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EFFECTS OF ECDYSTEROIDS ON THE GROWTH OF THE POST-TESTICULAR REPRODUCTIVE ORGANS IN THE SILKWORM, *BOMBYX MORI*

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Abstract—The post-testicular male reproductive organs of *Bombyx mori*, namely the accessory glands, seminal vesicles and ejaculatory duct, grow markedly in size throughout the 10-day pupal period. By employing PL 15.2, a monoclonal antibody produced against an accessory gland protein of the mealworm beetle *Tenebrio molitor*, we demonstrated that an organ-specific secretory antigen appears in the accessory glands of *B. mori* between the sixth and seventh day of pupal life. To investigate the control of reproductive maturation, we used transplantation and *in vitro* cultures. After organs from 1-day male pupae were implanted in female hosts of the same age, both growth and differentiation (appearance of PL 15.2 antigen) took place. Growth declined markedly with older hosts and no growth occurred in 4-day hosts. These results indicate that a humoral factor in young pupae promotes growth of the male organs. By using cultures of 1-day male organs, we showed that physiological levels of either ecdysone or 20-hydroxyecdysone stimulate growth *in vitro*, and that increased growth is correlated with longer exposure to hormone. In contrast, 2-deoxy-20-hydroxyecdysone did not promote growth. The combined results from culture *in vivo* and *in vitro* suggest that the large ecdysteroid peak known to occur in the early half of the *Bombyx* pupal period is necessary for the normal growth of the male reproductive organs.

Key Word Index: Silkworm, *Bombyx mori*, ecdysteroid, accessory gland, seminal vesicle, ejaculatory duct

INTRODUCTION

In many insect species, the male reproductive tracts secrete materials required to form a spermatophore (Davey, 1985) and specific factors which affect specific physiological events such as sperm activation, oöcyte maturation, oviduct contraction, oviposition and receptivity to males (Chen, 1984). Growth and differentiation of the post-testicular systems and (at least for some species) maintenance of their secretory activities, are under hormonal control (Chen, 1984; Raabe, 1986).

In the silkworm, *Bombyx mori*, the male reproductive system changes markedly during metamorphosis from larva to adult. The reproductive organs of the male include the paired testes, ducti deferentia, seminal vesicles, accessory glands and a single ejaculatory duct (Omura, 1938). The semen and secretions of the male reproductive tract contribute to the spermatophore which is assembled in the female bursa copulatrix at the time of mating (Omura, 1938). The spermatophore is an important site for the maturation of spermatozoa (Omura, 1938; Katsuno, 1977a, b; Kasuga *et al.*, 1987; Osanai *et al.*, 1986, 1987a, b). In addition, it has been shown that injection of aqueous extracts of the male reproductive organs into virgin females stimulated oviposition (Yamaoka and Hirao, 1977).

During the period when the post-testicular reproductive organs of *Bombyx mori* are growing so rapidly, radioimmunoassay has shown that there are pronounced changes in the titres of ecdysteroids (Calvez *et al.*, 1976; Kawasaki *et al.*, 1986; Tsuchida *et al.*, 1987). We are interested in the role of ecdysteroids in the growth and differentiation of the male reproductive tract of *B. mori*. In the present paper, we report the successful culture *in vivo* (transplantation) and *in vitro* of the organ complex composed of accessory gland, seminal vesicles and ejaculatory duct from the pupae, and we demonstrate that ecdysteroids promote the growth of these male reproductive organs.

MATERIALS AND METHODS

Animals and chemicals

Hybrid larvae from crosses of N 140 and C 145 strains of *B. mori* were reared on artificial diets (Yakult Co., Japan) at 25°C.

Acrylamide, ammonium persulphate, N,N′-ethylene-bis-acrylamide, Coomassie brilliant blue R-250, Tween-20, gelatine, goat anti-mouse immunoglobulin and horseradish peroxidase colour development reagent were purchased from Bio-Rad, U.S.A.; ecdysone, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, Tris (hydroxymethyl) aminomethane and SDS molecular weight markers from Sigma, U.S.A.; durapore (GVHP) from Millipore; Grace's medium and foetal bovine serum from Gibco Co., U.S.A. All other chemicals used were of reagent grade.

Tissue culture

All procedures were carried out under sterile conditions. Grace's medium containing 15% foetal bovine serum was used as a basal medium. All media were

sterilized by filtration through 0.45 μm filters prior to use. Pupae were surface-sterilized by dipping in 70% ethanol for 5 min and then blotted on tissue paper. The complex of the accessory gland, seminal vesicle and ejaculatory duct were dissected out in basal medium and freed from adhering fat body. After a thorough rinse in fresh basal medium, two or three of the organ complexes were transferred to Falcon organ culture dishes containing 5 ml of basal medium with or without hormones. The medium was not changed during the 2 days of organ culture.

