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EFFECTS OF VERTEBRATE HORMONES ON THE REPRODUCTIVE SYSTEM OF *ACHATINA FULICA* (GASTROPODA : STYLOMMATOPHORA)

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ABSTRACT

The effects of vertebrate hormones, namely, estradiol, testoviron, progesterone and human chorionic gonadotrophin (HCG) on the reproductive system of Achatina fulica *were studied. The snails were injected with these hormones. Histological changes in the reproductive and accessory sex organs, such as ovotestis, albumen gland, prostate gland and uterus were observed. The tissues were fixed in Bouin's fluid and processed for light microscopy. The study revealed that there was a marked increase in the number of oocytes in the ovotestis of snails injected with progesterone, HCG and estradiol when compared to those of the control groups. Testoviron seemed to exert the least effect on oocyte production.*

Histological changes were observed in the albumen gland of the snails injected with estradiol, progesterone, and HCG. The glands became larger in size and their albumen canal was filled with secretory material. The gland cells or secretory cells were large and contained globular secretory material in the cytoplasm.

INTRODUCTION

Evidence for an endocrine control of the reproductive system in invertebrates has been produced by many authors[1]. In the gastropods, most of the results indicated that the cerebral ganglia produced two hormones that controlled the male and female parts of the reproductive system[2]. In the basommatophoran pond snail, *Lymnaea stagnalis*, two endocrine centers control the female reproductive activity. The dorsal bodies, which are attached to the surface of the cerebral ganglia, produce a dorsal body hormone which controls directly or indirectly vitellogenesis, and the growth and differentiation of the female accessory sex organs[3-7]. The cerebral neuroendocrine caudodorsal cells release an ovulation hormone which stimulates the ovulation of the oocytes[8-11].

In the stylommatophoran slugs, *Agriolimax reticulatus*, vitellogenesis in the ovotestis and the growth and differentiation of the female accessory sex organs are stimulated by the hormone of the dorsal body[12]. In addition, in *A. reticulatus* and *Limax flavus*, egg-laying is controlled by a neurosecretory factor of the brain or cerebral ganglia[13]. In *Helix pomatia*, a brain factor has been demonstrated to stimulate the synthetic activity of the female accessory sex organs[14,15]. Chavadej *et al.*[16] reported that in *Achatina fulica*, the cerebral ganglion homogenate seemed to increase oocyte production while an optic tentacle homogenate increased the production of spermatozoa.

The biosynthesis of steroids and their possible function in relation to reproduction has been studied in a number of gastropods. Steroids and steroid-synthesizing enzymes have also

been demonstrated[17-23]. These studies primarily indicated the synthesis or the occurrence of steroids in gonads and digestive glands. Furthermore, in some stylommatophorans, dorsal bodies, buccal ganglia, eggs, seminal receptacles and albumen glands appear to contain steroids or their synthesizing enzymes[18,24-26]. In the slug, *Arion ater*, testosterone, 11-ketotestosterone and 17- β-hydroxy-progesterone were detected in the egg and estrone and estradiol-17 in the spermatheca[25]. Further, dehydroepiandrosterone and 11-ketotestosterone were detected in the gonad; pregnenolone, estradiol-17 and estrone in the albumen gland and estrone in the spermatheca of the slug, *Ariolimax californicus*[18-20].

In stylommatophorans, the steroids might be involved in the regulation of development, growth and activity of accessory sex glands. Castration experiments have shown that these processes were under control of gonad hormones[27-28]. In basommatophorans, on the other hand, castration of the gonad had no effect on the accessory sex glands[23]. Another possibility would be that gonadal steroids are locally involved in the regulation of a particular process, such as oogenesis, ovulation, spermatogenesis or spermiation. Aubry[29] demonstrated that in *L. stagnalis*, the injection of testosterone stimulated the male phase and inhibited the female phase while estradiol had the reverse effect; and progesterone stimulated both phases. Takeda[30] showed in the slugs *Deroceras reticulatus* and *L. flavus* that injection of estrogen stimulated egg-laying and reduced the rate of oocyte development; injection of androgen had the opposite effects. The injection of pituitary hormones accelerated growth and maturation of the gonad in *D. reticulatus*[31].

