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HUMORAL REGULATION OF THE CEREBRAL NEUROSECRETORY SYSTEM OF *RHODNIUS PROLIXUS* (STAL.) DURING GROWTH AND MOULTING

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INTRODUCTION

The evident temporal discontinuity of release and synthesis of secretions in the cerebral neurosecretory system during the moult cycle of insects, demonstrated in many species (see Gabe, 1966), indicates that secretion of its hormones is not continuous and implies some form of regulation of the system. By analogy with vertebrates, the obvious way by which such regulation could be achieved is by some form of feedback from a factor whose presence in the blood is stimulated by the neurosecretory system. Neuroendocrine centres have been invested with the ability to provide the endocrine system with an overall 'directive' which is adjusted in response to a diversity of inputs (Scharrer, 1959) and have been compared with error detectors whose output is regulated by comparison of the effects of their activity with a reference standard (Scharrer & Scharrer, 1963). They are therefore well suited for the reception of feedback stimuli. That the insect neurosecretory system is subject to such a feedback regulation during metamorphosis has been suggested in early reviews (Williams, 1950; Bodenstein, 1954), but these suggestions have apparently not been tested experimentally nor extended to non-metamorphic moulting. As observed by Wigglesworth (1970), 'The existence of feedback responses in the physiological working of the body is accepted as a fact of life and in the insect at least such responses have not been subjected to much detailed analysis.'

The moulting cycle generally requires only initiation in order to proceed to completion (discussion in Locke, 1970), although certain specific processes may require secondary cues (e.g. tanning of the cuticle; Cottrell, 1962; Fraenkel & Hsiao, 1965). The activities of the cerebral neurosecretory system after the initiation of moulting may therefore be expected to be regulated in accordance with the progress of events within the developing insect. These events are primarily responses to the moulting hormone, which may therefore be expected to be centrally implicated in the mediation of such a feedback mechanism. The present paper provides evidence for a humoral factor originating in the thorax or abdomen of fifth instar *Rhodnius*, which regulates the secretory and synthetic activities of the medial neurosecretory cells during the moulting cycle and which is probably part of a feedback mechanism regulating moulting.

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MATERIALS AND METHODS

All operations were performed on fifth instar *Rhodinus* which had been reared synchronously from the egg and given blood meals of roughly standard size (Steel & Harmsen, 1971).

Most of the operations required parabiosis with a decapitated insect. The technique was as follows. The partner from which only the body was required was anaesthetized with carbon dioxide, decapitated with a noose of surgical silk and a small crystal of phenyl thiourea was placed on the wound. The body was maintained in carbon dioxide while the partner was similarly anaesthetized, the tip of its head was amputated and the cut end was juxtaposed with the neck wound of the other insect. The join was then sealed with a mixture of beeswax and synthetic resin and gentle pressure was applied alternately to the two abdomens to check for leaks and to ensure continuity of the two haemocoels. Further enforced circulation of haemolymph by this procedure has been found unnecessary (Wigglesworth, 1934). In some controls both partners retained their brains, being joined at the tips of their heads.

Seventeen pairs of insects joined in parabiosis were prepared for each of the combinations described under Results (total 102 operated insects). These pairs were maintained at 26 °C and about 70 % relative humidity in separate jars on blotting paper. It was found unnecessary to restrict their movement. After 7 days the success of each operation was evaluated by the following criteria: active peristalsis in both heart and gut, normal posture, coordinated locomotion, a withdrawal reflex to squeezing the prothoracic legs with forceps and circulation of haemolymph through the connexion between the partners. From those individuals in which both partners (whether decapitated or not) fulfilled all these criteria (at least 10 of each 17 pairs) the heads were removed and fixed in Masson's modified Bouin's fluid (Foot, 1933).

The cerebral neurosecretory system was examined in serial sections stained in paraldehyde fuchsin (PAF), prepared as described previously (Steel & Harmsen, 1971).