Transplantation

Post-testicular male organs were removed from 1-day male pupae as described above. Small incisions were made with a razor blade in the ventral integument of the sixth segment of female pupae, and the male organ complexes were transplanted into the haemocoel of the female pupae with a Pasteur pipette. The incisions were sealed with paraffin wax.

Protein samples

Dissections were performed in cold phosphate-buffered saline (0.01 M sodium phosphate, 0.14 M sodium chloride, pH 7.0) and the organs collected in 1.5 ml microcentrifuge tubes. The bursa copulatrix including sperm and spermatophore, and spermatophragma were collected from female adults. Organs were disrupted with a Kontes homogenizer in 100–200 μl of ice-cold phosphate-buffered saline. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Monoclonal antibody

The PL 15.2 monoclonal antibody was prepared against an antigen in the tubular accessory glands of the mealworm beetle, *Tenebrio molitor* (Grimnes *et al.*, 1986a,b). According to the Amersham isotyping kit, this antibody is an immunoglobulin G1 with a *k* light chain. Ascites fluid was used for the experiments with proteins from *B. mori*.

Electrophoresis on SDS-polyacrylamide slab gels and Western blotting

Electrophoresis on SDS-polyacrylamide was carried out by the method of Laemmli (1970) with a 3% stacking gel and a 12% separating gel. After separation of the organ proteins on slab gels, the gels were electrophoretically blotted onto a durapore filter for 4 h at 15°C. The electrode solution consisted of 25 mM Tris base, 192 mM glycine, 20% methanol and 0.05% SDS. After blotting, the filter was immersed in 50 ml of TBST2 (10 mM Tris–HCl buffer, 0.5 M NaCl, 0.02% Tween 20, pH 7.5) containing 3% gelatin and shaken for 1 h at room temperature to block non-specific protein-binding sites. The filter was then incubated in 50 ml of TBST5 (10 mM Tris–HCl buffer, pH 7.5, 0.5 M NaCl, 0.05% Tween-20) containing 1% gelatine and PL 15.2 monoclonal antibody (1:1000 dilution) for 2 days at room temperature. After washing the filter four times at 5-min intervals with 100 ml of TBST2, the filter was incubated in 50 ml of TBST5 containing 1% gelatine and rabbit antimouse immunoglobulin G coupled with horseradish peroxidase for 2 days. Finally, the filter was washed four times at 5-min intervals with 100 ml of TBST2 and with 150 ml of the same buffer without Tween-20, and the sites of immunoglobulin G binding were visualized with 4-chloro-1-naphthol plus hydrogen peroxide.

RESULTS

Developmental morphology

In his classic work, Omura (1938) identified nine parts of the male reproductive system of adult silkworms (Fig. 1). For the present study, adjacent organs have been pooled to facilitate immunochemical scoring of development. Glandula pellucida and lacteola are considered together as the accessory gland; vesicula seminalis and ampulla ductus deferentis are designated the seminal vesicle; and the glandula spermatophorae, alba and prostatica are designated the ejaculatory duct. The accessory gland, seminal vesicle, and ejaculatory duct increase significantly in size over the pupal period (Fig. 2).

SDS-polyacrylamide gel electrophoresis and Western blotting of the proteins from male reproductive organs

Coomassie-stained gels of homogenates from each of the five major parts of the adult male tract (testes, vasa deferentia, accessory glands, seminal vesicles, and ejaculatory ducts) show the expected organ-specific patterns of protein banding [Fig. 3(A)]. In addition, we blotted parallel gels and probed with monoclonal antibodies developed against the male accessory gland proteins of *T. molitor*. One of these, PL 15.2, revealed two strongly-reacting antigens of about 20 and 23 kDa in the homogenates of the accessory glands, weakly-reactive bands of high molecular weight in the ejaculatory duct, but no reaction in homogenates of the testes, seminal vesicles, or vasa deferentia [Fig. 3(B)]. A strongly-reactive band was also seen at about 20 kDa in the spermatophragma, suggesting that the antigens in the accessory gland are secretory proteins and that the 20 kDa antigen in the spermatophragma originates probably from the 20 or 23 kDa antigen in the accessory gland.

Homogenates of the accessory glands from animals of increasing age, from 4-day pupae to adults, were subjected to electrophoresis on an SDS slab gel and to Western blotting (Fig. 4). In this age series, the first traces of the reactive 20 and 23 kDa antigens were first detected in 7-day pupae and strong accumulation of the proteins was seen in homogenates from 9-day pupae and older animals. The reactive antigens can serve as indices of differentiation.

