Hormonal Effect on Cultivated Insect Tissues V. The Role of Testis Proteins in Spermiogenesis of the Cabbage Armyworm, *Mamestra brassicae*, *in vitro* (Lepidoptera : Noctuidae)

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Testes obtained from the last instar larvae and the diapausing pupae of the *Mamestra brassicae* were cultivated in GRACE's medium containing α -ecdysone. Alpha-ecdysone was effective for the precocious maturation of the testes from the 6-day-old last instar larvae. When "naked" spermatocysts were co-cultured with intact or ruptured testis, or were cultivated in the "pre-conditioning medium", they underwent spermiogenesis. Disc electrophoretic analysis showed that two major protein bands were obtained from the medium after cultivation. Similar patterns of protein bands were also detected from the supernatant of homogenates of testes, or from the culture medium after cultivation of the ruptured testes for 6 days. It was suggested that these proteins were originated from testis wall. The role of the testis proteins in spermiogenesis *in vitro* is discussed.

INTRODUCTION

It is well known that in the cabbage armyworm spherical spermatocysts in the testes of non-diapausing pupae develop into elongated cysts several days after pupation, while the most developed cysts in diapausing pupae remain in the stage just before elongation. Premature pupation by allatectomy has been known to induce early spermatogenesis (FUKUDA, 1944; TAKEUCHI, 1969; MITSUI et al., 1976; FRIEDLÄNDER et al., 1981).

On the other hand, spermatocysts of the diapausing pupae can develop into elongated spermatids in *in vitro* culture (SCHMIDT and WILLIAMS, 1953). As factors to induce precocious spermiogenesis, KAMBYSELLIS and WILLIAMS (1971a,b) reported that the "naked" spermatocysts removed from diapausing pupae of the silkworm showed no detectable response to the added α - and β -ecdysone, but a rapid differentiation into spermatozoa took place when a "macromolecular factor" (MF) contained in blood was present in the culture medium. The MF, which has been said to be heat-labile and non-dialysable substance with a molecular weight of at least 30,000, is synthesized and secreted by haemocytes; and the MF-like activities could be obtained by the substitution of the MF with mammalian sera (KAMBYSELLIS and WILLIAMS,

1971a). Further, KISS and WILLIAMS (1975) reported the MF-like activity of the Cohn β -globulin factor III. On the other hand, YAGI et al. (1969) and TAKEDA (1972) could promote spermiogenesis of testes from the diapausing rice stem borer larvae and the slug moth pharate pupae respectively cultured *in vitro* in the media containing β -ecdysone, but free of insect blood, although their media contained fetal bovine serum which has been found to have the MF-like activities. YAGI and FUKUSHIMA (1975) succeeded to induce the spermiogenesis of "naked" spermatocysts in *Bombyx mori* by adding haemolymph to GRACE's medium. Recently, we reported that spermiogenesis occurred in GRACE's medium without ecdysone or MF if cultivated with an intact testis, and that the "naked" spermatocysts could develop into an elongated form in the medium in which the testis had been pre-cultured (SHIMIZU and YAGI, 1978).

In the present studies, we further investigated the factors and methods to induce precocious spermiogenesis *in vitro*.

MATERIALS AND METHODS

Insect. Testes taken from the last instar larvae and the diapausing pupae of *Mamestra brassicae* which were reared on an artificial diet under short day conditions at 23°C were used for cultures.

Cultivation of testis in the medium containing α -ecdysone. Testes were taken from the 1- and the 6-day-old last instar larvae, the 12-day-old prepupae and the 3-day-old diapausing pupae. The culture methods used in the present experiments were the same as those described previously (FUKUSHIMA and YAGI, 1975; SHIMIZU and YAGI, 1978). Seven days after the onset of cultivation the cultivated testes were torn open to examine the developmental status of spermatocysts, which were classified into 3 grades as previously described (SHIMIZU and YAGI, 1978). Alpha-ecdysone was dissolved in 99% ethanol and added to GRACE's medium without haemolymph or sera so as to make the final concentration of the hormone 5 μ g/ml.

