J. Insect Physiol., 1976, *Vol.* 22, *pp.* 1123 *to* 1125. *Pergamon Press. Printed in Great Britain.*

NEUROENDOCRINE CONTROL OF MOULTING CYCLE IN MEALWORMS: BIOASSAY OF MOULTING HORMONE

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(*Received* 30 *December* 1975; *revised* 12 *February* 1976)

Abstract—The moulting hormone content of mealworm homogenates was determined by injection of partially purified fractions into abdomens of mature larvae of *Musca domestica*. In mealworms with a 12-day interval between ecdyses, moulting hormone was at a maximum at 8 days.

INTRODUCTION

ACCORDING to the classical scheme for the control of apolysis, an increased titre of circulating moulting hormone leads to apolysis and deposition of new cuticle. Moulting hormones are presumed to be liberated from the prothoracic glands, which, in turn, are regulated principally by ecdysiotrophin produced by the median neurosecretory cells of the pars intercerebralis (references in WIGGLESWORTH, 1964; WYATT, 1971; DOAN, 1973; GILBERT and KING, 1973). During the larval stages of Lepidoptera and Diptera, fluctuations in the body content of moulting hormone (MH) have been demonstrated by bioassay (SHAAYA and KARLSON, 1965a, 1965b; KAPLANIS *et al.*, 1966; BARRIT and BIRT, 1970).

ROMER (1973) has recently investigated fluctuations of MH-activity in the larva of the mealworm, *Tenebrio molitor*. Romer homogenized ecdysial glands and oenocytes, and assayed the native or denatured total homogenates in the *Calliphora* test (ADELUNG and KARLSON, 1971). His results show detectable amounts of moulting hormone activity throughout the instar, and he concludes that there are two approximately equal peaks in the moulting hormone activity of ecdysial gland homogenates. It is difficult to reconcile the high 'background' level of moulting hormone activity (usually at least 50% of peak value) which Romer reports, with a classical scheme of fluctuating titres. ROMER (1973) suggested that the content of circulating hormone could be significantly altered by differential activity of a hormone inactivating system.

In more recent studies by DELBECQUE *et al.* (1975), larvae of *Tenebrio* were staged by the 'ocular' method of Stellwaag-Kittler (1954) and the β-ecdysone content was determined by gas chromatography and mass spectroscopy. The data reveal a single major peak in MH-activity and no sustained background activity.

In the present paper, we report the results from a re-examination of moulting hormone content of whole larval *Tenebrio*. Homogenates were differentially extracted to purify partially the ecdysones (OHTAKI *et al.*, 1968) and the extracts were assayed by injection into *Musca* larvae, which are more sensitive to ecdysones than are larvae of *Calliphora* (KAPLANIS *et al.*, 1966). In a subsequent paper (LACK and HAPP, 1976), we will examine the changing rates of amino acid incorporation into the medial neurosecretory cells of the pars intercerebralis over the interval between ecdyses.

MATERIALS AND METHODS

Mealworms, *Tenebrio molitor*, purchased from a commercial supplier, were maintained in our laboratories on a diet of Purina chick startena supplemented with potato as a source of moisture. All animals used had been in our animal room for at least two weeks. Manipulations were performed on sixth or seventh instar larvae.

Moulting hormone was assayed by injection of samples into mature larvae of *Musca domestica* (NIAMD strain). Adult flies were maintained on a diet of sucrose and powdered milk, and maggots were reared in plastic boxes containing CSMA diet.

Moulting hormone was extracted from mealworms by the technique of OHTAKI *et al.* (1968). Twenty animals (on each day of the instar) were homogenized in acetone: methanol (1:1) and the ecdysones were partially purified by their differential solubilities in alcohols, including their partition into the n-butanol epiphase. The final extract was stored in 100% methanol at $-20°C$, at a concentration of *ca.* two mealworm equivalents/ml. We did not attempt to separate the α- and β-ecdysones from one another.