Most studies on the effects of hormones on reproduction of pulmonates have been carried out in the basommatophoran, *L. stagnalis*, and various species of stylommatophoran slugs, but very few on stylommatophoran land snails. Hence, the objectives of the present investigation were to study the effects of hormones, human chorionic gonadotrophin (HCG), estradiol, testoviron and progesterone on reproduction of the giant African land snail, *A. fulica*. This was done by examining histological changes induced by these hormones in the ovotestis, albumen gland, uterus and prostate gland.

MATERIALS AND METHODS

Adult snails (shell length 4-5 cm) of *A. fulica* were collected from Chantabury Province in September, then brought into the laboratory of the Center for Applied Malacology and Entomology, Department of Biology, Faculty of Science, Mahidol University. The snails were maintained individually for two months in a white small plastic bowl, which contained ground coconut husks and was covered with a nylon-mesh lid. The snails were fed daily with cucumbers, lettuce, mushrooms, sweet potatoes and food additives.

Injection of hormones

Injection of snails was performed using the method of Takeda[30] during the dry season (November-March) when snails stopped laying eggs. Hormones used in these experiments were human progesterone, estradiol, testoviron, and HCG. These hormones were suspended in corn oil, except for HCG which was dissolved in saline solution. The hormones were injected into the foot-muscle of snails with a microsyringe. The concentration of hormones administered was 1 μg/g body weight. After seven days, these snails were treated with hormones at 2 μg/g body weight. After each injection, they were housed and fed as described above. The controls consisted of three groups : a non-injected group, a sham-injected corn oil group and a sham-injected NaCl group. There were four experimental groups : a progesterone-

injected group, an estradiol-injected group, a testoviron-injected group and an HCG-injected group. Twenty snails were used for each control and experimental group. The experiments were done in replicates.

At 14 days after the second injection of hormones, ten snails of each group were measured and weighed. Then, they were relaxed with menthol crystals for one hour and the shells were removed. Ovotestis, albumen gland, prostate gland and uterus were dissected from snails and examined histologically with the light microscope. The remaining snails were reared in the laboratory to observe oviposition.

Histological study

The whole organs of ovotestis, albumen gland, prostate gland and uterus were fixed in Bouin's fluid for 6 hours. Then, they were washed with 70% alcohol several times, and dehydrated in alcohol 70%, 80% (twice), 95% (twice), 100% and dioxane (three times), each step for 20 min. After dehydration, the tissues were embedded in paraplast and sectioned with a rotary microtome at 5 μm thickness. Sections were stained with Harris's hematoxylin and eosin, examined and photographed with an Olympus BH-2 light microscope. The numbers of oocytes in the ovotestis were counted using a counter.

RESULTS

The oviposition was not observed in any of the experimental groups of snails injected with progesterone, estradiol, testoviron and HCG. The results of the histological observation were as follows:

Ovotestis

Control groups : The ovotestis of the control groups consisted of 3-5 creamish-white lobes embedded in the digestive gland. Each lobe was made up of numerous small follicles or acini (Fig. 2A). Each acinus was lined with a basement membrane. The acinus always contained spermatozoa and all the stages of spermatogenesis could be observed within a single acinus (Fig. 2B). The oocytes which are large with prominent nuclei and nucleoli, on the contrary, were much less numerous and rarely seen (Fig. 2C). When the spermatozoa and oocytes were observed together in a single acinus, the spermatozoa were usually located in the center, whereas the oocytes were found on the periphery of the acinus. The average number of oocytes in the ovotestis was 30 (Fig. 1).

Experimental groups : The ovotestes of the snails injected with estradiol, progesterone, testoviron and HCG appeared very similar to those of the control groups. They consisted of 3-5 lobes with numerous acini. A large number of acini contain spermatids and spermatozoa (Fig.3A). However, there was a marked increase in the number of oocytes in the ovotestis of snails injected with progesterone (60 oocytes in ovotestis), HCG (58 oocytes in ovotestis) and estradiol (44 oocytes in ovotestis) when compared to those injected with testoviron (32 oocytes in ovotestis) and the control (30 oocytes in ovotestis) (Fig. 1). Fig.3B shows the oocytes in the ovotestis of snails injected with progesterone.

