Hormonal Effect on Cultivated Insect Tissues
III. Effects of α - and β -Ecdysone or Prothoracic Glands
on Spermiogenesis in Two Noctuid Insects
in vitro (Lepidoptera : Noctuidae)¹

Tokunin FUKUSHIMA² and Shigemi YAGI³

Laboratory of Applied Zoology, Faculty of Agriculture, Tokyo University
of Education, Komaba, Meguro-ku, Tokyo 153, Japan

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Experiments were carried to investigate the effects of α - and β -ecdysone or prothoracic glands in GRACE's medium on spermiogenesis of the diapausing pupae of the cabbage armyworm, *Mamestra brassicae* or the last instar larvae of the tobacco cutworm, *Spodoptera litura*. In cultures of the testes of these 2 species, spermiogenesis was promptly accelerated with α -ecdysone or prothoracic glands, however, no visible change occurred when β -ecdysone was added to the medium. The results demonstrated that α - and β -ecdysone acted on the development of spermatocysts in different ways, and that spermiogenesis could be promoted by α -ecdysone in GRACE's medium which contained neither insect haemolymph nor mammalian serum.

INTRODUCTION

Several attempts have been made, by using the cultivation of testes or spermatocysts to examine the stimulative effects of ecdysones on spermatogenesis, especially spermiogenesis of some lepidopterous insects *in vitro*. In previous experiments, YAGI et al. (1969) reported that spermiogenesis of the diapausing rice stem borer, *Chilo suppressalis* was directly promoted in an insect haemolymph-free medium, CSM-2F (MITSUHASHI, 1968) with the addition of β -ecdysone, although this medium contained chemically unknown substances such as fetal bovine serum instead of the insect haemolymph. In addition, similar results were obtained with this medium in diapausing slug moth pharate pupae, *Monema flavescens*; namely, the spermatocysts in intact testes developed to contain well differentiated spermatids with the addition of β -ecdysone and the "naked" spermatocysts were more sensitive than the intact spermatocysts (TAKEDA, 1972a, b).

On the other hand, it has been demonstrated that the "naked" spermatocysts taken from diapausing pupae of the silkworms, *Hyalophora cecropia* and *Samia cynthia*,

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² Present address: Chiba Cancer Center Research Institute, Department of Pathology, Nitona-cho, Chiba 280, Japan.

³ To whom reprint request should be sent.

were insensitive to α - and β -ecdysone *in vitro* but spermiogenesis was promptly accelerated when a macromolecular factor (MF) contained in haemolymph was added to ecdysone-free medium, and further, that the spermatocysts within intact testes only responded when both the MF and ecdysones were present in the medium (KAMBYSELLIS and WILLIAMS, 1971a, b). Thus, they suggested that the role of ecdysone was indirect and regulated the transfer of the MF to the germ cells. However, whether the action of ecdysone is direct or indirect has not been conclusively determined.

In the present studies, by the use of GRACE's medium which is chemically defined and contains neither insect haemolymph nor mammalian serum, we have tried to determine the effects of α - and β -ecdysone or prothoracic glands on spermiogenesis of two noctuid insects, the cabbage armyworm, *Mamestra brassicae* and the tobacco cutworm, *Spodoptera litura*, *in vitro*.

MATERIALS AND METHODS

Medium. As culture medium, only GRACE's medium was used. In each culture vessel, containing 0.04 ml of the medium, explants were incubated at 25°C in an incubator (CO₂ : O₂ = 1 : 1).

Testes. The testes taken from 3-day-old diapausing pupae of *M. brassicae* which were reared on an artificial diet under short day conditions at 20–22°C were mainly used for the cultures. Some experiments were also performed on 1-day-old final instar larvae of *S. litura* reared on an artificial diet under long day conditions at 25°C. Culture methods used in these experiments were the same as those mentioned previously (YAGI et al., 1969).

Seven days after the onset of cultivation, the testes were dissected with a pair of fine needles to examine and quantify the progress of spermiogenesis, which was classified into 5 grades as described previously by YAGI and FUKUSHIMA (1975).

Prothoracic glands. Active prothoracic glands obtained from 9-day-old larvae of the final instar destined for non-diapause (AGUI and YAGI, 1973) were cultivated for 2 days, and then to this culture testes were introduced and co-cultured for a further 7 days. The number of cultivated prothoracic glands used were 5 and 10.