Co-cultures with intact or ruptured testes. To investigate the role of testes in spermiogenesis, a testis was ruptured with a pair of fine needles and the liberated "naked" spermatocysts were co-cultured with a ruptured or with an intact testis in 0.02 or 0.1 ml of GRACE's medium.

Preparation of "pre-conditioning medium." The "naked" spermatocysts liberated from the testis were pre-cultured for 5 days with the testicular fluid which had flowed out from its own testis. Those cysts degenerated within 4 days from the onset of cultivation. This testicular fluid was named "pre-conditioning medium". Newly added spermatocysts were cultivated in the sole "pre-conditioning medium".

Cultivation of intact testes for electrophoresis. A testis from a 3-day-old pupa was explanted into GRACE's medium after rinsing three times with RINGER-TYRODE's solution. In each culture vessel, which contained 0.02–0.08 ml of GRACE's medium, one testis was cultivated for 1–6 days at 25°C in an incubator. Culture medium in which an intact testis had been cultivated was collected, while an intact testis was ruptured with a pair of fine needles on the paper to absorb testicular fluid from the testis. Thus collected samples were applied to disc electrophoresis.

Preparation of proteins from ruptured testes for electrophoresis. Forty pupal testes taken from the diapausing pupae were ruptured with a pair of fine needles in 0.9% NaCl

solution, and were rinsed twice with RINGER-TYRODE's solution to wash away the testicular fluid. These ruptured testes were homogenized in 0.5 ml of 0.05 M Tris-HCl buffer solution. The homogenates were then centrifuged at 3,000 rpm at 4°C for 10 min. The supernatant was applied to polyacrylamide disc electrophoresis.

Cultivation of ruptured testes for electrophoresis. Testes taken from the diapausing pupae were ruptured with a pair of fine needles and were rinsed twice with RINGER-TYRODE's solution. These ruptured testes were cultivated in 0.02 ml of GRACE's medium for 6 days. After cultivation, the medium was collected and applied to polyacrylamide disc electrophoresis.

Polyacrylamide gel electrophoresis. The following samples were analyzed by polyacrylamide gel electrophoresis to identify the basic proteins: 1) GRACE's medium, 2) culture medium after cultivation of an intact testis for 1–6 days, 3) testicular fluid after cultivation of spermatocysts for 1–6 days, 4) supernatant of the homogenates of testes, 5) culture medium after cultivation of ruptured testes for 6 days. Polyacrylamide gel electrophoresis was carried out on 7.5% polyacrylamide gels in 0.05 M Tris-glycine buffer at pH 8.3 as described by DAVIS (1964) and ORNSTEIN (1964). The electrophoresis was run at 5 mA per tube for 1–3 hr. The gels were stained with 1% amido black in 7.5% acetic acid solution and destained with 7.5% acetic acid. The destained gels were photographed, while the optical densities of the stained protein in the gels were recorded with a densitometer. All experiments were made in duplicate.

RESULTS

Effect of α -ecdysone on cultivated testis in vitro

Cultivated testes taken from the 1-day-old last instar larvae did not show any spermiogenesis even if α -ecdysone was present in the medium; on the contrary, those from the last stages showed accelerated spermiogenesis *in vitro* with the medium containing the hormone. However, little development occurred in the control testes cultivated without ecdysone; the testes survived for 7 days (Table 1).

It was found that the spermatocysts showed remarkable development when co-cultured with ruptured testes in the testicular fluid which had flowed out from its own

Table 1. Effect of α -ecdysone on cultivated testis of the cabbage armyworm, *M. brassicae*, *in vitro*

	Concentr. $\mu\text{g/ml}$	No. of exp.	Spermiogenesis ^a		
			S. C.	P. C.	E. C.
Last instar	5	14	14	0	0
(1-day-old)	0	14	14	0	0
Last instar	5	10	4	5	1
(6-day-old)	0	15	15	0	0
Prepupae	5	10	6	1	3
(12-day-old)	0	14	14	0	0
Diapausing Pupae	5	10	3	2	5
(3-day-old)	0	10	10	0	0

^a Testes were cultivated for 7 days with 0.02 ml of GRACE's medium containing α -ecdysone, then ruptured to examine the status of spermatocysts.