Albumen gland

Control groups : The albumen glands in mature snails of the control groups were light-yellow in color, with a rather oblong shape. They were composed of a large number of secretory follicles which opened into the main duct or albumen canal situated centrally (Fig. 4A). The

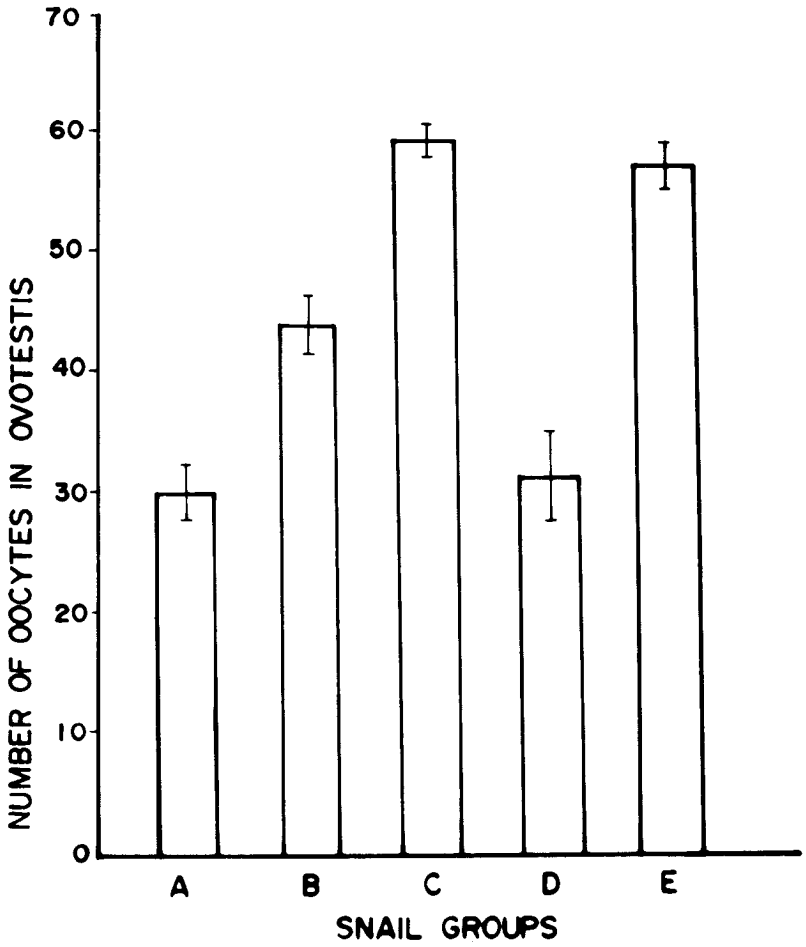

Fig. 1. Histograms showing numbers of oocytes in the ovotestis of *A. fulica.*

A = Non-injected group

B = Estradiol - injected group

