Hormonal Effect on Cultivated Insect Tissues

I. Effect of Ecdysterone on Cultivated Testes of Diapausing Rice Stem Borer Larvae (Lepidoptera: Pyralidae)

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(Received April 24, 1969)

The testes obtained from diapausing rice stem borer larvae were cultivated in the medium CSIM-2F, lacking any insect blood, in order to investigate the morphological changes of male germ cells in vitro, and it was ascertained that spermatogenesis was occasionally induced in the testes considerably a long time after the onset of cultivation. Further study was carried out to make sure the effect of a moulting hormone, ecdysterone, on the testes in vitro. From the results showing a remarkably rapid development of both testes and spermatocysts of the diapausing larvae, it was concluded that ecdysterone acted to promote spermatogenesis of the rice stem borer larvae.

INTRODUCTION

In their studies on cultivated testes of diapausing pupae of silkworms, Hyalophora cecropia and Samia cynthia in hanging drops of blood obtained from pupating larvae or developing adults of these species, SCHMIDT and WILLIAMS (1953) showed that spermatocytes developed promptly into spermatids.

On the other hand, LENDER and DUVEAU-HAGEGE (1962, 1963 a, b) succeeded in cultivating the gonads of the last instar larvae of wax moth Galleria mellonella even in a medium without the blood of this moth for seven days or longer, and further it was pointed out that the spermatogonia multiplied in the cultivated male gonads, and spermatocytes underwent meiosis resulting in the formation of spermatozoids. Recently MITSUHASHI (1965) reported that spermatocytes of rice stem borer, Chilo suppressalis, developed into spermatids in a medium which did not contain insect hemolymph.

By the use of MITSUHASHI's medium, we have also tried to cultivate the testes taken out from diapausing Chilo larvae, and further, the effect of ecdysterone (TAKEMOTO et al., 1967) obtained from Achyranthis Radix, on spermatogenesis of the borer has been investigated.

MATERIALS AND METHODS

Rice stem borer larvae used were reared on a semi-synthetic diet under aseptic condition (KAMANO and YUSHIKA, 1967), and the diapause was artificially induced

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Hormonal Effect on Cultivated Insect Tissues

by keeping them under a short photoperiod at 25°C. The testes provided for
cultivation were taken out when they reached 60–70 days of age of the last instar.

For the culture medium, CSM-2F (Mitsushashi, 1968) was used throughout
the experiments. The culture vessels were almost the same as those used by
Mitsushashi and Maramorosch (1964). The amount of medium in each vessel
 ranged from about 0.15 ml to 0.3 ml.

Firstly the in vitro development of spermatocysts or whole testes obtained from
diapausing larvae was investigated for a considerably long time, and secondly
the effect of ecdysterone on diapausing testes in vitro was observed for 7–20 days.
During the experiments, cultures were kept at 25°C, and the medium was changed
once a week. The status of spermatogenesis as well as the development of testes
as a whole was examined with an inverted phase contrast microscope.

Preliminary tests were performed to determine the optimum dosage of ecdy-
sterone which showed positive effects on cultivated testes, and it was revealed that
200 μg per ml of the hormone was adequate. One testis of a pair was used for
hormonal test and the other taken from the same individual at the same time was
used as a control. The major and minor axes of the testis were measured every
24 hours for 6 days and the growth rate of the testis was expressed as an index
number calculated by the following formula:

\[ V = L \times W^2 \]

Where V indicates the index number of the testis volume, L and W indicate the
major and minor axes of the testis in mm, respectively. Seven days after the
onset of cultivation, most of the testes were dissected with a pair of fine needles
to examine the progress of spermatogenesis.

RESULTS

Development of Spermatocysts in Vitro

In order to cultivate spermatocysts, testes of diapausing larvae were ruptured
in the medium by means of fine needles, and any morphological change of the
germ cells in vitro was pursued. The cysts which came into direct contact with
the medium were broken immediately into spermatocytes or small fragments. But
some spermatocysts still remained as masses in the ruptured testes, and later they
occasionally migrated out of these testes. Such spermatocysts, liberated from the
testes about 7 days or more later, were maintained well and occasionally developed
into elongated cysts (Fig. 1).

