ASSAY FOR ECDYSONE (MOLTING HORMONE) ACTIVITY USING THE
HOUSE FLY, MUSCA DOMESTICA L.

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ABSTRACT

Larvae of the house fly, Musca domestica L., were used as the test
insect in a modified assay for ecdysone. The house fly is easily reared
by using standard procedures and provides a high yield of organisms suitable
for bioassay. The quantity of α-ecdysone needed for about
~CF$

pupation in

the abdomens of ligated house fly larvae was 0.005-0.006 μg, one-third to
one-fourth the amount needed to effect the same response in the Calliphora
test.

INTRODUCTION

The ecdysones are steroid hormones that regulate molting and meta-
morphosis in insects (1,2). Although a number of physiological systems
may be used to detect ecdysone activity (1,3), the standard method of
assay for these molting hormones has been the ligated abdomens of larva
of the blowfly, Calliphora vicina Robineau-Desvoidy (= erythrocephela).
This assay, designated the Calliphora test, was the major assay used
throughout the research that resulted in the isolation of α-ecdysone from
the silkworm, Bombyx mori (L.) (4,5) and its characterization as 2β, 3β,
14α, 22R, 25-pentahydroxy-Δ7-5β-cholesten-6-one (6). However, during our
studies on the molting hormones of insects, we examined a number of insect
species as to their suitability as assay organisms and found that if larvae
of the house fly were substituted for Calliphora, we could obtain a
reliable bioassay and could also obviate certain disadvantages which we
found with Calliphora. This paper presents pertinent information for the
use of the house fly as an assay insect for molting hormone activity.

MATERIALS AND METHODS

Rearing of Insects. For these tests, we used a NAIDM-1498 strain of house flies reared by the CSMA procedure (7). The diet, scaled down proportionately to support the growth of about 1750 larvae, was prepared as follows: To each of three cylindrical glass jars (14.5 x 19.8 cm) lined with plastic bags containing 300 g of CSMA medium was added 660 ml of water in which had been dissolved 4.4 g of brewers yeast and 8.8 ml of Diamalt (8). After thorough mixing, the media were allowed to ferment for about 18 hours at 28° C (RH 50%). The following day, eggs were collected within one hour from 5- to 6-day-old adult house flies held on a dry diet mixture of sucrose, dry defatted milk, and yeast hydrolysate (5:4:1). The eggs were suspended in water, and 1750 ±50 eggs were added to each jar with a calibrated pipette. The larvae were removed from the medium for ligation 4 to 5 days later when at least 10% of the total population in the jar had pupated. First the larvae were concentrated in the bottom of the jar by slowly removing the upper, dried portion of the medium and the pupae. The remaining medium and larvae were transferred to a Berlese funnel attached to a glass container. When the larvae were exposed to the light, they migrated through the sieve of the funnel into the glass container. Then they were collected and rinsed thoroughly with water.

Ligation of Insects. The larvae were ligated on the 7th or 8th segment (9) with a single overhand knot by using 50 weight cotton thread. The knot was tightened but not to the extent that the insect could neither retract nor protrude its mouthparts. Ligations were usually begun in the morning and terminated by noon. Groups of 50 to 150 of the ligated organisms were placed in petri dishes (15.0 x 2.0 and/or 10.0 x 2.0 mm) that were lined with filter paper (10), slightly moistened with water to prevent dessication, and held at 28° C. The next morning the organisms which had pupated in the anterior end were removed and held about 5 to 6 hours before injection that same afternoon.

Injection of Insects. Injections were made as aqueous solutions of 0.8 to 5 µl, with 3 µl as the preferred volume, using a micrometer-driven syringe with a 28-gauge needle. Before injection, the insect and needle were wiped lightly with cotton moistened with 0.1% sodium hypochlorite. The needle was inserted just behind the ligature and extended posteriorly almost to the tip of the abdomen. The insect was ligated two segments behind the first ligation. After the injection, the larva was removed from the needle, and the second ligation was tightened. Controls injected with distilled water and an ecdysone standard prepared just before use were run concurrently in all tests. The next morning the organisms were evaluated according to the system for the Calliphora assay (1): A score of 1 for complete, 0.75 for marked, and 0.5 for slight. The results were expressed as a percentage of the number surviving.
RESULTS AND DISCUSSION

Other species of Diptera have been previously evaluated and compared with Calliphora. These insects were either less sensitive and/or otherwise unsuitable for the bioassay of ecdysone (1). We have found that the house fly, which we have used extensively in our nutritional and biochemical studies on sterols (11,12,13,14), is also useful as a test insect for molting hormone(s). A single container of 1750 larvae usually provided more than the necessary number of organisms for ligation. In 111 tests over a 2-year period, in which approximately 56,000 larvae were ligatured by 12 different operators, the overall average of organisms that pupated at the anterior end 24 hours after ligation was $20 \pm 8.5\%$ (S.E.) or about double that reported for Calliphora (1). In more experienced hands, however, yields of 35 to 40% with the house fly are not unusual. Also if insects are selected again 6 hours later, or 30 hours after ligation, the total yield may be as high as 55 to 65%.