C = Progesterone - injected group

D = Testoviron - injected group

E = HCG - injected group

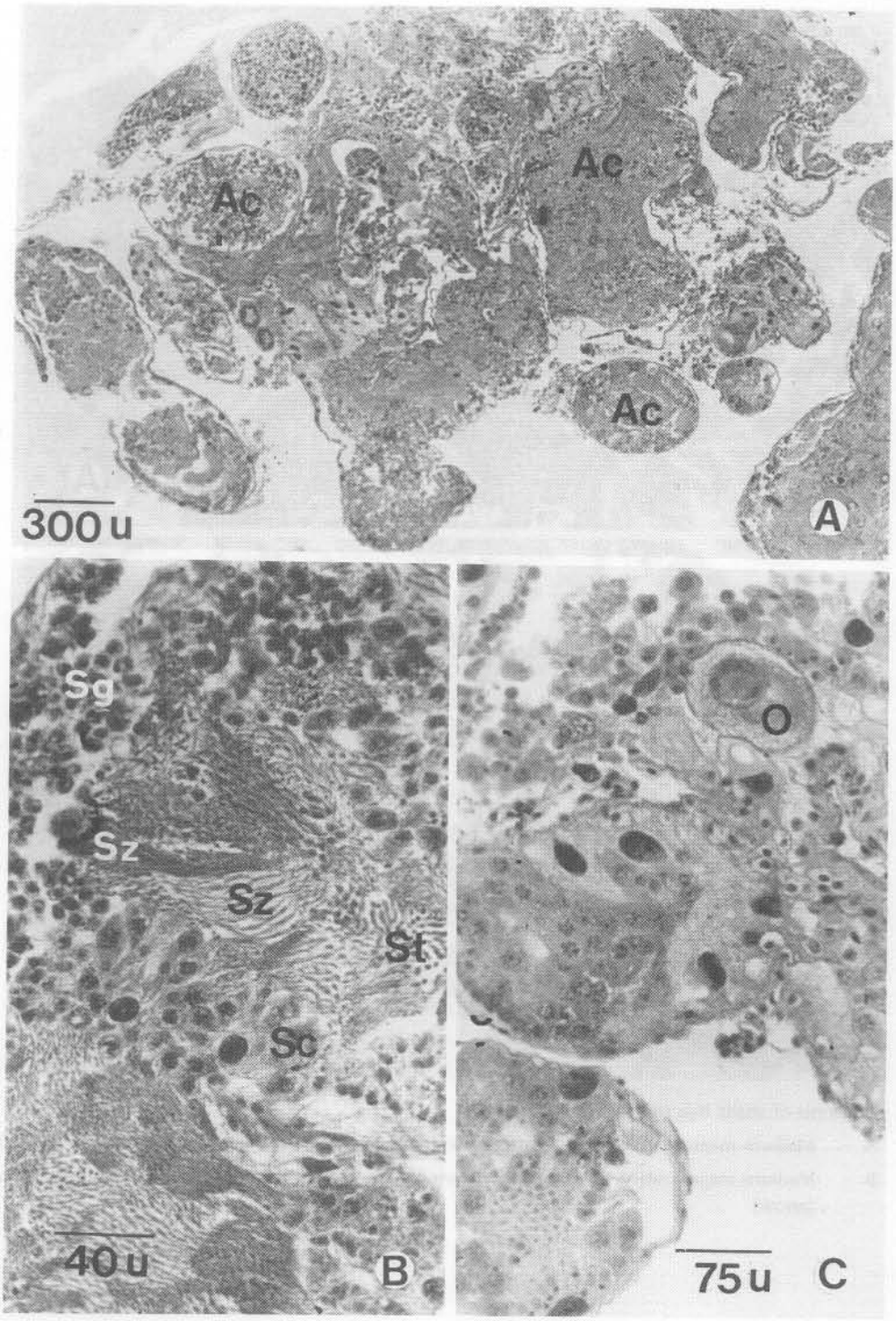

Fig. 2. Ovotestis of control snails.

A : Low magnification of the ovotestis of control snails. It is composed of numerous follicles or acini (Ac).

B : High magnification of an acinus, showing all stages of spermatogenesis : spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz).

C : Medium magnification of an acinus, showing a developing oocyte (O).

