J. Exp. Biol. (1974), 61, 155–171
[illegible] 1 plate and 8 figures
Printed in Great Britain

POTASSIUM-INDUCED RELEASE OF THE DIURETIC HORMONES OF *RHODNIUS PROLIXUS* AND *GLOSSINA AUSTENI*: Ca DEPENDENCE, TIME COURSE AND LOCALIZATION OF NEUROHAEMAL AREAS

BY S. H. P. MADDRELL AND J. D. GEE

The A.R.C. Unit of Invertebrate Chemistry and Physiology and Department of Zoology, Downing Street, Cambridge

(*Received* 6 *December* 1973)

SUMMARY

1. Exposure of neurohaemal areas to solutions of elevated K concentration (above 40 mM) causes a maximal release of diuretic hormone in *Rhodnius prolixus* and *Glossina austeni*.

2. An involvement of Ca in hormone release is indicated by the reduction caused by low concentrations of this cation (below 2 mM) or by the presence of Mn.

3. During prolonged treatment with K-rich solutions the rate of hormone release is initially high, but then declines. This response parallels that for Ca entry into squid giant axons during maintained potassium depolarization and suggests that the rate of Ca entry controls the rate of hormone release.

4. Tetrodotoxin did not reduce the potassium-induced release of the hormone, suggesting that K acts directly on the neurosecretory axon endings in the neurohaemal areas.

INTRODUCTION

Release of neurohormones at neurosecretory axon endings is thought to result from the depolarization associated with the arrival of action potentials. That this process is Ca dependent has been demonstrated in both vertebrate neurohaemal organs, for example, the neurohypophysis (Douglas & Poisner, 1964; Dreifuss, Grau & Nordmann, 1971, 1973; Uttenthal, Livett & Hope, 1971) and invertebrate ones, for example, the pericardial organs of the crab (Berlind & Cooke, 1968, 1971). In the vertebrate pituitary, hormone release can be evoked by depolarizing the neurosecretory cell membranes with solutions of elevated K concentration (Dreifuss *et al.* 1973) and there is indirect evidence that such treatment has a similar effect on the corpora cardiaca of insects (Gosbee, Milligan & Smallman, 1968; Normann, 1970). These investigations of insect corpora cardiaca were purely cytological, however, and did not include a quantitative study of the concentrations of K and Ca required for hormone release. The experiments described in the present paper use a sensitive bio-assay technique to demonstrate in a much more direct way the release of hormones, in this case the release of the diuretic hormones of the blood-sucking insects, *Rhodnius prolixus* and *Glossina austeni*. We also give the results of a quantitative investigation

of the effects of changes in the K and Ca concentrations of the bathing solution on the release of these two hormones.

Baker, Meves & Ridgway (1973*a*) recently showed that the rate of Ca entry into squid giant axons in response to maintained depolarization with K-rich solutions rises to a peak in a few seconds then falls steadily away. If neurohormone release is dependent on Ca entry, then such release ought to follow a similar time course during continued depolarization and in this paper we describe experiments to test this. As will be shown, the rate of neurohormone release indeed behaves in a similar way, reaching a maximum within a few seconds and then declining exponentially.

The technique used in this investigation involves the *in situ* depolarization of neurosecretory cells, a method used previously in insects only by Gosbee *et al.* (1968) who followed solely by histological means the effect of depolarization of cell membranes of the corpus cardiacum of *Periplaneta americana*. The particular advantage of the technique described here, which combines localized depolarization *in situ* by solutions of elevated K concentration with a method of biological assay of the results of such a depolarization, is that it allows the sites of release (neurohaemal areas) to be distinguished from regions where hormone may occur but is not released.

METHODS

Materials

For the experiments described, 5th-stage larvae of *Rhodnius prolixus* Stål, 1–2 weeks old were taken from a laboratory stock maintained at 27 °C. Tsetse flies, *Glossina austeni* (Newst.), were obtained as pupae from the Tsetse Research Laboratory, Langford, Bristol. The pupae were reared and the adults allowed to emerge in the light at 25 °C. They were then placed in separate glass tubes and kept in darkness at 12 °C. At this temperature, the flies live for periods of 2–7 days before use, without needing to be fed. Only teneral flies, i.e. flies which had not taken a blood meal, were used.

The basic solutions had the following compositions (mM): for experiments with *Rhodnius*, NaCl 129, KCl 8·6, $CaCl_2$ 2, $MgCl_2$ 8·5, NaH_2PO_4 4·3, $NaHCO_3$ 10·2, glucose 34; pH 6·7; for experiments with *Glossina*, NaCl 128·3, KCl 20·1, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 5·1, Na glutamate 3, malic acid 2·8, citric acid 1·8, glucose 10; pH adjusted to 7 with NaOH. K-rich solutions were made by increasing the amount of KCl at the expense of NaCl. Variations in Ca concentration in the range 0–4 mM required no compensating changes, but higher Ca concentrations were attained by decreasing the Mg content and reducing the amount of phosphate and bicarbonate buffering so as to prevent precipitation of insoluble calcium salts. Similar changes had to be made in solutions containing Mn^{2+} ions, again to prevent precipitate formation.

Hormone assay

The basic method used in this investigation has been the simple one of applying, under liquid paraffin, a small drop of solution to the part of the insect under test and then after a suitable interval placing a Malpighian tubule in the drop for a direct assay of its diuretic hormone content (Maddrell, 1969). Malpighian tubules of *Rhodnius* and *Glossina* are similar in that they secrete fluid only very slowly in the absence of th

