

HORMONAL CONTROL OF THE MALPIGHIAN TUBULES OF THE STICK INSECT, *CARAUSIUS MOROSUS*

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INTRODUCTION

Factors have been isolated from the central nervous system of the stick insect, *Carausius morosus*, which affect the rate of secretion of the Malpighian tubules (Gersch *et al.* 1960; Unger, 1965; Vietinghoff, 1967), but it has not yet been shown that these factors are of physiological importance *in vivo*. There is, however, an accumulation of stainable material in the corpora cardiaca of *Carausius* on dehydration (Pflugfelder, 1937), and Ramsay (1955*a*) found that the rate of secretion of tubules *in situ* varied according to the state of hydration of the insect. The control of the Malpighian tubules by a hormone originating in the nervous system, whose level in the haemolymph has been shown to vary, has been demonstrated in *Rhodnius* (Maddrell, 1963), *Dysdercus* (Berridge, 1966*a*) and *Periplaneta* (Wall & Ralph, 1964; Mills, 1967; Mills & Nielsen, 1967), and similar control is suspected in a number of other species. This paper reports the occurrence in *Carausius* of a diuretic hormone and examines its significance in the regulation of water balance.

MATERIALS AND METHODS

Single isolated tubules were used for the assay of diuretic activity. The technique for their preparation is essentially that described by Ramsay (1954). After removal from the insect, single tubules were transferred quickly to drops of saline (about 40 μ l.) under liquid paraffin in a plastic Petri dish. The surface of such dishes is only slightly wettable, so that the drop becomes anchored without spreading out. The proximal end of the tubule was drawn out of the drop, and urine was collected from a gash in the side. The volume of urine secreted was estimated from the diameter of the drop of urine, and the rate of urine secretion was calculated. Up to 20 tubules could be obtained from a single insect.

The saline used for dissection and as the bathing medium for isolated tubules is based on that used by Wood (1957). Its composition is: KCl 15.0 mM/l., NaCl 12.5 mM/l., KH_2PO_4 3.0 mM/l., NaOH 2.5 mM/l., MgCl_2 50.0 mM/l., CaCl_2 7.5 mM/l., glucose 185.5 mM/l. (pH 6.6).

Serum was prepared as described by Ramsay (1955*a*) and was stored at -10°C . until needed.

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The rate of secretion by isolated tubules was found to vary according to the time of year, the nature of the medium by which the tubules were surrounded (Ramsay, 1955*b*) and whether the insect was well-fed or fasting. In order to provide a reasonably standard assay for diuretic activity tubules were taken only from well-fed, gravid adult female insects. There is also a variation in rate of secretion among the tubules taken from a single insect, so that only those tubules were used whose mean rate of secretion was greater than 1.5 nl./min.

Tissues whose diuretic activity was to be assayed were ground up with a small glass pestle and mortar in a few μ l. of distilled water to rupture the neurosecretory cells and membrane-bound granules (Pérez-González, 1957). An equal volume of twice-strength saline was then added to restore the osmotic pressure and ionic concentrations. Breis thus prepared were added to the drops bathing tubules whose rate of secretion had been recorded during the previous hour. A brei was said to have a diuretic effect if the rate of urine secretion during the hour following its addition was greater than during the previous hour.

Results are plotted \pm S.E.

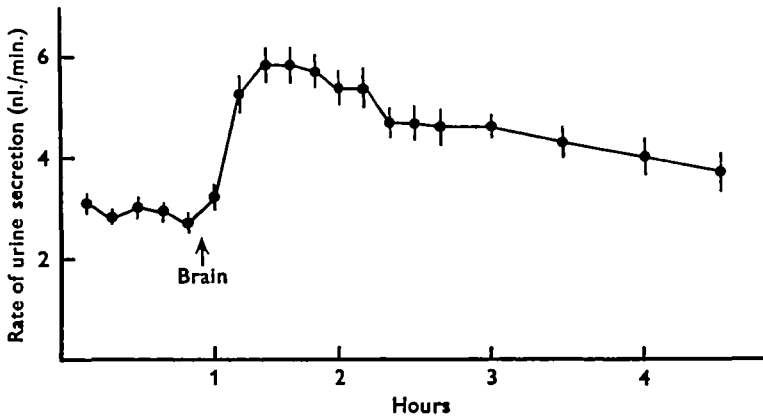


Fig. 1. The effect upon the rate of urine secretion of the addition of a brain brei (indicated by an arrow) to give a final concentration of 2 brains/100 μ l. (mean of 12 tubules).