RATIONALE OF EXPERIMENTAL DESIGN

The moulting cycle in *Rhodnius* is initiated by feeding. It has been shown (Steel & Harmsen, 1971) that this stimulus in the fifth instar precipitates rapid release of stainable neurosecretory material from the brain; material stored in Type 2a cells is mobilized and discharged, resulting in the sequential conversion of these cells to Types 3, 2 and 1 as release continues. The phase of release following feeding is thus characterized by the absence of Type 2a cells and continues for 6–8 days. By day 8 the rate of synthesis exceeds that of release and the Type 2 and 1 cells produced earlier gradually reappear as Type 2a. After this time the brain is no longer necessary for moulting (Wigglesworth, 1934). If the mechanism responsible for the slowing of release and onset of synthesis at the approach of the 'critical period' is humoral, it should be possible to accelerate these changes in the neurosecretory system by exposing the brain of an insect at an early stage in the phase of release (1 day after feeding) to haemolymph from an insect which has reached the 'critical period' (8 days after feeding).

Insects at 1 day after feeding were therefore joined in parabiosis to insects at 8 days

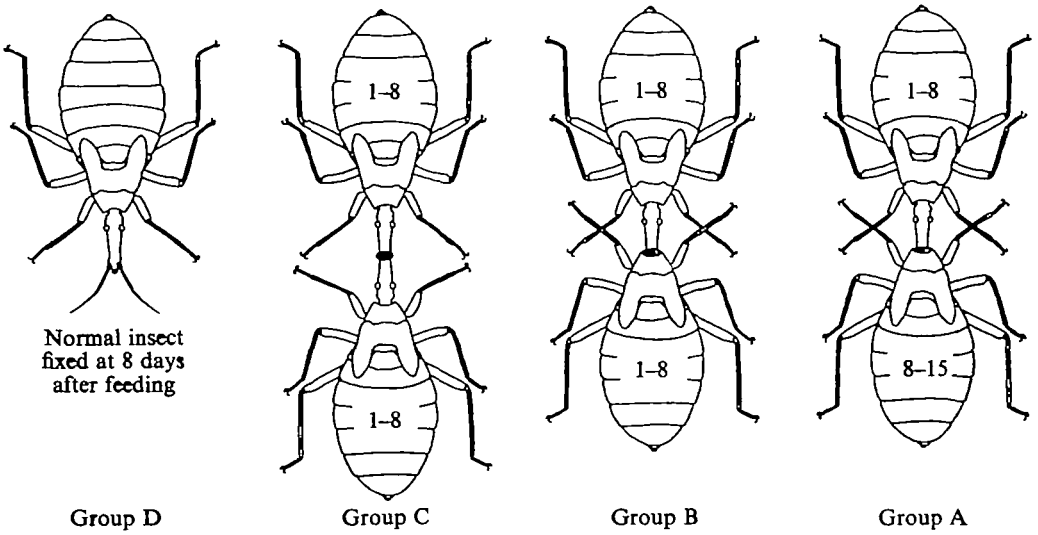

Fig. 1. Schematic diagram of the types of operations employed. The numbers on the abdomens indicate the period of time (in days after feeding) between the operation and fixation of the brain. Further description in the text.

after feeding which had been decapitated in order to obviate any possibility of interaction between neurosecretions from the brains of the two insects. The only known endocrine centres remaining in decapitated *Rhodnius* are the prothoracic glands which produce the moulting hormone (Wigglesworth, 1952). The neurosecretory system was examined 7 days after the operation, i.e. at effectively 8 days after feeding (Fig. 1, Group A). Selection of these times provides two advantages, as follows. First, the process of attenuation of release from cell Types 3, 2 and 1 is complete by day 8 in normal insects, but Type 2a cells, which appear only as the result of resynthesis, have not yet begun to accumulate; thus it is possible to detect either an inhibition of release or an acceleration of resynthesis with relative simplicity. Secondly, the decapitated insect undergoes parabiosis between 8 and 15 days after feeding, which, being immediately after the critical period, may be presumed to be the time at which any factor in the haemolymph would be at its most effective.

