ISOLATION AND PURIFICATION OF THE DIURETIC HORMONE FROM RHODNIUS PROLIXUS

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Abstract—Extracts of Rhodnius prolixus mesothoracic ganglionic masses were subjected to column chromatographic separation procedures. The eluates were assayed biologically for diuretic hormone activity.

Gel filtration through columns of polyacrylamide gel was found to separate soluble diuretic activity into two distinct zones. Poor recovery was obtained with respect to the activity of the crude extract. No diuretic activity was found to co-chromatograph with a sample of 5-hydroxytryptamine. When samples of haemolymph from freshly fed Rhodnius were chromatographed under similar conditions only the second zone (low molecular weight) was found to have diuretic activity. It is this low molecular weight zone which would more reasonably qualify as the physiologically active diuretic hormone. Likewise, the diuretic activity released from isolated mesothoracic ganglionic masses by K\(^+\)-rich Ringer's solution showed only low molecular weight activity after chromatography.

INTRODUCTION

The normal control of diuresis in a variety of insects has been reviewed by Maddrell (1971), but only in the American cockroach Periplaneta americana (Goldbard et al., 1970) and the locust Schistocerca gregaria (Mordue and Goldsworthy, 1969) have attempts been made to purify and characterize the diuretic hormones (DH).

Aqueous extracts of the mesothoracic ganglionic mass (MTGM) of Rhodnius prolixus have been shown (Maddrell, 1963) to contain a DH. Some similarities between the properties of 5-hydroxytryptamine (5-HT) and Rhodnius DH were noted by Maddrell et al. (1969), although later evidence (Maddrell et al., 1971) indicated that 5-HT is probably not the natural hormone for the Malpighian tubules of Rhodnius.

In Rhodnius most of the hormone is contained in the MTGM, and is released into the haemolymph after taking its blood meal. The present work describes progress towards the isolation and purification of DH from MTGM extracts, haemolymph, and from MTGM where the release of DH is induced by K\(^+\)-rich Ringer's solution.
MATERIALS AND METHODS

*Rhodnius prolixus* were used from a laboratory culture maintained at 27°C and 60% r.h.

Bioassay of the diuretic activity was performed using isolated distal secretory portions of Malpighian tubules of *Rhodnius* according to the procedure described by Maddrell (1969). Diuretic activity was estimated quantitatively in the bioassay by dilution of active material to a known final concentration giving the threshold of maximal response, i.e. typically a secretion of 1 μl/hr per tubule. In order to estimate the efficiency of extraction and the distribution of DH in the various sources from one *Rhodnius*, a diuretic hormone unit (DH unit) was established. One DH unit is defined as the minimum amount of DH contained in 1 μl of active solution required to elicit a maximal response from one Malpighian tubule.

The Ringer’s solution used had the following composition in mM/l. distilled water: D-glucose, 34·4; NaCl, 129; KCl, 8·6; CaCl₂, 2·0; MgCl₂, 8·6; NaHCO₃, 10·2; NaH₂PO₄, 4·3.

Sources of diuretic hormone

(a) *Mesothoracic ganglionic mass*. In order to determine the best extractant for the diuretic hormone, MTGM were dissected out and homogenized in a small glass pestle and mortar with ethanol; methanol; a mixture of chloroform and methanol (3:1 by volume); 0·01 M hydrochloric acid; 0·01 M ammonium acetate buffer pH 6·9; 0·1 M phosphate buffers ranging from pH 5·8 to 8·0 or distilled water. After low-speed centrifugation at 3500 rev/min for 15 min the supernatants were removed, dried in a vacuum, and resuspended in Ringer’s solution for bioassay.

(b) *Head and thorax*. Large-scale extractions were prepared from the head and thorax of 100 adult *Rhodnius* by freezing the excised material in liquid nitrogen and pulverizing in a stainless steel press. The resulting powder was suspended in distilled water (10 ml) at 4°C, ultrasonicated for 1 min using a Schuco Disembrator, and centrifuged at 3500 rev/min for 15 min in a M.S.E. 4L centrifuge at 4°C. After removal of the supernatant, the residue was resuspended in distilled water (10 ml) and the procedure thrice repeated. The combined supernatants were freeze-dried in aliquots and stored at −20°C, and subsequently redissolved in water for chromatography.