RESULTS

1. *The source of the diuretic factor*

Diuretic activity was found in breis of brain (Fig. 1), corpora cardiaca (Fig. 2) and sub-oesophageal ganglion. The amount of activity which could be extracted from each of these sources was similar, but from the weights of these tissues, given in Table 1, it can be seen that the activity per unit weight is greatest in the corpora cardiaca. Activity was also found in serum (Fig. 3) and in the ganglia of the ventral nerve cord, but not consistently in any one ganglion. The swellings of the lateral nerves of the medial nervous system, the corpora allata and thoracic muscle were without activity.

An attempt was made to determine in which part of the brain activity was located. The brains of both *Carausius morosus* and *Sipylus* sp. were used. Extracts of the brain and corpora cardiaca of the latter species have a diuretic effect on *Carausius* Malpighian tubules similar to that exerted by *Carausius* brain and corpora cardiaca, but

the neurosecretory area of the pars intercerebralis is more easily visible in the whole brain than in *Carausius*. No activity was detected in the deutero- or trito-cerebrum of either species, but the optic lobes and both the medial and lateral parts of the

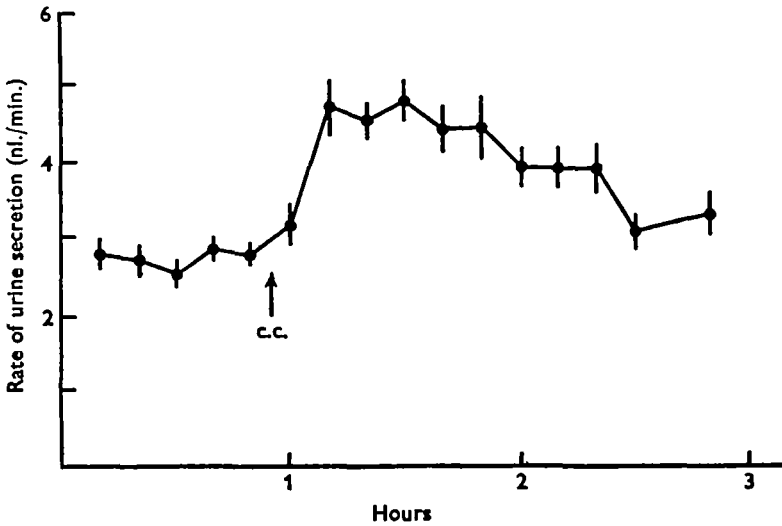


Fig. 2. The mean diuretic response of 13 tubules to the addition of a brei of corpora cardiaca to give a final concentration of two pairs/100 μ l.