Ecdysones. Two kinds of ecdysones, namely α - and β -ecdysone were used in the present experiments. The α -ecdysone was dissolved in 99% ethanol and added to the medium to make the final concentrations of the hormone 0.5 and 5 μ g/ml. The solvent, which had a final concentration of 1/1000 had no effects on the development of the spermatocysts. The β -ecdysone was dissolved directly in the medium and the hormone concentrations were adjusted to 0.3, 3.0, 15.0 and 30.0 μ g/ml respectively.

RESULTS

When the testes taken from 3-day-old diapausing pupae of *M. brassicae* were cultivated in GRACE's medium, no development occurred although they survived well for 7 days or longer (Table 1). On the contrary, if the cultures were prepared in the medium containing α -ecdysone, spermiogenesis was promptly accelerated and many elongated spermatocysts were observed in the testes after 7 days cultivation; the higher dose of 5 μ g/ml of α -ecdysone was more effective than 0.5 μ g/ml of the

Table 1. EFFECTS OF α - AND β -ECDYSONE ON SPERMIOGENESIS OF THE CABBAGE ARMYWORM, *M. brassicae*, *in vitro*

Ecdysones	Dose ($\mu\text{g/ml}$)	No. of exp.	Spermiogenesis in medium ¹				
			—	±	+	++	+++
α -ecdysone	0.5	8	0	0	7	1	0
	5.0	10	0	0	0	3	7
β -ecdysone	0.3	11	11	0	0	0	0
	3.0	10	10	0	0	0	0
	15.0	8	8	0	0	0	0
	30.0	8	8	0	0	0	0
Control ²	—	10	10	0	0	0	0

¹ Testes were cultivated for 7 days.

— : spherical spermatocysts only; ± : mixture of spherical spermatocysts and pyriform cysts;
 + : elongated spermatocysts are less than 10%; ++ : elongated spermatocysts are more than 10 % but less than 50%; +++ : elongated spermatocysts are more than 50%.

² GRACE's medium only.

Fig. 1. Spermatocysts of *M. brassicae* 7 days after the onset of cultivation of intact testes in GRACE's medium with 5 $\mu\text{g/ml}$ of α -ecdysone (A), 0.3 $\mu\text{g/ml}$ of β -ecdysone (B), or 10 prothoracic glands (C). (X 40)

hormone treatment (Fig. 1A, Table 1). In the case of the treatment of β -ecdysone, however, no visible change was observed when the hormone concentration ranged from 0.3 to 30.0 $\mu\text{g/ml}$ (Fig. 1B, Table 1). Similar results were obtained when the testes taken from 1-day-old last instar larvae of *S. litura* were cultivated in medium containing α - or β -ecdysone; that is to say, spermiogenesis was rapidly promoted by the addition of 5 $\mu\text{g/ml}$ of α -ecdysone, but 3 $\mu\text{g/ml}$ of β -ecdysone had no effect on the development of spermatocysts *in vitro* (Table 2).

Further experiments were made to determine the effects of active prothoracic glands on spermiogenesis of the *Mamestra* pupae *in vitro*. With an increase in the number of co-cultured prothoracic glands, there was a progressive increase in the development of the spermatocysts (Fig. 1C, Table 3).

Table 2. EFFECTS OF α - AND β -ECDYSONE ON SPERMOGENESIS OF THE TOBACCO CUTWORM, *S. litura*, *in vitro*

Ecdysones	Dose ($\mu\text{g/ml}$)	No. of exp.	Spermiogenesis in medium ¹				
			—	$\pm$	+	++	+++
α -ecdysone	5.0	4	1	0	1	2	0
β -ecdysone	3.0	4	4	0	0	0	0
Control ²	—	4	4	0	0	0	0

^{1,2} See Table 1.Table 3. EFFECTS OF PROTHORACIC GLANDS (PG) ON SPERMOGENESIS OF THE CABBAGE ARMYWORM, *M. brassicae*, *in vitro*

No. of PG ¹	No. of exp.	Spermiogenesis in medium ²				
		—	$\pm$	+	++	+++
5	10	2	0	7	1	0
10	3	0	0	0	3	0
Control ³	10	10	0	0	0	0

¹ PG were pre-cultured for 2 days, and then they were co-cultured with testes for a further 7 days.^{2,3} See Table 1.

