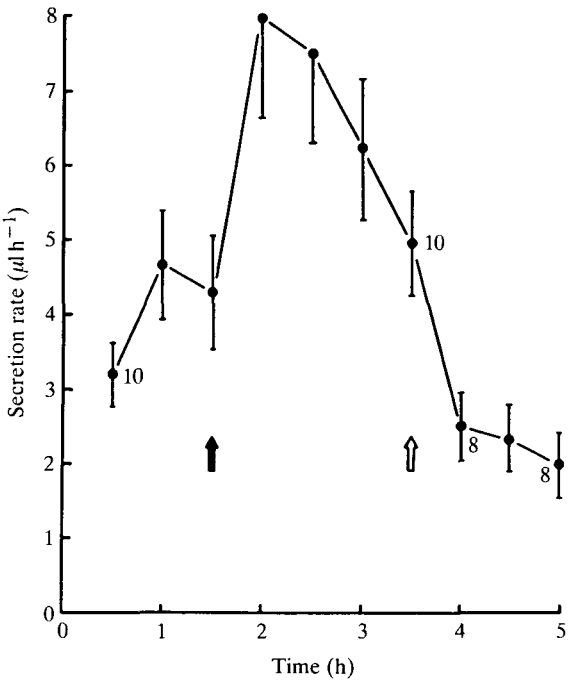

Fig. 4. Effect of corpus cardiacum (CC) homogenate on urine secretion rate. 1·0 pair of CC added to bath at filled arrow. CC removed with three rinses of fresh saline at open arrow. Vertical bars represent ±1 S.E. and the number of trials is given.

more than 60–90 min after cyclic AMP treatment. IBMX produced a nearly identical effect (data not shown). When the cyclic AMP concentration in the bath was reduced to 0·1 mmol l^{-1}, no effects were observed, either on muscle contraction or fluid transport. Proctolin, at what was expected to be a supramaximal dose (67 nmol l^{-1}, Miller, 1983), had the reverse effect in that the small piece of gut surrounding the cannula became very active, rippling and twisting, and the rate of ureter contractions increased. There was, however, no effect on the rate of urine formation (Table 1).

Other ganglia

Fig. 6 compares the stimulatory effects of other ganglia to that of the CC. All of the ganglia tested showed a small (0·01–0·02 CC-equivalents) increase in secretion

Table 1. *Effects of cyclic AMP and proctolin on urine secretion rates*

Drug	Secretion rate (μl h^{-1}) Control	Stimulated
Cyclic AMP (1·0 mmol l^{-1})	1·90 ± 0·24 (N = 3)	2·72 ± 0·26
Proctolin (67 mmol l^{-1})	1·70 ± 0·36 (N = 3)	1·65 ± 0·78

Values are mean ± 1 S.E.

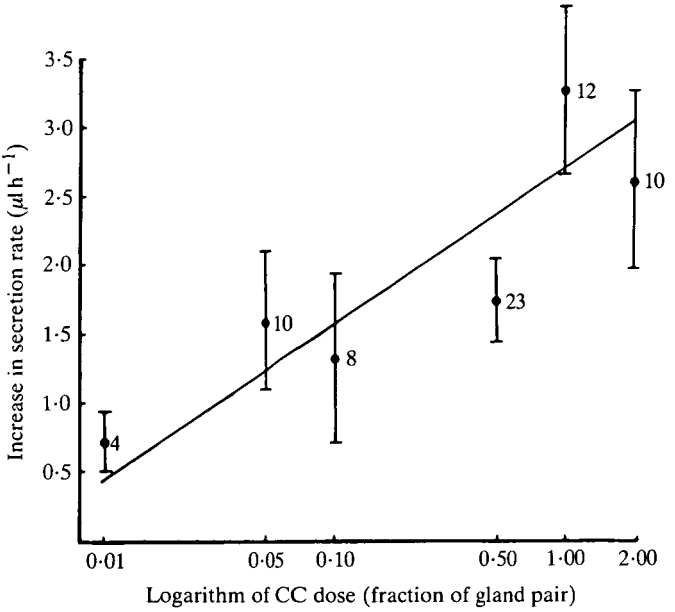

Fig. 5. Dose–response curve for corpus cardiacum (CC) homogenate showing the maximum change in urine secretion rate for each dose. Each point represents the mean (±1 S.E.) for the number of trials indicated. The slope ($\mu l\,h^{-1}$) is 1·02 log (CC dose)+2·60. The correlation coefficient (r) is 0·898.

rate during the first 15 min after addition, following which the PI- and TAG-stimulated preparations showed a decline in secretion rate, whereas the secretion rate of the SOG-stimulated preparations remained elevated until rinsed. In all cases, rinsing with fresh saline caused an initial drop in secretion rate, followed by recovery (PI, SOG) or return to baseline rate (TAG).