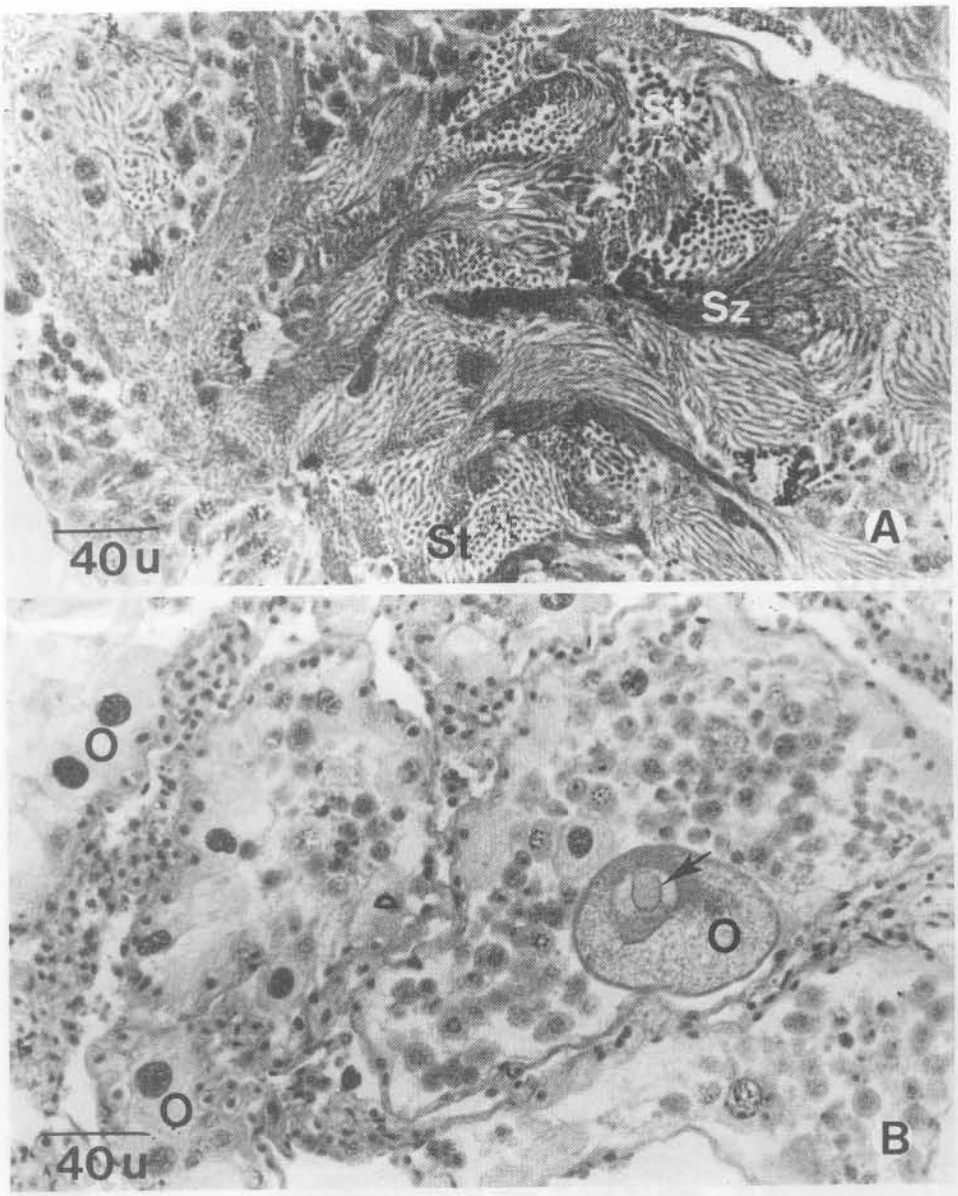

Fig. 3. Ovotestis of snails injected with progesterone.

A : Medium magnification of an acinus, showing numerous spermatids (St) and spermatozoa (Sz).

B : Medium magnification of an acinus, showing young and developing oocytes with distinct nucleoli (arrow).

secretion of the gland emptied into the albumen canal. It was observed that there was no secretory material in the canal (Fig. 4B). The canal was lined by a ciliated columnar epithelium supported by a thin coat of circular muscle. The follicles of the albumen gland were round or oval in shape (Fig. 4B). They were held together by thin strands of connective tissue. Each follicle consisted of a number of gland cells (10-13 cells in small follicle; 25-30 cells in large follicle) arranged around a small central lumen (Fig. 4C). The cytoplasm of the cells contained very little globular secretory material. There were two types of cells in the follicle : the large secretory cells and the small ciliated cells. The large secretory cell had a broad basal region in which a large granular nucleus was located, and they tapered towards the lumen (Fig. 4C). The small ciliated cells were wedged between the large secretory cells and located close to the lumen. They contained small oval nuclei (Fig. 4C).

Experimental groups : The albumen glands of the snails injected with progesterone, estradiol and HCG were light-yellow in color. The gland was round to oval in shape. The albumen canal, both the main duct and side ducts were filled with secretory material (Fig.5A). The large secretory cells around the lumen had undergone hypertrophy. The cells were large and active in secretion (Fig. 5B). They contained large portions of cytoplasm filled with globular secretory material. The nuclei of these cells were small because they were pushed to the cell periphery at the base (Fig. 5B). In the snails injected with testoviron, the activity of the secretory cells was less pronounced. The albumen canal contained little secretory material (Fig. 6A). The secretory cells contained secretory material in the cytoplasm but it was not in globular form (Fig. 6B).

Prostate gland

The prostate gland is an elongated organ that lies close to the uterus. The prostate glands seemed to be least affected by the vertebrate hormones. Hence, the prostate glands of the control snails and those of the snails injected with vertebrate hormones (estradiol, progesterone, testoviron and HCG) appeared similar (Fig. 7). They were light-yellow in color and consisted of numerous lobes. Each lobe was composed of many prostate acini (Fig. 7A). Each acinus was round to tubular in shape with gland cells arranged around a central lumen (Fig. 7B). The prostate secretion was discharged into the lumen and then into the prostate canal. There were two types of gland cells which could be distinguished by their staining affinity. The first type contained eosinophilic granules in the cytoplasm (Fig. 7C). The second type contained clear granules in the cytoplasm (Fig. 7C). The nuclei of these gland cells were located at their bases (Fig. 7C). There were more gland cells with clear granules than those with eosinophilic granules. Colloidal substance was present in the prostate canal (Fig. 7A).