The principle control arrangement is identical with Group A except that both partners are of the same age (Fig. 1, Group B) and serves to distinguish any effects on the neurosecretory system due to surgical injury from those due to humoral changes. A second group of operated controls (Fig. 1, Group C) is the same as Group B save that both insects retain their heads. This combination was prepared for comparison with Group B to determine whether the cytological changes in the neurosecretory system are altered by the possible dilution of circulating neurosecretory substances which might result from the sharing of one brain between two bodies. Groups A, B and C are compared with unoperated individuals of the same age (8 days after feeding) drawn from the same population of insects (Fig. 1, Group D).

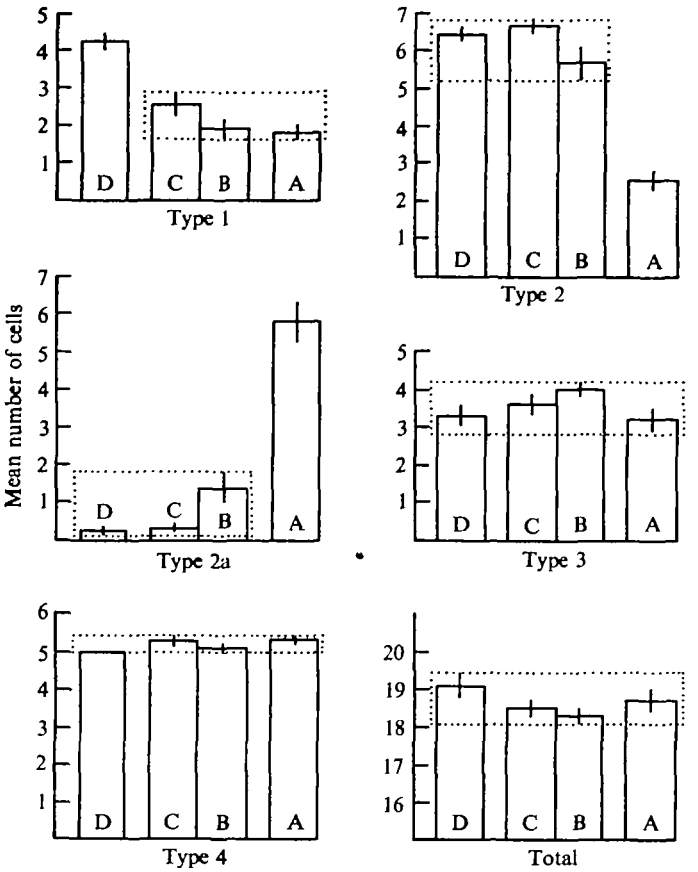

Fig. 2. Effects of parabiosis operations on the numbers of the five types of PAF-positive cells in the medial neurosecretory cell group on one side of the brain of fifth instar *Rhodnius*. The letter in each column denotes the type of treatment by the code adopted in Fig. 1. Vertical lines at the top of each column indicate the standard error. Dotted boxes enclose those groups among which no significant variation occurs in the number of the cell type considered (see Tables 1 and 2).

RESULTS

Examination of unoperated insects at 1 day after feeding confirmed that the behaviour of the neurosecretory system up to the time of the operations was as described previously (Steel & Harmsen, 1971): Type 2a cells had decreased in number almost to zero and had been replaced by an equivalent number of Type 3 cells, showing that the material stored in Type 2a cells prior to feeding had been mobilized and was in the process of release.

The mean numbers of each of the five cell types in each medial group resulting from the four treatments of Fig. 1 are shown in Fig. 2. It is obvious that the numbers of the cell types vary between treatments, but as the operated controls differ from both experimental and normal insects, the existence of more than one effect of treatment is apparent. It is first necessary to establish which of the cell types show significant variation in their number between treatments before proceeding to determine the nature of particular differences.

