J. exp. Biol. (1975), 63, 391–401
With 5 figures
Printed in Great Britain

THE CONTROL OF DIURESIS IN THE TSETSE FLY *GLOSSINA AUSTENI:* A PRELIMINARY INVESTIGATION OF THE DIURETIC HORMONE

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(*Received* 24 *February* 1975)

SUMMARY

The rate of secretion of the Malpighian tubules of *Glossina austeni* is controlled by a diuretic hormone. This hormone is present in the nervous tissue of the fly together with a degradative enzyme that can be inactivated by boiling. It is demonstrated that the Malpighian tubules are able to destroy the diuretic hormone; they may therefore participate in the control of diuresis. The diuretic hormone appears to be a heat-stable, non-dialysable, alcohol-soluble molecule, containing amino acid, glucose and sialic acid residues.

INTRODUCTION

The ingestion of a blood meal by the tsetse fly *Glossina austeni* is followed by a period of rapid diuresis (Lester & Lloyd, 1928; Gee, 1975). The control of this process must be both rapid and precise for the first drops of urine appear even before the fly has finished feeding, and yet diuresis must end at the right moment or the very high rates of excretion would soon lead to desiccation and death. It has also been demonstrated that the extent of diuresis must be controlled to take account of the degree of dehydration of the fly before feeding and the size of the blood meal (Bursell, 1960).

The release of the diuretic hormone (DH) of *G. austeni* has been shown to occur at fine neurosecretory axon endings close to the Malpighian tubules (Maddrell & Gee, 1974). Such an arrangement is in keeping with the ability of *Glossina* rapidly to switch on diuresis in response to the ingestion of a blood meal. Experiments reported in the present communication demonstrate that the fly also possesses a mechanism for switching off diuresis after the removal of unwanted water from the blood meal and following the cessation of DH release.

The control of diuresis in insects has been investigated in several species and has been reviewed by Maddrell (1971). In some cases attempts have been made to purify and characterize DH – for example, in *Rhodnius prolixus* (Aston & White, 1974), in *Periplaneta americana* (Goldbard, Sauer & Mills, 1970) and in *Schistocerca gregaria* (Mordue & Goldsworthy, 1969). However, the precise character of any

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insect DH is as yet unknown. Preliminary studies of the properties and structure of the DH of *Glossina austeni* are reported here.

MATERIALS AND METHODS

Adult *Glossina austeni* Newstead were used throughout these investigations. The flies were reared by the method previously described (Gee, 1975). Isolated Malpighian tubules were set up as *in vitro* preparations using the technique described by Ramsay (1954).

The isolated tubule preparation was used as a bioassay for DH. The tubules secrete only very slowly when bathed in control saline solution but in the presence of DH they begin to secrete rapidly. Solutions were therefore assayed for DH by applying them to isolated tubules and observing any changes in the rate of secretion.

The bathing medium had the following composition (mM): NaCl 128·0, KCl 20·0, $NaH_2PO_4.2H_2O$ 5·0, $CaCl_2.6H_2O$ 2·0, $MgCl_2.6H_2O$ 2·0, glucose 10·0, Na glutamate 3·0, malic acid 2·75, citric acid 1·75. The pH was adjusted to 7 with NaOH.

Unless otherwise stated, the source of the DH in the extracts was the thoracic ganglion. This was homogenized in a small glass mortar with approximately 10 μl of bathing medium using a ground-glass pestle. The resulting brei was transferred to a larger volume of the bathing medium in a plastic centrifuge tube, to which was also added the solution used to wash the mortar. The tube was placed in a water bath at 100 °C for 2 min. The solution was then centrifuged at approximately 13500 *g* for 1 min using a Beckman 152 Microfuge, to remove tissue debris and proteins precipitated by boiling. The supernatant was decanted off to form the final hormone extract and this was stored at −15 °C.

Solutions to be dialysed were placed in narrow-bore (6 mm diam.) Visking dialysis tubing. Microdialysis was performed using an 'Electrothermal' ultra-micro dialysis cell which enabled the solutions on either side of the dialysis membrane to be assayed. All dialysis experiments were performed at 4 °C.

Trypsin was obtained from BDH Chemicals, Ltd. All other enzymes were obtained from Sigma Ltd.

