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EXCRETION IN THE BLOOD-SUCKING BUG, *RHODNIUS PROLIXUS* STÅL.

III. THE CONTROL OF THE RELEASE OF THE DIURETIC HORMONE

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INTRODUCTION

The profuse diuresis of freshly fed 5th-stage larvae of *Rhodnius* is caused by the release into the haemolymph of a diuretic hormone from the fused ganglionic mass in the mesothorax, probably from hormone-rich bodies assumed to be neurosecretory cells, lying posteriorly in the mass (Maddrell, 1962, 1963*b*). The present communication investigates the control of the release of the hormone.

MATERIALS AND METHODS

Fifth-stage larvae of *Rhodnius prolixus* from a laboratory culture were used in the experiments. Most of the methods used have been described earlier (Maddrell, 1963*b*, 1964).

RESULTS

It seemed reasonable to suppose that the release of the hormone was stimulated nervously or humorally or both. The possibility of nervous control was examined first. Various of the nervous connexions to the ganglionic mass (Fig. 1) were cut, the insects were fed and the amount of urine subsequently produced was measured as described elsewhere (Maddrell, 1964). After diuresis the insects were dissected and the success of the operation was checked. The results of these experiments are displayed in Table 1. Clearly, no nervous information from the head or thorax is involved in the diuretic response. Cutting the nerves to the abdomen, however, abolished the response. Núñez (1963) attributed the absence of diuresis in such insects to the sectioning of the axons of the neurosecretory cells responsible for the release of the diuretic hormone. Two lines of evidence support a different explanation. Recent experiments with ligated fed insects (Maddrell, 1963*b*) have shown that diuretic hormone can only be found in haemolymph samples collected from parts of the insect that include the fused ganglionic mass in the mesothorax. Neurosecretory cells with axons release their products at the ends of the axons (Herlant-Meewis & van Damme, 1962); if cutting the abdominal nerves in *Rhodnius* does interrupt the neurosecretory pathway involved in the release of the diuretic hormone, then the cell bodies of the neurosecretory cells must lie in the abdomen and the axons run into the gangli-

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onic mass in the mesothorax. If such were the case, the aggregations of hormone-rich material visible at the back of the ganglionic mass (Maddrell, 1963*b*) would be the swollen endings of the neurosecretory cell axons and not the cell bodies. However, examinations of transverse and longitudinal sections of mesothoracic ganglia stained to reveal neurosecretory material (paraldehyde-fuchsin method of Cameron & Steele, 1959) showed that each of the hormone-containing masses in the ganglionic mass has a nucleus and is a neurosecretory cell body. Since the hormone is released from the ganglionic mass which contains the neurosecretory cells responsible for its manufacture, the absence of diuresis in insects with denervated abdomina is not a result of the interruption of the neurosecretory pathway. Probably, the absence of diuresis in these insects is due to interruption of the path by which the release of the hormone is stimulated, especially since severing any of the other nervous connexions with the