Table 1. *Results of non-parametric analysis of variance of the data of Fig. 2*

Cell Type	Type 1	Type 2	Type 2a	Type 3	Type 4	Total
Computed H	8·15	26·07	25·00	4·79	3·99	3·83
χ^2 corresponding to $P = 0{\cdot}05$	7·82	7·82	7.82	7·82	7·82	7·82
χ^2 corresponding to $P = 0{\cdot}001$	16·27	16·27	16·27	16·27	16·27	16·27
P that means are homogeneous*	$< 0{\cdot}05$	$< 0{\cdot}001$	$< 0{\cdot}001$	$> 0{\cdot}05$	$> 0{\cdot}05$	$> 0{\cdot}05$

The Kruskal-Wallis statistic, H, is distributed as χ^2 for large samples. χ^2 values obtained from tables of Fisher & Yates (1957).

* Probability that the observed numbers of the cell type considered are not significantly affected by the treatments A, B, C and D.

The results of non-parametric analysis of variance of the data of Fig. 2 according to the method of Kruskal & Wallis (1952) are summarized in Table 1. Values of $P > 0{\cdot}05$ are considered compatible with the null hypothesis that observed variations between treatments are random. Such values of P were obtained in the cases of cell Types 3 and 4 and of the total number of stainable cells. Significant values of H were obtained in the cases of cell Types 1, 2 and 2a. It is concluded that parabiosis significantly affects the numbers of cell Types 1, 2 and 2a in the medial group but is without affect on the numbers of cell Types 3 and 4 or on the total number of stainable cells. Significant changes in the numbers of three of the five cell types without change in the total number of cells imply that the conversions between cell types occurring between 1 and 8 days after feeding vary between the four treatments. In order to determine which of the treatments is responsible for the changes in the number of cell Types 1, 2 and 2a, individual comparisons of the numbers of these three cell types between the four treatments were made. These revealed two qualitatively different effects which will be presented separately.

(*a*) *The neurosecretory system in operated controls*

Both groups of operated controls differ from normal insects (Fig. 2). This observation suggests that the neurosecretory system is influenced by surgical injury. Table 2 shows that the number of Type 1 cells in all three operated groups differs significantly from normal. However, there are no differences in the number of Type 1 cells between the operated groups. The mean number of Type 1 cells in Groups A, B and C are all similar but all lower than the mean for Group D (Fig. 2). It is concluded that all three types of parabiosis operations significantly reduce the number of Type 1 cells by about two cells in each medial group independently of whether or not the partners are at the same stage of development. No other differences between control-operated and normal insects were found.

The absence of significant differences between the neurosecretory systems of the two types of operated controls (compare Groups B and C in Table 2) permits two deductions. First, that the magnitude of the above reduction in numbers of Type 1 cells is equal in all groups of operated insects and thus a similar reduction in Group A brains may be inferred. Secondly, that the neurosecretory system is unaffected by connexion of its supply of haemolymph to two bodies instead of one.

Table 2. *Effects of the four treatments on the mean numbers of the cell types found to vary significantly in Table 1*

Groups compared	Group A	Group B	Group C
	Type 1 cells		
Group B	0·06	—	—
Group C	3·83	2·37	—
Group D	13·00 $P < 0·001$	7·75 $P < 0·01$	9·20 $P < 0·01$
	Type 2 cells		
Group B	10·02 $P < 0·001$	—	—
Group C	15·14 $P < 0·001$	1·88	—
Group D	13·16 $P < 0·001$	2·05	1·79
	Type 2a cells		
Group B	12·55 $P < 0·001$	—	—
Group C	15·11 $P < 0·001$	3·07	—
Group D	13·30 $P < 0·001$	3·14	0·05

Experimental treatments are lettered according to the code of Fig. 1. The H value for each comparison is given, together with the corresponding value of P where $P \leqslant 0·05$.