RESULTS

When the Malpighian tubules of *G. austeni* were removed during rapid diuresis and set up in control saline their rate of secretion soon fell to a very low level. However, the tubules recommenced rapid secretion on the addition of an extract of thoracic ganglia to the bathing medium. It was apparent that the rate of secretion of the tubules was controlled by some exogenous factor, but no diuretic activity could be detected in haemolymph taken from flies during rapid diuresis. The small amount of haemolymph present (Tobe & Davey, 1972) necessitated collection from several flies in order to obtain a volume sufficient to bathe an isolated tubule. During collection, degradative enzymes in the haemolymph (Maddrell & Gee, 1974) may have inactivated any hormone present and oxidation of tyrosine may have made the haemolymph toxic to the tubules. The haemolymph was therefore unsatisfactory as a source of DH.

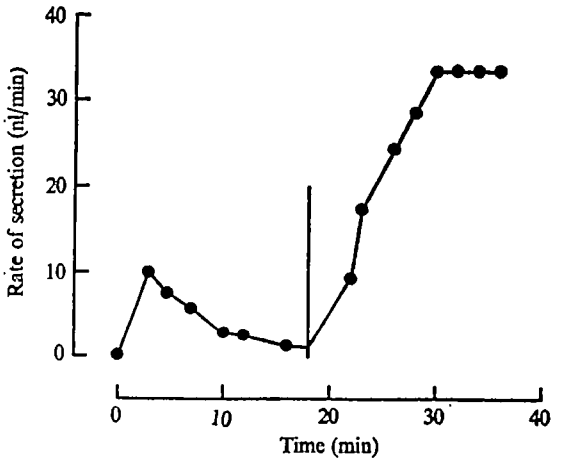

Fig. 1. The rate of secretion of an isolated Malpighian tubule of *G. austeni* bathed by an unboiled homogenate of thoracic ganglia (5/100 μl) and the effect of replacing this with a boiled homogenate (5 ganglia/100 μl) at the time indicated by the vertical line.

Table 1. *The effect of boiled tissue homogenates on the rate of secretion of isolated Malpighian tubules of* Glossina austeni

Organ or tissue	Organ or tissue concentration (per 100 μl bathing medium)	Rate of secretion (% maximum rate in 10^{-3} M cyclic AMP, average of 2 determinations)
Thoracic ganglion	4	100
Brain	5	89
Fat body (containing fine abdominal nerves)	From 2 insects	77·5
Preabdominal nerve-trunks	From 20 insects	86·5
Malpighian tubules	8	0
Gut	2	0
Cuticle (abdominal tergites)	From 2 insects	0
Flight muscle	From 2 insects	0

The nervous tissue of the fly proved a more satisfactory source of DH. Boiling the extracts was found to stabilize diuretic activity (Fig. 1). However, as the addition of a small amount of unboiled homogenate to a stable active extract soon caused the loss of all diuretic activity, it was concluded that boiling inactivated an enzyme which otherwise degraded the DH. Diuretic activity was found throughout the nervous system (Table 1), though release could be induced by K-rich solutions only at neurosecretory axon endings in the abdomen, and it was concluded that these were the neurohaemal area for DH in *Glossina* (Maddrell & Gee, 1974).

In other insects – for example, in *Rhodnius* (Maddrell, 1964*a*), *Dysdercus* (Berridge, 1966) and *Carausius* (Pilcher, 1970) – it has been demonstrated that the Malpighian tubules are able to destroy DH and thereby are able to control the level of DH in the haemolymph. To investigate the ability of the Malpighian tubules of *Glossina* to inactivate DH, intact tubules were incubated under liquid paraffin with an extract of thoracic ganglia. Each 25 μl of extract was incubated with 12 tubules. The solution was assayed for DH after 30 and 60 min. To show that any loss was not due to the inherent instability of the hormone or to its solution in liquid

Table 2. *The ability of intact Malpighian tubules of* Glossina austeni *to remove diuretic activity during incubation with thoracic ganglion extract*

Incubation	Time (min)	Loss of diuretic activity (% decrease in rate of secretion of a single tubule isolated into the solution before and after incubation)
12 tubules with 25 μl extract	30	22·3
12 tubules with 25 μl extract	60	66·8
25 μl extract (control)	60	7·0