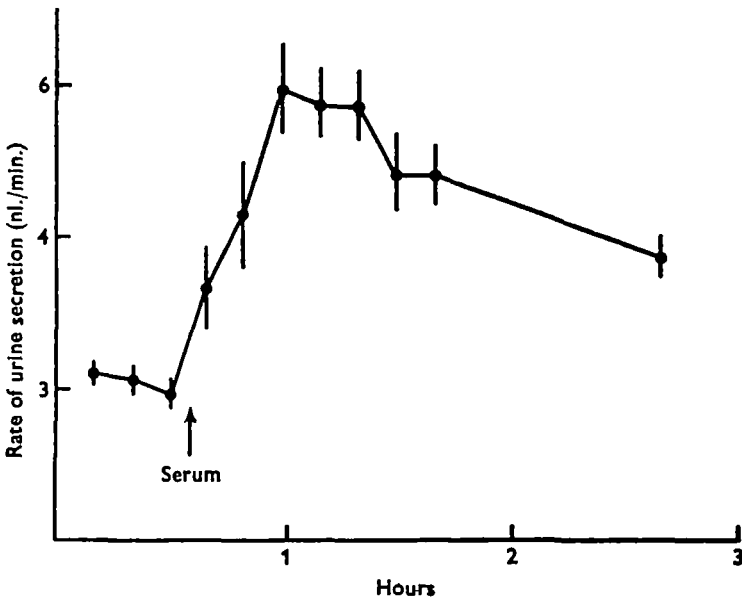


Fig. 3. The mean diuretic response of eight tubules to the addition of an equal volume of serum to the drop bathing the tubules.

protocerebrum contained extractable diuretic activity. The distribution of diuretic activity within the brain is shown in Fig. 4. Since both the corpora cardiaca and the protocerebral neurosecretory area contain extractable diuretic activity, and passage of

neurosecretory material from the protocerebral neurosecretory cells to the corpora cardiaca has been demonstrated in *Leucophaea* (Scharrer, 1952), it seems likely that the diuretic activity of the corpora cardiaca is due to the material passing from the active area of the brain to them for storage and release, rather than to a secretory product of the intrinsic glandular cells of the corpora cardiaca.

Table 1. *Comparison of the diuretic activity which can be extracted from the brain, corpora cardiaca and sub-oesophageal ganglion*

Tissue	Wet weight (mg.)	Relative activity/mg.
Brain	0.6660	1.0
C.c. (+c.a.)	0.0298	22.4
S.o.g.	0.1244	5.3

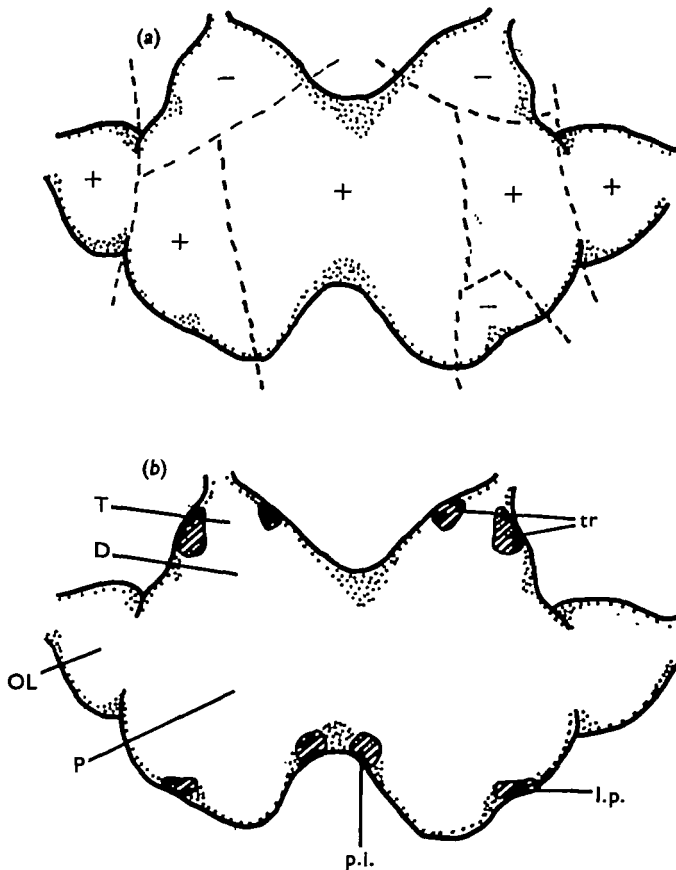


Fig. 4(a). Distribution of diuretic activity within the brain. + = Diuretic activity present; - = diuretic activity absent. (b) The positions of the neurosecretory areas of the brain (indicated by shading) (after Raabe, 1959). T = tritocerebrum; D = deutocerebrum; P = protocerebrum; OL = optic lobe; tr = neurosecretory area of tritocerebrum; l.p. = lateral area of neurosecretory cells of protocerebrum; p.i. = neurosecretory area of pars intercerebralis of protocerebrum.