One arrives at the proper tightness of the ligature only through experience; a too loosely tied ligature results in insects that pupate in both the posterior and anterior end. When the ligature was tightened so the insect could not retract or protrude its mouth parts following ligation, the number of test organisms was reduced to a fraction of that obtained with a normal ligature, and an increased number of the insects pupated only posteriorly to the ligature (Table 1). It is not currently understood why the overtight ligature has these effects.

Low yields also result from using larvae that are too young. The data in Table 2 relate the percent pupation of the total population to
Table 1
The effect of normal and tight ligation on anterior and posterior pupation in house fly larvae

<table>
<thead>
<tr>
<th>Ligation</th>
<th>Percent pupation</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
<td>Posterior</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>34.0</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Tight</td>
<td>4.3</td>
<td>10.0</td>
<td></td>
</tr>
</tbody>
</table>

*Average values from 31 groups of 100 house fly larvae per group with normal and tight ligation compared concurrently on each group of test organisms.

the percent of ligated organisms that developed a puparium at the anterior end. No major difference was found in the average percent yield (17-20%) from populations in which 11 to 90% of the insects had previously pupated. However, in those containers in which 0 to 10% of the population had pupated, the yield of organisms for bioassay was reduced about one-half. In our work, we used larvae in which 10% or more of the total population had pupated.

Table 2
Percent pupation of house fly larvae at time of ligation and percent yield of test insects

<table>
<thead>
<tr>
<th>Pupation (%)</th>
<th>Number of Tests</th>
<th>Number of Insects ligated</th>
<th>Yield of test insects* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>8</td>
<td>4100</td>
<td>11 ±8</td>
</tr>
<tr>
<td>11-30</td>
<td>16</td>
<td>9800</td>
<td>20 ±7</td>
</tr>
<tr>
<td>31-50</td>
<td>18</td>
<td>10600</td>
<td>19 ±6</td>
</tr>
<tr>
<td>51-75</td>
<td>26</td>
<td>11300</td>
<td>19 ±9</td>
</tr>
<tr>
<td>76-93</td>
<td>16</td>
<td>3400</td>
<td>17 ±7</td>
</tr>
</tbody>
</table>

*Mean ± standard error.
The organisms that pupated at the anterior end 24 hours after initiation of ligation were held under observation for 5 to 6 hours for spontaneous pupation in the posterior end, prior to the selection of insects for injection. In 141 control tests, spontaneous posterior pupation was less than 1.0% under the conditions described. We chose to inject the organisms the day after ligation for the following reasons: 1. The shorter time interval required for the detection of spontaneous posterior pupation in the ligated house fly larva, 2. reduction in the time required for assay, and 3. reduction in the mortality that results from a longer holding period.

The number of organisms used for assay was dependent on the object of the test. Five insects were adequate to survey extracts or fractions for activity or for the "bracketing in" process. However, for quantitative measurements, 20 to 25 insects were required. As reported for Calliphora (15), comparisons were best made within a test, and comparisons between tests must be made by using ecdysone standards. The use of a standard has been emphasized for Calliphora (15). For more precise assays, we preferred to use two standards, one which gave a response of about 60% and another which produced a response of about 20%. The response of controls treated with ecdysone served as an indicator of the physiological state of the test organisms and provided a basis for comparing the results with those from previous or subsequent tests.

The response of the house fly to various dosages of pure α-ecdysone isolated from the tobacco hornworm (16) is summarized in Table 3. The amount of hormone that gave about 60% pupation of the test insects was 0.005-0.006 μg (= 1 house fly unit); the amount of ecdysone required for
an equivalent response with Calliphora is about 0.02 µg (15,16) or three to four times that for the house fly.

Table 3
Percent response of ligated house fly larvae injected with various dosages of α-ecdysone*

<table>
<thead>
<tr>
<th>Dose** (µg)</th>
<th>Response*** (% ± SE)</th>
</tr>
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<tbody>
<tr>
<td>0.002</td>
<td>23 ±13</td>
</tr>
<tr>
<td>0.004</td>
<td>42 ±5</td>
</tr>
<tr>
<td>0.005</td>
<td>51 ±4</td>
</tr>
<tr>
<td>0.006</td>
<td>61 ±8</td>
</tr>
<tr>
<td>0.008</td>
<td>68 ±4</td>
</tr>
<tr>
<td>0.012</td>
<td>81 ±3</td>
</tr>
</tbody>
</table>

*Isolated from the tobacco hornworm, 1 Calliphora unit = approx. 0.02 µg (16).

**In 3 µl.

***Average values from 3 replicates with 25 insects at each dose. Percent pupation on container 10-25%; average weight of larvae 28 ±2 mg.

For the past several years the house fly assay has been used in our laboratory to follow steroid metabolism in insects, to determine the titer of ecdysone(s) in the developmental stages of several species of insects, and recently in the isolation of two ecdysones from the tobacco hornworm (16). In certain aspects of our work, the Calliphora test and the house fly assay were compared and the Calliphora test has also been used to confirm certain results obtained with the house fly assay. The use of the house fly was found to greatly simplify the assay for ecdysone, and in our hands has provided an assay that was as reliable as the Calliphora test.
ACKNOWLEDGMENT

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REFERENCES

10. Reeve-Angel filter paper grade 230, size 9 cm and 15 cm. Mention of a company or a proprietary product does not necessarily imply endorsement by the U. S. Department of Agriculture.