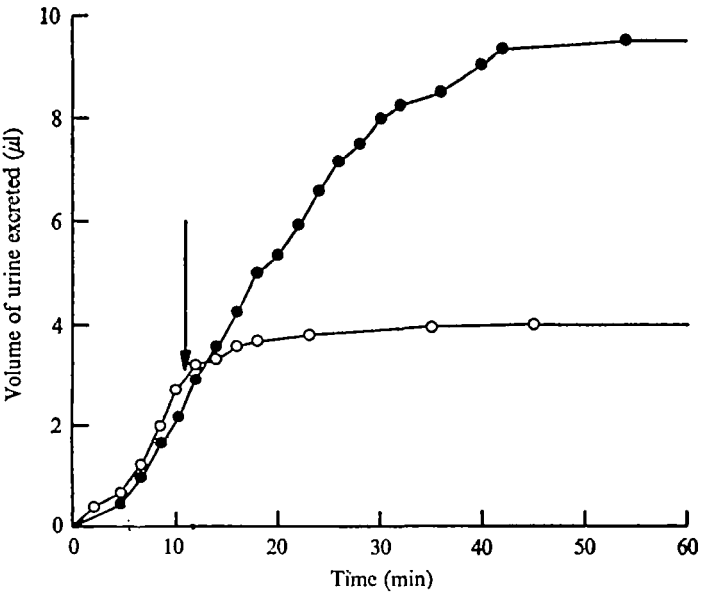

Fig. 2. A comparison of the volume of urine produced by a single *G. austeni* ligatured at the neck 10 min after taking a blood meal (○) with the volume produced by an unligatured fly (●) which had taken a similar meal. The arrow marks the time at which the ligature was applied.

paraffin, a control drop of extract was stored under liquid paraffin for the duration of the experiment and then assayed for DH. It is apparent from the results shown in Table 2 that the Malpighian tubules are capable of inactivating DH. The tsetse fly therefore has two methods of removing DH from the haemolymph – by the action of enzymes in the haemolymph itself and by the action of the target organs, the Malpighian tubules.

To demonstrate the removal of DH from the haemolymph *in vivo*, the release of hormone was halted during rapid diuresis by clamping a pair of fine forceps across the neck of the fly to form a tight ligature. Fig. 2 shows the effect of such a ligature – rapid excretion was soon halted. This contrasts with the situation in *Rhodnius* where excretion is unaffected by the complete removal of the head after feeding (Maddrell, 1963). An examination of the contents of the abdomen of the fly 35 min after th

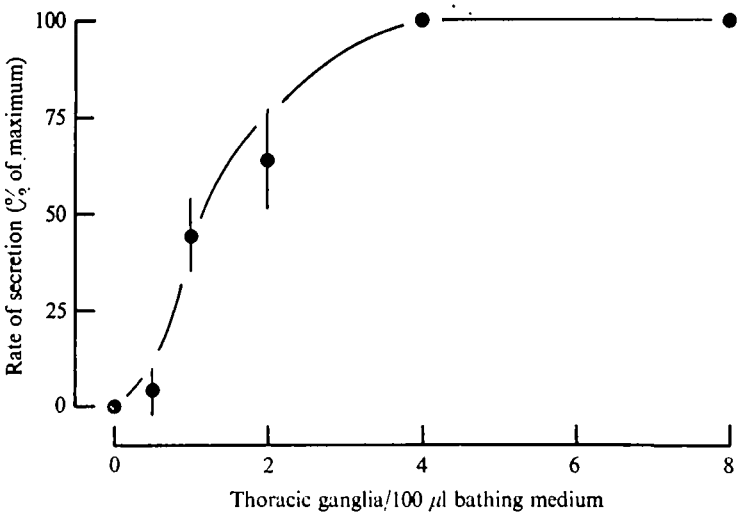

Fig. 3. Dose/response curve for the stimulation of secretion by the Malpighian tubules of *G. austeni* by homogenates of thoracic ganglia.

application of the ligature revealed a copious haemolymph and an absence of fluid in the rectum. It would appear that the ligature caused a build-up of haemolymph by preventing rapid secretion of the Malpighian tubules, for no fluid had collected in the rectum. This was most probably brought about by an inhibition of the release of DH. In the absence of DH release, any hormone remaining was rapidly inactivated (Fig. 2).