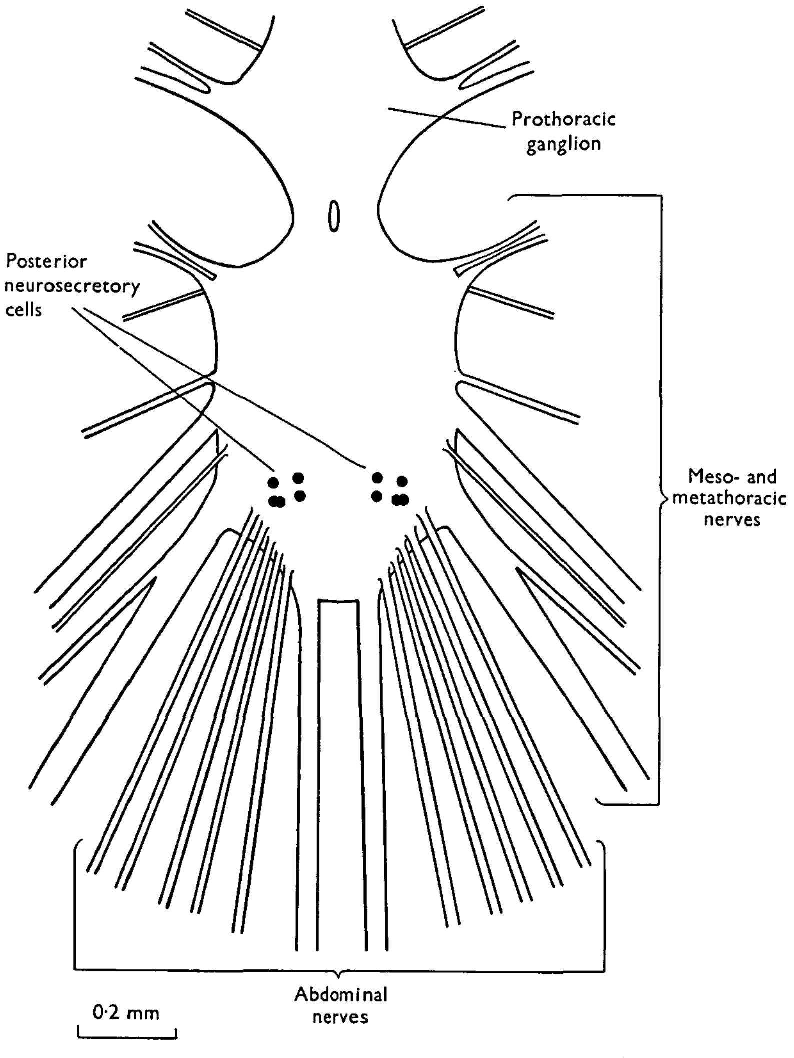

Fig. 1. Dorsal view of the nervous connexions to the mesothoracic ganglionic mass.

ganglionic mass does not impair the diuretic response (Table 1). It seems reasonable to conclude from these experiments that afferent nervous information from the abdomen is necessary for an effective release of the diuretic hormone at feeding.

If the response depends solely on afferent nervous information from the abdomen, then it should be possible to initiate diuresis in decapitated insects. To test this possibility, 30–50 μl. of Ringer's solution diluted to the concentration of mammalian blood ($\Delta = 0{\cdot}59^\circ$ C.) and at room temperature was injected into the mid-gut of each of several decapitated insects. To do this, a small hole was cut in the abdominal tergum and a small part of the mid-gut was extruded and waxed in position. A fine glass tube was then inserted through a hole cut in the gut wall and, after it had been sealed in with wax, Ringer's solution was blown through it into the lumen of the mid-gut. Provided that there were peristaltic waves passing back along the gut (Maddrell, 1964), there was a release of the diuretic hormone as judged by the accumulation in the rectum of 5–15 μl. of clear urine in the next hour (twelve successes in eighteen attempts). The injection of Ringer's solution direct into the haemocoel had no such effect and so the swelling of the rectum was not merely caused by osmotic movement of fluid into the rectum from the diluted haemolymph. This experiment demonstrates that neither feeding nor the presence of the head nor a change in temperature is essential to the diuretic response.

Table 1. *The effect on subsequent diuresis of cutting various of the nervous connexions to the ganglionic mass*

Nerves cut	Extent of diuresis (% by weight of the meal) Mean ± S.D.	No. of insects
None (controls)	44·4 ± 5·5	27
Connectives to the prothoracic ganglion	41·3 ± 8·2	27
All meso- and metathoracic nerves	37·6 ± 9·3	10
All abdominal, meso- and metathoracic nerves	Nil	7
All abdominal nerves	Nil	10

There seem to be two possible sources of the stimulus which gives rise to diuresis. The first is the fall in osmotic concentration of the haemolymph after feeding (Maddrell, 1964). To test this possibility, six insects were fed with blood made hypertonic to their haemolymph ($\Delta_{\text{meal}} = 0{\cdot}84^\circ$ C., $\Delta_{\text{haemolymph}} = 0{\cdot}75^\circ$ C.). These insects produced a flow of urine at a rate which was not much slower than normal, and eventually excreted urine equivalent on the average to 17% of the meal, even though measurements of the osmotic concentration of the haemolymph showed that it was rising when normally it would have been falling. Similar results were obtained from ten insects fed with blood made isotonic to the haemolymph, though in these insects the extent of diuresis was more nearly normal. The diuretic response, therefore, is not initiated by osmoreceptors.

The second possible source of stimulus is the mechanical effect on the abdomen of ingesting the meal. There is already some evidence in support of this idea. Diuresis at the maximum rate starts within 2–3 min. from the time that the insect starts to feed, so that within this time effective stimulation must occur, the hormone being released and transported to the tubules at a concentration sufficient to elicit the maximum response from the tubules. Probably only a mechanical event could occur early enough to stimulate the release of the diuretic hormone. The initially dorso-ventrally flattened abdomen distends visibly within 15–30 sec. from the time that the insect starts to ingest fluid, so that this change would be early enough to account for the prompt release of the hormone.