DISCUSSION

The results of the present experiments showed that spermiogenesis of both *Mamestra* and *Spodoptera* was rapidly promoted *in vitro* either by the addition of α -ecdysone in the medium or by being co-cultured with active prothoracic glands. In this case, there seems to be little possibility that MF, as noted in the INTRODUCTION, was attached to the surface of the explanted testes or that the walls of the testes were injured because the explants were rinsed in Ringer-Tyrode's solution several times and were treated very carefully during cultivation. Thus, it is suggested that MF may not be needed to accelerate spermiogenesis of these 2 species *in vitro*, contrary to saturniia pupae (KAMBYSELLIS and WILLIAMS, 1971a, b). As previously mentioned, in *Chilo* (YAGI et al., 1969), a similar result was obtained in that there occurred an apparent change in the peritoneal sheath of the explanted testes by the addition of ecdysones or prothoracic glands. Besides, it was observed in the *Mamestra* testes obtained from 3-day-old diapausing pupae that the spermatocysts occasionally elongated within several days when the peritoneal sheath of the testes was ruptured to liberate the spermatocysts in the medium (SHIMIZU and YAGI, unpublished). Therefore, it is assumed that the peritoneal sheath may play a role in the development of spermatocysts.

An interesting result was that β -ecdysone had no effect on the development of spermatocysts *in vitro*. This is the first report which shows the ineffectiveness of β -ecdysone although several experiments have been carried out to demonstrate the effects of ecdysones, especially β -ecdysone on cultivated testes or spermatocysts, in some lepidopterous insects (YAGI et al., 1969; KAMBYSELLIS and WILLIAMS, 1971a, b; TAKEDA, 1972a, b). In all the experiments that have been reported, insect haemo-

lymph, mammalian sera or other chemically unknown substances were used in the media. Consequently, these substances may have together with β -ecdysone, promoted spermiogenesis *in vitro*.

In the present studies, it was ascertained that the development of spermatocysts was rapidly accelerated not only by α -ecdysone but also by the prothoracic glands. In *Mamestra*, AGUI and FUKAYA (1973) showed that the development of wing discs was initiated by ecdysones such as α -, β -ecdysone, ponasterone A and cyasterone but not rubrosterone, and further among these hormones at a concentration of 1 μ g/ml, α -ecdysone alone could induce the development of wing discs similar to that observed *in vivo*. Moreover, recently, the hormone released from the cultured prothoracic glands in *Bombyx* or *Manduca* larvae was identified as α -ecdysone (CHINO et al., 1974; KING et al., 1974). Therefore, it is highly possible that the prothoracic glands in *Mamestra* secrete α -ecdysone in the medium and this hormone directly stimulates spermiogenesis *in vitro*. Many investigators have also demonstrated the different action of α - and β -ecdysone on various target organs or cells *in vitro*. According to MANDARON (1973), α -ecdysone and inokosterone were able to induce complete and normal metamorphosis in cultured leg discs of *Drosophila*, whereas β -ecdysone inhibited the subsequent development of the discs. In addition, COURGEON (1972) demonstrated that α -ecdysone stimulated mitosis in *Drosophila* cells *in vitro* but β -ecdysone did not. In contrast, others have suggested that β -ecdysone was the active form of the hormone and the other ecdysone analogs such as α -ecdysone were apparently prohormones (KING, 1972a, b; CHIHARA et al., 1972.; MARKS, 1973; KING and MARKS, 1974). Furthermore, KING (personal communication) found that the testes of *Manduca* can convert α - to β -ecdysone *in vitro*. Even if we accept this view, we do not know why the added β -ecdysone was not able to induce the spermiogenesis in our *in vitro* systems.

It was pointed out that the donor's age, namely the developmental stage of the germ cells was very important to investigate the mechanism of the hormonal control of spermatogenesis (DOANE, 1973; KURODA, 1974). In fact, we observed that there was little initiation of spermiogenesis when *Mamestra* testes from 6-day-old last instar larvae destined for diapause were cultivated in GRACE's medium containing α -ecdysone (YAGI et al., unpublished).

Further studies will be needed to clarify whether or not spermiogenesis is directly accelerated by ecdysone without containing MF and what the differences are between the action of α - and β -ecdysone on the development of germ cells.

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REFERENCES

- AGUI, N. and M. FUKAYA (1973) Effects of moulting hormones and prothoracic glands on the development of wing discs of the cabbage armyworm (*Mamestra brassicae* L.) *in vitro*. *Appl. Ent. Zool.* **8**: 73-82.
 AGUI, N. and S. YAGI (1973) Relationship between endogenous ecdysone titer and *in vitro* activity of prothoracic glands of *Mamestra brassicae*. *Appl. Ent. Zool.* **8**: 239-240.