Properties of the diuretic hormone

Extraction of *Glossina* DH in a stable form requires the homogenate of nervous tissue to be boiled. A dose–response curve for the product of this normal extraction procedure is shown in Fig. 3. However, since stable extracts can only be produced by boiling, it is not possible to tell if hormonal activity unstable to boiling is lost during extraction. After extraction, the hormone is able to withstand boiling at 100 °C for 10 min and freezing at −15 °C for 3 months without further loss of activity. Activity is also preserved during evaporation to dryness of DH in Ringer's solution and its subsequent resuspension in distilled water. These properties are similar to those of the heart-accelerating, hyperglycaemic and diuretic factors extracted from the corpora cardiaca of cockroaches and locusts (Davey, 1961; Steele, 1961; Mordue & Goldsworthy, 1969). The DH of *Rhodnius* is also stable at −20 °C, though it loses almost all its activity when boiled (Aston & White, 1974).

Stable extracts with diuretic activity were also produced by homogenizing brains or thoracic ganglia in 80% ethanol. After centrifugation at 13500*g* for 1 min, both the supernatant and centrifugate, which had been resuspended in distilled water, were evaporated to dryness at 60 °C. When they were redissolved in bathing medium, DH could be detected only in the supernatant fraction. The fact that extracts in ethanol are stable without boiling provides further evidence of the enzymatic nature of the degradative mechanism, which is inactivated by 80% ethanol. DH is also soluble in absolute methanol. It can be extracted by methanol from dried powder

prepared by evaporating to dryness an extract of thoracic ganglia in distilled water. The yield of hormone is considerably reduced but this may in part be due to the film of lipids that, on evaporation of the methanol, is deposited over the residue. This makes it difficult for the aqueous solvent, in which the extract must be dissolved before it can be assayed for DH, to penetrate to the water-soluble substances below. The diuretic factor extracted from locust corpora cardiaca is also soluble in methanol (Mordue & Goldsworthy, 1969), as is *Rhodnius* DH (Aston & White, 1974), though less than 5% of the total DH available for aqueous extraction in the latter is methanol-soluble.

During dialysis against Ringer's solution for 24 h using both dialysis tubing and the micro-dialysis cell, diuretic activity was retained within the dialysis membrane. The membrane of the dialysis cell had a pore size of 2·4 nm, equivalent to a molecular weight of 1200, and the DH presumably therefore has a molecular weight greater than this value. During dialysis the diuretic activity of the extract was reduced. This may have been due to adherence of DH to the dialysis membrane, since the effectiveness of a control extract stored in a plastic tube for the same period at the same temperature was not reduced. Dialysis against distilled water for 24 h inactivates DH and it is perhaps significant that when blowfly haemolymph containing bursicon is dialysed against distilled water a white precipitate is formed with which much of the tanning activity is associated (Cottrell, 1962).

Gel filtration of hormone extract on Biogel P2 proved unsuccessful, because the hormone could not be eluted from the column.

Incubation of diuretic hormone with enzymes

Extracts of thoracic ganglia were incubated at 30 °C for 60 min with the following enzymes: trypsin, chymotrypsin, α-amylase, neuraminidase, DNase, RNase and lipase. Incubation was terminated by placing the incubates in boiling water for 2 min and the solutions were assayed for DH using isolated Malpighian tubules. For each enzyme the following controls were performed (1) extract alone was incubated and then boiled; (2) extract was incubated with denatured (boiled) enzyme and then boiled; (3) active enzyme was added to the extract which was then boiled immediately. In the presence of an excess of the degradative enzymes all the hormone was inactivated instantaneously and there was no difference between control (3) and the normal incubation. The concentrations of enzymes required were established by adding low concentrations of the enzymes to isolated tubules secreting in the presence of DH. By gradually increasing the concentration of enzyme, the concentration suitable for an incubation experiment was determined (Fig. 4). Inhibition of secretion was not caused by any adverse effect of trypsin on the secreting cells *per se*, because if trypsin was washed off and the bathing drop was replaced by fresh extract, the cells responded normally and the rate of secretion returned to the previously high level (Fig. 4). By similar experiments it was demonstrated that the other enzymes likewise had no adverse effects on the Malpighian tubules.