To test this idea directly, air was blown into the mid-gut in each of thirty unfed insects. In only four or five of these insects was there any evidence at all of a release of the diuretic hormone and even in these cases only very small quantities ($< 0 \cdot 5$ μl.) of urine accumulated in the rectum. It is thought that the following points explain the relative failure of these experiments.

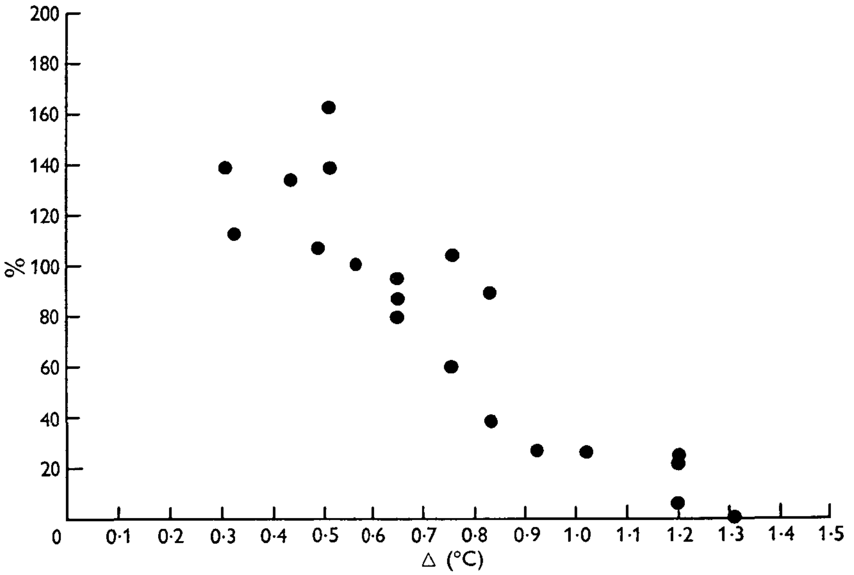

Fig. 2. The change in sustained maximum rates of secretion by isolated Malpighian tubules brought about by changes in the osmotic concentration of the haemolymph. Ordinate, sustained maximum rate of secretion after a change in concentration (100% = rate in unaltered haemolymph). Abscissa, osmotic concentration of altered haemolymph. Sufficient diuretic hormone was added to the haemolymph to ensure that its concentration was always well above that needed to elicit the maximum rate of secretion by the tubules.

(*a*) Even when insects were injected with hypotonic Ringer's solution ($\Delta = 0 \cdot 59^\circ$ C.), the rate of urine production was only 10–30% of that in intact fed insects, suggesting that injection was only moderately successful in simulating the sensations occurring during feeding.

(*b*) Of twenty insects injected with isotonic Ringer's solution ($\Delta = 0 \cdot 75^\circ$ C.), only two produced urine even though ten insects fed with the same Ringer's solution all subsequently produced a profuse flow of urine.

(*c*) Experiments showed that the sustained maximum rate of secretion by isolated sets of Malpighian tubules was slower in haemolymph whose concentration had been increased by mixing it with concentrated Ringer's solution and was faster in haemolymph diluted with distilled water (Fig. 2). Incidentally, this probably explains why diuresis in insects fed on artificially concentrated blood is slower than normal.

It may well be, therefore, that when insects are artificially inflated there is a reduced amount of hormone released and this is relatively ineffective unless the concentration of the haemolymph decreases.