Changes in urine osmotic concentration

When tubule preparations were placed in bathing saline, the osmotic pressure (OP) of the urine was initially hypo-osmotic to the bath by 5–10 mosmol l^{-1}. Urine OP gradually increased with time so that after 90–120 min, the urine was hyperosmotic to the bath by an equivalent amount and this differential was maintained for the remainder of the experiment (Fig. 7A). This pattern was also observed during the control period of CC-stimulated preparations, the urine initially being slightly hypo-osmotic to the bath and gradually becoming hyperosmotic at 90 min (Fig. 7B). However, the addition of 1·0 pair of CC caused a steady decrease in the OP of the urine for the first hour following stimulation, after which the OP steadied at 11–12 mosmol l^{-1} hypo-osmotic to the bath. When the homogenate was rinsed off, the urine OP rose to the extent that it was isosmotic to the bath 90 min after the rinse. The fact that the urine becomes hypo-osmotic to the bath during the time that secretion rates have doubled strongly suggests that solute is reabsorbed by either the lower Malpighian tubules or the ampulla.

Changes in urine elemental composition

The concentrations of Na, K and Mg in the urine of control preparations are presented in Fig. 8. Na and K are shown because of their known importance to fluid transport, and Mg because the pattern of change, although not the absolute concentration, was very similar for Mg, sulphur and Ca. In control preparations (Fig. 8A), the Na concentration was rather low and showed no decline with time, although the variability was high. At least part of this variability can be attributed to measurement techniques, as the relatively small size of the Na peak and its location on the rising shoulder of the background make it difficult to measure accurately in low concentrations using EDS alone. The K concentration showed a slow, steady decline with time. If potassium transport were indeed the driving force for fluid secretion under control conditions, its concentration in the urine should remain constant, although the absolute quantity transported would be less. As the amount of transported K decreases and the rate of fluid secretion falls, however, equilibration times within the tubule increase, which might explain the overall decline in K concentration.

The initial Mg concentration in the urine (17 mmol l^{-1}; Fig. 8A) was considerably higher than in the saline (10 mmol l^{-1}). This suggests either active Mg export or considerable fluid reabsorption in the lower tubule/ampulla. Sulphur (presumably in the form of sulphate ions) showed a similar ratio in that the initial urine

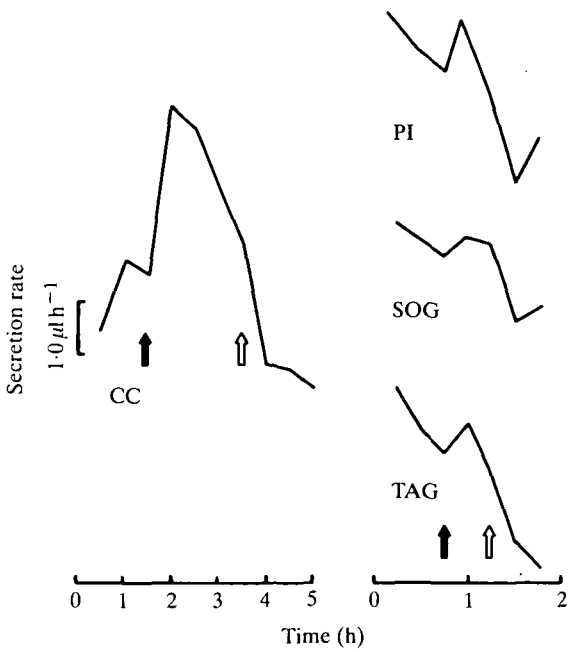

Fig. 6. Comparison of the changes in secretion rate caused by homogenates of corpus cardiacum (CC), pars intercerebralis (PI), suboesophageal ganglion (SOG) and terminal abdominal ganglion (TAG). Homogenates added to bath at filled arrow and washed off with three rinses of fresh saline at open arrow. y-axis units are identical for all tracings.