After these preliminary experiments, extract was incubated with enzymes at the following concentrations (mg/ml): trypsin 0·001; chymotrypsin and α-amylase 0·01; neuraminidase, DNase, RNase and lipase 0·1. Incubation with trypsin, chymotrypsin and α-amylase destroyed 100%, and neuraminidase 90%, of the diuretic

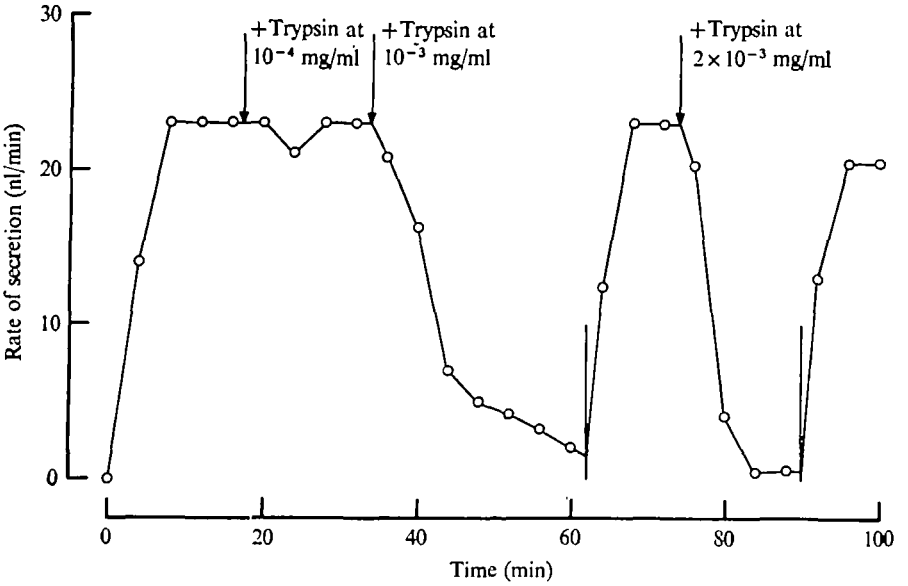

Fig. 4. The effect of trypsin on the rate of secretion of a tubule isolated into a bathing drop containing extract of thoracic ganglia (5/100 μl). The arrows mark the times at which trypsin was added to give the concentrations shown and the vertical lines mark the times at which the bathing drop was replaced by fresh extract of thoracic ganglia.

activity in 60 min. Incubation with neuraminidase for 20 min and 40 min destroyed 28% and 58% of the activity respectively. In the control experiments for these enzymes the diuretic activity of the extracts was not affected. DNase, RNase and lipase caused no decrease in diuretic activity either in incubation experiments or when added directly to isolated tubules secreting in the presence of DH.

Neuraminidase is known to attack hormone receptor sites (Wooley & Gommi, 1964). To demonstrate that the presence of neuraminidase was not preventing the tubules from responding to DH, a 10 μl aliquot of concentrated hormone was applied to an isolated preparation in which secretion had been halted by the addition of neuraminidase. The tubule responded with a temporary increase in the rate of secretion (Fig. 5). When neuraminidase was washed off and the bathing drop was replaced by fresh extract, the rate of secretion returned to, and was maintained at, the previously high level (Fig. 5).

From the effect of these enzymes on the diuretic activity of extracts we may deduce that the disruption of the following bonds inactivates *Glossina* DH: peptide bonds between amino acids involving the carboxyl groups of arginine and lysine (trypsin), peptide bonds where the carboxyl function is donated by an aromatic amino acid (chymotrypsin), $\alpha(1-4)$ bonds between glucose residues in a polysaccharide chain (amylase) and bonds between *N*-acetyl neuraminic acid and sugar residues (neuraminidase). In summary, we may propose that DH contains amino acids, glucose and *N*-acetyl neuraminic acid (sialic acid) residues and that these play some part in the functioning of the hormone.

