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NEUROENDOCRINE CONTROL OF MOULTING CYCLE IN MEALWORMS: HISTOLOGY AND AUTORADIOGRAPHY OF CEPHALIC COMPONENTS

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Abstract—Fluctuations in the activities of the cephalic neuroendocrine system of larval mealworms (*Tenebrio molitor*) have been investigated by autoradiographic and histological techniques. Shortly after ecdysis, the proteinaceous granules in the cytoplasm of the 'A' type medial neurosecretory cells of the brain undergo a marked increase in numbers per cell and in chromophilia; both numbers and stain density reach a maximum about two-thirds of the way through interval between ecdyses and then both decline precipitously. The cyclic histological changes are nicely correlated with overall patterns of protein synthesis, as demonstrated by incorporation of ^{3}H-amino acids into the medial cells. Paradoxically no cyclicity was observed downstream: no fluctuations in numbers or chromophilia of stainable inclusions were detected in the axons of the medial neurosecretory cells, in the corresponding efferent nerves to the corpora cardiaca, or within the cardiaca themselves. Mechanisms are proposed to account for this apparent paradox, and the patterns of protein synthesis within the 'A' type medial neurosecretory cells are correlated with previously determined fluctuations in moulting hormone activity.

INTRODUCTION

DEVELOPMENTAL hormones coordinate a multitude of biosynthetic and morphological events in the succession of moulting cycles which occur as an insect progresses from first instar larva to reproductively mature adult. Both titre of ecdysone in the whole animal and also the intensity of cephalic neurosecretory activity fluctuate during insect development (e.g. SHAAYA and KARLSON, 1965; KAPLANIS *et al.*, 1966; FEIR and WINKLER, 1969). Numerous experiments have established that the medial neurosecretory cells (MNSC) of the brain somehow control ecdysone titre (e.g. FUKUDA, 1944; WILLIAMS, 1947, 1952; STUMM-ZOLLINGER, 1951). The mechanism is presumed to be by means of cyclic release of neurosecretion from the MNSC which results in a surge in the titre of ecdysone (HERLANT-MEEWIS and PAQUET, 1956; DELERMA, 1956).

For a demonstration of the cyclicity within the MNSC, three criteria are of particular significance: (1) the amounts of stainable neurosecretion present at various points in the instar, (2) the rates of synthesis of neurosecretory proteins within short intervals of the instar, and (3) the rates of release of neurosecretion from the cell bodies themselves and from the various structures downstream (axons of the MNSC, nervi corpora cardiaca I (NCCI) and the corpora cardiaca). The first of these three criteria has been most commonly monitored by histological techniques and the corresponding rates of synthesis and release inferred from changing stain density at successive points in the period between successive ecdyses. If cyclicity occurs, then the density of stainable neurosecretion might be expected to fluctuate as material is first stored, and later released, at appropriate times in the developmental program. As more species have been examined, some of the histological data have failed to support the prevailing belief in the cyclicity of the MNSC. In a cockroach (*Blatta orientalis*) Fuller (1960) detected fluctuations in the amounts of stainable materials in the individual neurosecretory cells, but could not discover co-ordinated changes among the several MNSC. In the bug, *Rhodnius prolixus*, STEEL and HARMSEN (1971) were unable to demonstrate cyclic release of neurosecretory material from the MNSC over the course of a larval instar. Attempts to resolve such difficulties have included application of radio tracers and subsequent autoradiography. Thus, HIGHNAM (1962) showed that in *Locusta*, the more densely staining medial cells readily incorporated labelled cysteine. And yet, GILLOT *et al.* (1970) could not demonstrate any correlation between the density of the cytoplasmic staining in the MNSC of *Melanopus sanguinipes* and the incorporation of labelled cysteine.