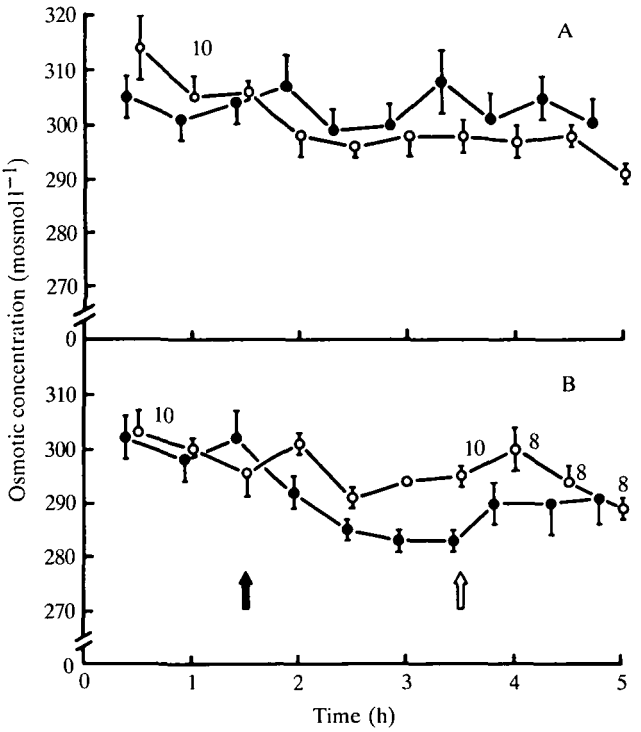

Fig. 7. Comparison of osmotic concentrations for saline bath (○) and urine (●). Vertical bars represent ±1 S.E. and the number of replicates is given. (A) Controls; (B) effects of homogenates of corpus cardiacum (CC). 1·0 pair of CC added to bath at the filled arrow and rinsed off with three changes of fresh saline at open arrow.

concentration (40 mmol l^{-1}) was twice that of the saline (20 mmol l^{-1}), as was the case for Ca (7·5 *vs* 3·5 mmol l^{-1}). When the preparations were stimulated with 1·0 pair of CC, those elements which are presumed to enter the tubule lumen passively showed a large drop in concentration in the urine as can be seen in Fig. 8B for Mg (17 to 6 mmol l^{-1}). Sulphur and Ca also showed decreased concentrations in the urine following stimulation but to a lesser extent than observed for Mg. Blowfly Malpighian tubules are known actively to transport sulphate ions (Knowles, 1975), and in mosquitoes both sulphate and magnesium ions are actively transported by an inducible mechanism (Maddrell & Phillips, 1975*a*, 1978; Phillips & Maddrell, 1974). It is possible, therefore, that the three divalent ions (Ca^{2+}, Mg^{2+}, SO_4^{2-}) are being actively transported by the cricket tubules. A more conservative explanation, however, is that the divalent ions are moving into the tubule lumen passively and that the apparent increase in their concentration is due to fluid recovery by the lower tubule/ampulla, since there is evidence to indicate that solutes, at least, are being recovered (Fig. 7B). The experimental design used in this study does not provide evidence to distinguish between the two possibilities.

The K concentration increased from a prestimulation value of approximately 20 mmol l^{-1} to a maximum of 33 mmol l^{-1} 1 h following the addition of CC, whereas