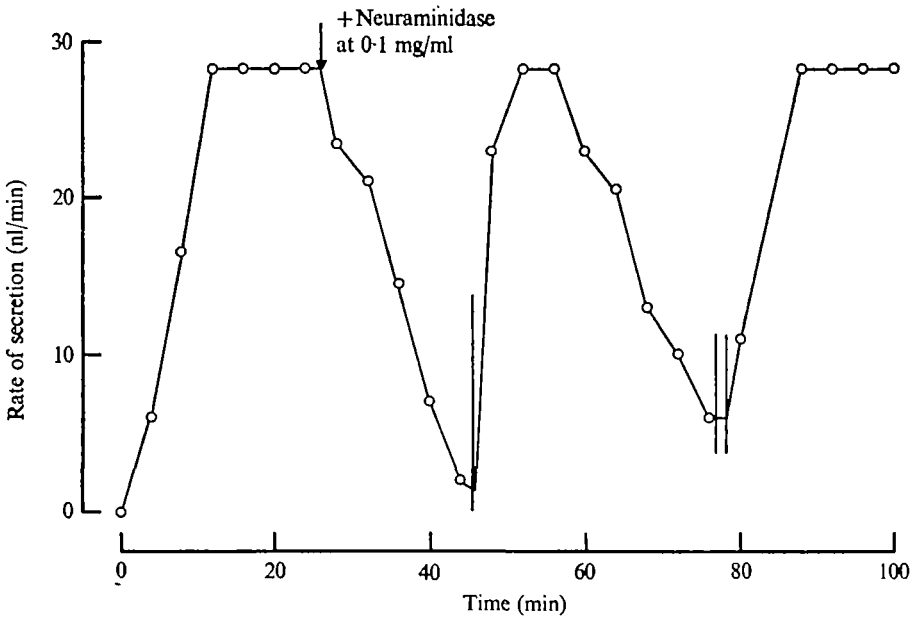

Fig. 5. The effect of neuraminidase on the rate of secretion of a tubule isolated into a bathing drop of extract of thoracic ganglia (5/100 μl). Neuraminidase was added at the time marked by the arrow. Ten μl of concentrated extract (20 ganglia/100 μl) was introduced into the bathing drop at the time indicated by the single vertical line. The bathing drop was removed, the tubule was washed with Ringer's solution and a drop of fresh ganglion extract was added at the time indicated by the double vertical lines.

DISCUSSION

Though it was not possible to demonstrate the presence of DH in the haemolymph of *G. austeni*, a substance with diuretic activity was extracted from its nervous system. It is necessary that any insect that employs a DH to control its rate of excretion should be able to terminate the action of that hormone or permanent diuresis would cause desiccation. Indeed, the promotion of untimely release of DH, leading to death by desiccation, has been suggested as the action of certain insecticides (Maddrell & Casida, 1971; Casida & Maddrell, 1971). The ability of insect Malpighian tubules to destroy DH has been demonstrated in *Rhodnius* (Maddrell, 1964*a*), *Dysdercus* (Berridge, 1966) and *Carausius* (Pilcher, 1970), and it is apparent that the tubules of *Glossina* are capable of reducing the titre of DH in the haemolymph in a similar fashion. There has been no previous demonstration of a specific breakdown enzyme apparently manufactured by the nervous system and released into the haemolymph where it is able to destroy DH (Madrell & Gee, 1974), though the presence of such an enzyme in *Rhodnius* has been postulated by Aston & White (1974).

On the basis of these preliminary investigations it is possible to construct a hypothesis to explain the control of diuresis in the tsetse fly. During feeding, stretch receptors in the gut or body wall are stimulated; the presence of such receptors in *Glossina* was predicted by Maddrell (1964*b*). Afferent impulses are transmitted from these receptors to the brain, from where efferent impulses are dispatched which eventually invade the neuroaxonal endings causing an influx of Ca^{2+} which stimulates

the release of DH by exocytosis (Maddrell & Gee, 1974). It is postulated that the brain forms part of this reflex arc, since the application of a ligature at the neck of the fly apparently stops the release of DH. Release of hormone will normally continue as long as the receptors remain stretched. Therefore, if DH is not destroyed almost as rapidly as it is released, its titre in the haemolymph will rise continuously during diuresis and release of hormone will have to stop well in advance of the termination of excretion to allow sufficient time for the titre of DH in the haemolymph to be reduced below the threshold of tubule stimulation. It would be difficult to make such a system as precise as is required in *Glossina*. More precise control would be possible if the hormone were destroyed at a rapid and approximately constant rate, enabling the concentration of DH in the haemolymph to be determined by varying the rate of release. The rate of release will, in turn, depend on the information received by the brain from stretch receptors monitoring the distension of the gut or body wall. As soon as the meal is sufficiently concentrated, the stretch receptors will become quiet and this will result in an immediate decline in DH release. The concentration of DH in the haemolymph will soon be reduced by the action of the Malpighian tubules and the degradative enzyme in the haemolymph, and since the hormone–receptor interaction is readily reversible, as can be seen from the rapid response and recovery to DH of the Malpighian tubules *in vitro*, diuresis will rapidly come to an end.