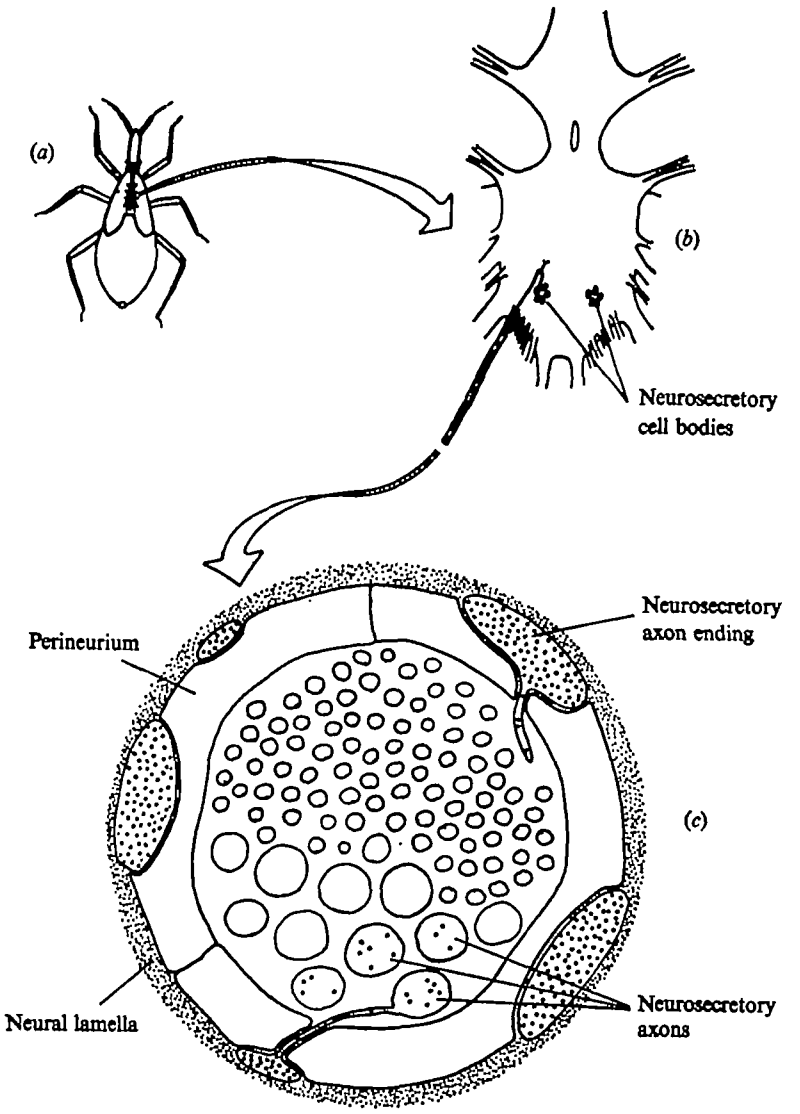

Fig. 1. Diagram to show where in *Rhodnius* the diuretic hormone is released into the circulating haemolymph. (*a*) The position and size of the mesothoracic ganglionic mass in relation to the rest of the insect. (*b*) The position in the mesothoracic ganglionic mass of the neurosecretory cell bodies which synthesize the diuretic hormone and the course of one of their axons along an abdominal nerve. (*c*) A cross-section of one of the abdominal nerves to show, lying outside the perineurium, the neurosecretory axon endings from which the diuretic hormone is released. Note the branches to these endings from the protected neurosecretory axons which run longitudinally, deeper within the nerves.

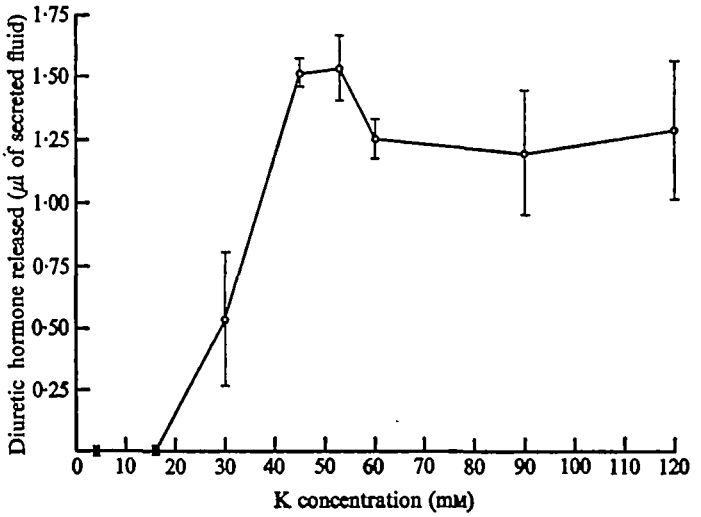

Fig. 2. The effect of the K concentration of the bathing solution on the quantity of diuretic hormone released from the mesothoracic ganglionic mass and abdominal nerves of *Rhodnius*. Each point on the graph is the mean of several determinations and the length of the vertical lines attached to each point indicates the extent of the standard error of the mean. The Ca concentration of the bathing solution was held constant at 2 mM.

respective diuretic hormones but promptly start to secrete at a high rate when they come into contact with these hormones.

RESULTS

A. *Experiments on* Rhodnius prolixus

The diuretic hormone of *Rhodnius* is secreted by neurosecretory cell bodies which lie in the posterior portion of the mesothoracic ganglionic mass; the cell bodies can be removed and extracts of them prove to contain large amounts of diuretic activity (Maddrell, 1963). Axons of these cells run forwards in the ganglionic mass and, after giving off a collateral branch which enters the neuropile, they turn and run back out of the mass in the peripheral abdominal nerves (Maddrell, 1966). In these nerves the axons branch and terminate in a large number of bulbous endings situated outside the perineurium and separated from the haemolymph only by the acellular neural lamella. In freshly fed insects these endings show ultrastructural evidence of hormone release; ligature experiments also point to them as the site of diuretic hormone release (Maddrell, 1966). Fig. 1 summarizes these findings in diagrammatic form. In the experiments now to be described, the mesothoracic ganglionic mass of *Rhodnius* together with lengths of the abdominal nerves still attached has been treated with a variety of solutions to establish the ionic requirements for hormone release.

Diuretic hormone release induced by elevated potassium concentrations

In these experiments isolated thoracic ventral nerve cords of *Rhodnius* were bathed in 5 μl drops containing various K concentrations and were left for 1 h. The ganglia and their attached nerves were then removed, the drops made up to 100 μl w