Transplantation

To determine whether humoral factors in the pupa might be required for growth and differentiation of the male organs, complexes of the accessory glands, seminal vesicles, and ejaculatory ducts were transplanted from 1-day male pupae into the female pupae of various age. After implantation, the organ complexes were allowed to remain in the female hosts for 10 days, which corresponds to the normal pupal period. As shown in Fig. 5, the male organs grew well in female hosts. Furthermore, the final size of the transplants decreased with increasing age of the

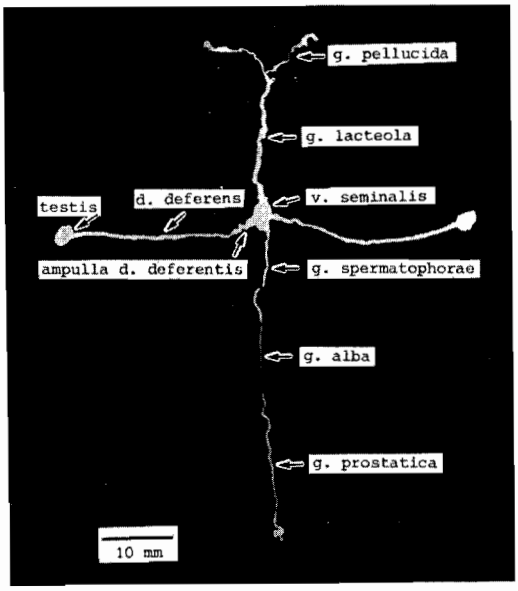

Fig. 1. Male adult reproductive system of the silkworm, *Bombyx mori*.

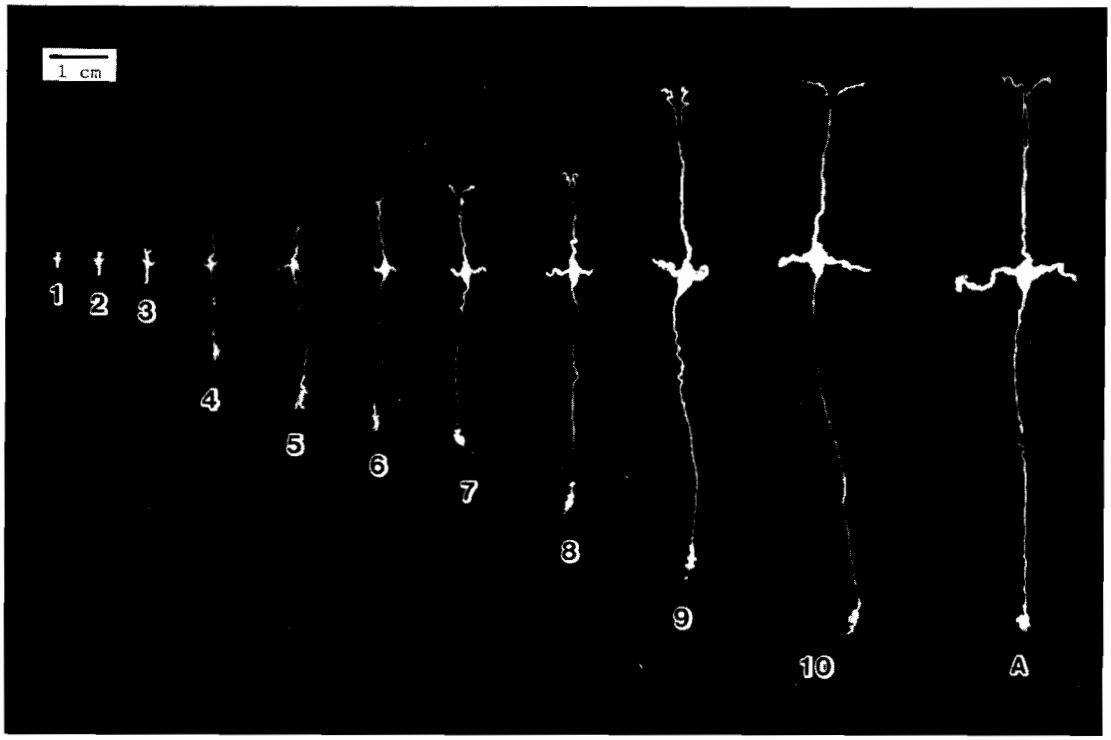

Fig. 2. Growth of the male reproductive organs (accessory gland, seminal vesicle and ejaculatory duct) of the silkworm during the pupal period. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10-day old pupae, A = Adults.