The experiments reported here demonstrate that *Glossina* DH is soluble in water and alcohols, it is stable from -15 to 100 °C and it is non-dialysable. It shows certain similarities to *Rhodnius* DH which is water-soluble, non-dialysable and stable at low temperatures (Aston & White, 1974) and *Schistocerca* DH which is heat-stable and is soluble in water and methanol (Mordue & Goldsworthy, 1969). The amount of tissue that must be homogenized to produce an active extract of *Glossina* DH (threshold response at 0·5 thoracic ganglia/100 μl bathing medium) is a factor of ten greater than that required for the DH of *Rhodnius* (0·07 mesothoracic ganglionic masses/100 μl; Maddrell, 1963) and that of *Dysdercus* (0·05 m.n.s.c./100 μl; Berridge, 1966). However, activity may be destroyed by the action of the degradative enzyme and by boiling during the extraction of *Glossina* DH.

Many vertebrate neurosecretory hormones are known to be peptides or polypeptides. In some cases – for example, the hormones of the neurohypophysis – their precise molecular structures have been determined and they have been synthesized *in vitro*. It has been proposed that some insect neurosecretory hormones are also polypeptides. The hyperglycaemic, heart-accelerating and diuretic hormones of *Periplaneta* (Davey, 1961; Steele, 1963; Brown, 1965; Goldbard *et al.* 1970), the heart-accelerating and diuretic factors of *Schistocerca* (Mordue & Goldsworthy, 1969) and the DH of *Rhodnius* (Aston & White, 1974) have all been described as peptides or polypeptides, though only on the basis of inactivation by proteolytic enzymes. *Glossina* DH is similarly inactivated by the proteolytic enzymes trypsin and chymotrypsin but it is also sensitive to α-amylase and neuraminidase (sialidase). Previously attempts have been made to inactivate insect hormones by the use of sialidase, but the tanning hormone of *Sarcophaga* (bursicon) (Cottrell, 1962) and the brain hormone of *Bombyx* (Yamazuki & Kobayashi, 1969) were not affected by this enzyme. There are no previous reports of attempts to inactivate insect hormones with amylase,

though Yamazuki & Kobayashi (1969) did find that their most active preparation of *Bombyx* brain hormone contained about 15% glucose. However, they did not establish that glucose contributed to the action of the hormone.

With a structure containing amino acids, glucose residues and sialic acid, the DH of *Glossina* has all the components of a sialoglycoprotein. Such molecules are found in the vertebrate nervous system in regions rich in ganglia such as the caudate nucleus, cerebellar grey matter, pons and medulla oblongata of the vertebrate brain (van Nieuw Amerongen & Roukema, 1973), and it is perhaps significant that the source of the DH used in the present investigations was also a ganglion – the thoracic ganglion. However, we know from experiments, in which DH release was promoted by K-rich solutions, that *Glossina* DH is not released from the thoracic ganglion (Maddrell & Gee, 1974). In *Rhodnius*, DH extracted from the mesothoracic ganglionic mass occurs in three different molecular forms, though only one of these (the low-molecular-weight form) is released and this appears to be the true hormone (Aston & White, 1974). Other neurosecretory hormones are known to be attached to macromolecules during storage; vasopressin, for example, is attached to neurophysin from which it dissociates when released from the neurosecretory cells. However, in the case of *Glossina* DH it is clear from the action of specific enzymes on its diuretic activity, that amino acids, glucose and sialic acid must be present for the hormone to be able to cause an increase in the rate of secretion of isolated Malpighian tubules. Though these components have only been demonstrated in hormone extracted from the thoracic ganglion, it would seem essential that they are also present when the naturally released DH reaches the receptor sites on the Malpighian tubules *in vivo*.

I would like to thank the Tsetse Research Laboratory, Langford, for supplying tsetse fly pupae, Dr M. J. Berridge for supervision of the research and Dr P. A Langley for reading the manuscript. The work was supported by a Research Studentship awarded by the Science Research Council.

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