Uterus

Control groups : The uterus is a long hollow organ lying parallel to the prostate gland. The uterine canals contained some colloid (Fig. 8A). The uterine wall was composed of three layers : the inner, middle and outer layers (Fig. 8A). The inner layer lining the lumen was narrow and composed of a simple ciliated columnar epithelium. The cells were relatively short and their oval nuclei were located at the base (Fig. 8B). The middle layer was wide and composed of numerous gland cells surrounded by a lamina propria. Most of the gland cells were large and had round to oval shapes. They were highly vacuolated with their nuclei located at the periphery (Fig. 8B). The outer layer consisted of circular and longitudinal smooth muscles (Fig. 8B). This layer was rather thick and covered with connective tissue.

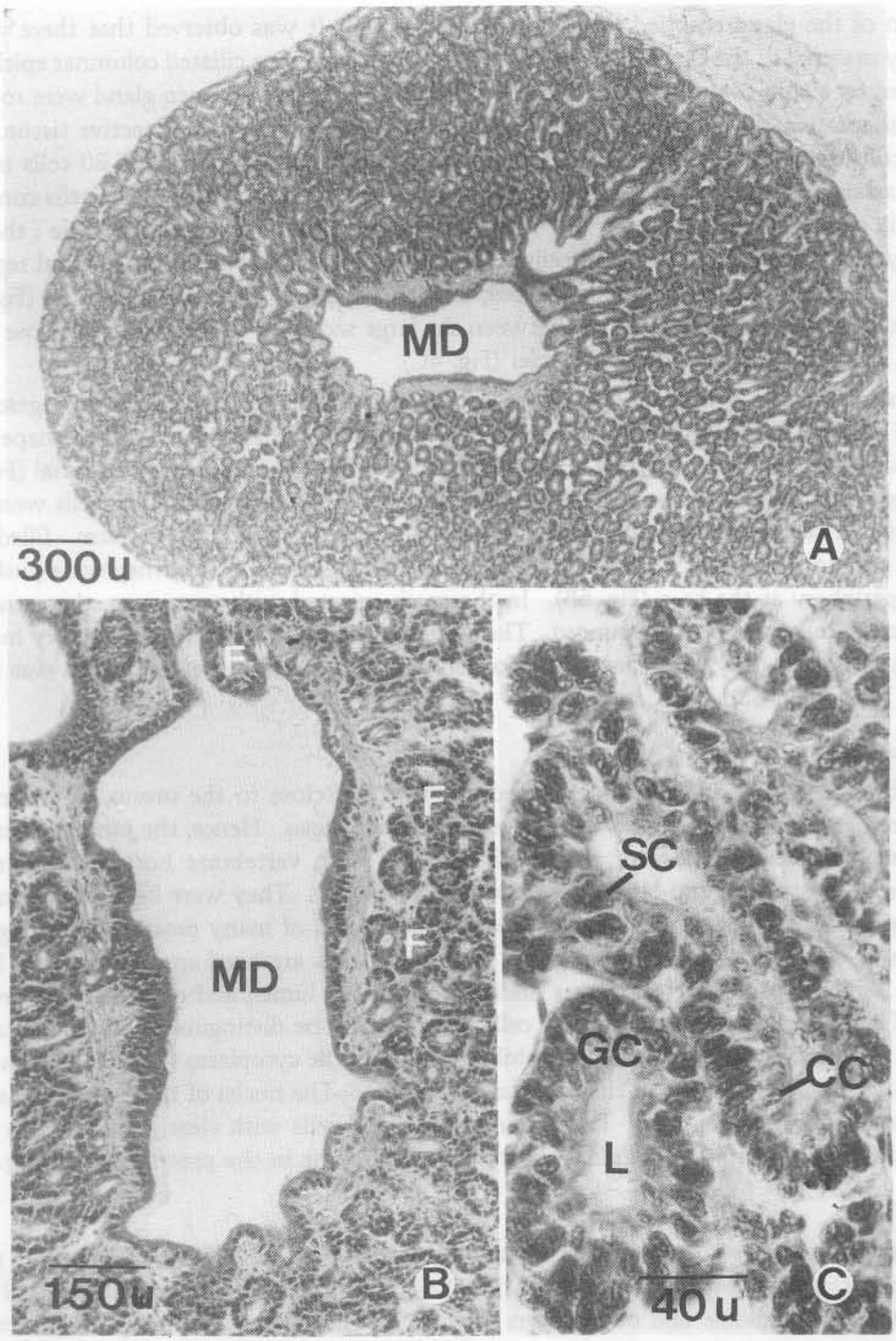

Fig. 4. Albumen gland of control snails.