2. *The diuretic response*

The response of Malpighian tubules of *Carausius* to the addition of a brei of brain or corpora cardiaca or of serum is shown in Figs. 1-3. The threshold for diuresis is approximately 0.15 brains or pairs of corpora cardiaca per 100 μ l. At this concentration, in about 50% of tubules the rate of urine secretion is raised for 1 hr., but in the remainder the rate rises only briefly and the mean rate for the second hour is less than for the previous hour. Above the threshold concentration both the duration of diuresis (that is, the time taken for the rate of secretion to return to the level found before the addition of the brei) and the maximum rate of secretion occurring during diuresis (expressed as a percentage of the rate during the hour before the addition of the brei) increase with increasing concentration of brei, but reach a maximum somewhere in the order of 0.5 brains or pairs of corpora cardiaca per 100 μ l.

3. *Inactivation of the hormone*

The decline of rate of urine secretion which occurs towards the end of diuresis might be due to the deterioration of the tubules, which occurs in tubules bathed by wholly artificial media for many hours (Ramsay, 1955*b*), or to a fall in the concentration of the hormone in the medium surrounding the tubules. The fact that there is a maximum duration of diuresis (about 5-6 hr.) which is only rarely exceeded even with very high concentrations of hormone would appear to support the former hypothesis. However, a similar maximum duration is observed when the tubules are bathed by a medium of 1 part serum: 3 parts saline, that is, under conditions in which Ramsay (1955*b*) has shown that tubules survive with little deterioration in their performance. Moreover, it is possible to elicit a second, though smaller, stimulation of urine secretion by the addition of a second dose of hormone after the end of the first diuresis. Thus the end of diuresis cannot have been due entirely to the deterioration of the tubules, but must have been due also to the reduction of the hormone concentration. Reduction of hormone concentration can also be demonstrated by allowing a tubule, bathed by a drop of saline containing diuretic hormone, to secrete until the end of diuresis. If the tubule is then removed, and a second tubule placed in the drop in place of the first, the second tubule is not stimulated. If, however, the first tubule is removed before the end of diuresis the second tubule is stimulated when it is placed in the drop. This fall in the concentration of the hormone is not due to spontaneous decay, for the hormone is stable at room temperature for several days (Pilcher, 1969).

The hormone does not appear to pass unchanged into the urine. Tubules were set up in drops of saline containing a high concentration of hormone (20-30 times threshold), and urine was collected from these tubules throughout the period of diuresis. To this urine a saline was added which brought the concentration of the ions in urine to the levels present in normal saline. Tubules set up in drops of this adjusted urine secreted no faster than tubules in adjusted urine collected from untreated tubules. The addition of the saline involved a sixfold dilution of the urine, but the initial concentration of hormone in the bathing medium was 20-30 times the threshold concentration, and by the end of diuresis the concentration of the hormone in this drop must have fallen to near threshold, so that it seems unlikely that the hormone

was not detected in the urine simply because of dilution. It is also not possible to detect diuretic hormone in the urine of *Rhodnius* (Maddrell, 1964a).

Since the hormone concentration in the bathing drop falls, and since active hormone does not pass into the urine, it seems probable that the tubule cells inactivate the hormone. This inactivation may also help to explain why the threshold for detectable response by *Carausius* tubules is so high. *Rhodnius* tubules are able to respond to *Carausius* diuretic hormone (Maddrell, Pilcher & Gardiner, 1969), but at a concentration well below threshold for response by *Carausius* tubules (S. H. P. Maddrell, unpublished results). At these low concentrations *Rhodnius* tubules respond with a small diuresis of long duration. *Rhodnius* tubules respond to low doses of *Rhodnius* hormone, which is a different substance from *Carausius* hormone (Maddrell *et al.* 1969), with a small diuresis of short duration. This is also thought to be due to the rapid breakdown of the hormone by the tubules (Maddrell, 1964a). It may be that this small diuresis caused by a small amount of *Carausius* hormone can only be detected in *Rhodnius* tubules because the tubules are unable to inactivate *Carausius* hormone, with the result that the diuresis is prolonged. *Carausius* tubules, on the other hand, inactivate the hormone rapidly, and no response is detectable at these lower concentrations.