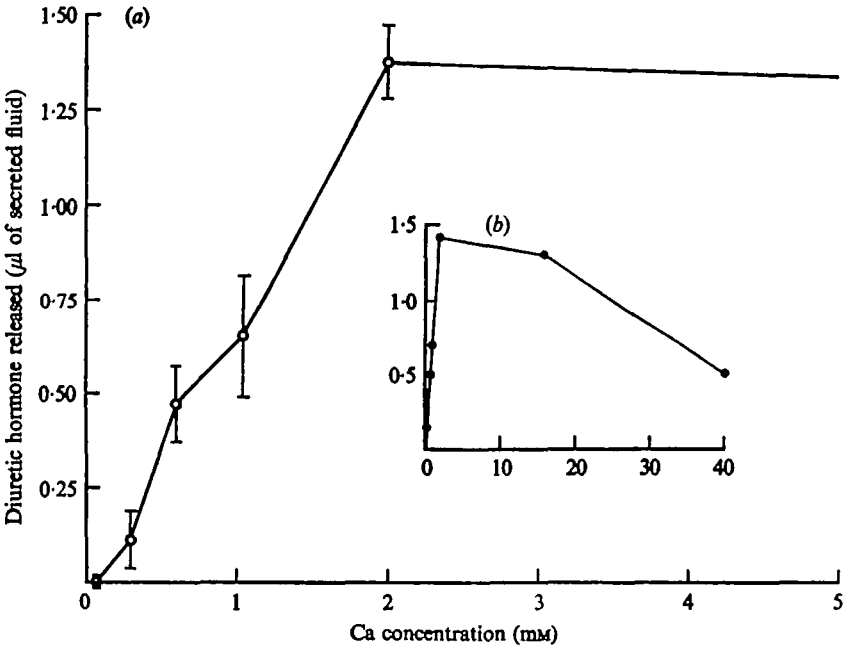

Fig. 3. The quantities of diuretic hormone released by treating the mesothoracic ganglionic mass and abdominal nerves of *Rhodnius* with solutions containing 70 mM-K and different concentrations of Ca. (*a*) Ca concentrations 0–5 mM, and (*b*) Ca concentrations 0–40 mM.

standard Ringer and a Malpighian tubule isolated into each. The volume of fluid secreted by the tubule during the next hour was taken as a measure of the diuretic activity in the drop of solution. The results obtained at differing K concentrations are shown in Fig. 2. Clearly, potassium concentrations of more than 45 mM lead to sufficient release of diuretic hormone into 100 μl to cause a Malpighian tubule to secrete at its maximum rate for 1 h. Treatment with 30 mM-K caused a reduced release of hormone, while 16 mM-K caused no measurable release of hormone.

Effects of Ca on K-induced diuretic hormone release

In the experiments described above, the Ca concentration was held constant at 2 mM. Since the release of neurohormones and neurotransmitters in other systems is known to depend on Ca^{2+} ions (Douglas & Poisner, 1964; Berlind & Cooke, 1968, 1971) it was of interest to test the effects of altering the Ca concentration on diuretic hormone release induced by high potassium concentrations. For these experiments the K concentration was held at 70 mM and the Ca concentration varied. As before, the pro- and mesothoracic ganglia together with the proximal abdominal nerves were isolated into a 5 μl drop of the test solution and after 1 h the nervous tissue was removed, the drop made up to 100 μl with standard Ringer and a Malpighian tubule introduced into the drop to gauge its diuretic hormone content. The results are set out in Fig. 3 from which it emerges that no measurable hormone release occurs in Ca-free conditions, that as little as 0·25 mM-Ca allows some hormone release, but that 2 mM-Ca is required for a maximum release. At very high Ca levels there is some indication that hormone release is somewhat reduced.

Effects of Mn^{2+} ions on K-induced diuretic hormone release

In recent work on other systems, it has been possible to block Ca entry using Mn^{2+} ions (see, for example, Baker Meves & Ridgway, 1973*b*; Dreifuss *et al.* 1973); it is therefore interesting to test the effects of such agents on diuretic hormone release to see if a similar inhibition occurs. These experiments were complicated by the fact that Mn^{2+} ions at concentrations higher than about 3 mM adversely affect the secretory performance of Malpighian tubules. In an attempt to overcome this difficulty, ganglia isolated as before were immersed for 2 min in small drops of Mn-containing, K-rich solutions. The drops were then diluted with Mn-free solution to bring the final Mn concentration down to 2·5 mM. In control experiments, ganglia were immersed for 2 min in drops of a Mn-free, K-rich solution. These drops were then diluted with a suitable quantity of Mn-containing solution, again to give a final Mn concentration of 2·5 mM. As before, a Malpighian tubule was placed in each drop and its subsequent secretion followed as a measure of the diuretic activity of the drop. The results of these experiments showed that, at 7 mM-Mn, hormone release was apparently little affected, though because the presence of Mn in the test drop makes the response of Malpighian tubules to diuretic hormone less consistent it is only possible to conclude that 7 mM-Mn has no large effect on hormone release. At 20 mM-Mn there was a more marked effect (in 50% of cases, there was no detectable hormone release). At 50 mM-Mn the effect was considerable; tubules placed in 40 μl. drops secreted only 94 ± 59 nl (mean $\pm$ S.E.) in 1 h compared with 757 ± 237 nl secreted by tubules in 1 h in control experiments. Clearly Mn^{2+} ions can depress the release of diuretic hormone from the mesothoracic ganglionic mass and its abdominal nerves, so that it seems very probable that Ca entry into the axon endings is required for diuretic hormone release in *Rhodnius*.

Time course of induced release of diuretic hormone

To gain some impression of how quickly the diuretic hormone can be released from the nervous system, ganglia isolated as before were immersed for periods ranging from 3 sec to 1 min in 10 μl drops of a solution containing 70 mM-K and 2 mM-Ca. The drops were then assayed for diuretic activity by introducing a Malpighian tubule into each and observing the quantity of fluid secreted by the tubules. The results are shown in Fig. 4; they show that considerable amounts of hormone are released in response to an exposure to 70 mM-K of as little as 10 sec.

By immersing a single ganglion for short periods into successive drops of K-rich solution in turn it becomes clear that while there is an almost immediate and large release of hormone into the first one or two drops, succeeding immersions have a good deal smaller effect (Fig. 5). It appears that release occurs in two phases; an early fast phase of short duration which rapidly gives way to a more prolonged phase of release going on at a slower rate.