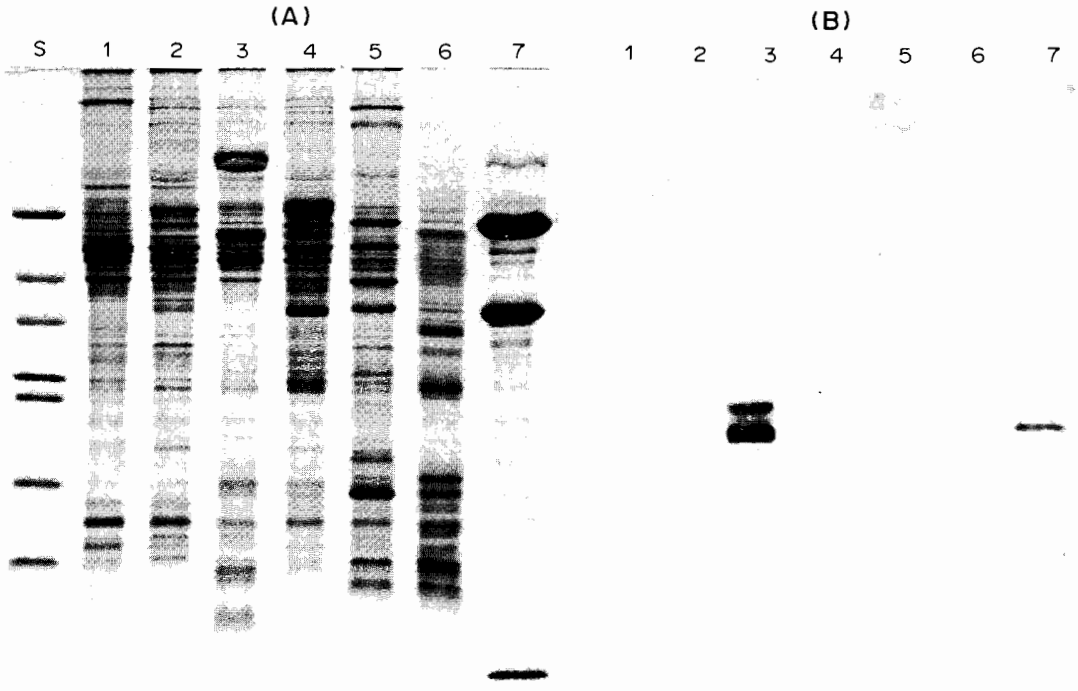

Fig. 3. SDS-polyacrylamide slab gel electrophoresis and corresponding immunoblot of the male adult reproductive organ homogenates. Samples were collected from males just after adult ecdysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel (200 V for 4 h) and transferred electrophoretically to durapore (GVHP, Millipore). Protein sample of 100 μg was applied to each lane. The durapore sheet was incubated with PL 15.2 antibody followed by rabbit anti-mouse immunoglobulin G coupled with peroxidase, and the site of immunoglobulin G binding were visualized with 4-chloro-1-naphthol plus hydrogen peroxide. Lane S, Molecular weight marker (Sigma, Bovine albumin, 66,000; Egg albumin, 45,000; Glyceraldehyde-3-phosphate dehydrogenase, 36,000; Carbonic anhydrase, 29,000; Trypsinogen, 24,000; Trypsin inhibitor, 20,100; α-Lactalbumine, 14,200); 1, testis; 2, ductus deferentis; 3 accessory gland; 4, seminal vesicle; 5, ejaculatory duct; 6, bursa copulatrix including spermatozoa and spermatophore (collected from female adults 45 min after the beginning of copulation); 7, spermatophragma (collected from female adults 45 min after the beginning of copulation).