Table 2. *The rate of urine secretion by tubules set up in media containing serum from fed or fasting insects*

Serum	Number of tubules	Mean rate of urine secretion (nl./min.)
Fed	13	3.12 ± 0.28
Fasting	17	2.41 ± 0.20

Table 3. *The rate of urine secretion by tubules from fed or fasting insects, isolated in saline*

Insect	Number of tubules	Mean rate of urine secretion (nl./min.)
Fed	13	1.29 ± 0.13
4-6 day fasting	17	0.52 ± 0.09

2. *The effect of fasting on the level of diuretic hormone in the haemolymph*

In order to test whether the level of diuretic hormone in the haemolymph varies in response to the state of hydration of the insect, tubules were set up in a medium containing 3 parts saline: 1 part serum taken from either well-fed or fasting dehydrated insects, and the rate of urine secretion during the first hour was noted (Table 2). Serum from fed insects supports urine secretion at a greater rate than does serum from fasting insects. It is possible that this difference might be due to the level of hormone in the serum.

Tubules taken from fed and fasting insects were set up in saline, and it was found that those from the fed insects secreted urine faster than those from fasting insects (Table 3). *Carausius* tubules *in situ* also secrete somewhat faster in fed than in fasting insects (Ramsay, 1955a) and it seems likely that the rate of secretion by tubules *in vitro* reflects their rate *in vivo*, and thus the level of hormone to which they were

exposed before removal from the insect. It is worth noting that tubules taken from *Dysdercus* on the third day of the fifth instar, when the blood contains diuretic hormone, secrete urine when set up in saline, whereas those taken on the sixth day, when the blood has no diuretic activity, are inactive (Berridge, 1966a).

The appearance of tubules also changes during fasting. In well-fed insects the tubules are translucent and distended, but during the course of fasting they become gradually filled with white granules. This change in appearance is correlated with their rate of secretion. Both the change of appearance and the fall-off of rate which occur during fasting can be reversed by subsequent feeding. Three insects were kept in jars without food for 96 hr. One of these insects (A) was then killed and examined, and the rate of urine secretion by a number of its tubules in saline was determined. A ligature was then tied between the thorax and abdomen of one of the two remaining insects, and each was put in a jar containing food. After 48 hr., both were killed and examined, and the rate of urine secretion by the tubules determined. The changes associated with fasting—low secretory rate; white, densely granular Malpighian tubules; fluid-containing midgut; reduced blood volume leading to flaccidity of the abdomen—which are presumed to have occurred when the insect was fasting, are reversed by feeding in the control insect (C). In the ligatured insect (B), on the other hand, food only passed along the gut as far as the ligature, and the blood volume was only restored in the thorax. The abdomen contained little blood and remained flaccid. The secretory activity and appearance of the tubules remained characteristic of fasting, dehydrated insects. The sheath of the nerve cord did not appear to have been constricted, so it seems unlikely that the nervous tissue itself had been damaged. It therefore seems that the blood rather than the nervous system plays a critical role in restoring the activity of the Malpighian tubules after fasting. The rate of urine secretion by the tubules of these three insects is shown in Table 4.

Table 4. *The rate of urine secretion by tubules from the three experimental insects (see text)*

Insect	Mean rate of urine secretion by tubules isolated in saline (nl./min.)
A	0.43
B	0*
C	1.48

* Rate too low to measure.