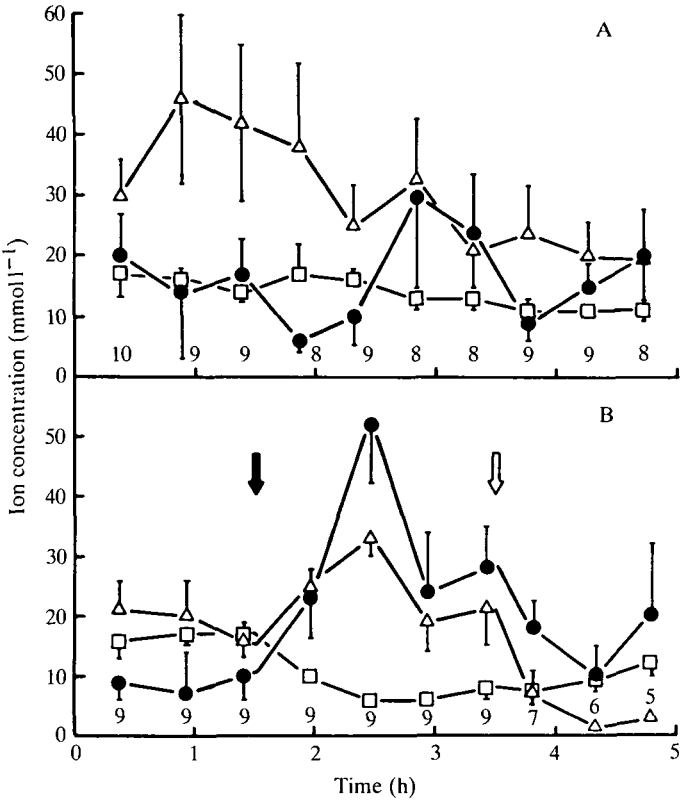

Fig. 8. Comparison of urine concentrations of Na (●), K (△) and Mg (□). Vertical bars represent ±1 S.E. and number of replicates is given. (A) Controls; (B) effects of homogenates of corpus cardiacum (CC). 1·0 pair of CC added to bath at the filled arrow and removed with three rinses of fresh saline at the open arrow.

the Na concentration rose from 10 to 50 mmol l^{-1} during the same period (Fig. 8B). The five-fold increase in urine Na compared to the less than two-fold increase in K suggested that the former may be the dominant cation in stimulated fluid transport. The possibility remained, however, especially in view of the decreased OP of the urine, that K was being actively reabsorbed by the lower tubule/ampulla. To address this point, a number of individual tubules were incubated *in vitro*. Even in relatively large volumes of oxygen-saturated saline, such tubules were not viable for more than 45–60 min, so it was not possible to use a tubule as its own control. Accordingly, tubules were set up in saline drops containing 1·0 pair of freshly homogenized CC. The Na concentration was identical in both single tubule and whole preparations (Table 2). K and Cl concentrations in individual tubules were, if anything, slightly lower than in the whole preparations, clearly demonstrating that substantial reabsorption of these elements was not occurring in this system.

The Cl concentration in the urine, as measured by EDS, ranged from 60 to 110 mmol l^{-1}. These values were essentially the same for both control and experimental preparations. Under both conditions, variability between samples was

Table 2. *Elemental concentrations (mmol l^{-1}) in urine from CC-stimulated tubules*

Element	Single tubules	Whole preparations
Na	50 ± 10	52 ± 10
K	24 ± 5	33 ± 3
Cl	95 ± 6	110 ± 8
	(N = 5)	(N = 9)

Values are mean ± 1 S.E.
CC, corpus cardiacum.

sufficiently high to mask any pattern of change between stimulated and unstimulated preparations.

Sodium-free saline

When the saline bathing whole preparations was replaced with a nominally Na-free saline, the secretion rate quickly decreased by more than 60 % (Fig. 9). Rinsing with normal saline produced a slow return towards the control secretion rate. Several preparations were stimulated with 1·0 pair of CC 1 h after transfer to Na-free saline but showed no change in secretion rate.

DISCUSSION

The Malpighian tubule system of the cricket appears to offer a number of advantages for the examination of the action of putative diuretic and antidiuretic factors. Providing that the requirements for oxygen and temperature are met,

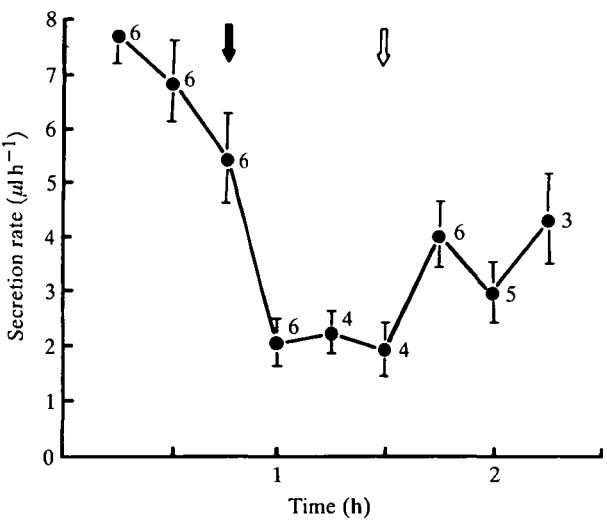

Fig. 9. Effects of sodium-free saline on urine secretion rate. Normal saline replaced with three rinses of nominally sodium-free saline at filled arrow and removed with three rinses of fresh normal saline at open arrow. Vertical bars represent ±1 S.E. and number of replicates is given.

preparations remain viable for at least 6 h. Even at the end of this time the tubules remain irritable in that the secretion rate will approximately double with the addition of CC homogenate (J. H. Spring & S. R. Hazelton, unreported observations). The unusual architecture of the Malpighian tubule system also makes it possible to collect useful amounts of urine for analysis.