In the present paper we report the results of an analysis of biosynthetic patterns in the MNSC over

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the interval between ecdyses in the larval mealworm, *Tenebrio molitor*. Earlier histological studies on this species had suggested that stainable materials are found in the MNSC throughout the larval instar, but that fluctuations occur, such that stain density is highest before and after ecdysis and lowest midway between ecdyses (ARVY and GABE, 1953, 1954). Data from other beetle larvae, namely, those of *Leptinotarsa decemlineata* and *Hydrous piceus*, also indicate cyclicity in the MNSC (ARVY and GABE, 1953; DELERMA, 1956). The work reported below utilized autoradiographic techniques to define with some precision the intervals of peak amino acid incorporation (and presumably protein sysnthesis) in the MNSC of larval *Tenebrio*. Fluctuations in incorporation of amino acids are examined in context: compared with patterns of uridine incorporation (presumably a precursor of ribonucleic acid), considered in concert with the variation in stainable materials in the neuroendocrine system; and correlated with the whole body titre of moulting hormone activity (LACK and HAPP, 1976).

MATERIALS AND METHODS

Young mealworms (*Tenebrio molitor* L.) were purchased from a commercial supplier and maintained in our laboratory on a diet of Purina chick startena supplemented with potato as a source of moisture. All animals used for experimental purposes had been in our animal room for at least 2 weeks. Manipulations were performed on sixth or seventh instar larvae.

Histology

Neuroendocrine complexes were dissected from mealworms at various times over the course of a single instar. Brains and corpora cardiaca were fixed in modified Susa and stained with paraldehyde fuchsin. Tissues were sectioned at 9 μm. In addition, neuroendocrine complexes were fixed in performic acid and stained whole in depression slides according to the resourcin fuchsin technique of ITTYCHERIAH and MARKS (1971).

Autoradiography

(1) *Amino acid incorporation.* Protein synthesis was assayed by injecting, *in vivo*, a solution of four tritiated amino acids containing a total of 10 μCi/g wt of mealworm tissue. Each tritiated amino acid comprised one-quarter of the total radioactive solution. The following amino acids were used: glycine-2-^{3}H (specific activity = 2.2 Ci/m-mole, L-serine-3-^{3}H (specific activity = 6.3 Ci/m-mole), L-valine-^{3}H (specific activity = 31.6 Ci/m-mole) and DL-leucine-4,5-^{3}H (specific activity = 16.4 Ci/m-mole). All tritiated amino acids were purchased from Amersham/Searle.

All injections were performed with a 28 gauge Hamilton microsyringe, with the aid of a dissecting microscope. The syringe was inserted ventro-laterally through the third inter-segmental membrane and tritiated amino acids (2 μl) were injected into the first thoracic segment. After injection, the wounds were immediately sealed with paraffin and the mealworms were individually placed in 60 mm Petri plates containing filter paper. All animals were incubated for a period of 3 hr $\pm$ 2 min. Animals that showed signs of haemolymph loss were discarded. Brains were then dissected from surviving larvae and washed three times in a hypotonic solution of the same cold amino acids for 30 min.

The brains were fixed in aqueous Bouin's (0.5% trichloroacetic acid replaced the 5% acetic acid) and stained with resourcin fuchsin for whole mounts (ITTYCHERIAH and MARKS, 1971). Brains were sectioned at 3 μ. Autoradiographs were stored for two weeks at 0°C and were then processed in the usual manner (POLLISTER, 1969). Grains were counted with the aid of an ocular grid divided into squares of 5 μm^2. To determine the differential incorporation into neurosecretory cells relative to other larval brain tissues, we have used counts over other neurons in the region surrounding the 'A' type cells as background. Consequently, the counts of silver grains over extraneous brain tissue were subtracted from the counts over the cytoplasm of the 'A' cells in order to get a more accurate estimation of differential incorporation of amino acids into the 'A' cells.

A cold control (i.e. stained but not injected with tritiated amino acids) was similarly processed together with the test slides. Control slides show that prestaining with resourcin fuchsin did not induce spontaneous development of silver grains. Additional control brains (injected with tritiated amino acids) were incubated for 60 min at 37°C in 0.05 M phosphate buffer (pH 8.9) containing 0.1 mg/ml trypsin. These brains were then processed in parallel with the other experimental material.