The experiments described so far do not eliminate the possibility that the variation of some constituent of serum other than the diuretic hormone is the cause of the variations in tubule activity. The rate of urine secretion by the isolated Malpighian tubules of *Carausius* depends upon the concentration of potassium in the external medium (Ramsay, 1955b), so that low rates of urine secretion might be accounted for by reduced potassium in the haemolymph. Hoyle (1954) found that the level of potassium in the haemolymph of the locust can fall by as much as 50% in food deprivation. The fall of potassium in the blood of *Carausius* is, however, relatively small (Ramsay, 1955a). The greatest fall in blood potassium observed by Ramsay

(1955*a*) was not enough to account for the reduction in rate of secretion, on the basis of the relationship between blood potassium and rate of secretion found by Ramsay (1955*b*). The osmotic pressure of the haemolymph, which would also affect the rate of urine secretion (Ramsay, 1954), does not change significantly during fasting (Ramsay, 1955*a*). The concentration of metabolites in the haemolymph during fasting has not been investigated. Berridge (1966*b*) has shown that sucrose and lactose are unable to support urine formation by the Malpighian tubules of *Calliphora*. In a few experiments on *Carausius* tubules, in which the glucose normally present in the saline was replaced by sucrose or lactose, no consistent difference in rate of urine secretion was seen, at least during the first few hours. Thus if *Carausius* tubules are also unable to utilise sucrose and lactose, the effect of lack of these metabolites is too slow to account for the marked difference seen during the first hour between tubules in serum from fed and fasting insects. It therefore seems unlikely that differences in concentrations of these metabolites can account for the greater rate of urine secretion in fed animals, but the possibility that other metabolites are involved cannot finally be eliminated. If the differences are in fact due to differences in hormone titre, as seems likely, the ligation experiment indicates that the hormone is released anterior to the ligature, that is, in the head or thorax. Insects ligatured between head and thorax do not feed, so it has not been established whether the hormone is released in the head. The high concentration of the hormone in the corpus cardiacum, an established neurohaemal organ (Scharrer, 1952), suggests that this may well be the site of hormone release.

3. *Causes of variation in the level of hormone in the blood*

The level of hormone in the blood could be altered by variations in the rate at which it enters the blood (that is, in synthesis and release of hormone) or in the rate of removal from the blood, or in both. Diuretic activity is present in the brain, corpora cardiaca and sub-oesophageal ganglion of both fed and fasting insects, and no difference has been detected in the size of response of tubules to extracts from fed or fasting insects. This may indicate either that no differences occur or that the assay is not sensitive enough to detect the changes which occur. There is considerable variation in the response of tubules to a constant dose of hormone, and it would thus not be surprising if small differences in hormone content were not detected. The corpora cardiaca of *Carausius* were, however, assayed on the more sensitive tubules of *Rhodnius*, but no difference could be detected in the size of the response to extracts from fed or fasting insects (S. H. P. Maddrell, unpublished results). In *Rhodnius*, as much diuretic hormone can be extracted from the mesothoracic ganglionic mass immediately after diuresis as immediately before (Maddrell, 1962), and it thus seems that release causes very little reduction in the amount of hormone present. If inhibition of hormone release occurred during dehydration, this might cause inhibition of synthesis, so that the amount of hormone present could remain constant. Pflugfelder (1937), however, observed an accumulation of stainable material in the corpora cardiaca during desiccation, but this may represent some substance other than the diuretic hormone.