The extreme sensitivity of *Acheta* tubules to oxygen deprivation is rather atypical. Isolating individual tubules or groups of tubules in a drop of saline under oil has been the standard practice for monitoring Malpighian tubule activity *in vitro* for more than 30 years (Ramsay, 1954). If the Malpighian tubule system of the cricket is isolated in this manner, the tubules no longer move and urine production ceases after 30–45 min (J. H. Spring & A. D. Hyatt, unpublished observations), a time course very similar to that reported here for single tubules. The original method described by Ramsay (1954) included an air bubble held in close apposition to the bathing droplet in order to maintain an adequate supply of oxygen to the tubules. This precaution has been justifiably eliminated as unnecessary for most subsequent studies of tubule function (see Maddrell, 1980), although, as the present study clearly demonstrates, the ability of Malpighian tubules from different insect species to function unimpaired under oil for prolonged periods cannot be taken for granted.

As with many other *in vitro* preparations, the cricket Malpighian tubules never attain a true steady-state condition; the rates of fluid and ion transport continue to decline slowly with time. However, the pseudo-steady-state is a stable condition and provides a suitable baseline for the bioassay of stimulatory and inhibitory agents. The approximately two-fold increase in tubule secretion rate caused by CC-homogenate is of the same magnitude as hormonally induced diuresis in a number of other insects such as mosquitoes (Williams & Beyenbach, 1983) and locusts (Proux, Rougon & Cupo, 1982; Rafaeli & Mordue, 1982). The tubules are sensitive to doses as low as 0·01 pair of CC and reach a maximum stimulation with 1·0 pair, values very comparable with those reported for the locust (Morgan & Mordue, 1983), mosquito (Williams & Beyenbach, 1983), butterfly (Nicolson, 1976) and desert beetle (Nicolson & Hanrahan, 1986).

All of the diuretic hormones (DH) reported to date are low molecular weight peptides, although they may be stored as larger polymers (reviewed by Aston & Hughes, 1980), and the diuretic factor in the cricket is likely to have a similar structure. All of the known DHs are mimicked by the second messenger cyclic AMP. In *Acheta*, the dual action of cyclic AMP on muscles and tubules complicates the measurement of its stimulatory effect. The addition of cyclic AMP to the bathing medium stopped all muscle contraction, allowing the ampulla and ureter to distend with accumulated fluid. Nevertheless, urine secretion did increase greatly, although not to the extent observed with CC homogenate. Clearly, cyclic AMP is stimulating accelerated fluid secretion. That the two processes of muscle contraction and urine formation are not directly interrelated is further substantiated by the effect of proctolin. In spite of greatly increased tubule movement and more rapid ureter peristalsis, the rate of urine secretion remained unchanged. Although relaxation of the Malpighian tubule system musculature can hinder the flow of urine, rapid

contraction cannot promote the flow unless the rate of secretion has also been elevated.

Other putative sources of diuretic material (PI, SOG, TAG) produce slight but measurable increases in urine formation. The stimulations are quite low (0·01–0·02 CC-equivalents) and in some instances appear to be relatively transient. These observations are consistent with the report by Morgan & Mordue (1984) that the locust diuretic hormone is present in the brain, SOG, TAG and other ventral ganglia. However, they find levels of DH 15–25 times higher than in the corresponding ganglia in crickets. This discrepancy may be partly the result of using crude homogenates since the brei might contain antagonistic factors or degradative enzymes. Chromatographic isolation of the bioactive factors will be necessary to compare activities quantitatively.

Primary urine formation is almost invariably isosmotic to the haemolymph (reviewed by Bradley, 1985). O'Donnell & Maddrell (1983) have demonstrated that, at least in the case of *Rhodnius*, an osmotic gradient of 3 mosmol l^{-1} (about 1 %) is sufficient to drive stimulated fluid transport. Under some circumstances, such as the hormonally stimulated diuresis in *Rhodnius*, the lower portion of the tubule can selectively reabsorb solutes, resulting in a change in ion composition and a urine which is strongly hypo-osmotic to the bathing medium (Maddrell & Phillips, 1975*b*, 1976). When *Acheta* tubules are maintained *in vitro* for extended periods, the urine is hyperosmotic to the medium by 5–10 mosmol l^{-1}, which is consistent with the accepted model for tubule function. When the tubules are stimulated with CC homogenate, the OP drops to 10–12 mosmol l^{-1} hypo-osmotic to the bath. While not as dramatic as the 100 mosmol l^{-1} decrease observed in *Rhodnius* tubules (Maddrell & Phillips, 1975*b*, 1976), this decrease obviously indicates that considerable solute reabsorption is occurring either in the lower tubule, the ampulla or both. Ultrastructural observations (Parker & Spring, 1985) suggest that the proximal few millimetres of the tubule and the ampulla both have the columnar cells typical of insect reabsorptive epithelia.