The lack of clear-cut proof of the idea that diuresis is caused by a mechanical change at feeding makes it necessary to describe other less direct experiments. The possibility that an increase in internal pressure controls diuresis is examined first because certain of the points established help to support the general argument. To test this possibility, five freshly fed insects were decapitated and about 60 μl. of the blood meal were squeezed out through the neck so that there was no longer any excess internal pressure. Diuresis continued at the normal rate in each case for longer than 2 hr. This cannot be explained on the basis that hormone already released into the haemolymph was sufficient to maintain diuresis for as long as this, because, in experiments in which the flow of haemolymph from the source of the diuretic hormone to the Malpighian tubules was prevented (Maddrell, 1963*b*), diuresis at the normal rate did not persist for longer than 15 min. It was still possible that the initial pressure increase was adequate to sustain the continued release of the diuretic hormone which occurs during diuresis (Maddrell, 1964). To examine this possibility, reference was made to some earlier experiments in which freshly fed insects were ligated at the junction of the thorax and abdomen (Maddrell, 1963*b*). In these insects it was found that there had been a continued release of the diuretic hormone into the haemolymph of the thorax. Either the initial supply of nervous information from the sense organs in the abdomen was sufficient to maintain the release of the hormone, or the ligature did not prevent nervous information from passing through it. To decide between these alternatives, eight freshly fed insects were each ligated as before and the abdomen was then cut off just behind the ligature. Samples of haemolymph were removed from the thorax 1–1½ hr. later and tested for diuretic activity using preparations of isolated Malpighian tubules (Maddrell, 1963*b*). In each case the haemolymph was found to contain no detectable activity. It seems reasonable to conclude that a constant supply of nervous information must reach the ganglionic mass from the abdomen to sustain the release of the hormone. It is now possible to conclude from the fact that fed insects having no excess internal pressure continue to produce urine that diuresis is not a response to the internal pressure in the abdomen.

As a test of the idea that the stretching of the abdomen is responsible for diuresis, one would like to be able to feed the insect without allowing the abdomen to distend and yet to allow the other changes associated with feeding, such as the dilution of the haemolymph, to develop. The following experiment came close to doing this. Five unfed insects each had a small part of the mid-gut extruded through a small hole cut in the abdominal wall where it was waxed in position to prevent the loss of haemolymph. The insects were weighed and after the protruding part of the gut had been gashed the insects were allowed to feed. In this way, they sucked large quantities of blood which leaked out of the abdomen without distending it much. This treatment makes the insects insatiable (Maddrell, 1963*a*) and they were kept feeding for 30 min. to allow the full development of all the changes associated with feeding except, of course, the distension of the abdomen. The insects were then weighed again to find out how much blood had been retained in the abdomen, and the total amount of urine produced in the next 3–4 hr. was measured. In no case was the amount of urine produced any greater than would have been expected for normal insects taking meals of the same small size as that retained. This is in spite of the fact that normal insects take similar amounts of blood in only 2–3 min. and a full meal in about 12 min. As a control experiment, ten insects were treated similarly but the protruding part of the gut was waxed over and left intact. In each of these insects feeding and diuresis were normal. This experiment strongly suggests that diuresis is controlled by the distension of the abdomen rather than by changes in the composition of the haemolymph.

The following experiment provides support for this suggestion. Because it was possible to decapitate fed insects and squeeze blood out of the gut without stopping diuresis, it seemed reasonable that similar experiments involving the removal of practically the whole of the blood meal could be performed without the fear that the nervous shock would check diuresis. Six freshly fed insects were treated in this way and were left with 20–25 mg. of blood in the gut. In no case did the subsequent diuresis exceed 7 μl. (a small amount of diuresis would be expected because of the diuretic hormone in the haemolymph at the time of the operation). The same result came from a similar experiment involving rather less drastic treatment in which six fed insects, each of which had a part of the gut wall protruding from the abdominal wall, were deflated by squeezing blood out through a hole cut in the gut wall. It seems probable that the deflation of the abdomen removes the stimulus which controls diuresis.

In summary, the evidence supporting the notion that proprioception is responsible for diuresis is as follows:

(1) Neither a fall in osmotic concentration of the haemolymph nor a change in temperature nor a change in pressure is essential to the response.

(2) The stimulation received in the abdomen must occur very soon after feeding begins; the abdomen distends visibly almost as soon as ingestion begins.

(3) The stimulation from the abdomen must be continuous; the stretch receptors in the abdomen of *Rhodnius* are known to adapt very slowly, if at all (Van der Kloot, 1961).

(4) The results of two series of experiments strongly suggest that diuresis is controlled by the distension of the abdomen. In this connexion it is relevant to point out that in intact insects the extent of diuresis is proportional to the size of the meal (Maddrell, 1964).

It seems reasonable to conclude that abdominal stretch receptors provide the nervous information which is essential to the initiation and maintenance of the release of the diuretic hormone.