DISCUSSION

Extracts of the protocerebrum and corpora cardiaca have been shown to have a marked diuretic effect upon the Malpighian tubules of *Carausius*, while the corpora allata are inactive. This is also the case in *Dysdercus* (Berridge, 1966a), *Schistocerca* (Highnam, Hill & Gingell, 1965; Mordue, 1969) and *Anisotarsus* (Núñez, 1956). In *Rhodnius* also, the brain contains extractable diuretic activity (Maddrell, 1963). Electrocoagulation of the median neurosecretory cells of the protocerebrum causes distension of the abdomen of *Locusta* (Cazal & Girardie, 1968), *Gryllus* (Girardie, 1966) and *Calliphora* (Thomsen, 1952), suggesting the involvement of these cells in promoting diuresis, but the corpora cardiaca of *Locusta* and *Gryllus* contain anti-diuretic factors (de Bessé & Cazal, 1968). The brain and corpora cardiaca of *Periplaneta* contain an antidiuretic factor (Wall & Ralph, 1964; de Bessé & Cazal, 1968), and both diuretic and antidiuretic factors have been demonstrated in the corpora allata (Mills, 1967; Wall & Ralph, 1964). The corpora allata of *Apis*, too, contain a diuretic factor, while the corpora cardiaca contain an antidiuretic one (Altman, 1956). Whereas a diuretic factor has been demonstrated in the corpora cardiaca of *Carausius*, the corpora cardiaca of another phasmid, *Clitumnus*, have been shown to contain an antidiuretic substance (de Bessé & Cazal, 1968). The swellings of the lateral nerves of the medial nervous system, which have the structure of neurohaemal organs (Brady & Maddrell, 1967; Raabe, 1965), contain an antidiuretic factor in certain species (de Bessé & Cazal, 1968). It appears that there are marked differences between species both in the organs which may be involved in the control of urine secretion and in the factors which they are thought to produce.

It is uncertain why the optic lobes of the brain contain diuretic activity. It is possible that at least some of the activity of this region is due to 5 HT, which is known to occur in *Carausius* central nervous system (Gersch *et al.* 1963), and which stimulates urine secretion (Maddrell *et al.* 1969). The failure of BOL to block the action of brain extracts indicates, however, that all the diuretic activity of the brain is not due to 5 HT (Maddrell *et al.* 1969).

Until the diuretic hormone has been isolated and characterized chemically it is not possible to be certain that it is the same active factor which is found in the brain, corpora cardiaca, sub-oesophageal ganglion and serum. However, the similarity between the effects of all of these on secretory rate, ionic composition of the urine and trans-wall potential (D. E. M. Pilcher, 1970, in preparation) suggests that the active factor in serum is the same as, or very similar to, that in the other tissues. The occurrence of *Carausius* diuretic factor in the haemolymph is important in deciding whether it is a hormone of physiological significance. According to the classical definition a hormone must be released into the circulatory system in response to relevant physiological stimuli, and thus reach its target organ. Although de Robertis' (1964) concept of the unitary nature of neurohumoral mechanisms has led to wide acceptance of the idea that there is no fundamental difference between active factors released from the nervous system into the general circulation and those released into a restricted space close to the target organ, any factor which affects the Malpighian tubules of *Carausius* must enter the general circulation, since the tubules are not known to be innervated. The diuretic factor which can be extracted from the brain and corpora cardiaca of

Carausius appears to fulfill the criteria for a hormone, since its level in the haemolymph varies between fed and fasting, dehydrated insects, and the activity of tubules from fed and fasting insects *in vitro* can be related to Ramsay's (1955*a*) observations on tubules *in situ*.

It is possible to envisage a mechanism whereby the activity of the tubules could be controlled by this hormone in *Carausius*. As long as the animal has access to food the hormone would be released into the circulation at a rate equal to that at which it was being removed by the activity of the tubules and perhaps other tissues. When the insect is no longer able to feed, release of hormone would be reduced or cease, while the tubules would continue to inactivate the hormone remaining in the haemolymph. This is very similar to the mechanism proposed for *Rhodnius* (Maddrell, 1964*a, b*) and *Dysdercus* (Berridge, 1966*a*).