S. C. : spherical spermatocysts, P. C. : pyriform spermatocysts, E. C. ; elongated spermatocysts.

Table 2. Effect of testes on spermiogenesis of the cabbage armyworm, *M. brassicae*, *in vitro*^a

Stages	Treatments ^b	Medium (ml)	No. of exp.	Days after cultivation	Spermiogenesis ^c		
					S. C.	P. C.	E. C.
Prepupae (12-day-old)		— ^d	4	1	4	0	0
				3	1	1	2
				6	0	0	4
	— ^d	6	1	— 6	0	0
				3	— 1	0	0
				6	— —
	0.02	8	2	7	0	1
				4	4	1	3
				6	1	4	3
	0.02	13	2	13	0	0
				6	8	1	4
				7	—4(0)	0(0)	8(1)
Diapausing Pupae (3-day-old)		0.02	10	2	6	4	0
				4	0	0	10
				6	0	0	10
	0.1	5	2	— 5	0	0
				4	— 1	0	1
				6	— —	0	1

^a Testis was ruptured with fine needles, and liberated "naked" spermatocysts were co-cultured with intact or ruptured testis for 7 days.

^b Small and large circles show "naked" spermatocysts and testis, respectively. Large circle with an arrow indicates ruptured testis.

^c S. C., P. C., E. C. : see Table 1.

—, number of culture vessels containing degenerated spermatocysts.

*, indicates degenerated spermatocysts only.

(), spermiogenesis in testis after 7 day cultivation.

^d Only testicular fluid was used.

testis, or with intact or ruptured testes in GRACE's medium (Table 2 and Fig. 1). However, the "naked" spermatocysts alone were immediately broken into small fragments or degenerated after 2–3 days cultivation. When a 5 times greater volume of GRACE's medium was used, the spermatocysts failed to develop into elongated cysts.

Effect of testes on spermiogenesis in vitro

Next, the role of testes themselves in the development of spermatocysts was examined. When "naked" spermatocysts were cultivated in the "pre-conditioning medium" in which cysts had been pre-cultured for 5 days, they developed to elongated cysts after 4 days cultivation. However, "naked" spermatocysts degenerated in the GRACE's medium without such pre-conditioning treatment (Table 3).

Effect of "pre-conditioning medium" on spermiogenesis in vitro

Since the pre-cultured medium was effective in stimulating the elongation of

Fig. 1. Elongated spermatocysts co-cultured with a ruptured testis. Te: testis $\times 125$.

Fig. 2. Disc acrylamide electrophoretic patterns of three samples and densitometric recordings of gel separation patterns. A: GRACE's medium, B: culture medium after cultivation of testis for 3 days, C: testicular fluid from the testis after cultivation for 3 days.

Table 3. Effect of pre-conditioning medium on spermiogenesis of *M. brassicae*, *in vitro*^a

	Medium ml	Pre-culture days	No. of exp.	Days after cultivation	Spermiogenesis ^b		
					S. C.	P. C.	E. C.
Degenerated spermatocysts	trace ^c	5	9	2	—7	1	0
				4	—1	0	6
				6	—	0	7
Control ^d	0.02	5	8	2	—4	0	0
				4	*	*	*

^a After “naked” spermatocysts were pre-cultivated for 5 days, others were added to the “pre-conditioning medium”.

^b S. C., P. C., E. C., — and *; see Tables 1 and 2.

^c See preparation of “pre-conditioning medium” in MATERIALS and METHODS. Newly added spermatocysts were cultivated in the “pre-conditioning medium” without GRACE’s medium.