The major shortcoming of using a complex preparation such as the cricket tubule system is revealed by the ion analyses. EDS analysis of all major and minor elements (Na, K, Cl, Mg, sulphur, Ca) accounts for only 65 % of the observed urine OP (J. H. Spring & S. R. Hazelton, unreported observations). Although we consider our EDS analysis to be only semi-quantitative, comparisons of standards and urine samples with flame-emission spectroscopy seldom revealed variations greater than 10–15 % for Na and K. This is not sufficient to account for the discrepancy between urine OP and measured solute concentrations. In *Schistocerca*, proline in the luminal fluid provides the major source of metabolic fuel for the hindgut and to accommodate this requirement the tubules actively secrete proline at concentrations of up to 38 mmol l^{-1} (Chamberlin & Phillips, 1982*a*,*b*). Bicarbonate secretion is normally considered to be the domain of the hindgut (Bradley & Phillips, 1977; Strange, Phillips & Quamme, 1982); however, given the relatively high concentration of bicarbonate in our saline (initially 10 mmol l^{-1}) even passive movement of this ion would contribute significantly to the OP. Potential contributions of amino acids and

bicarbonate notwithstanding, there remains a discrepancy between the urine OP and the measured solute concentrations. Furthermore, Mg, sulphur and Ca are all present in the urine (at least initially) at nearly twice the bath concentrations. The most conservative hypothesis consistent with these data is that there is considerable recycling of the major ions and water within the tubule system. This would account for the relatively low concentrations of Na and K in the urine, the elevated concentrations of Mg, sulphur and Ca, and the unassigned solute component. During CC-stimulated diuresis, the measured elements account for nearly 85 % of the observed OP. Also, the concentrations of Mg, sulphur and Ca drop to lower than bath levels, which is consistent with their being passively transported into the lumen. One major contribution of the diuretic factor in the CC homogenate may simply be the inhibition of water and ion reabsorption, although some ion recycling must still occur for the urine to be hypo-osmotic.

One further consideration of stimulated urine formation is the change in the relative concentrations of Na and K. CC homogenate increases urine Na level five-fold and K level less than two-fold. Elemental analysis of the urine from single tubules and whole preparations clearly shows that this concentration change is not simply due to the rapid reabsorption of K, as occurs in *Rhodnius* (Maddrell & Phillips, 1976), but to increased transport of Na into the tubule lumen. Traditionally, the blood-feeding insects (e.g. *Rhodnius*, Gupta, Hall, Maddrell & Moreton, 1976; *Aedes*, Williams & Beyenbach, 1983) show elevated Na concentrations in the urine following a blood meal, whereas phytophagous insects (e.g. *Carausius*, Pilcher, 1970; *Pieris*, Nicolson, 1976; *Locusta*, Morgan & Mordue, 1983) show essentially no alteration in the Na:K ratio in the urine during diuresis. Nicolson & Hanrahan (1986) recently reported that a diuretic factor present in the brain, CC and prothoracic ganglion of the desert beetle, *Onymacris plana*, causes the Na concentration in the urine to double, but K still remains the dominant cation (186 mmol l^{-1} K compared to 45 mmol l^{-1} Na). The only previously recorded exception to this pattern is the dragonfly larva, where the ionic composition of the urine is identical to that of the haemolymph (Nicholls, 1985). The situation in *Acheta* is clearly different because the resting urine is K-rich, whereas the stimulated urine is Na-rich, a change that closely resembles the switch-over observed in blood-feeders. Crickets, however, are not faced with the periodic ingestion of a massive NaCl load, but like locusts, are primarily herbivorous and therefore are feeding on a K-rich food source. According to existing models, this should favour K as the primary ion for tubule secretion (Phillips, 1981). It was not possible to address this problem through ion substitution experiments because in *Acheta*, as in the locust (Morgan & Mordue, 1981) and the dragonfly (Nicholls, 1985), tubule function is greatly inhibited by removal of Na from the bathing medium. Thus, at present, these observations remain unexplained.

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