A : Low magnification, showing general morphology. Numerous follicles are arranged around the main duct (MD) of the albumen canal.

B : Medium magnification of follicles (F) around the main duct (MD).

C : High magnification of follicles showing gland cells (GC) around the central lumen (L). The gland cells are large secretory cells (SC) and small ciliated cells (CC).

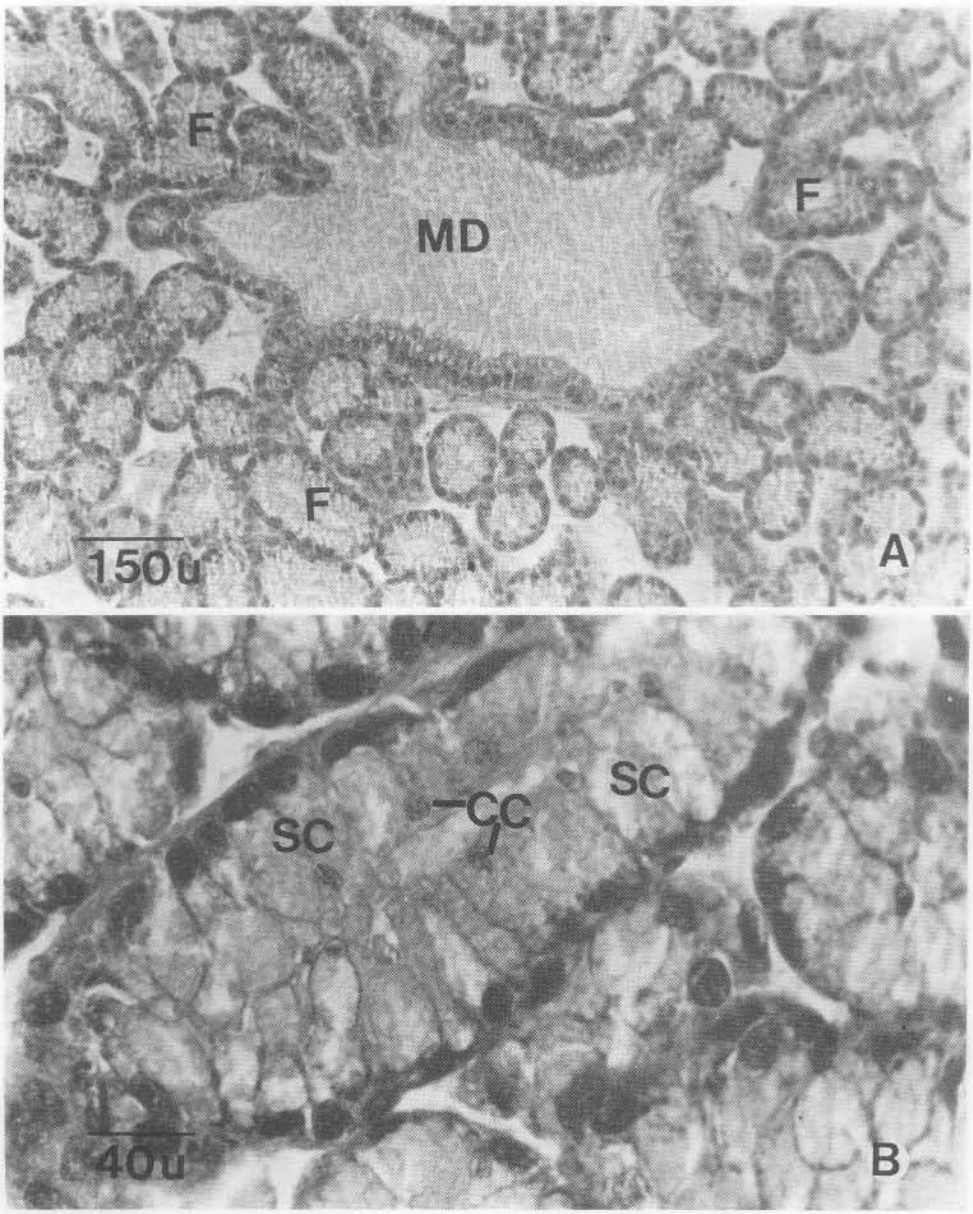

Fig. 5. Albumen gland of snails injected with HCG.