(*b*) *The neurosecretory system exposed to haemolymph from older insects*

Three statistically significant abnormalities are found in the neurosecretory system of Group A insects. The first is the reduction by about two in the number of Type 1 cells noted above, which is independent of changes in the haemolymph. The second is a highly significant difference in the number of Type 2 cells found in Group A brains compared with both operated controls and normal insects ($P < 0·001$ in all three cases) (Table 2). The third abnormality is complementary to the second and consists of a highly significant difference between the number of Type 2a cells in Group A brains compared with all the other groups ($P < 0·001$ in all three cases).

The numbers of Type 2 cells and of Type 2a cells are both unaffected by the operational procedure (compare Groups B and C with Group D in Table 2) but are profoundly affected by the stage of development of the partner (compare Groups A and B). It is concluded that exposure to haemolymph from insects at 8–15 days after feeding reduces the number of Type 2 cells in each medial group on day 8 by an average of four cells and increases the number of Type 2a cells by between four and five cells (Fig. 2).

DISCUSSION

Effects of surgical injury

All the neurosecretory cell types found in operated insects were normal and no damage to any tissue was evident. Detailed quantitative analysis of the neurosecretory system of control-operated insects revealed only very minor differences from that of normal, unoperated insects. The effects of injury are trivial in comparison with those mediated by the haemolymph. The quantitative effects of control operations on the numbers of the various cell types must first be established so that they may be distinguished from humoral effects.

The only significant difference found between normal and control-operated insects is a reduction in the number of Type 1 cells by about two cells in operated insects. Since all the other cell types contain more PAF-positive material than do Type 1 cells, the two absent Type 1 cells must possess more material after 7 days parabiosis than they do in normal insects. Marginal increases (in no case greater than an average of one cell) occurred in the numbers of cell Types 2, 2a and 3 in different groups of operated insects (Fig. 2). It therefore seems that the two cells which would normally be of Type 1 on day 8 are present variably as Type 2, 2a or 3 at the end of 7 days parabiosis. It has been shown (Steel & Harmsen, 1971) that the first 8 days after feeding are characterized principally by the release of material from Type 2a cells which is manifest as a sequential conversion of Type 2a to 3 to 2 to 1. Type 1 cells are thus at this time the end-product of release via this pathway. The presence in operated insects of an average of two cells apparent as Type 2, 2a or 3 which would otherwise be of Type 1 therefore indicates a slight inhibition of release of material via this route. The variability of the fate of the Type 1 cells is compatible with attribution of the effect to surgical injury, which presumably varies somewhat between individuals. The absence of significant differences between control-operated groups of insects indicates that the general level of injury is the same in different groups.

Effects of haemolymph from older insects

Parabiosis of an insect at 1 day after feeding joined to an insect at 8 days after feeding for 7 days results in the above effects of surgical injury in addition to a decrease in the number of Type 2 cells by about four cells and an increase in the number of Type 2a cells by about five cells. It is therefore clear that the haemolymph of the older insect induced changes in the cytological appearance of the neurosecretory system.

Interpretation of the significance of the humorally induced changes requires first that the results be corrected for the effect of surgical injury. The retention of one or two cells as Type 2 or 2a in Group A brains as the result of surgical injury would tend to exaggerate the decrease in Type 2 cells and/or reduce the extent of the increase of Type 2a cells which cannot be attributed to injury. Either way, when the injury effect is allowed for, the decrease in number of Type 2 cells and increase in Type 2a cells become equal (4–5 cells in each case). In the absence of changes in any of the other cell types, this result indicates the conversion of Type 2 cells to Type 2a in Group A brains alone.