(2) *Uridine incorporation.* Brains were dissected from ten mealworms on each day of the instar. Mealworms were anesthetized in saline and injected, *in vivo*, with 10 μCi H^3-uridine/g weight of mealworm tissue (specific activity = 5000 mCi/m-mole). For each mealworm the dosage of injected ^{3}H-uridine was individually calculated.

After injection the wounds were immediately sealed with paraffin. All larvae were incubated for a period of 3 h $\pm$ 2 min. Brains were then removed and washed three times in an isotonic solution of cold uridine for a period of 1 hr. Staining and histological preparations were identical to the ^{3}H-amino acid experiment.

Slides were incubated for three weeks at 0°C. All grain counts were determined at 1000× magnification with the aid of an ocular grid subdivided into squares of 5 μm^2.

Control slides showed that prestaining did not induce spontaneous silver grain development. Control slides were processed in parallel with the test slides.

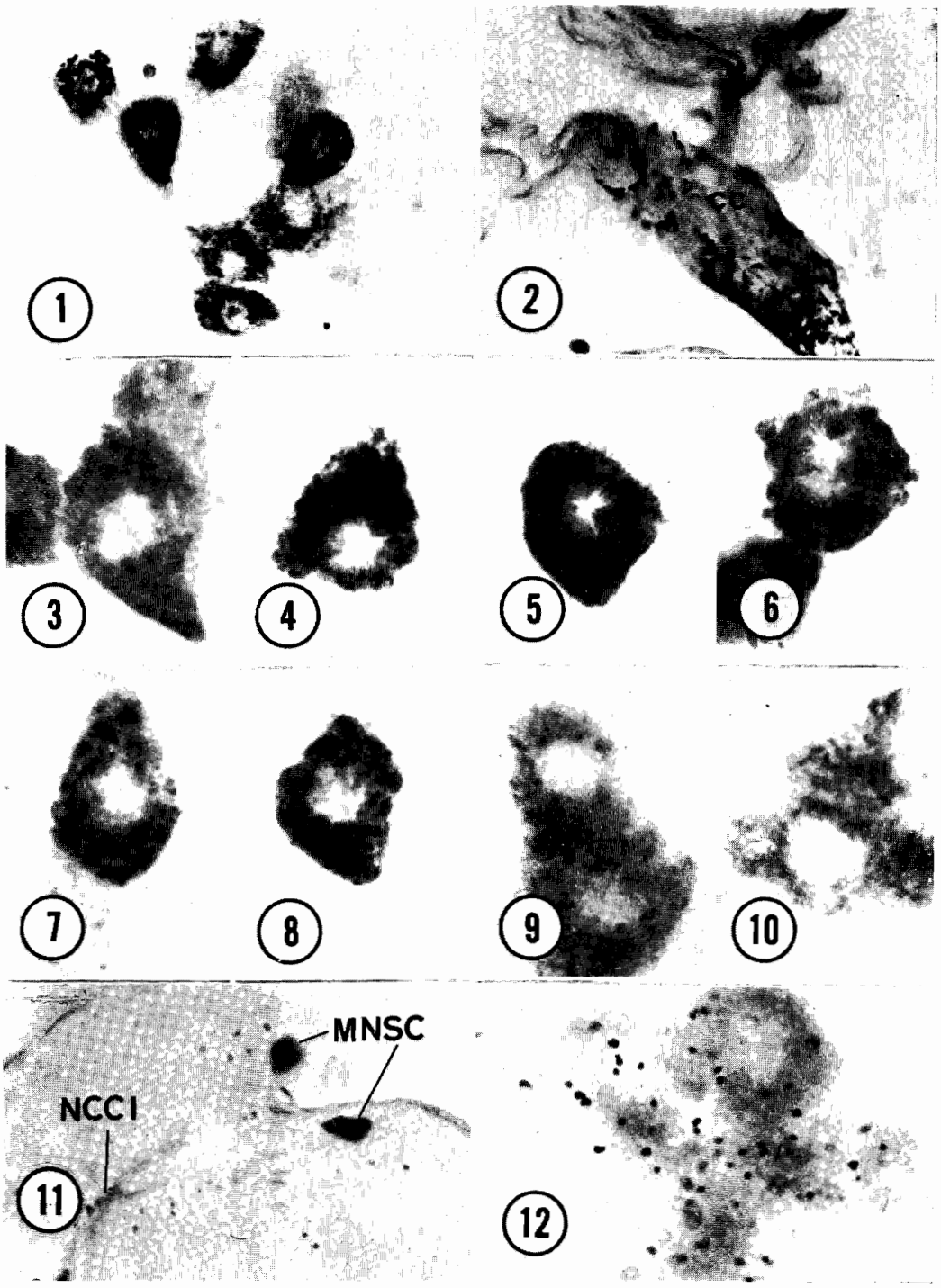

Fig. 1. The "A" type neurosecretory cells of the pars intercerebralis of a mealworm larva. Brain was stained with paraldehyde fuchsin.

Fig. 2. The corpora cardiaca of a larva (5 days after ecdysis) is filled with neurosecretion.

Figs. 3–10. Medial neurosecretory cells of the pars intercerebralis. Over the course of a single instar period, medial cells show changes in cytoplasmic grain size and stain density. Tissue fixed in modified Susa, embedded in paraffin and stained with paraldehyde fuchsin. Fig. 3. Newly ecdysed larvae. Fig. 4. One day after ecdysis. Fig. 5. Three days. Fig. 6. Five days. Fig. 7. Seven days. Fig. 8. Nine days. Fig. 9. Eleven days. Fig. 10. Twelve days.

Fig. 11. Medial neurosecretory cells (MNSC) and their axons which merge to form the nervi corpora cardiaca (NCCI).

Fig. 12. Autoradiograph of medial neurosecretory cells in par intercerebralis of mealworm larva at 6 days after ecdysis.

RESULTS

Histology

Under our rearing conditions [23°C, 12.12 (L:D) photoperiod], the interval between successive ecdyses of 5th and 7th instar larval mealworms was 12 days. Apolysis occurred on or about day 10 of the period between ecdyses.