In most cases, a few days were required for the spermatocysts of 70–100μ in
diameter to reach the mature state of 120–150μ, from when in the medium the
elongated cysts developed. At the time of elongation of the cysts, they were
different in appearance from the normal ones in vitro; namely the surface of the
former seemed to be rough. But the sperm formation was almost completed in
such cysts as mentioned above (Fig. 2).

Cultivation of Testes

The total number of testes used for this experiment was 113 and they were
divided into 10 preparations. During the continuous cultivation of testes obtained
Fig. 1. Spermatogenesis induced about one week after onset of cultivation. A, B, C, and D show the development of spermatocysts liberated from testis (Te) 8, 9, 10, and 11 days after onset of cultivation respectively; arrows indicate continuous observation of the same spermatocyst (×40).

Fig. 2. Sperm-formation in vitro (×150).

from diapausing larvae, it was observed in 39 testes that various epithelial-like cells migrated from the surface of the testes or vas deferens, and began to make cell sheets around the explants. The cell migration occurred mostly about 2-3 weeks after the onset of cultivation. The fixation of the testes on the glass surface with cell sheets seemed to be favourable for the maintenance of the cultures. After a while, the peritoneal sheaths of some testes became transparent, so that the development of germ cells was easily recognizable through these sheaths. As shown in Fig. 3, the development of spherical spermatocysts into elongated ones which contained spermatids was observed in 6 out of the 113 cultures. Generally, the elongation of cysts promptly occurred when the diameter of the cysts reached about 120-150µ as mentioned before. In the cultures which were maintained for a fairly long time
Fig. 3. Spermatogenesis observed in cultivated testis. A, B, C, and D show the development of the spermatocysts 30, 31, 32, and 33 days after onset of cultivation respectively; arrows indicate continuous observation of the same spermatocyst (×100).

Fig. 4. Spermatogenesis induced a long time after the culture was set up. Scy: Spermatocyst liberated from testis (Te), Ecy: Elongated cyst developed in the medium (×140).
two testes survived for a year or longer. With the lapse of time the cultivated testes were partially ruptured, and resulted in the liberation of spermatocysts which developed into elongated ones within several days. The surface of these elongated cysts was as smooth as that of the ones in vivo (Fig. 4).

**Bioassay of Medium**

Although the medium CSM-2F did not contain any insect blood, we felt it might be necessary to test hormonal action of some organic components of the medium, which were chemically unknown, namely TC-yeastolate, lactalbumin hydrolysate, Bacto-peptone, and fetal bovine serum. These substances were mixed together in the same ratio as in the medium, and assays were made, by means of injection, on the isolated abdomens of house fly larvae and rice stem borer larvae as well. Doses applied were 10 µl of the mixed components in the case of house fly and 10 µl or 30 µl in the case of rice stem borer. The results indicated that these substances did not act as moulting hormone so far as the doses applied were concerned, whereas the aqueous solution of ecdysterone at the concentration of 0.01 µg per 10 µl showed remarkable moulting hormone action (Table 1). It may be added, 30 µl of the mixed components is equivalent to the amount contained in the medium, i.e. 150 µl of CSM-2F.

<table>
<thead>
<tr>
<th>Test object</th>
<th>Substances injected</th>
<th>Dose</th>
<th>No. animals</th>
<th>No. pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated abdomen of rice stem borer larvae**</td>
<td>Ringer-Tyrode's soln.</td>
<td>10 µl</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Components of medium*</td>
<td>10 µl</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 µl</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ecdysterone</td>
<td>0.01 µg/10 µl</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>Isolated abdomen of house fly larvae***</td>
<td>Ringer-Tyrode's soln.</td>
<td>10 µl</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Components of medium*</td>
<td>10 µl</td>
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<tr>
<td></td>
<td>Ecdysterone</td>
<td>0.01 µg/10 µl</td>
<td>44</td>
<td>23</td>
</tr>
</tbody>
</table>

* Mixture of TC-yeastolate, lactalbumin hydrolysate, Bacto-peptone, and fetal bovine serum.
** Each test object was about 77 mg in weight.
*** Each test object was about 17 mg in weight.