The location of the abdominal stretch receptors

To find out which parts of the abdomen were involved, the course of the abdominal nerves was traced. Each lateral half of abdominal segments 1–5 inclusive is supplied by one nerve from the ganglionic mass (Fig. 3). The first abdominal segment has no sternite and the nerves supplying it run directly to the dorsal surface. Two larger central nerves leaving the mass each supply the lateral halves of segments six and seven as well as the compressed terminal segments and the rectum. It was relatively simple, therefore, to open the mesothorax ventrally and to denervate different parts of the abdomen. To find out what effect this had on diuresis, the operated insects were fed and any urine produced was collected and measured. The insects were then dissected to check the success of the operation and the volume of urine found in the rectum was added to that actually voided. The total amount of urine produced depended on the number of half-segments that had been denervated rather than on their identity; any three or four half-segments could be deprived of their nervous supply with little effect on subsequent diuresis, but the extent of diuresis was much reduced when more half-segments were involved (Fig. 4). Apparently, nervous information from any one half-segment plays just about as important a part as that from any other in the stimulation of the release of the diuretic hormone. This result is circumstantial evidence in support of the idea that stretch receptors control diuresis, for Van der Kloot discovered (1961) that there are two stretch receptors in each abdominal segment, one on each side.

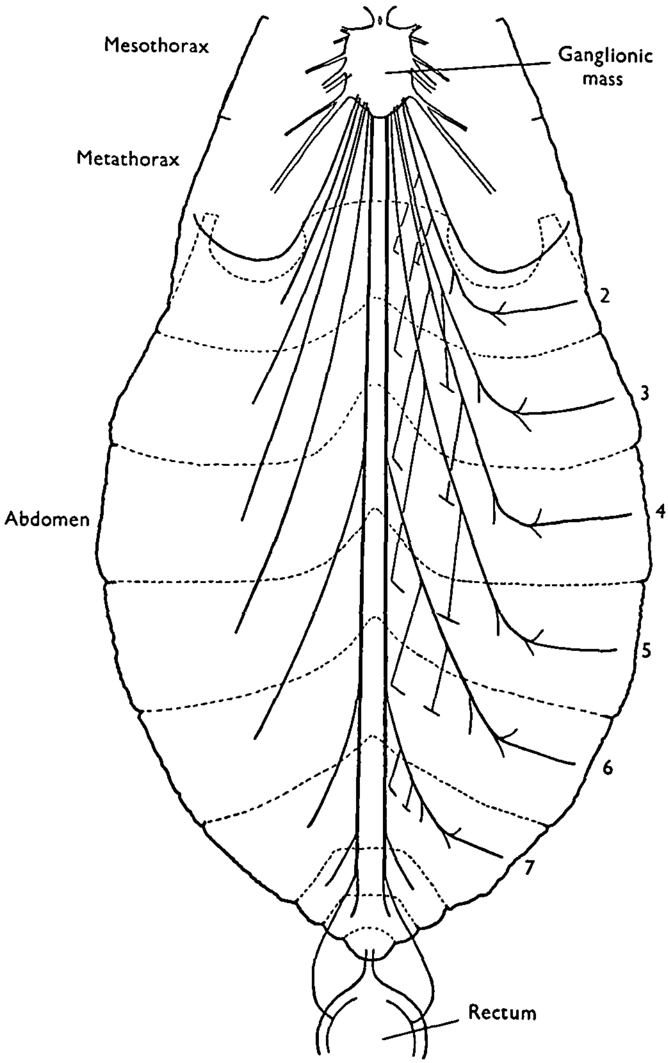

Fig. 3. The abdominal nervous supply. For the sake of clarity, the course of the nerves is traced further on the right-hand side than on the left.

If the receptors were on the gut wall, as seemed possible from experiments in which diuresis was initiated by inflating the gut with Ringer's solution, there should be nerves running from the gut to the abdominal wall in each abdominal segment. A careful search failed to reveal the presence of such connexions, hence it appears unlikely that the receptors are in the walls of the mid-gut.