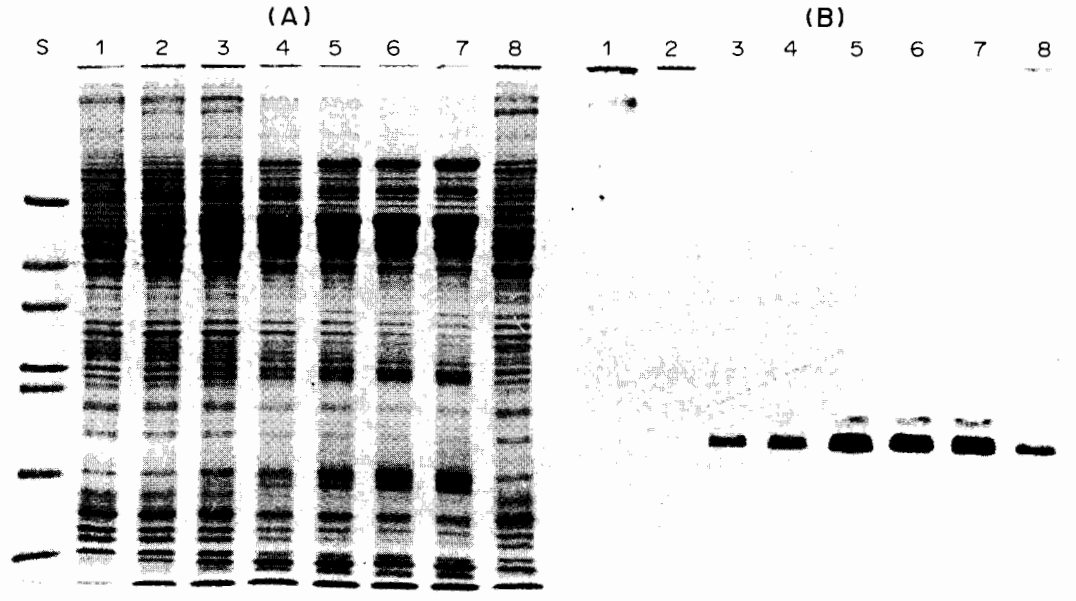

Fig. 4. SDS-polyacrylamide slab gel electrophoresis and corresponding immunoblot of homogenates of the accessory glands at various ages. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel (200 V, 4 h) and transferred electrophoretically to durapore (GVHP, Millipore). Protein sample of 100 μg was applied to each lane. Lane S, Molecular weight marker (Sigma); 1, 4-day pupa; 2, 6-day pupa; 3, 7-day pupa; 4, 8-day pupa; 5, 9-day pupa; 6, 10-day pupa; 7, unmated adult; 8, adult 45 min after the beginning of copulation.

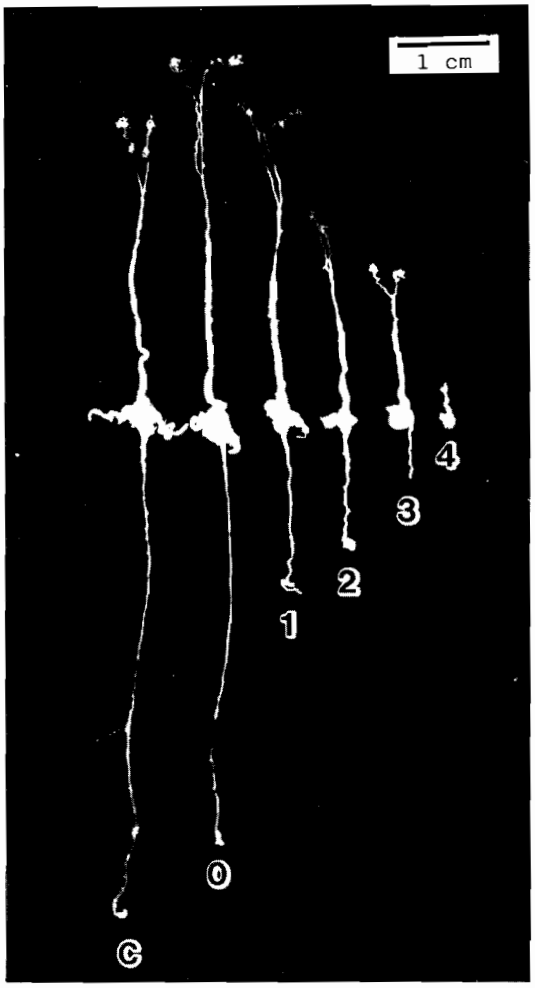

Fig. 5. Organ complexes of accessory gland, seminal vesicle and ejaculatory duct developed in the female pupae. The organ complexes from 1-day male pupae were transplanted into 0, 1, 2, 3 and 4-day female pupae.