By observing how long it takes to cause measurable releases of diuretic hormone into considerably larger drops of solution, one can follow the rate of release during more prolonged treatments with K-rich solutions. Fig. 6 sets out the results of a series of such experiments and show how the rate of release gradually slows down: at 0·1 min, hormone is released at a rate sufficient, if it were maintained, to 'satura-'

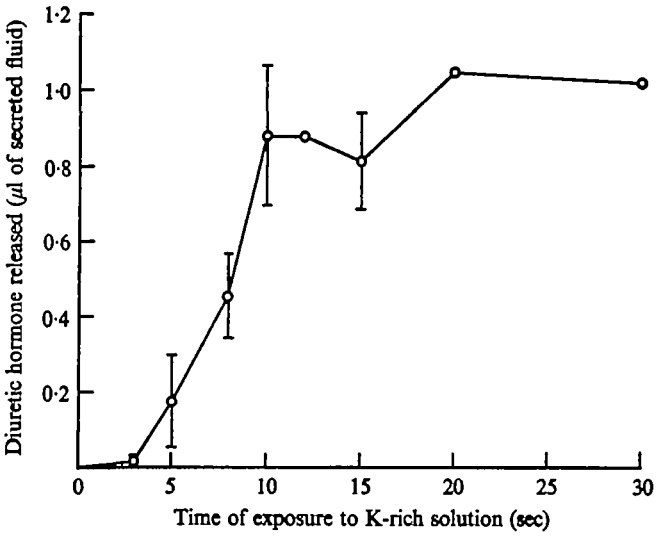

Fig. 4. The quantities of diuretic hormone released from release sites in *Rhodnius* in response to exposures of different lengths of time to 5 μl drops of a solution containing 70 mM-K and 2 mM-Ca. Vertical lines attached to a point represent the extent of the standard error of the mean of several determinations.

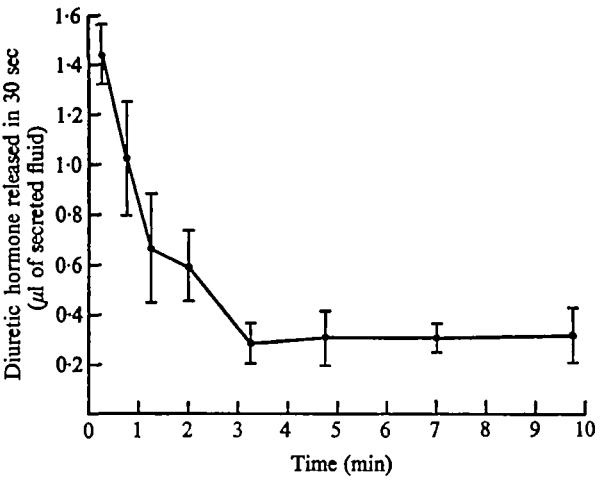

Fig. 5. The quantities of diuretic hormone released in 30 sec periods from the mesothoracic ganglionic mass and abdominal nerves of *Rhodnius* during continuous treatment with a solution containing 70 mM-K and 2 mM-Ca. Each point on the graph is the mean of several determinations and the vertical lines attached to the points indicate the extent of the standard error of the mean.

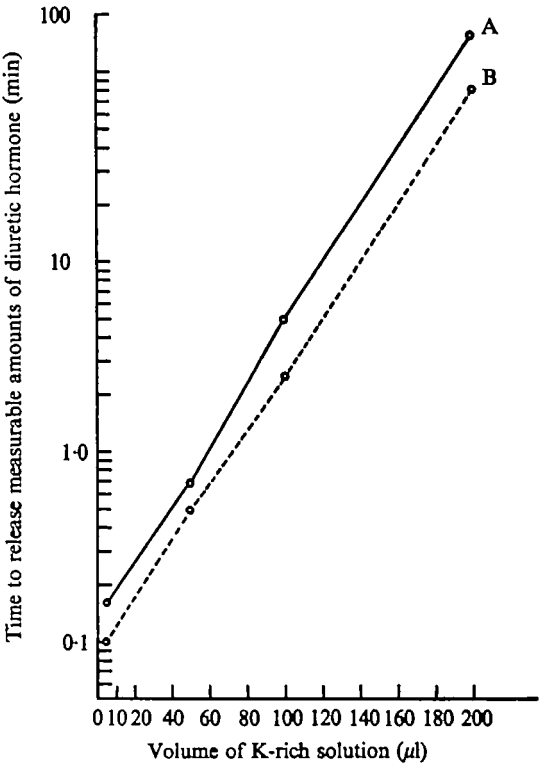

Fig. 6. The effect of the volume of a drop of K-rich solution bathing a mesothoracic ganglionic mass and abdominal nerves of *Rhodnius* on the time taken to release sufficient diuretic hormone to elicit (A) the maximum rate of secretion, or (B) half the maximal rate of secretion of a Malpighian tubule subsequently placed in the drop.

about 170 μl of solution in a minute; at 1 min the rate is enough to saturate 32 μl of solution in a minute, while at 10 and 100 min the rate of release has fallen to one which would in a minute saturate only 3 and 0·3 μl respectively.

The use of K-rich solutions to locate sites of hormone release

There is good evidence in *Rhodnius* that the natural release of the diuretic hormone occurs from neurohaemal areas situated on the surfaces of the proximal lengths of the peripheral abdominal nerves (Maddrell, 1966). It is of interest to see if hormone release induced by K-rich solutions also goes on at these sites. To test this, mesothoracic ganglionic masses were isolated, the abdominal nerves cut off and the masses then treated with K-rich solutions. No detectable hormone release occurred although, of course, these ganglionic masses contain the actual neurosecretory cells responsible for the synthesis of the diuretic hormone.

In a parallel series of experiments, decapitated insects were dissected open and all the central nervous system removed, together with as much as possible of the peripheral nervous system leaving only the peripheral abdominal nerves. Immersing these carcasses in K-rich solutions caused a prompt appearance in these solutions of large amounts of diuretic hormone. Similar experiments with insects in which only t

peripheral abdominal nerves were removed, failed to demonstrate measurable releases of diuretic hormone.

These experiments demonstrate that K-rich solutions will only cause diuretic hormone release in *Rhodnius* from the neurohaemal areas for this hormone, suggesting that this technique might be used in the location of neurohaemal areas for other hormones.

The experiments described have shown that K depolarization of intact ganglionic masses will not cause release of diuretic hormone unless the neurohaemal areas are attached. If, however, a cut is made through the perineurium so that K-rich solution can penetrate into the interior of the ganglionic mass, large amounts of hormone are released. This will occur even in masses lacking the peripheral nerves containing the neurohaemal areas for diuretic hormone release. It seems likely that release in this case must come from the cell bodies themselves.