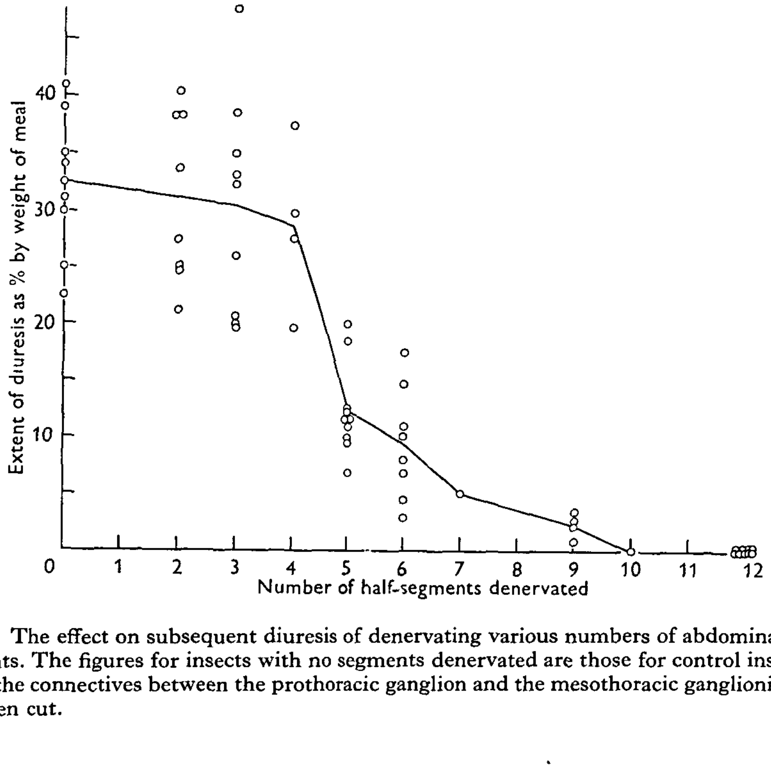

Fig. 4. The effect on subsequent diuresis of denervating various numbers of abdominal half-segments. The figures for insects with no segments denervated are those for control insects in which the connectives between the prothoracic ganglion and the mesothoracic ganglionic mass had been cut.

To find likely sites for the receptors, the course of the abdominal nerves was followed farther (Fig. 5). Each segmental nerve supplies two branches to the longitudinal intersegmental muscles, a branch to the spiracle, four branches to the abdominal wall, a branch to each of the three vertical tergosternal muscles and a branch to supply the tergal wall and the longitudinal intersegmental muscles on it. It is known that stretch receptors in insects are usually to be found in association with strands of connective tissue or muscle (Finlayson & Lowenstein, 1958). The intersegmental muscles and tergosternal muscles are, therefore, possible sites. Probably, the receptors are not in the intersegmental muscles, because they are stretched at a later stage than would account for the very prompt release of the diuretic hormone. The same reasoning suggests that the receptors are not in the abdominal wall, which, although it is stretched, is stretched too late. In contrast, the tergosternal muscles lie just next to the anterior segment of the mid-gut, which accommodates the ingested blood and clearly must stretch these muscles as soon as feeding begins (Fig. 6).

To decide between these alternative sites, the abdomen of the unfed insect was painted with 'New-Skin', a commercial preparation of pyroxylin in organic solvents. This treatment mechanically prevented the abdomen from increasing in area when the stretch receptors responsible for the control of diuresis must be sensitive to the vertical distension of the abdomen and that they are probably in the tergosternal muscles.