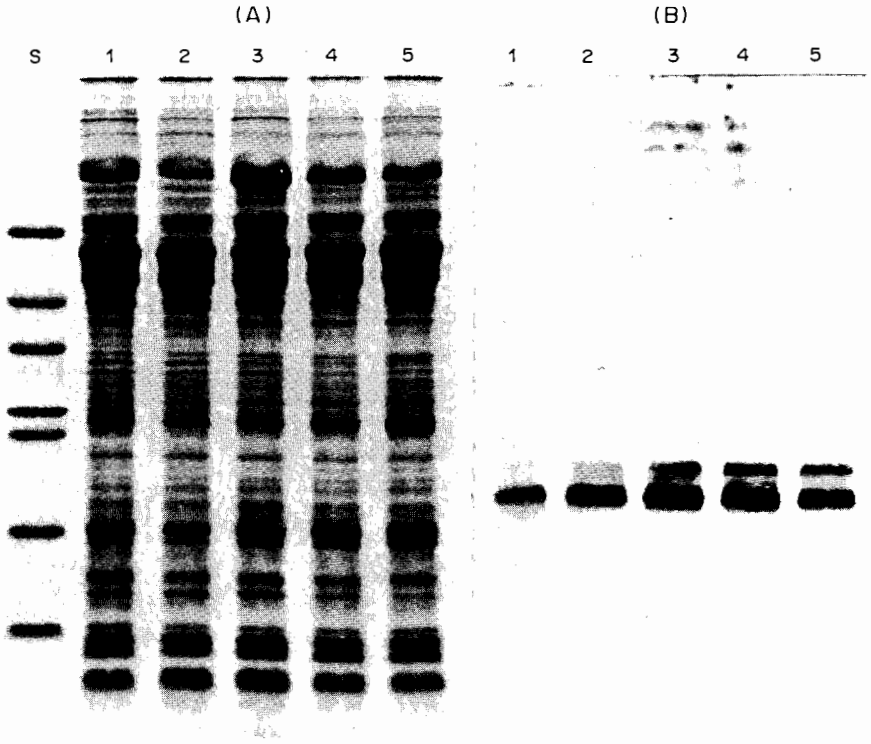

Fig. 6. SDS-polyacrylamide slab gel electrophoresis and corresponding immunoblot of accessory glands developed in female pupae. Organ complexes of accessory gland, seminal vesicle and ejaculatory duct from 1-day male pupae were transplated to 0, 1, 2 and 3-day female pupae (see Fig. 5). Protein sample of 100 μg was applied to each lane. Lane S, molecular weight marker (Sigma), 1, control (developed normally in male pupae), 2, 3, 4 and 5, transplanted into 0, 1, 2 and 3-day female pupae, respectively.

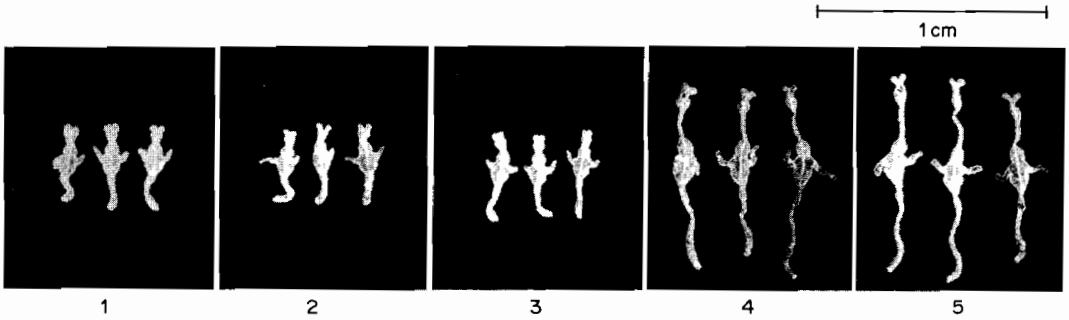

Fig. 7. Effect of 20-hydroxyecdysone on the growth of accessory gland, seminal vesicle and ejaculatory duct *in vitro* culture. The organ complexes were dissected out from 1-day pupae and cultured in Grace's medium containing 15% foetal bovine serum with 20-hydroxyecdysone for 2 days (1, without 20-hydroxyecdysone; 2, 0.005 μg/ml; 3, 0.05 μg/ml; 4, 0.5 μg/ml; 5, 5 μg/ml).

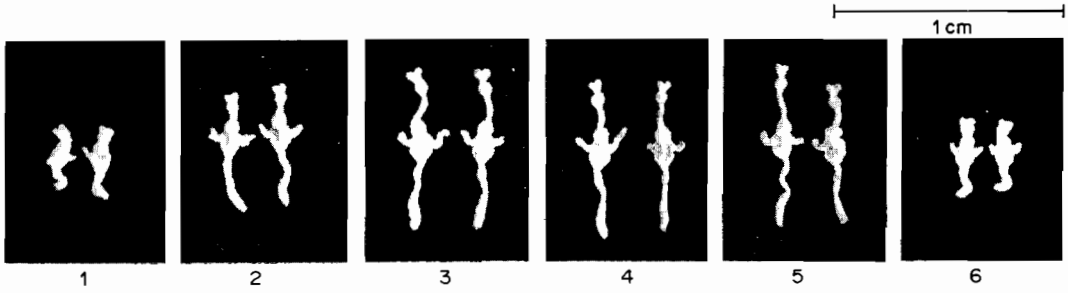

Fig. 8. Effects of ecdysone or 2-deoxy-20-hydroxyecdysone on the growth of accessory gland, seminal vesicle and ejaculatory duct *in vitro* culture. The organ complexes were dissected out from 1-day pupae and cultured in Grace's medium containing 15% foetal bovine serum with ecdysone or 2-deoxy-20-hydroxyecdysone for 2 days (1, without hormone; 2, 0.05 μg ecdysone/ml; 3, 0.5 μg/ml; 4, 5 μg/ml; 5, 50 μg/ml; 6, 50 μg 2-deoxy-20-hydroxyecdysone/ml).