Between 8 and 10 of the 'A' type neurosecretory cells can be recognized in the brain of most mealworms (Fig. 1). No striking divergence from this pattern was seen among the various larval instars. Characteristically, the soma of each 'A' type MNSC is $32 \times 18\ \mu$ and its spheriod nucleus is about $7\ \mu$ in diameter. We found no satisfactory criteria for dividing the various 'A' cells into sub-classes, neither on the basis of shape, staining characteristics, nor position. The perikarya of the 'A' type MNSC of each hemisphere lie close together in the posterior dorsal region of the pars intercerebralis. The axons of the MNSC run forward in the upper region of the neuropile, cross over, and descend in the anterior region of the brain. They exit from the brain in the NCCI and pass to the paired corpora cardiaca. These neurohaemal organs lie ventral to the brain and dorsolateral to the digestive tract, as noted by ARVY and GABE (1954).

Four to five larval brains were sectioned and examined at each day of the period between ecdyses. The 'A' type medial cells are paraldehyde fuchsin-positive, and resourcin fuchsin-positive throughout the entire period. However, there is a distinct cyclicity in the staining properties of the 'A' type MNSC as shown by paraldehyde–fuchsin staining. This cyclicity is seen as a change in both the size and density of the cytoplasmic content. During the first 6 days after ecdysis, the cytoplasmic content of the 'A' type MNSC progressively stains more deeply and the size of the granule greatly enlarges (Figs. 3 to 6). From day 7 through day 12, the size of the granules progressively decreases; there is a corresponding decrease in the stain density of the cytoplasm (Figs. 7 to 10). Variation among animals is negligible. These cyclic changes could not be correlated with any changes in the size of the perikarya, or of the nucleoli; nor could this cyclicity be correlated with changes in the amounts of stainable material found within the axons arising from the medial cells. There are no apparent differences in the amount of paraldehyde fuchsin-positive material in the medial cell axon or in the NCCI during the entire instar period (Fig. 11). Furthermore, this paraldehyde fuchsin-positive material can readily be traced from the medial cells to the corpora cardiaca in both whole mount preparations and serial sections.