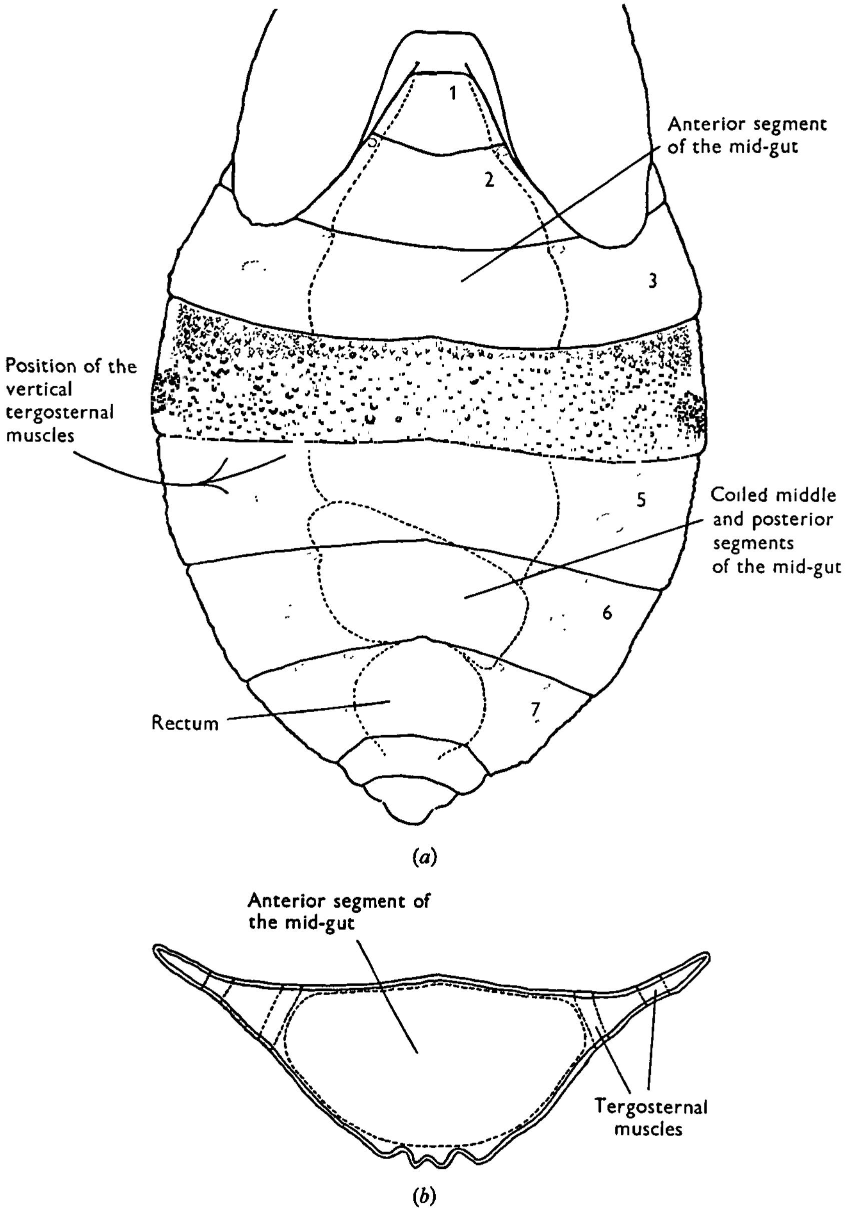

Fig. 6. (*a*) Plan view, (*b*) cross-section (between segments four and five) of the abdomen to show the position of the mid-gut in relation to that of the tergosternal muscles. The dorsal surface of segment four is drawn in detail to show the unpigmented patches of cuticle over the insertions of the tergosternal muscles.

Subsequent to these discoveries, Prof. W. G. Van der Kloot informed me that he had found that the abdominal stretch receptors in *Rhodnius* were activated by the upward movement of the dorsal surface of the abdomen. He also did a few experiments to try to locate the receptors by tracing out the abdominal nerve, cutting the branches while trying to keep recording from the sense organ. On the basis of these few experiments, he concluded that the stretch receptors were in the tergosternal muscles. Since this agrees with the above suggestion, which is based on different evidence, there seem to be good grounds for concluding that the nervous information essential to diuresis originates in stretch receptors in the tergosternal muscles.

DISCUSSION

The experiments reported in this paper do not preclude the possibility that there is some stimulus other than the stretching of the abdomen necessary to the release of the diuretic hormone. But since it has been shown that the inflation of the gut of decapitated insects with Ringer's solution is sufficient to cause diuresis, so that neither feeding nor the presence of the head is essential to the response, such a possibility is perhaps unlikely. It is at least the simplest hypothesis that diuresis in *Rhodnius* is a reflex response to the distension of the abdomen caused by the swelling of the mid-gut.

Some recent papers (Núñez, 1962, 1963) contain observations which appear to be incompatible with this idea. However, Dr Núñez has since informed me that he has repeated some of his experiments feeding his experimental animals on heparinized blood instead of as earlier on blood treated with oxalate. He now finds that diuresis is *not* affected by decapitation or by cutting the ventral nerve cord just anterior to the mesothoracic ganglionic mass. He suggests that *Rhodnius* larvae may be more sensitive to surgery after meals of blood treated with oxalate. Essentially his results now support the ideas presented in this paper.

Núñez (1963) also found that when the ventral nerve cord was cut anterior to the mesothoracic ganglionic mass the insects produced no urine when fed. In the author's experiments, this operation had very little effect on diuresis (Maddrell, 1963*a*, and the present paper). A clue as to a possible reason for this discrepancy comes from Núñez's control experiments in which he operated upon twelve insects but left the nervous system untouched. When these insects were fed they accumulated an average of 11 mg. of urine in the rectum (its elimination was prevented by blocking the anus). However, the possibility that this control operation itself may have had an effect on diuresis was, apparently, not examined. In experiments to test this point, each of ten intact insects accumulated under similar conditions not less than 35 μl. (average 43 μl.) of urine in the rectum. Even in five insects with the nerve cord cut an average of 30 μl. of urine accumulated in the rectum. In addition, in all these insects, large amounts of urine had been forced forwards into the mid-gut. It seems possible, therefore, that Núñez's technique, even in the absence of neurotomy, had impaired diuresis, which may explain why he found that insects with the ventral nerve cord cut produced no urine when fed.