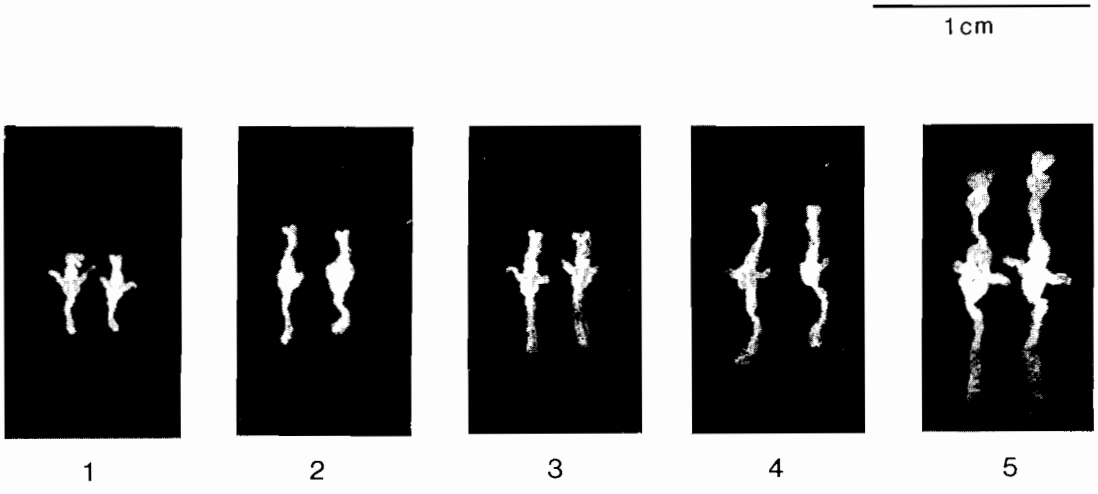

Fig. 9. Effects of duration of 20-hydroxyecdysone exposure on the growth of accessory gland, seminal vesicle and ejaculatory ducts *in vitro* culture. The organ complexes were dissected out from 1-day pupae and cultured in medium containing 20-hydroxyecdysone (5 μg/ml). 20-Hydroxyecdysone was washed out at 0 (1), 3 (2), 6 (3), 24 (4) and 48 (5) h after the beginning of the incubation, and the organ complexes were then cultured for the balance of the 48-h experiment in medium without hormone.

female recipients. When organ complexes were transplanted into 4-day or older female hosts, they did not grow in size, and we were unable to recover them from the hosts.

To determine whether the male organs would differentiate as well as grow in a female host, we used PL 15.2 on Western blots from male accessory glands transplanted into female hosts of 1, 2, and 3 pupal days. Reactive antigens were detected in all homogenates of male implants (Fig. 6). We could carry out this experiment successfully for male accessory glands transplanted into female hosts of older than 3 days, since transplanted organ complexes were so difficult to recover from older hosts.

Effects of ecdysteroids in vitro

To investigate directly the importance of ecdysteroids to maturation of the male tract, complexes of the accessory glands, the seminal vesicles and the ejaculatory duct from 1-day pupae were exposed to varying concentrations of ecdysteroids (10^{-8} M–10^{-4} M) for 2 days *in vitro*. No significant growth was seen in controls without hormones. As the dose of 20-hydroxyecdysone exceeded 10^{-6} M, stimulation of growth was evident (Fig. 7). For ecdysone, a concentration of 10^{-7} M promoted some growth but the effect was more marked at 10^{-6} M (Fig. 8). In contrast, 2-deoxy-20-hydroxyecdysone had no effect on the growth of the organs at concentrations of up to 10^{-4} M (Fig. 8).

To investigate the effect of increasing duration of exposure to hormone, the male organs from 1-day pupae were cultured *in vitro* for 48 h periods. In each case, the glands were explanted into media containing 20-hydroxyecdysone (10^{-5} M) and the hormone washed out with basal media at various times thereafter. As shown in Fig. 9, growth of the organ complex was greater with increasing duration of exposure to ecdysteroid.