Although considerable numbers of granules, presumably representing neurosecretion, are found in the peripheral regions of the corpora cardiaca, no cyclicity in granule size or density was observed during the present study (Fig. 2).

Autoradiography: amino acid incorporation

Amino acids were incorporated into insoluble materials of the MNSC at all times. Of the 125 larval brains examined, 120 showed incorporation into the MNSC to be greater than that of control brain tissue in the same animal. Furthermore, there was a clear pattern in the varying rates of incorporation (Fig. 13), a cycle which closely parallels the changes reported above in the staining intensity of the 'A' type MNSC.

Just after ecdysis, and over days 8 to 13, tritiated amino acids were incorporated at relatively low levels.

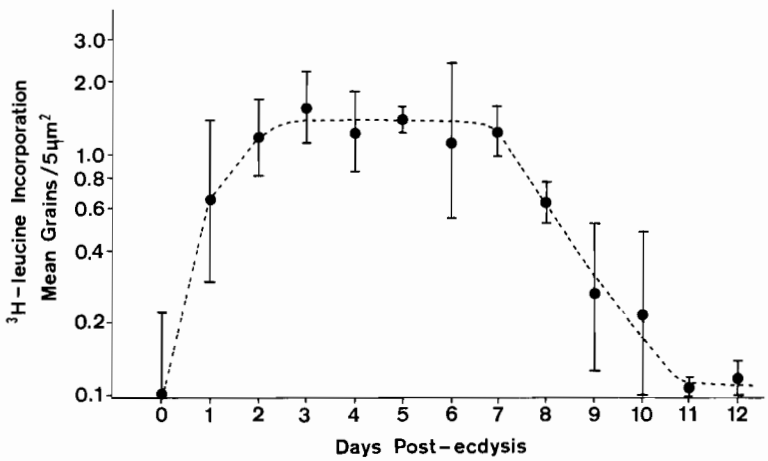

Fig. 13. Grain counts for ^{3}H-amino acid incorporation into the 'A' type medial neurosecretory cells in the interval between larval ecdyses. 8–10 brains were observed at each day. The mean grain counts are expressed as silver grains per $5\ \mu m^2$. The samples are heteroskedastic: Bartlett's test yielded X^2 of 150.62. The appropriate log transformation gave homogeneous variances and allowed an estimate of mean grain counts and 95% confidence intervals. The latter are indicated by vertical bars.

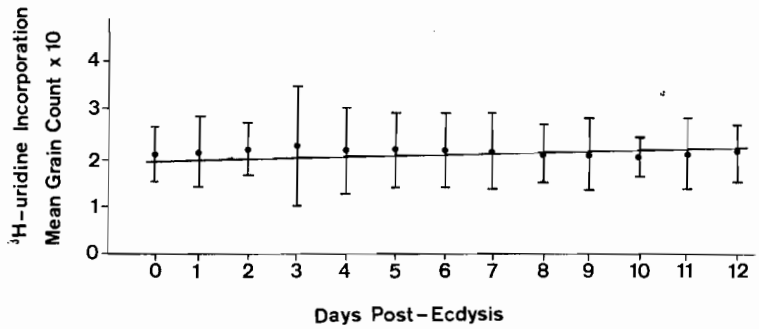

Fig. 14. Incorporation of tritiated uridine into the medial neurosecretory cells. The vertical bars indicate standard error.

From day 1 through day 8, tritiated amino acids were rapidly incorporated.

Differences between the degrees of tritium incorporation are significant as demonstrated by determination of 95% confidence intervals after logarithmic transformation (SOKAL and SNEATH, 1969).

Autoradiography: uridine incorporation

Medial cell incorporation of ^{3}H-uridine did not significantly differ over the period between successive ecdyses (Fig. 14).

Analysis of variance of cytoplasmic grain counts suggested that the mean values did not differ at the $P < 0.05$ level. Regression analysis suggested that the regression line plotted by the least squares method is described by the equation $Y = 1.7 \pm 0.003 \times$.