Since the size of the blood meal determines whether or not a larva of *Rhodnius* will subsequently moult (Wigglesworth, 1934), it is perhaps not surprising that the impact of the meal upon the insect seems to be largely dependent on the response of the abdominal stretch receptors. Thus not only does this response control the release of the prothoracotropic hormone, but it also regulates the size of the meal (Maddrell, 1963*b*) and controls the release of the diuretic hormone.

It is of some interest that there seems to be in many insects a relation between an increase in the volume of the body and subsequent diuresis. For example, a profuse flow of urine follows feeding in other insects which gorge themselves with blood such as *Triatoma infestans*, the mosquito (Wigglesworth, 1953) and the tsetse fly (Lester & Lloyd, 1928; Bursell, 1960). In the tsetse fly, diuresis was normal in flies that ingested solutions as concentrated as 1·25 % NaCl solution (≡ Δ of 0·74° C.) and there was some diuresis after the ingestion of more concentrated solutions (Lester & Lloyd, 1928). The diuretic response in the tstese fly might well, therefore, be controlled by stretch receptors as in *Rhodnius*.

The system in *Rhodnius* is closely similar to that in larvae of the beetle, *Anisotarsus cupripennis* (Núñez, 1956). Núñez's work on this insect provided the first good evidence for a diuretic hormone in insects. As in *Rhodnius*, it was shown that the hormone in *Anisotarsus* is released in response to sensory information received in the abdomen. In one experiment, Núñez injected 1 % NaCl solution into the haemolymph, whose concentration was not much higher (≡ 1·16–1·67 % NaCl solution). The insects were able to excrete the excess volume although the system has normally to deal with water taken up through the surface of the animal. This suggests that the animal perceives the extra volume by stretch receptors and not by osmoreceptors. Núñez (1961) has also shown that there is a rapid formation of urine in *Schistocerca cancellata* following the injection into the haemocoel of large quantities of nearly isotonic Ringer's solution.

Ramsay (1953) showed that in the larvae of *Aëdes aegypti* the fluid leaving the Malpighian tubules has two possible fates; either it is passed forward into the mid-gut and is resorbed there or it is carried back by peristaltic movements of the hind-gut and is voided through the anus. When more haemolymph was injected into the haemocoel, the rate of peristalsis of the hind-gut increased so that more fluid was excreted. Ramsay interpreted this as a direct effect of proprioceptors via the central nervous system on the movements of the hind-gut. However, these results do not preclude the possibility that the rate of tubular secretion had also increased, though equally they provide no evidence in support of such an idea. Whatever the mechanism, this mosquito larva excretes fluid faster when the volume of the haemolymph is increased.

Cottrell (1962) found that newly emerged adult *Calliphora* excrete about 7 % of the body weight *after* the insect has expanded by swallowing air, and that most of the excretion occurs in the first 3 hr. after expansion. Rapid excretion also follows ecdysis in the silkworm (Shimizu, 1931) and the locust (Lee, 1961).

Whether these instances of diuresis following increase in the volume of insects are more than coincidental remains to be seen. Conceivably, an increase in the volume of the body might always cause a fast flow of secretion from the Malpighian tubules. If the flow of tubule fluid were released from the insect without taking into account the osmotic concentration of the haemolymph, the system would be of little use to the insect. However, regulation of rectal resorption now established for the locust (Phillips, 1964*a*, *b*) is probably common to most insects and so the concentration and quantity of urine actually eliminated can be adjusted by the rectum, depending on the needs of the insect. This is not to deny the Malpighian tubules any role in the regulation of the volume of the haemolymph, because the rectum can only release a copious flow of urine when fluid enters it at a high rate.

SUMMARY

1. The diuresis of freshly fed larvae of *Rhodnius* is controlled by afferent nervous information from the abdomen.

2. This information is supplied by abdominal stretch receptors sensitive to vertical distension of the abdomen. It is most probable that the receptors are in the vertical tergosternal muscles, a trio of which occurs towards each lateral edge in abdominal segments 2–7 inclusive.

3. It is suggested that diuresis in *Rhodnius* is a reflex response to the distension of the abdomen brought about by the swelling of the anterior part of the mid-gut during feeding.

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