^d GRACE’s medium alone was kept at 25°C for 5 days.

Fig. 3. Densitometer traces of disc electrophoresis patterns. A: supernatant of the homogenates of the ruptured testes. B: culture medium after cultivation of the ruptured testes for 6 days.

“naked” spermatocysts, “pre-conditioning medium” was analyzed by electrophoresis. Although GRACE’s medium did not show any protein band, the culture medium after cultivation of testes for 3 days showed two major protein bands, a and b (Fig. 2A,B,C). Therefore, it seemed likely that these proteins derived from the cultivated testes themselves. Further experiments showed that similar patterns of proteins were also detected from supernatant of the homogenates of testes in diapausing pupae (Fig. 3A), or from the culture medium in which ruptured testes had been cultivated for 6 days (Fig. 3B). Therefore, it is confirmed that these proteins are originated from the testes and liberated into the medium *in vitro*.

DISCUSSION

The present experiments show that the response of spermatocysts to α -ecdysone *in vitro* is depended on the age of the testes. The spermatocysts obtained from 6-day-old larvae (prepupae) of the last instar showed spherical secondary meiosis cysts (128

cells). Since endogenous ecdysone titer increased from 6 days (prepupae) and became maximum 9 days after the last larval ecdysis (AGUI and YAGI, 1973), it appears that the prepupal testis had already received hormonal action. This was also confirmed by the fact that the donor's age is important, for no development took place if gonads were taken from larvae younger than the prepupal stages (DOANE, 1973).

"Naked" spermatocysts developed into elongated ones when they were cultivated with ruptured or intact testis with or without the use of GRACE's medium or cultivated in "pre-conditioning medium" (Tables 2 and 3). However, when 0.1 ml of GRACE's medium was used, the spermatocysts failed to develop into elongated cysts because of dilution effects. These results suggest that in the medium there may be some unknown substances which have a role in development of the spermatocysts or in their maintenance *in vitro*. Previous experiments showed that the "naked" spermatocysts could develop into an elongated form in medium in which the testis had been pre-cultured for 5 days (SHIMIZU and YAGI, 1978). From these results, it is highly possible that the testes may produce MF-like substances.

Using peroxidase as a tracer, the presence of a blood/germ cell barrier in the insect has been found in the locust, *Locusta migratoria* (MARCAILLOU and SZÖLLÖSI, 1975, SZÖLLÖSI and MARCAILLOU, 1977), and in the moth, *Anagasta kuehniella* (SZÖLLÖSI et al., 1980). Furthermore, MARCAILLOU and SZÖLLÖSI (1978) reported that the penetration of horseradish peroxidase decreases or ceases when high levels of endogenous ecdysteroids are detected in the haemolymph by radioimmunoassay. Similarly, JONES (1978) postulated that ecdysone treatment *in vitro* prematurely stimulated the formation of the barrier in the testicular follicles of grasshoppers, *Schistocerca gregaria*. These results do not seem to agree well with the ecdysone-MF hypothesis of KAMBYSELLIS and WILLIAMS (1971a, b).

From the present experiments, it is suggested that the testis synthesizes proteins and liberates them into the follicles and testicular fluid. This led us to the idea that the testis wall may be at least one of the origins of the MF-like substances, and that spermiogenesis may be induced by cooperation of the testis wall and spermatocysts; the function of ecdysone may be to alter the penetrability of the testis wall (KAMBYSELLIS and WILLIAMS, 1971a, b) and thereby to facilitate the release of proteins from it. Indeed, the testis wall was changed after exposure to ecdysone *in vitro* so that it became transparent (YAGI et al., 1969). Further studies will be needed to clarify whether these elongated spermatocysts in the present experiments have nuclei or not, since the apyrene spermatozoa form long bundles of flagella which appear very similar to those of the eupyrene spermatozoa (LEVIATAN and FRIEDLÄNDER, 1979).

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