Effects of tetrodotoxin on diuretic hormone release

The results of the experiments so far described on undamaged parts of the nervous system show that treatment with K-rich solutions causes a diuretic hormone release which is dependent on the entry of Ca^{2+} ions into the nervous system. Before one can assume that these events occur at the neurosecretory axon endings, one must exclude the possibility that other axons are depolarized by the elevated K concentrations and they in turn stimulate the neurosecretory axons to release the diuretic hormone. This is most easily tested by treatment with tetrodotoxin (TTX) which blocks action potentials and so effectively isolates the neurosecretory axon endings from any such processes. A series of experiments in which ganglia, isolated as before, were treated with K-rich solutions containing TTX at a concentration of 3×10^{-5} M showed that undiminished release of diuretic hormone occurred. It is very likely then that the events described do occur at the neurosecretory axon endings and are not initiated elsewhere in the nervous system.

B. *Experiments on* Glossina austeni

A preliminary survey of tissues of *Glossina* by bio-assay for diuretic hormone located diuretic activity in homogenates of the brain, the thoracic ganglion and in nerves which pass out from the latter into the abdomen (as shown in Fig. 7). Activity was also found in samples of abdominal fat body but all other tissues were inactive. The technique outlined on p. 162 was used to demonstrate which of these regions was the actual site of hormone release.

The use of K-rich solutions to locate sites of hormone release

Since isolated Malpighian tubules of *Glossina* are not able to maintain rapid secretion in solutions of very high K and correspondingly low Na concentrations, a Ringer with a K concentration of 40 mM was chosen to attempt to promote diuretic hormone release and thereby locate the neurohaemal area.

Hormone release could not be detected when isolated thoracic ganglia together with attached preabdominal nerve trunks but without the fine abdominal nerve branches were placed in 10 μl drops of K-rich Ringer's solution bathing isolated Malpighian

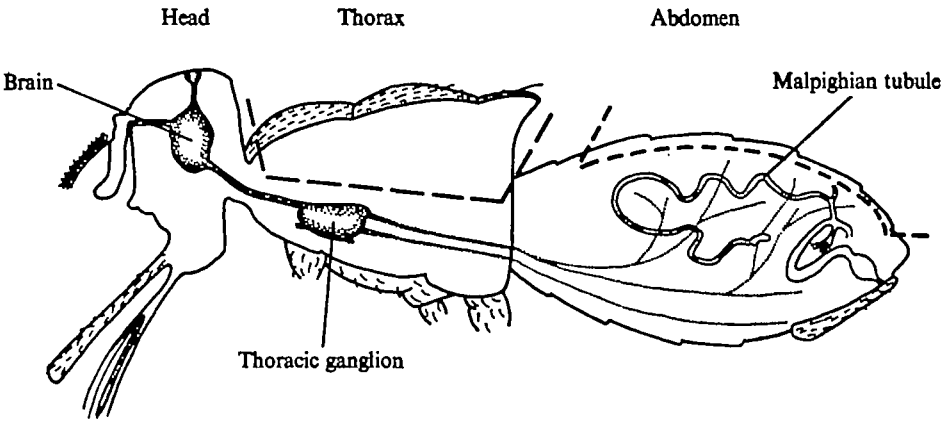

Fig. 7. Diagrammatic view of a sagittal section through *Glossina austeni* to show the thoracic ganglion and the nerves which run from it into the abdomen and ramify in the neighbourhood of the Malpighian tubules. Also indicated (heavy dashed lines) are the lines of cuts made to open the thorax and abdomen from the dorsal side.

tubules. Similarly, diuretic hormone release did not occur when 10 μl drops of K-rich solution were applied to the ganglion *in situ* under liquid paraffin (to allow the solution access to the ganglion, the dorsal wall of the thorax and much of the flight muscle was first removed as shown in Fig. 7). However, an assay of K-rich solution which had been circulated over the abdominal tissues under liquid paraffin proved positive. This result suggests that the neurohaemal area for diuretic hormone in *Glossina* is located within the abdomen.

To follow this up, ultrastructural studies were made of samples of abdominal tissue. Electron micrographs of these regions, for example Fig. 9, show bundles of fine nerves containing the membrane-bound vesicles characteristic of neurosecretory axons, ramifying throughout the fat body and often occurring in close proximity to the Malpighian tubules. The axons are usually enveloped by glial sheaths but in places they emerge and the axonal membranes are then separated from the surrounding haemolymph only by the basement membrane. At such places release of the contents of the membrane-bounded vesicles into the haemolymph could occur. Though uch axons may carry hormones other than diuretic hormone, these ultrastructural studies demonstrate the presence of neurosecretory axons in areas which the depolarization studies show are capable of releasing diuretic hormone.

Unfortunately, the rate of secretion of isolated tubules in K-rich solutions into which diuretic hormone has been released is not as stable in *Glossina* as in *Rhodnius*. The rate tends to reach a peak and subsequently decline with a variable time course. Examples are shown in Fig. 8. This makes a quantitative analysis of the effectiveness of any solution in promoting hormone release rather more difficult in *Glossina* than in *Rhodnius*. However, the distinction between those solutions into which hormone had been released and those into which it had not was very clear and within the former category a gradation of effectiveness could be established.

The apparently transient nature of the response of the isolated tubules to diuretic hormone is probably due to the release of a breakdown enzyme together with the hormone. This enzyme, which *in vivo* probably regulates the hormone titre of

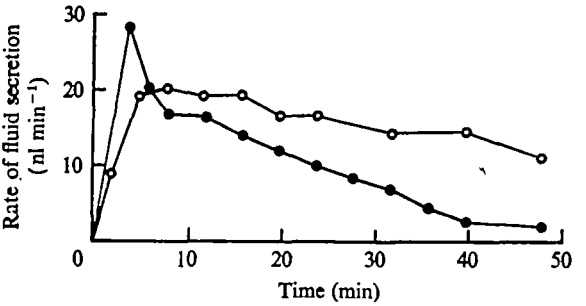

Fig. 8. Rates of fluid secretion by two Malpighian tubules of *Glossina*, each isolated into a drop of K-rich solution previously in contact for 1 min with the neurohaemal area for the diuretic hormone.

haemolymph by balancing breakdown of hormone with its release, appears to be present in each part of the nervous system of *Glossina* where diuretic activity has been demonstrated by homogenization experiments. It is also present in the haemolymph and so the abdominal tissues must be washed with a Ringer's solution of low K concentration to remove this enzyme before hormone release is stimulated by K-rich solutions.