**Hormonal Effect on the Development of Testes**

A group of 31 testes of diapausing larvae was treated with ecdysterone, and another group of 31 was kept as control. One testis of a pair enlarged within a few days without exception in the medium containing ecdysterone only when the other testis of the pair, which had been kept in the medium without ecdysterone, maintained itself fairly well for a week (Fig. 5A). Such effects of ecdysterone, as mentioned above, were not observed when the control testis degenerated (Fig. 5B). This phenomenon seemed to suggest that the hormonal effect on testes deeply...
Hormonal Effect on Cultivated Insect Tissues

Fig. 5. Typical examples showing development of testes in the medium. A: A pair of testes showing daily increase in volume in the medium with ecdysterone and maintenance of same volume of testis in the medium without ecdysterone. B: A pair of testes showing daily decrease in volume in the medium with or without ecdysterone.

Fig. 6. Cultivation of testes of a diapausing larva. A: A testis 4 days after onset of cultivation in the medium without ecdysterone. B: The same in the medium with ecdysterone (×70).

depended upon the physiological state of the testes or their host larvae. Fig. 6 shows an example in which a testis cultivated for 4 days in the medium with ecdysterone increased in volume 2 times as large as the control, and the peritoneal sheath of the testis treated with the hormone became transparent. When the
enlarged testis was ruptured 7 days after cultivation, well developed spermatids were observed (Fig. 7), whereas in case of testis without hormone treatment, most spermatocysts were spherical in form and did not develop further than the state at which they were at the beginning of cultivation. In some cultures subjected to the hormone, cell migration from the testes was observed as early as 7 days after cultivation, and further, the muscles attached to the explanted testes as well as testes themselves continued active contraction for a week or longer. On the contrary, in control cultures, no contraction was observed although cell migration occurred about 20 days after cultivation.

**DISCUSSION**

From the results of the present study it was ascertained that the majority of spermatocysts failed to develop into elongated cysts in the medium CSM-2F, however, some cysts which were liberated from cultivated testes that were ruptured but survived for several days or longer in the medium, succeeded in spermatogenesis, although the elongated cysts appeared abnormal in shape. An interesting fact is that the elongated spermatocyst showed quite normal shape only when they were released from the testes which were kept in the medium as long as 20 days or longer. This seems to suggest that germ cells may need considerable time to adapt to the artificial medium, and complete normal spermatogenesis. In fact, as mentioned before, two testes were able to survive for more than one year and continued to liberate spermatocysts which developed into elongated cysts containing spermatids.

On the other hand, ecdysterone caused remarkable growth and development of both testes as a whole and spermatocysts contained in them. This leads us to consider that ecdysterone added to the medium was responsible for the morphological changes of the male gonads, because the medium proved to be free of molting hormone activity. Therefore, it seems to be reasonable to assume that the development of germ cells of diapausing rice stem borer is largely
Hormonal Effect on Cultivated Insect Tissues

affected by the moulting hormone as known in vivo (Fukaya and Mitsuhashi, 1961; Yagi et al., unpublished). A further possible consideration leads us to assume that the development of the testes may be induced by some physiological changes in the peritoneal sheath caused by moulting hormone. There may be at least two evidences that make this assumption reasonable: 1) There occurred an apparent change in the peritoneal sheath of the testis by the addition of ecdysterone. 2) Spermatocysts occasionally developed into elongated cysts within several days even in the medium without ecdysterone, if they were liberated from the ruptured testis a considerably long time after cultivation.

Since Goldscheidt (1915, 1916) observed the spermatogenesis of Hyalophora cecropia in vitro, various media containing insect hemolymph have been used to cultivate insect testes. As mentioned before, Schmidt and Williams (1953) were the first investigators who demonstrated that spermatogenesis of some insects was initiated by the presence of the prothoracic gland hormone. Later, insect testes or male germ cells were successfully cultivated in media without insect hemolymph (Demal, 1961; Lender and Duveau-Hagege, 1962, 1963 a, b; Mitsuhashi, 1965) and this phenomena was interpreted as that some substances contained in the medium might fulfil the role of the prothoracic gland hormone (Mitsuhashi, 1965). However, it could be said from the present experiments that the medium CSM-2F has so far shown no activity of moulting hormone. Furthermore, it was ascertained that the medium has no action to induce rapid spermatogenesis in vitro. Further studies may be needed to clarify the mechanism which concerns the regulation of spermatogenesis.

ACKNOWLEDGEMENTS

We wish to thank Dr. M. Kobayashi, Sericultural Experiment Station, for kindly supplying us the ecdysterone used in this study. Thanks are also due to Dr. J. Mitsuhashi, Division of Entomology, National Institute of Agricultural Sciences, for his invaluable suggestions.

REFERENCES