The conversion of Type 2 cells to Type 2a, which was induced humorally in this experiment, occurs also in normal brains, where it is regarded as the principle

cytological index of synthesis of neurosecretion (Steel & Harmsen, 1971). Moreover, this conversion is the dominant activity in the neurosecretory system of normal insects throughout the time period (8–15 days after feeding) when decapitated insects were found to be active in inducing it. In contrast, this conversion is never normally encountered in the period 1–8 days after feeding, for during this period release of material is occurring at a high rate (Steel & Harmsen, 1971); at day 8 in normal insects (Group D, Fig. 2) Type 2 cells are numerous and Type 2a cells are still few. Thus it appears that the mechanism responsible for the conversion of Type 2 to Type 2a between days 8 and 15 in normal insects is capable of inducing the same effect in younger insects in which it never otherwise occurs. The mechanism evidently mediates its effect via the haemolymph.

Group A brains produced 5–6 Type 2a cells during the 7 days parabiosis. This is a greater number than is ever found in normal insects during the fifth instar; production of this number of cells normally requires 20 days (Steel & Harmsen, 1971). There seem to be two alternative explanations for this tripled rate of production of Type 2a cells.

The rate of release between 1 and 8 days is normally great enough to prevent the production of Type 2a cells (Steel & Harmsen, 1971). If this release was unimpaired in Group A, production of Type 2a cells at three times the rate found in normal insects even when the rate of release is low would require an immense rate of synthesis. There is no evidence that such a rate is possible.

The second, and more conservative, explanation is that synthesis occurred in Group A brains at the normal rate for an insect at 8–15 days after feeding and the presence in these brains of more Type 2a cells than normal is due to the simultaneous inhibition of release from the neurosecretory system. Were this so, much of the material normally released between 1 and 8 days would remain in the cells so that less material would have to be synthesized by them before they would reappear as Type 2a. Synthesis at the normal rate would then produce more Type 2a cells per unit time.

It is therefore suggested that the massive conversion of Type 2 cells to Type 2a cells in Group A insects is a result of both stimulation of resynthesis and inhibition of release of neurosecretion. The fact that synthesis and the attenuation of release also occur simultaneously during days 4–8 after feeding in normal insects (Steel & Harmsen, 1971) implies that the mechanisms by which these two processes are regulated are closely interrelated. The primary effect of the humoral factor may be exerted on either process and it is not possible to determine which from the present experiments.

Nature of factor(s) mediating the effects

It is well known that digestion of the blood meal, development of the fat body and the numerous synthetic activities characteristic of the pre-moult period are all much further advanced by 15 days after feeding than at 8 days. The induced changes in the behaviour of the neurosecretory system may therefore be a response to the richer nutritional environment provided by the 8- to 15-day insect. Coles (1965) found that although amino acid levels in the haemolymph of fifth instar *Rhodnius* remained roughly constant throughout the moulting cycle, a continuous increase occurred in total blood protein during the first 10 days after feeding. The neurosecretory system may be sensitive to the concentration of one or more of these proteins.

A more attractive hypothesis is that the humorally induced changes in the neurosecretory system are produced by the moulting hormone, ecdysone. There is considerable circumstantial evidence in favour of this view. Wigglesworth (1952) found that the histological signs of secretory activity in the prothoracic glands of fifth instar *Rhodnius* reach a peak at 7–12 days after feeding. The decapitated insects producing the factor therefore contained maximally active prothoracic glands. The attenuation of release and onset of resynthesis of stainable neurosecretory material follow the same time-course in normal insects as the rise in activity of the prothoracic glands; completion of the former coincides with the peak of the latter. There is no other known endocrine centre in decapitated *Rhodnius* which is active at this time. If the changes described by Wigglesworth (1952) reflect the ecdysone concentration in the haemolymph, the brains in which attenuation of release and onset of resynthesis were induced prematurely had been exposed to haemolymph containing a prematurely high titre of ecdysone. It is unlikely that the ecdysone titre would be materially reduced by parabiosis to an insect containing a low endogenous titre of the hormone; indeed, it is known that an inactive prothoracic gland can be activated either by parabiosis to a second insect containing active glands (Williams, 1952) or by injection of ecdysone (Siew and Gilbert, 1971).