Effects of varying K and Ca concentrations on diuretic hormone release

In these experiments the dorsal wall of the abdomen was removed under liquid paraffin as shown in Fig. 7. The tissues were washed with a solution of low K concentration and the test solution was then applied as a 10 μl drop which was circulated through the abdomen for 1 min before being assayed for diuretic hormone.

The results obtained at differing K concentrations but with the Ca concentration held constant at 2 mM are shown in Table 1*a*. Potassium concentrations above 40 mM were equally effective at causing hormone release. A reduced amount of release occurred at 30 and 25 mM but concentrations below this caused no detectable release of diuretic hormone.

With a test solution containing 40 mM-K and using the same experimental technique, a second series of experiments was performed in which the Ca concentration of the test solution was varied. Maximal release of diuretic hormone took place into solutions containing 2 and 4 mM-Ca. Reduced release occurred in 1·5 mM-Ca but no detectable release took place in 1 mM-Ca. These results are summarized in Table 1*b*.

Owing to the release of breakdown enzyme, the period of contact of the test solution with the neurohaemal area was only 1 min in *Glossina* compared to 1 h in *Rhodnius*. Release of hormone can therefore only be detected if sufficient Ca is present for rapid release of hormone, whereas in *Rhodnius* slow release at low Ca concentrations can also be detected.

The experimental technique used to obtain these results differed in one major respect from that used in the experiments on *Rhodnius*. In the latter case the 5 μl drop of test solution was made up to 100 μl with standard Ringer's solution before introducing a Malpighian tubule, thereby approximately standardizing the composition of the medium bathing the tubules during assay for diuretic hormone. In the present experiments on *Glossina* the 10 μl drop of test solution was transferred directly to

Table 1. *The effectiveness of various solutions in promoting release of diuretic hormone from the neurohaemal area of* Glossina austeni

(*a*) Effects of different K concentrations

Concentration of Ca (mM) ...	2					
Concentration of K (mM)	10	20	25	30	40	60
Release of diuretic hormone	−	−	+	++	+++	+++

(*b*) Effects of different Ca concentrations

Concentration of K (mM) ...	40			
Concentration of Ca (mM)	1	1·5	2	4
Release of diuretic hormone	−	++	+++	+++

The amount of diuretic hormone released into a 10 μl drop in 1 min is indicated as follows: +++, maximum release; ++, reduced release; +, very reduced release; −, no release.

Table 2. *The effect of varying the potassium and calcium concentration of the bathing medium on the response of isolated Malpighian tubules of* Glossina *to diuretic hormone*

Concentration of K^+ (mM) ...	40	40	20
Concentration of Ca^{2+} (mM) ...	1	2	2
Release of diuretic hormone ...	−	+++	−
Threshold concentration of diuretic hormone for tubule response (thoracic ganglia/100 μl)	0·5	0·6	0·5
Concentration of diuretic hormone for 50% maximum tubule response (thoracic ganglia/100 μl)	1·0	1·0	1·25

an isolated tubule. It is therefore possible that the effects observed in the results are due more to altering sensitivity of the assay system as the ionic concentration of the medium bathing the tubules changes, rather than to the altering effectiveness of the test solutions in promoting hormone release. To distinguish between these two alternatives, concentrated homogenates of the thoracic ganglion were produced in differing Ringer's solutions, boiled to denature the breakdown enzyme, diluted in their respective Ringer's solutions and applied to isolated tubules. The results of this experiment are shown in Table 2.

The sensitivity of the tubules to the hormone does not appear to alter as the composition of the Ringer's solution is changed.

The time course of induced release of diuretic hormone

As with the release of diuretic hormone into solutions of elevated K concentration in *Rhodnius*, the diuretic hormone of *Glossina* could be detected in drops of K-rich solutions which had bathed the neurohaemal area only for very short periods of time. 10 μl drops of Ringer's solution with a K concentration of 40 mM and a Ca concentration of 2 mM were irrigated through the exposed abdominal tissues under liquid paraffin for varying periods of time and then assayed for hormone. Hormone could be detected in drops that had been in contact with these tissues for 10 sec, 30 sec, 1 [illegible]

[redacted] 3 min. For drops in contact for 10 min and 25 min the assay was negative, presumably the breakdown enzyme released initially with the hormone having by then reduced the hormone concentration in the irrigating drop below the threshold concentration for tubule response.

By monitoring the amounts of hormone released in the first and second minutes of treatment with K-rich solution it was possible to demonstrate that in *Glossina* the initially high rate of hormone release declines rapidly during depolarization. A 10 μl drop of K-rich solution was applied to the neurohaemal area for 1 min and then replaced by a similar drop for a further minute. The drops were assayed separately for diuretic hormone. Hormone could only be detected in the first drop, suggesting that the rate of hormone release declined during the first minute of depolarization to a rate insufficient to produce a detectable concentration of hormone in the second drop.

Effect of Mn on K-induced diuretic hormone release

10 μl drops of Ringer containing 4 mM-Mn in addition to 40 mM-K and 2 mM-Ca were irrigated through the abdominal tissues under liquid paraffin for 1 min. No diuretic hormone could be detected in such drops and increasing the irrigation time to 3 min did not alter the result. On checking whether these results were due to a decreased sensitivity of the assay system in the presence of 4 mM-Mn, it was found that the threshold for tubule response to thoracic ganglion homogenate in such a Ringer's solution was indeed increased. This effect could account for the apparent absence of release of diuretic hormone. To demonstrate that Mn^{2+} ions do inhibit hormone release a different technique was adopted in which the abdominal tissues were bathed with 4 mM-Mn, K-rich Ringer's solution for 1 min before applying a second 10 μl drop of K-rich solution containing no Mn. Under these conditions, hormone could be detected only in the second drop. This result is the reverse of that previously obtained when Mn was not present in the first drop. 4 mM-Mn must have prevented hormone release during the first minute of depolarization or have decreased its rate to such an extent that rapid release could still occur during a second minute of depolarization by a K-rich solution. In *Glossina*, as in *Rhodnius*, it appears that Ca must enter the axon endings for diuretic hormone to be released.

Effect of tetrodotoxin on K-induced diuretic hormone release

Using the standard technique for inducing hormone diuretic release in *Glossina*, a 10^{-5} M solution of tetrodotoxin in K-rich Ringer's solution caused release of diuretic hormone in a manner indistinguishable from that observed when a K-rich solution without tetrodotoxin was used. It would appear that the induced release of diuretic hormone by solutions of elevated K concentration in *Glossina* is caused by the depolarization of the neurosecretory axon endings and not by nerve impulses originating from a region elsewhere in the nervous system where axons may have been depolarized by the K-rich solution.