- CHIHARA, C. J., W. H. PETRI, W. H. FRISTROM and D. S. KING (1972) The assays of ecdysones and juvenile hormones on *Drosophila* imaginal disks *in vitro*. *J. Insect Physiol.* **18**: 1115–1123.
- CHINO, H., S. SAKURAI, T. OHTAKI, N. IKEKAWA, H. MIYAZAKI, M. ISHIBASHI and H. ABUKI (1974) Biosynthesis of α -ecdysone by prothoracic glands *in vitro*. *Science* **183**: 529–530.
- COURGEON, A. -M. (1973) Effects of α - and β -ecdysone on *in vitro* diploid cell multiplication in *Drosophila melanogaster*. *Nature* **236**: 250–251.
- DOANE, W. W. (1973) Role of hormones in insect development. In “*Developmental Systems: Insects*” vol. 2 (ed. S. J. Counce and C. H. Waddington), pp 291–497. Academic Press, London.
- KAMBYSELLIS, M. and C. M. WILLIAMS (1971a) *In vitro* development of insect tissues. I. A macromolecular factor prerequisite for silkworm spermatogenesis. *Biol. Bull.* **141**: 527–540.
- KAMBYSELLIS, M. and C. M. WILLIAMS (1971b) *In vitro* development of insect tissues. II. The role of ecdysone in the spermatogenesis of silkworms. *Biol. Bull.* **141**: 541–552.
- KING, D. S. (1972a) Ecdysone metabolism in insects. *Am. Zool.* **12**: 343–345.
- KING, D. S. (1972b) Metabolism of α -ecdysone and possible precursors by insects *in vivo* and *in vitro*. *Gen. Comp. Endocrinol. Suppl.* **3**: 221–227.
- KING, D. S. and E. P. MARKS (1974) The secretion and metabolism of α -ecdysone by cockroach (*Leucophaea maderae*) tissues *in vitro*. *Life Sciences* **15**: 147–154.
- KING, D. S., W. BOLLENBACHER, D. W. BORST, W. V. VEDECKIS, J. D. O’CONNOR, P. I. ITTYCHERIAH and L. I. GILBERT (1974) The secretion of α -ecdysone by the prothoracic glands of *Manduca sexta* *in vitro*. *Proc. Nat. Acad. Sci. USA* **71**: 793–796.
- KURODA, Y. (1974) Spermatogenesis in pharate adult testes of *Drosophila* in tissue cultures without ecdysones. *J. Insect Physiol.* **20**: 637–640.
- MANDARON, P. (1973) Effects of α -ecdysone, β -ecdysone and inokosterone on the *in vitro* evagination of *Drosophila* leg discs and the subsequent differentiation of imaginal integumentary structures. *Develop. Biol.* **31**: 101–113.
- MARKS, E. P. (1973) Deposition of insect cuticle *in vitro*: Differential responses to α - and β -ecdysone. *Gen. Comp. Endocrinol.* **21**: 472–477.
- MITSUHASHI, J. (1968) Tissue culture of the rice stem borer, *Chilo suppressalis* WALKER (Lepidoptera: Pyralidae). III. Effects of temperatures and cold-storage on the multiplication of the cell line from larval hemocytes. *Appl. Ent. Zool.* **3**: 1–4.
- TAKEDA, N. (1972a) Effect of some phytoecdysones on the spermatogenesis of the slug moth prepupa, *Monema flavescens* WALKER. *Appl. Ent. Zool.* **7**: 37–39.
- TAKEDA, N. (1972b) Effect of ecdysterone on spermatogenesis in the diapausing slug moth pharate pupa, *Monema flavescens*. *J. Insect Physiol.* **18**: 571–580.
- YAGI, S. and T. FUKUSHIMA (1975) Hormonal effect on cultivated insect tissues. II. Effects of juvenile hormone on spermiogenesis of the silkworm, *Bombyx mori* L., *in vitro*. *Appl. Ent. Zool.* **10**: 77–83.
- YAGI, S., E. KONDO and M. FUKAYA (1969) Hormonal effect on cultivated insect tissues. I. Effect of ecdysterone on cultivated testes of diapausing rice stem borer larvae. *Appl. Ent. Zool.* **4**: 70–78.