Possible mode of action of the factor

The ability of implanted corpora allata to produce accumulation of granules and enlargement of the nuclei of the medial neurosecretory cells of adult *Calliphora* led Thomsen & Lea (1969) to conclude that the corpus allatum influences the synthesis of neurosecretory material. In the present experiments, decapitation removed the corpus allatum from the *Rhodnius* which caused resynthesis in their partners; thus, the corpus allatum could be the source of a factor regulating the synthesis of neurosecretion in *Rhodnius* during the brain-dependent period only if stimulated to do so by the factor produced in the thorax or abdomen. That the factor may act on the brain via the corpus allatum is supported by the finding that ecdysone injections stimulate RNA synthesis in this gland (Siew & Gilbert, 1971). But the effects described by both the above teams may themselves be indirect effects.

There is some precedent for a direct effect of ecdysone on the central nervous system, for Haskell & Moorhouse (1963) found that an extract of *Bombyx* pupae containing ecdysone influenced the discharge patterns of central neurones of *Schistocerca;* further, the accumulation of stainable neurosecretory material beyond normal levels found in *Leucophaea* brains cultured *in vitro* can be offset by ecdysterone (Marks, Ittycheria & Leloup, 1972). If the neurosecretory system possesses a direct sensitivity to the hormone during the moult cycle *in vivo*, the present experiments constitute evidence of a feedback regulation of the endocrine system controlling moulting. In *Rhodnius* the moult cycle is initiated by nervous stimuli at feeding (Wigglesworth, 1934; van der Kloot, 1961) which provoke a rapid release from the brain of neurosecretory material (Steel & Harmsen, 1971) which presumably contains the prothoracotropic hormone which stimulates production of the moulting hormone (Wigglesworth, 1939, 1952). The present work suggests that the moulting hormone acts back on the brain where release is attenuated and resynthesis stimulated as its titre in the haemolymph rises.

If ecdysone does indeed regulate the behaviour of the neurosecretory system during the moulting cycle, it would appear that in both the normal situation and in parabiosis release of neurosecretions occurs at low ecdysone concentrations and is inhibited during rising concentrations, whereas resynthesis occurs at high ecdysone concentrations and is stimulated during rising concentrations. A falling titre of ecdysone would thus permit initiation of another phase of release and then synthesis of neurosecretions; moulting cycles would follow each other in continuous succession (unless arrested by other factors), each being initiated shortly before ecdysis of the previous instar, as in *Locusta* (Clarke & Langley, 1963). However, other regulatory mechanisms must also be involved, for the above proposals do not account for the eventual cessation of synthesis or the prolonged absence of release in the unfed *Rhodnius*.

SUMMARY

1. In normal fifth instar *Rhodnius* the cytological changes occurring in the medial neurosecretory cells (MNC) of the brain are very different before and after the 'critical period' for decapitation.

2. When a decapitated insect which has reached the 'critical period' (8 days after feeding) is joined in parabiosis to an insect with an intact cerebral endocrine system and which has not yet reached the 'critical period' (1 day after feeding) the MNC of the younger insect are induced to switch over from their normal sequence of cytological changes to those characteristic of the older insect. The induced changes do not occur in normal insects of the same age or in insects joined in parabiosis to others of the same age.

3. The nature of the changes indicates that release of stainable neurosecretory material is inhibited and its synthesis stimulated by the older insect. It is inferred that the haemolymph of insects which have reached the 'critical period' contains a factor which induces in the MNC an inhibition of release and a stimulation of synthesis. These are the events which occur in the MNC of normal insects at the critical period.

4. Considerable circumstantial evidence suggests that the factor is ecdysone. It may act either directly on the brain or on the corpus allatum. Its contribution to a feedback regulation of the endocrine system controlling growth and moulting is discussed.

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