(c) *Haemolymph*. Haemolymph was collected from adult *Rhodnius* 5 to 20 min after being fed. Between 10 and 20 μl were normally obtainable from each insect.

(d) *Incubation of mesothoracic ganglionic masses in K⁺-rich Ringer’s solution*. Isolated MTGM were incubated routinely at 27°C for 2 hr in K⁺-rich Ringer’s solution having the following composition in mM/l. distilled water: D-glucose, 34·4; NaCl, 8·6; KCl, 129; CaCl₂, 8·6; MgCl₂, 2·0; NaHCO₃, 10·2; NaH₂PO₄, 4·3. Care was necessary in order to remove the MTGM intact and undamaged together with short lengths of peripheral nerves attached to the posterior part of the MTGM, in order to obtain the maximum release of DH.
(e) **Haemolymph from insecticide poisoned insects.** The insecticide Zectran (4-dimethylamino-3,5-xylyl methyl carbamate) was applied topically to *Rhodnius* (500 µg/g) in 5 µl acetone. Haemolymph was collected from treated insects during the paralytic phase (approximately 3 hr after treatment).

**Gel filtration chromatography**

P-60, P-30, and P-2 (100–200 mesh) polyacrylamide gels (Bio-Rad Labs.) were packed in columns (Pharmacia or Whatman) and eluted with Ringer's solution sterilized by passage through a Millipore filter at 4°C. The eluates were monitored for u.v. absorbance at 280 nm using an LKB Uvicord II photometer and fractions collected using an LKB Ultrorac automatic fraction collector. Chromatographic conditions used to purify the DH are described in the legends accompanying the appropriate figures.

**RESULTS**

Previous work on the diuretic hormone (DH) of *Rhodnius* was done by Maddrell (1963, 1969) using homogenates of the mesothoracic ganglionic mass (MTGM). Our preliminary experiments, in order to determine the solubility and stability of crude MTGM extracts, showed that DH was best extracted by aqueous solvents. Of the organic solvents tried, only methanol extracted some DH activity (<5 per cent of the total). However, when the residue remaining after organic solvent extraction was re-extracted with water and bioassayed, no loss of DH activity had occurred. Distilled water proved the best extractant for DH and so was used in later experiments especially as it presented least difficulty for freeze drying and for compatibility with the bioassay. A fourfold aqueous extraction of the head and thorax extracted >95 per cent total recoverable DH activity.

Freeze-dried head and thorax extracts retained diuretic activity for at least 6 months at −20°C, but lost all activity after a week in aqueous solution. This result was obtained at room temperature or at 4°C for extracts dissolved in phosphate buffer pH 7.0, pH 8.0, or pH 9.0. Instability of the hormone was also demonstrated in active haemolymph taken from freshly fed *Rhodnius* which showed little activity after 2 hr at room temperature. Active haemolymph and freeze-dried extracts lost 99 per cent DH activity when heated at 100°C for 1 min. In highly concentrated extracts of MTGM (one MTGM in 50 µl water) trace amounts of DH activity could be detected after such treatment.

Dialysis of a MTGM extract at 4°C against 1 l. of Ringer's solution for 20 hr resulted in only a small loss of DH activity from the dialysis bag. The majority of activity is, therefore, non-dialysable.