Cold controls showed that the staining of brain tissues prior to emulsion coating does not initiate spontaneous silver grain development.

DISCUSSION

Cyclicity of the medial neurosecretory cells

The production of ecdysiotrophic neurohormone has been attributed, at least in part, to the 'A' type MNSC of the pars intercerebralis of insects (VAN DER KLOOT, 1960). In larval *Tenebrio*, the 'A' type MNSC correspond in number, size, and staining reactions, to the Type 1 cells found in *Blaps* (FLETCHER, 1969). Cyclic changes in size and density of proteinaceous cytoplasmic granules were previously reported to occur in 'A' cells of the mealworm (ARVY and GABE, 1953, 1954), and these earlier findings were confirmed in the present study. Similar changes in stain density have been reported in *Carausius morosus* (HERLANT-MEEWIS and PAQUET, 1956), *Leptinotarsa decemlineata* (ARVY and GABE, 1954) and *Hydrous piceus* (DELERMA, 1956). It is dangerous to draw conclusions about the extent of biosynthetic activity with a neurosecretory cell solely on the basis of histological changes. Using radiocysteine, HIGHNAM (1962) found that the more densely stained neurosecretory cells in the brain of the adult locust rapidly incorporated labelled cysteine, but these cells released the product at a very low rate. In contrast, incorporation was low but release was high in lightly staining medial cells. In a later study, GILLOT *et al.* (1970) could not find any correlation between medial cell chromophilia and incorporation of labelled cysteine.

To determine whether the cyclic fluctuations in stain density in the 'A' cells of larval *Tenebrio* do, in fact, reflect differences in protein biosynthesis (and therefore presumably ecdysiotrophin biosynthesis), we have utilized autoradiography. Our results with larval *Tenebrio* differ from those obtained in adult locusts by both HIGHNAM (1962) and GILLOT *et al.* (1970). In larval *Tenebrio* the intensity of protein synthesis nicely parallels the changes in stain density of cytoplasmic granulation. We believe that both of our sets of results reflect cyclic increases in the synthesis of protein for export, and we expect that an ecdysiotrophin is one of these proteins.

But why is there an apparent discrepancy between the mealworm and locust systems? One might argue that following the divergence of the neuropteroid and orthopteroid lines which occurred in the paleozoic era, the neuroendocrine mechanisms of the two groups have come to differ significantly. We find this explanation unappealing. We suspect that the explanation of the discrepancy lies in three interrelated factors: differences in developmental stages between the locust (HIGHNAM, 1962) and *Tenebrio* (present study), different choices of labelled precursors, and perhaps most important, the lack of specificity of the staining reaction used for neurosecretory materials. The *Locusta* were adults whereas the *Tenebrio* were larvae. Stage-specific differences in secretions of the brain have been reported; for example, adult brains of *Rhodnius* do not trigger moulting cycles in larvae (WIGGLESWORTH, 1963). Autoradiography in *Locusta* involved only cysteine administration, whereas we injected a mixture of four amino acids; thus, the precursor spectrum (and presumably product spectrum) which we sample is rather more catholic. Finally, several types of neurohormones are thought to be synthesized in the medial cells of the

pars intercerebralis (e.g. THOMSEN and MOLLER, 1963; FRAENKEL and HSIAO, 1965); the histological stains might fail to distinguish between these or might merely be staining protein carriers common to all (GABE, 1966).

The failure to demonstrate fluctuation in the rates of RNA synthesis was disappointing. It might reflect failure of the bulk of our precursor to penetrate the cytoplasmic pool of nucleoside triphosphates (HUMPHREYS, 1973). Alternatively, the onset of synthesis of hormone-specific messenger RNA might simply be obscured by the much higher on-going synthesis of other mRNA's, rRNA's and tRNA's needed for cellular maintenance.