Whilst the mechanism for the hormonal control of tubule activity may be basically similar, there are differences in detail between *Carausius* and *Rhodnius* which reflect the differences in water-balance problem in the two species. *Rhodnius* faces the problem of excreting large volumes of fluid immediately after its blood meals, and of then conserving water for a long period until the next meal. In contrast with this, *Carausius*, a herbivore, spends much of its time on or near the food plant, so that food may be more or less continuously available. Thus periods of fasting are likely to be shorter than in *Rhodnius*, and *Carausius* does not need to dispose rapidly of large volumes of fluid. It has merely to maintain a fairly low rate of secretion most of the time, but be able to reduce the rate if necessary when food is scarce. Thus *Carausius* might be expected to be secreting diuretic hormone more or less continuously, whereas *Rhodnius* releases it only after its rare meals (Maddrell, 1964*b*). The difference between the problems of these two species is also reflected in the size of the diuretic response. If active haemolymph is added to *Rhodnius* tubules, there is a hundredfold increase in rate of urine secretion, whereas serum from fed *Carausius* causes only a doubling of rate. The threshold for response in 50% of *Carausius* tubules is around 0.15 pairs of corpora cardiaca/100 μ l. and for *Rhodnius* tubules about 0.02 mesothoracic ganglia/100 μ l. This difference in threshold may indicate differences in the amount of hormone present in the tissues, or in the sensitivity of the tubules, but in either case shows that the mesothoracic ganglion of *Rhodnius* contains a quantity of hormone capable of causing a far greater diuretic response than the combined brain, corpora cardiaca and sub-oesophageal ganglion of *Carausius*.

Whereas a rapid onset of diuresis, caused by diuretic hormone released close to the tubules from peripheral abdominal nerves (Maddrell, 1966), may be essential to *Rhodnius*, disturbances of the water balance of *Carausius* can probably be corrected over a longer period. The diuretic hormone appears to be released from the anterior end of the body, possibly from the corpora cardiaca, and to pass back to the tubules in the haemolymph.

The role of the Malpighian tubules in ionic and osmotic regulation can not be considered without reference to the activity of the rectum. Ramsay (1958) pointed out that the passage of all the small molecules present in the haemolymph into the urine, followed by selective resorption of metabolically useful substances in the rectum, is analogous to the process of ultrafiltration and resorption in the vertebrate nephron. Under the influence of the diuretic hormone urine of the same ionic composition is

secreted at a greater rate (D. E. M. Pilcher, in preparation), which without selective resorption in the rectum would achieve neither ionic nor osmotic regulation. The capacity for rectal resorption varies according to the state of hydration in *Schistocerca* (Phillips, 1964*a, b, c*) and there is evidence for hormonal control of the rectum in this species (Mordue, 1969) and in *Periplaneta* (Wall, 1967) and *Locusta* (Cazal & Girardie, 1968). Neurohormones C₁ and D₁ affect rectal resorption as well as urine secretion in *Carausius* (Vietinghoff, 1966, 1967), but in addition have been found to affect the frequency and amplitude of the cockroach heart (Gersch *et al.* 1960), the activity of the cockroach nerve cord (Gersch & Richter, 1963) and the colour of *Carausius* hypodermis (Gersch *et al.* 1960). It therefore seems likely that these substances are of pharmacological significance only in some of these tissues.

It is possible that the rectum and Malpighian tubules are controlled independently of each other, so that the various ions, water, metabolically useful small molecules and waste products may be retained or lost to a varying extent. It may be that the Malpighian tubules are controlled by the level of the diuretic hormone in the blood, while the rectal pads, which are relatively inaccessible to the haemolymph (Gupta & Berridge, 1966; Oschman & Wall, 1969), might be controlled by a substance released from the neurosecretory endings which have been demonstrated in rectal tissue (Gupta & Berridge, 1966; Oschman & Wall, 1969).

SUMMARY

1. Urine secretion by isolated Malpighian tubules of *Carausius* is accelerated by a diuretic hormone which can be extracted from the brain, corpora cardiaca and suboesophageal ganglion.
2. The level of this hormone in the haemolymph varies according to the state of hydration of the insect.
3. The hormone is inactivated by the tubules, and a mechanism is proposed whereby the tubules might be controlled by the hormone *in vivo*.

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