DISCUSSION

Comparison with other systems and other animals

The results of these experiments on the ion requirements for release of the diuretic hormones of *Rhodnius* and *Glossina* accord with similar experiments on other systems in other animals. For example, work on the isolated neurohypophysis of a vertebrate has shown that hormone release depends on calcium entry following depolarization of the axon endings. Release can be electrically stimulated, in which case TTX blocks it, or it can be evoked by K-rich solutions when TTX has no effect (Dreifuss *et al.* 1971). Treatment with Co^{2+} or Mn^{2+} ions at 1 mM causes a 50% decrease in both Ca entry and hormone release (Dreifuss *et al.* 1973). Berlind & Cooke (1968, 1971) in experiments on the cardio-acceleratory hormone of the pericardial organs in the crab showed that Ca^{2+} ions were required for release evoked by electrical stimulation and that TTX blocks such release. They did not test the effects of K-rich solutions.

Until now there has, however, been no comparable work on insects. Gersch, Richter, Böhm & Stürzebecher (1970) claimed to have shown release of hormone from the corpora cardiaca of *Periplaneta americana* after treating them with solutions of elevated K concentrations. But, since they could get release into a solution containing K at the concentration at which it occurs in the haemolymph, the hormone release they obtained was not caused by K depolarization. Better, albeit indirect, evidence of K-induced hormone release comes from the work of Gosbee *et al.* (1968), who showed a depletion of paraldehyde-fuchsin positive material from the corpora cardiaca of *Periplaneta americana* after treatment with a solution containing 134 mM-K, and from the work of Normann (1970) who found abundant ultrastructural evidence of neurohormone release in corpora cardiaca of *Calliphora erythrocephala* treated with a solution containing 100 mM-K.

Time course of hormone release

Under continued depolarization with a K-rich solution, the release of diuretic hormone from its neurohaemal area is initially at an extraordinarily high rate before it falls away (see pp. 160 and 166). The initial rate is so high that, in *Rhodnius*, as much hormone as the insect can normally release in 3 h is produced within 30 sec. This can probably be explained by the continuous nature of the depolarization caused by treatment with K-rich solutions. If, as is likely, natural hormone release results from the depolarization associated with action potentials, then only during the relatively small fraction of the time when the membrane of the axon is actually depolarized will release occur and so the rate of release will be much slower than during a maintained depolarization.

As a very large release of hormone can be evoked in such a short period of time, it seems likely that a great deal of neurosecretory material (at least sufficient for the whole period of diuresis) must be stored within the axon endings. Treatment for 30 sec for example, would probably not give time for significant amounts of the neurosecretory material in the axons to move into the endings. It is no surprise, then, that after a natural release of the diuretic hormone in the 3 h after a meal, the mesothoracic ganglionic mass contains just as much extractable diuretic activity as it does bef[illegible]

diuresis (S. H. P. Maddrell, unpublished observations); the natural release can be achieved purely from neurohormone stocks already in the neurohaemal areas.

The rapid decline in hormone release during maintained depolarization has striking parallels with the decline in Ca entry during similar maintained depolarizations of squid giant axons (Baker *et al.* 1973*a*). Because diuretic hormone release fails in the absence of Ca and is blocked by Mn, it is very likely that release of diuretic hormone requires calcium entry. It is probable, then, that the fall-off in release during K depolarization is caused by the progressive failure of Ca entry. However, one cannot exclude the possibility that the decline in hormone release is due to a depletion of the stores of hormone in the axon endings. Returning a ganglion and attached nerves of *Rhodnius* from a K-rich solution to a solution containing 8·6 mM-K for a few minutes causes a recovery of the ability to release diuretic hormone at a high rate on subsequent retreatment with a K-rich solution and this, too, is very similar to the recovery of Ca entry rate in squid giant axons. However, a rest period may well have allowed replenishment of the hormone stores in the axon endings, so that one still cannot conclude that the decline in rate of hormone release is caused by a reduction in Ca entry, though this is the most attractive explanation of the facts.

K depolarization as a means of locating neurohaemal areas

Treatment of different areas of the intact nervous systems of *Rhodnius* and *Glossina* with K-rich solutions only causes release of diuretic hormone from the respective neurohaemal areas. That this should be the case is not at all surprising because it is only neurosecretory axon endings in neurohaemal areas which are not protected by the perineurium (see Fig. 1, for example). Treatment with K-rich solutions would be expected to depolarize neurones beneath the perineurium only very slowly indeed (cf. Treherne & Pichon, 1972). Unprotected neurosecretory axon endings, however, would be depolarized almost instantly and, as we have seen, hormone release occurs within seconds. Provided treatment is not prolonged, and since most hormone release occurs in the first minute or so there is no reason to prolong it, the use of K-rich solutions is a most useful probe technique for locating neurohaemal area or organs.

An example of a particular controversy which might be resolved using this technique is whether the heart accelerating factor of insects is released into circulation from the corpora cardiaca, or from the lateral cardiac nerves which run from the corpora cardiaca to the heart, or is not released into circulation at all but acts as a neurotransmitter at nerve endings on the muscles of the heart. In the cockroach, *Periplaneta americana*, both the corpora cardiaca and the lateral cardiac nerves contain comparable amounts of an extractable factor which will stimulate the rate of beating of isolated cockroach hearts (Johnson & Bowers, 1963), and both sites contain ultrastructural evidence of release of neurosecretory material (Miller & Thomson, 1968). On this evidence it is not clear if the heart stimulant is released into circulation as a hormone and if it is, which site is its release area. K depolarization separately of the two areas followed by bio-assay on an isolated heart should clearly show whether either area is the release site for a cardioacceleratory hormone.

K depolarization as a means of investigating ultrastructural events accompanying hormone release

One of the problems of investigating the ultrastructural events accompanying hormone release is that natural release occurs relatively slowly. As a result ultrastructural evidence of hormone release is encountered only relatively rarely in an examination of a neurohaemal area or organ. K depolarization (due to its enormous accelerating effect on hormone release) should provide abundant instances of release events in such a system. Indeed, Normann (1970) found that treatment with a K-rich solution greatly increased the numbers of exocytotic profiles visible in the corpora cardiaca of *Calliphora erythrocephala*. However, he exposed these corpora cardiaca for 2 min (to a solution containing 5 mM-Ca and 100 mM-K); the results of the present experiments suggest that a much shorter pretreatment, of only a few seconds duration, with a K-rich solution before fixation would provide the greatest concentration of release events in the fixed tissue.