In order to test the resistance of the DH to proteolytic enzymes the freeze-dried extract was incubated with trypsin, chymotrypsin and pronase for 2 hr. The activity disappeared after treatment with these proteolytic enzymes, suggesting that the DH present in these extracts contained peptide bonds.
Comparison of DH activity obtained from various sources

The table lists a typical set of results giving the percentage of DH activity obtained from various sources. Percentages are calculated on the basis that 100 per cent activity is found in the aqueous head and thorax extract. Although the aqueous head and thorax extracts contained the highest percentage of DH activity,

<table>
<thead>
<tr>
<th>Source</th>
<th>Source sample volume (μl) per insect</th>
<th>Dilution required to give threshold of maximal response</th>
<th>DH units % Total per insect activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) MTGM homogenate</td>
<td>50</td>
<td>30 times</td>
<td>1500 75</td>
</tr>
<tr>
<td>(b) Head and thorax extract</td>
<td>100</td>
<td>20 times</td>
<td>2000 100</td>
</tr>
<tr>
<td>(c) Haemolymph</td>
<td>10–20</td>
<td>2 times</td>
<td>20–40 1–2</td>
</tr>
<tr>
<td>(d) Incubation of MTGM in K+-rich Ringer's solution</td>
<td>10</td>
<td>20 times</td>
<td>200 10</td>
</tr>
<tr>
<td>(e) Haemolymph from Zectran poisoned insects</td>
<td>10</td>
<td>Undiluted sample gave 50% maximal response</td>
<td>5 &lt;1</td>
</tr>
</tbody>
</table>

after centrifugation at 100,000 g for 1 hr, it was found that 80 per cent of the activity sedimented with insoluble particulate material. Using these centrifugation conditions as our criterion of solubility it appeared that only 20 per cent of the DII activity remained in the supernatant.

Incubation of mesothoracic ganglionic masses in K+-rich Ringer's solution

Gosbee et al. (1968) demonstrated the depletion of stainable neurosecretory material from the corpora cardiaca of P. americana when bathed in a Ringer's solution containing high concentrations of potassium. Preliminary experiments in which five MTGM from Rhodnius were incubated in K+-rich Ringer's solution for 2 hr at 27°C resulted in the release of DH activity into the Ringer's solution. No release of activity was detected in a control incubation experiment with normal Ringer's solution. Further experiments with K+-rich Ringer's solution showed that Ca2+ was involved with DH release. Increasing the CaCl2 from 2.0 to 8.6 mM with KCl at 129 mM significantly improved the release of DH activity into the Ringer's solution. Using this technique 10 per cent of the total DH activity was released after 2 hr (see Table 1). Fig. 1 shows the results of a time course study of hormone release. The highest amount of DH activity present in the Ringer’s solution was found after incubation for 2 hr.

Gel filtration chromatography

Starting with extracts of Rhodnius head and thorax or MTGM various gel filtration columns were used to purify the DH.

Biogel P-30. Fig. 2 illustrates a typical elution profile of a head and thorax extract 100,000 g supernatant showing two zones of DH activity. The majority
of activity resided in the high molecular weight zone and was completely excluded from the gel. Only small amounts of activity were present in the low molecular weight zone, which proved to be unstable with complete loss of activity after 4 hr at room temperature.

![Graph](image1)

**Fig. 1.** Time course study of diuretic hormone activity released from mesothoracic ganglionic masses in K⁺-rich Ringer's solution. Each point is a mean of at least three determinations. I-shaped bars denote standard error.

![Graph](image2)

**Fig. 2.** Biogel P-30 polyacrylamide gel chromatography of a head and thorax extract (100,000 g supernatant). Pharmacia column size 4.8 × 1.6 cm; flow rate 0.14 ml/min; sample size 0.5 ml; fractions collected at 5 min intervals. Bands show zones of DH activity.
Biogel P-60. A typical elution profile for a head and thorax extract 100,000 g supernatant is shown in Fig. 3. High molecular weight activity again chromatographed with the void volume fractions (suggesting a molecular weight greater than 60,000). The observed ratio of high : low molecular weight DH activity.

![Biogel P-60 chromatography of a head and thorax extract](image)

**Fig. 3.** Biogel P-60 chromatography of a head and thorax extract (100,000 g supernatant). Pharmacia column size 6.2 x 1.6 cm; flow rate 0.14 ml/min; sample size 0.3 ml; fractions collected at 5 min intervals. Bands show zones of DH activity.