DISCUSSION

There have been few studies on the structure and function of the male reproductive tract of *Bombyx mori*. The early and careful histological work of Omura (1938) defined the organs in the system. Omura's suggestions that the secretion of the glandula prostatica activated spermatozoa was confirmed by Katsuno (1977a, b). A recent fascinating series of papers from Osanai's laboratory have described a metabolic pathway for delivering energy to the spermatozoa from degradation of protein to 2-oxoglutarate via arginine. The enzymes involved in the pathway (Arg-C endopeptidase, exopeptidase and arginase) are derived from the male reproductive tract (Aigaki and Osanai, 1985; Aigaki *et al.*, 1987; Kasuga *et al.*, 1987; Osanai and Aigaki, 1984; Osanai *et al.*, 1986, 1987a, b).

The present paper demonstrates that when exposed to a humoral factor present only in young pupae up to 4 days of age, the post-testicular male organs of *B. mori* begin to grow and the growth continues in the older pupae when the humoral factor is absent. As previously shown by radioimmunoassay, ecdysteroid titres in the haemolymph increase after pupation, reach a maximum at about 3 days, and then decline to undetectable levels toward adult eclosion (Calvez *et al.*, 1976; Kawasaki *et al.*, 1986; Tsuchida *et al.*, 1987). In addition, it has been shown that there is no significant sexual difference in the haemolymph ecdysteroid titre (Kawasaki *et al.*, 1986), although a clear difference in ecdysteroid titre between male and female has been reported in whole body samples (Hanaoka and Ohnishi, 1974). The activity of the humoral factor coincides with the peak of ecdysteroid titre in the pupal haemolymph of *B. mori*. Furthermore, the incubation *in vitro* of male organs with ecdysteroids demonstrated a direct effect of 20-hydroxyecdysone and ecdysone on their growth. The concentrations of ecdysone (10^{-6} M) and of 20-hydroxyecdysone (10^{-6} M) that promote growth *in vitro* are physiological, below the peak levels in the early pupa (6×10^{-6}–10^{-5} M) (Calvez *et al.*, 1976; Kawasaki *et al.*, 1986; Tsuchida *et al.*, 1987). In addition, we found that a relatively high concentration of 2-deoxy-20-hydroxyecdysone, which has been identified in the ovary of *B. mori* (Ohnishi *et al.*, 1981), had no apparent effect on the growth of organ complexes *in vitro*. This final result confirms that the ecdysone and 20-hydroxyecdysone are acting as a growth-promoting hormone rather than merely as steroid nutrients required by the cultured organs.

The hormonal control of growth and differentiation of male reproductive tracts has been extensively reviewed by Davey (1985) and Raabe (1986). In the male accessory glands of young pupal *T. molitor*, ecdysteroids promote growth by accelerating the cell cycle (Szopa *et al.*, 1985; Yaginuma *et al.*, 1988) and also differentiation by making the glands competent to produce the enzyme, trehalase, and adult-specific antigens (Grimnes and Happ, 1987; Yaginum and Happ, 1989). In *T. molitor*, the ecdysteroid effect on cell cycling and that on trehalase induction differ from one another in three respects: (1) concentration of hormone required *in vitro*, (2) the time required for hormone action *in vitro*, and (3) the interval between hormone administration and patency of response. Acceleration of cell cycling is effected at lower hormone levels and shorter exposures that does trehalase induction. Furthermore, acceleration of cell cycling coincides with the hormone peak in the mid-pupa (days 3–5, Yaginuma *et al.*, 1988) while the effect on trehalase production (Yaginuma and Happ, 1988) and on adult-specific antigens (Grimnes and Happ, 1987) lags several days and begins at the time of adult ecdysis when ecdysteroid levels are low.

As shown in the present study, in *B. mori* as in *T. molitor*, the appearance of the differentiation-specific proteins lags until several days after that fall of the pupal ecdysteroid peak. When male organs of *B. mori* were transplanted into 3-day female pupae, they did not exhibit full growth (Fig. 5) and yet antigen levels were indistinguishable from normal controls (Fig. 6). It may be that concentrations and time of exposure required for growth effects are different from those required for the differentiation effect, but this can be confirmed for *B. mori* only with more detailed studies.

In the present study, we showed that PL 15.2, a monoclonal antibody developed against an antigen in the accessory glands of the male mealworm beetle, recognized epitopes in organ-specific proteins of male silkmoths. This particular epitope is also found in the

tracheal cuticle of *T. molitor* (Grimnes *et al.*, 1986b) and in the assembly zone of the cuticle of pupal *Drosophila melanogaster* (Wolfgang *et al.*, 1987). It may be that this and other such monoclonal antibodies (Grimnes and Happ, 1986; Grimnes *et al.*, 1986a, b; Shinbo *et al.*, 1987) will be generally useful as probes for comparative studies of reproductive biochemistry and development of insects.

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