If this turns out to be the case then this technique would be valuable for pretreating such tissues as the corpora cardiaca of cockroaches, where the omega-shaped exocytotic profiles so characteristic of hormone release in other cases are encountered so rarely that it is reasonably held that hormone release may occur by some other method (Scharrer, 1968).

K depolarization as a means of obtaining 'clean' samples of hormones

Because it is only neurohaemal areas which are affected by short treatments with K-rich solutions, only neurohormones are released into such solutions. Moreover, since the events at release, although they are greatly speeded up, are probably more or less the same as they are in a natural release, the neurohormones obtained are likely to be in the form in which they are circulated (i.e. not in a bound or stored form). On both counts, samples of neurohormone produced by K depolarization are 'cleaner' than samples obtained, say by homogenizing particular parts of the central nervous system. One can be reasonably certain, for example, that any physiological effects possessed by such an active solution are attributable to hormones in it rather than to substances which normally are never found in circulation.

It is possible too, that the technique may find a use in the isolation and purification of particular hormones – again because the hormone samples resulting from K depolarization are relatively pure and most likely in the form in which they occur in circulation. The one drawback is that the yield of hormone is smaller than that from homogenizing pieces of the central nervous system. In *Rhodnius*, for example, 5 % of the total extractable hormone is released by 5 min of K treatment but only 10 % after 100 min treatment. It is possible that a series of very short exposures to K-rich solutions interspersed with somewhat longer recovery periods in solutions of normal K concentration might materially increase the yield, but this awaits further investigation.

We would like to thank Drs J. E. Treherne, R. J. Aston and A. F. White for their helpful advice in the early stages of this work, B. O. C. Gardiner for technical assistance and the Tsetse Research Laboratory, Langford for a supply of tsetse fly pupae.


AM
L
PN
H
BL
NL

J.D.G. was in receipt of a Research Studentship awarded by the Science Research Council and wishes to thank Dr M. J. Berridge for supervision of his research.

REFERENCES

BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973*a*). Calcium entry in response to maintained depolarization of squid axons. *J. Physiol.* **231**, 527–48.

BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973*b*). Effects of manganese and other agents on the calcium uptake that follows depolarization of squid axons. *J. Physiol.* **231**, 511–26.

BERLIND, A. & COOKE, I. M. (1968). Effect of calcium omission on neurosecretion and electrical activity of crab pericardial organs. *Gen. comp. Endocr.* **11**, 458–63.

BERLIND, A. & COOKE, I. M. (1971). The role of divalent cations in electrically elicited release of a neurohormone from crab pericardial organs. *Gen. comp. Endocr.* **17**, 60–72.

DOUGLAS, W. W. & POISNER, A. M. (1964). Stimulus-secretion coupling in a neurosecretory organ: the role of calcium in the release of vasopressin from the neurohypophysis. *J. Physiol.* **172**, 1–18.

DREIFUSS, J. J., GRAU, J. D. & NORDMANN, J. J. (1971). Release processes for neurohormones and the secretion of neurotransmitters. *Experientia* **27**, 1110.

DREIFUSS, J. J., GRAU, J. D. & NORDMANN, J. J. (1973). Effects on the isolated neurohypophysis of agents which affect the membrane permeability to calcium. *J. Physiol.* **231**, 96–8*P*.

GERSCH, M., RICHTER, K., BÖHM, G.-A. & STÜRZEBECHER, J. (1970). Selektive Ausschüttung von Neurohormonen nach elektrischer Reizing der Corpora Cardiaca von *Periplaneta americana in vitro*. *J. Insect Physiol.* **16**, 1991–2013.

GOSBEE, J. L., MILLIGAN, J. V. & SMALLMAN, B. N. (1968). Neural properties of the protocerebral neurosecretory cells of the adult cockroach, *Periplaneta americana*. *J. Insect Physiol.* **14**, 1785–92.

JOHNSON, B. & BOWERS, B. (1963). Transport of neurohormones from the corpora cardiaca in insects. *Science, N.Y.* **141**, 264–6.

MADDRELL, S. H. P. (1963). Excretion in the blood-sucking bug, *Rhodnius prolixus* Stal. I. The control of diuresis. *J. exp. Biol.* **40**, 347–56.

MADDRELL, S. H. P. (1966). The site of release of the diuretic hormone in *Rhodnius* – a new neurohaemal system in insects. *J. exp. Biol.* **45**, 499–508.

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

MILLER, T. & THOMSON, W. W. (1968). Ultrastructure of cockroach cardiac innervation. *J. Insect Physiol.* **14**, 1098–104.

NORMANN, T. C. (1970). The mechanism of hormone release from neurosecretory axon endings in the insect *Calliphora erythrocephala*. In *Aspects of Neuroendocrinology* (ed. W. Bargmann and B. Scharrer), pp. 30–42. Berlin, Heidelberg and New York: Springer-Verlag.

SCHARRER, B. (1968). Neurosecretion. XIV. Ultrastructural study of sites of release of neurosecretory material in Blattarian insects. *Z. Zellforsch. mikrosk. Anat.* **89**, 1–16.

TREHERNE, J. E. & PICHON, Y. (1972). The insect blood-brain barrier. *Adv. Insect Physiol.* **9**, 257–313.

UTTENTHAL, L. O., LIVETT, B. G. & HOPE, D. B. (1971). Release of neurophysin together with vasopressin by a Ca^{2+} dependent mechanism. *Phil. Trans. R. Soc.* B **261**, 379–80.

EXPLANATION OF PLATE

Fig. 9. Section of a fine branch of an abdominal nerve running close to a Malpighian tubule of *Glossina austeni*. Note the neurosecretory axon endings (•) which contain neurosecretory vesicles and which lie outside the perineurium (PN) separated from the haemolymph (H) only by the acellular neural lamella (NL). The nerve lies close to the Malpighian tubule wall which has a structure typical of a fluid secreting epithelium with basal infoldings (BI) and, facing the lumen (L), apical microvilli (AM) containing long mitochondria. × 8000.