![Biogel P-60 chromatography of haemolymph](image)

**Fig. 4.** Biogel P-60 chromatography of haemolymph from freshly fed *Rhodnius* (10,000 g supernatant), and of a mesothoracic ganglionic mass (MTGM) extract (100,000 g supernatant) superimposed. Whatman column size 9 x 1 cm; flow rate 0.14 ml/min; sample size 0.25 ml; fractions collected at 3 min intervals. Bands show zones of DH activity.
was approximately 10:1. Chromatography of a MTGM extract 100,000 g supernatant on a Whatman column gave a similar result as illustrated in Fig. 4.

The existence of two chromatographically distinct zones of DH activity from head and thorax or MTGM extracts led us to investigate the chromatographic properties of the DH found in the haemolymph of a freshly fed *Rhodnius* and the DH released from MTGM by K⁺-rich Ringer’s solution. A sample of the latter was chromatographed on an identical Whatman P-60 column as used above. This showed only one zone of activity which was eluted in identical fractions to the low molecular weight material found in the MTGM extracts. Also a sample of haemolymph (250 μl) from freshly fed *Rhodnius*, centrifuged at 10,000 g for 20 min to remove blood cells, was then chromatographed on an identical P-60 column. Bioassay of the fractions again revealed only low molecular weight activity, but this time eluting with a slightly lower elution volume, i.e. in fractions 14 to 16 (Fig. 4). This discrepancy in the elution volume could be explained by the fact that it was necessary to overload the column with haemolymph in order to obtain sufficient activity for bioassay. Accordingly, when MTGM were incubated in a mixture of ‘inactive’ haemolymph (from an unfed *Rhodnius*) and K⁺-rich Ringer’s solution (1:1 by volume) DH activity was again found to emerge in fractions 14 to 16. Therefore, irrespective of the source of hormone, the low molecular weight zone of DH activity on P-60 is chromatographically similar.

**Biogel P-2.** Chromatography of a MTGM extract 100,000 g supernatant showed two zones of DH activity, as illustrated in Fig. 5. In addition to the two zones of high and low molecular weight activity trace amounts of activity were sometimes found in fractions 23 and 24.

A sample of haemolymph (250 μl) from a freshly fed *Rhodnius*, centrifuged at 10,000 g for 20 min and then chromatographed on a P-2 column, showed a zone of low molecular weight activity (Fig. 5) in fractions 12 to 14 corresponding to the same low molecular weight active fractions in the MTGM extract. Again there were indications of trace DH activity in fraction 23. Chromatography on Biogel P-2 of a sample of DH released from MTGM by K⁺-rich Ringer’s solution gave results similar to those obtained from haemolymph DH.

In order to compare the above results with two low molecular weight compounds known to have DH activity, 5-hydroxytryptamine (5-HT) and cyclic 3',5'-adenosine monophosphate (cyclic AMP) were separately chromatographed on the same P-2 column. 5-HT was found not to co-chromatograph with any previously mentioned zones of DH activity (see Figs. 4, 5), although cyclic AMP chromatographed closely with the low molecular weight fractions 13 and 14.

**DISCUSSION**

We have noted that the DH has poor solubility in all except aqueous solvents and that extracts of MTGM were largely non-dialysable. This, coupled with the disappearance of DH activity in extracts after treatment with trypsin, chymotrypsin, and pronase, led us to believe initially that the DH of *Rhodnius* was a high molecular weight polypeptide. Investigations by MORDUE and GOLDSWORTHY (1969) on the
locust DH indicated that this hormone is also likely to be a polypeptide, whereas in the American cockroach, the DH has been partially purified by Goldbard et al. (1970) from extracts of the terminal abdominal ganglion, and a molecular weight greater than 30,000 has been proposed for it in this species.