Export of the neurosecretory materials

HIGHNAM and WEST (1971) have suggested that cyclicity in the titre of circulating neurosecretory materials could occur in the presence of continuous biosynthesis of these materials if either the axons or the corpora cardiaca served as storage depots and pulsed their contents out into the haemolymph according to some storage release program. In larvae of *Rhodnius* (STEEL and HARMSEN, 1971) and *Locusta* (CLARK and LANGLEY, 1962) the histological evidence does suggest storage in axons or cardiaca and periodic release. But in mealworms, we see no histological patterns consistent with such cyclic storage or release in either the axons of the medial cells or within the corpora cardiaca.

Is the lack of apparent cyclicity in the NCCI and the cardiaca of mealworms inconsistent with our data on cyclic synthesis in the somata of the medial cells? We see no real conflict, and suspect that there is a simple explanation. First, we suspect that the axons and the corpora cardiaca act as though they were 'conduits' which must be full in order for neurosecretion to flow from the somata to the haemolymph. Addition at the axon hillock is assumed to be correlated with release of the previously synthesized material from the corpora cardiaca (We do not mean to imply a hydrostatic mechanism, however). Secondly, we believe that the biosynthesis of neurosecretory material, and its movement from the soma to the axon, are two separate processes which may be independently controlled. During the early part of the instar (Days 0, 1 after ecdysis), synthesis accelerates to exceed export considerably, and thus chromophilia increases. In the plateau phase, (days 2–6) synthesis remains at a high level, but export is also high—and so stain density is consistently high. Finally, over the latter phase of the instar, export exceeds synthesis and thus, chromophilia of the medial cell declines. Unfortunately, we do not have data on the titre of ecdysiotrophic neurohormone in circulation and can only infer its probable levels from the fluctuations in neurosecretory cell activity and ecdysone content of whole insect homogenates (LACK and HAPP, 1976).

Little evidence is available which shows a direct and precise correlation between fluctuations in circulating ecdysiotrophins and surges in ecdysone content in larval insects. ISHIZAKI (1969) could find no correlation between the titres of the two hormones in larvae of *Bombyx mori*. In our previous paper (LACK and HAPP, 1976), the bioassay data reveal the major peak in ecdysone content of mealworm larvae occurs at 8 to 10 days after ecdysis.

Both protein synthesis in the MNSC and moulting hormone content vary in a cyclic manner, but the peaks are successive, not coincident. Synthesis of neurosecretory protein precedes the increase in the moulting hormone activity. Such a pattern is quite consistent with the classical hypothesis that synthesis and release of ecdysiotrophin by the brain triggers a surge in circulating ecdysone. In other insect species, ecdysiotrophin appears to function to enhance RNA synthesis within the prothoracic gland. Increased RNA synthesis precedes ecdysone increase in saturniid moth pupae (OBERLANDER *et al.*, 1965), and it has been reported that brain implants can elevate the level of RNA synthesis in the prothoracic gland of *Periplaneta americana* (GERSCH and STURZBECHER, 1970). DOANE (1973) has suggested that the synthesis of specific messenger RNA by the prothoracic gland should require an early and sustained presence of ecdysiotrophin in order to allow sufficient time for the synthesis of protein, which in turn, will synthesize the β-ecdysone. Thus Doane attributes the function of ecdysiotrophin to stimulating and maintaining the complex cellular machinery necessary for the synthesis of the product of the prothoracic gland. In larval *Rhodnius*, ecdysiotrophin appears to be released over a considerable period of time, both before the rise of the titre of moulting hormone and even after the peak of moulting hormone activity (STEEL and HARMSEN, 1971). Such a sustained release is consistent with our results on mealworm larvae. What turns off the production of ecdysiotrophin? According to our data, the biosynthetic activity of the MNSC falls sharply before ecdysis, and in fact, the fall coincides with the rise in ecdysone titre. It seems quite possible that high levels of circulating ecdysone (or even sustained high titre of ecdysiotrophin itself) may negatively feed back on the MNSC to dampen their rate of protein synthesis. Such negative feedback has been suggested by MARKS *et al.* (1972). Certainly such control mechanisms deserve greater experimental analysis.

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