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The assay technique was essentially that of KAPLANIS *et al.* (1966). One-half to one ml of methanolic extract was evaporated to dryness in a stream of nitrogen and resuspended in 50 or 100 μl of distilled water. All injections were 3 μl in volume. Each was delivered from a 10 μl Hamilton microsyringe with a 28 gauge needle into the ligatured abdomen of the larva. Controls included injections of 70% methanol, 5% methanol, 5% methanol containing β-ecdysone (Mann Laboratories, ecdysterone), distilled water, and distilled water containing β-ecdysone.

Moulting hormone activities of our test extracts were scored by two indices, termed the sclerotization index (SI) and the pupation index (PI). After carefully excluding all false positives due to surgical injury, we scored each assay animal as positive (detectable sclerotization) or negative (no sclerotization). The percentage of animals scored positive for each group is the sclerotization index. Whenever active extracts induced complete sclerotization and contraction of the isolated abdomen (equivalent to full pupation), the animals were scored positive for pupation and the percentage of positives per test was the pupation index.

RESULTS AND DISCUSSION

Isolated abdomens of mature larval *Musca domestica* sclerotize when injected with an aqueous solution containing 6 ng of β-ecdysone. When injections are successful, both the anterior and posterior parts of the bioassay animal appear red-brown. The cuticle has a flaky texture. The abdominal region is rounded and shows no sign of haemolymph loss. When viewed under a dissecting microscope, the cuticle is sufficiently transparent for the normal movement of haemolymph to be seen within the isolated abdomen. Dissection reveals a clear haemolymph, devoid of melanin. The fat body remains intact and exhibits no sign of decomposition.

Table 1 shows the result of the control experiments. In 22 sham injected abdomens, there was no sign of moulting hormone activity. Wounding, due to insertion of the syringe, did not appear to stimulate the sclerotization process. Careful removal of the syringe minimized haemolymph loss, and resulted in negligible signs of dehydration. The fat body remained intact.

Table 1. Control experiments for the bioassay of mealworm extracts in isolated abdomens of mature larval *Musca domestica*

Test	No. of injections	No. of surviving animals	SI*	PI*
Sham	22	22	0	0
Distilled water	25	23	0	0
5% Methanol	10	7	0	0
5% Methanol + 6 ng β-ecdysone	12	12	75% 33%	
Distilled water + 6 ng β-ecdysone	78	63	74	47

* SI Sclerotization Index.
PI Pupation Index.
See text for further explanation of these indices.

To determine whether the solvents used in the extraction, purification, and storage of test samples might initiate sclerotization, groups of 15 assay animals were injected with standard quantities (3 μl) of 70% methanol, absolute methanol, or butanol. In all the abdomen discolored within a few minutes (methanol) or hours (butanol). On the following morning, the animals appeared to be dehydrated. Dissection of the abdomen revealed decomposed fat body but the abdominal cuticle remained unsclerotized.

Since the extracts of *Tenebrio* larvae were stored in methanol, we were concerned that traces of methanol (persisting after evaporation) might interfere with the bioassay or mask the effects of ecdysone. To test this possibility, a solution of 5% methanol:95% water was injected into 10 assay abdomens. The data (Table 1) show good survival, and the seven surviving animals showed no signs of sclerotization. Death was attributed to haemolymph loss in the other three cases. When 6 ng of β-ecdysone were included in the 5% methanol injection, 9 of the 12 animals showed signs of sclerotization and 4 abdomens appeared to pupate. As these results are very similar to those with ecdysone in distilled water (Table 1), we concluded that minor methanol contamination would not interfere with our assay.

To determine whether significant loss occurred during extraction and purification, commercial β-ecdysone was processed in the same manner as were extracts of mealworms. The extraction technique did not inactivate β-ecdysone, although there was a loss of approximately 20%.

The bioassay of mealworm extracts in the ligatured abdomens of larval *Musca domestica* revealed the peak in moulting hormone activity (both by the sclerotization index and the pupation index) at eight days after ecdysis. Lesser activity was detectable at 9 and 10 days (Fig. 1). Within the limits of the technique, no moulting hormone activity was detected at any other time within the instar.