Maddrell (1963) has shown that in Rhodnius the majority of diuretic hormone activity is contained in the posterior part of the mesothoracic ganglion mass, localized in large neurosecretory cells, and that its release into the haemolymph is in response to stretch receptors stimulated by abdominal distension following feeding (Maddrell, 1964, 1966). The results of the purification techniques described in this paper indicate that the DH obtainable from the MTGM is present in three different molecular forms: soluble high molecular weight (greater than 60,000), soluble low molecular weight (less than 2000), and a particulate form which could be sedimented by 100,000 g centrifugation which accounted for about 80 per cent of the DH activity in MTGM extracts. Neurosecretory material is known to occur in the form of granules and this could account for the large amount of insoluble DH activity. Also it is possible that the soluble DH is capable of binding to membrane fragments, or that hormone containing vesicles are formed during tissue extraction. Any of these possibilities would require high-speed centrifugation to sediment such activity. All attempts to render this bound DH soluble, by treatment with sodium deoxycholate or organic solvents, were unsuccessful. Treatment involving the use of dilute hydrochloric or formic
acid also failed, mainly due to the difficulty of removing all traces of acid from samples prior to bioassay.

The demonstration of DH activity distributed unequally between three different molecular forms poses the question as to which of them is the ‘true’ hormone. For a substance to qualify as a DH it must be found to occur naturally in the haemolymph at a concentration which has a physiological effect on the Malpighian tubules. Our studies on the DH released into the haemolymph of *Rhodnius* after a blood meal indicate that DH activity from this source is chromatographically similar to the low molecular weight form obtained from MTGM extracts. Therefore we consider the low molecular weight form qualifies best as the ‘true’ DH. This view is confirmed by similar results from MTGM in K⁺-rich Ringer’s solution incubation experiments in which low molecular weight activity is found to be released.

The hormone found in the haemolymph and incubation medium proved to be unstable, little biological activity being detectable after 2 hr. This could be due to the inherent chemical instability of the hormone or the presence of degradative enzymes in the haemolymph, possible released with the DH. Such enzymes would provide *Rhodnius* with an efficient means of destroying its DH in order to avoid the consequences of desiccation from prolonged hormone action. However, attempts made to destroy any such enzymes by heat treatment also destroy most of the DH.

The results of experiments where Malpighian tubules have been used to assay MTGM or head and thorax crude extracts need careful interpretation since such preparations contain 98 per cent or more high molecular weight material, the properties of which may be considerably different from the low molecular weight hormone. Also nervous tissue may contain amines which could have a diuretic or even antidiuretic effect (Maddrell et al., 1971). However, no DH activity found in the haemolymph or incubation medium was found to co-chromatograph with 5-HT. This does not exclude the possibility that the low molecular weight hormone could be an aggregation of 5-HT with a small molecule such as adenosine triphosphate as has been shown to occur by Pletscher et al. (1971). Cyclic AMP also has DH activity and has been shown to be an intracellular second messenger in many different hormone systems including the *Rhodnius* DH (Maddrell et al., 1971). It is interesting to note that cyclic AMP chromatographed closely with the low molecular weight hormone on Biogel P-60 and P-2.

It has been shown (Maddrell, 1966) that the site of DH release in *Rhodnius* is from the swollen neurosecretory axon endings near the surface of the peripheral abdominal nerves close to where they join the MTGM, and there is electron microscopic evidence (Maddrell, 1966) that exocytosis could be involved in DH release. Reference to Table 1 shows that large quantities of hormone remain stored in the MTGM whereas just sufficient hormone is released during normal diuresis to elicit a maximal response from the Malpighian tubules. It would be consistent with our findings to consider that the high molecular weight forms serve as a storage of DH, whilst the low molecular weight form is that which is
released into the haemolymph to bring about diuresis. Experimentally induced release of DH was achieved under conditions of membrane depolarization using high potassium levels. Although this resulted in the least contaminated sample of hormone, only 10 per cent of the reservoir of activity could be released in this way. This situation seems to be analogous to the in vitro release of vasopressin from the rat neurohypophysis (Thorn, 1966) where about 5 per cent of the extractable vasopressin is released by high concentrations of potassium in the incubation medium.

Now that the compartmentalization of the DH in Rhodnius has been clarified, work can progress on the low molecular weight hormone with a view to determining its chemical structure.

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REFERENCES