Larval endopterygotes have been characterized by a single, usually low peak in moulting hormone activity (DOANE, 1973). In larvae of *Tenebrio*, our data indicate that this peak occurs at day 8 to 10, somewhat before apolysis, which takes place at day 10. Since 6 ng of Mann ecdysterone corresponds to one-eighth mealworm equivalent, the apparent ecdysterone content of a single mealworm is about 50 ng. The average live weight of a mealworm at this state was 108 mg. Therefore, the peak ecdysone is *ca.* 400 μg/kg of live weight, a value that falls within the range of ecdysone content found in other species (GILBERT and KING, 1973).

ROMER (1973) reported a progressive increase in

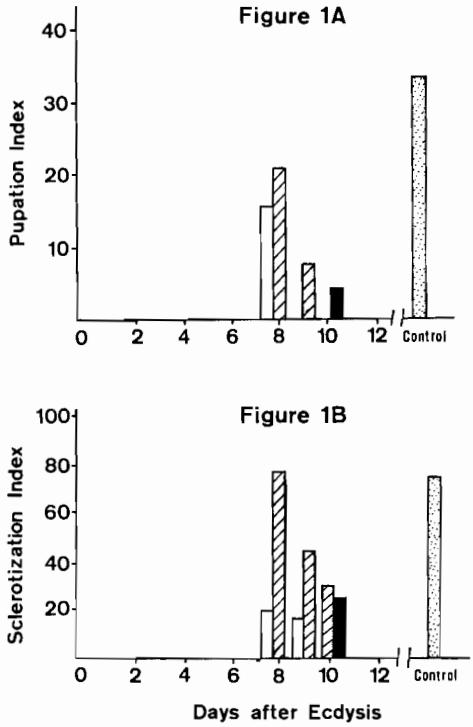

Fig. 1. Moulting hormone activity in mealworm extracts. At least twenty isolated abdomens of *Musca* were injected with extracts of larval *Tenebrio* from each day. Three dose levels were employed representing 1/32, 1/16, and 1/8 of a mealworm equivalent per 3 μl injection. Higher dose levels were impractical for the concentrated mealworm extract could not be completely suspended in the 3 μl vehicle. Even at the highest dose (1/8 mealworm equivalent), no activity was detected at days 1 to 7 or on day 11 and 12. Dose levels: □ 1/32 mealworm equivalent; ▨ 1/16 mealworm equivalent; ■ 1/8 mealworm equivalent.

moulting hormone activity toward the latter part of the interval between ecdyses in *Tenebrio*. In addition, he reported a marked peak just after ecdysis. We do not fully understand the discrepancy, but part of the answer may lie in differences in technique. We used partially purified moulting hormone fractions from larvae, whereas Romer used gross homogenates of ecdysial glands. Furthermore, Romer utilized a *Calliphora* bioassay while we employed *Musca* as assay animals. It is possible that *Musca* and *Calliphora* might exhibit species differences in sensitivity to a heterogeneous mixture of 'puparium-building substances' in extracts of mealworms.

Our data agree substantially with the results of DELBECQUE *et al.* (1975), which show a major peak of ecdysone in the latter portion of the interval between ecdyses and also show none of the 'background' activity in the early portion of that interval. DELBECQUE *et al.* (1975) also reported a secondary peak in ecdysone titre 2 to 3 days before the major peak. We did not detect the secondary peak. The discrepancy may reflect differing sensitivities of the assay methods, or perhaps stem from the fact that DELBECQUE *et al.* (1975) utilized *last* instar larvae whereas our animals were younger.

Acknowledgements—This study was supported in part by grants from the National Science Foundation (GB-36284) and the National Institute of Health (GM-21711) to G.M.H. We thank KIRBY MAXWELL and JANELLE SIMON for typing this manuscript. Dr. ROBERT GOODFELLOW, currently at Fordham University, kindly supplied the *Musca* culture.

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