A : Medium magnification of the main duct (MD) of the albumen canal filled with secretory material. F= follicle.

B : High magnification of follicles showing large secretory cells (SC) containing globular secretory material. CC= ciliated cell.

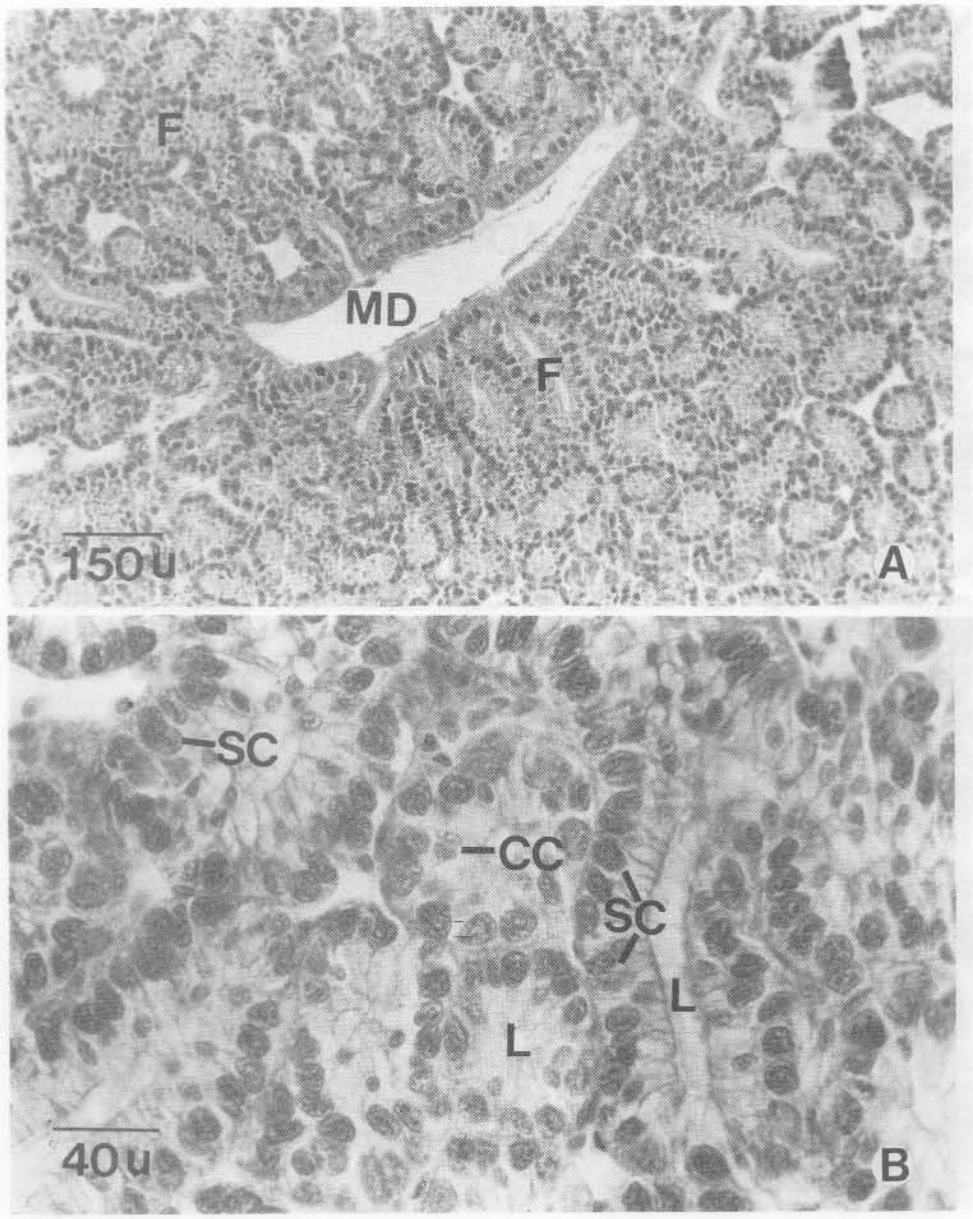

Fig. 6. Albumen gland of snails injected with testoviron.

A : Medium magnification, showing the main duct (MD) of the albumen canal with little secretory material. F = follicle.

B : High magnification of follicles showing gland cells around the lumen (L). SC = secretory cell, CC = ciliated cell.

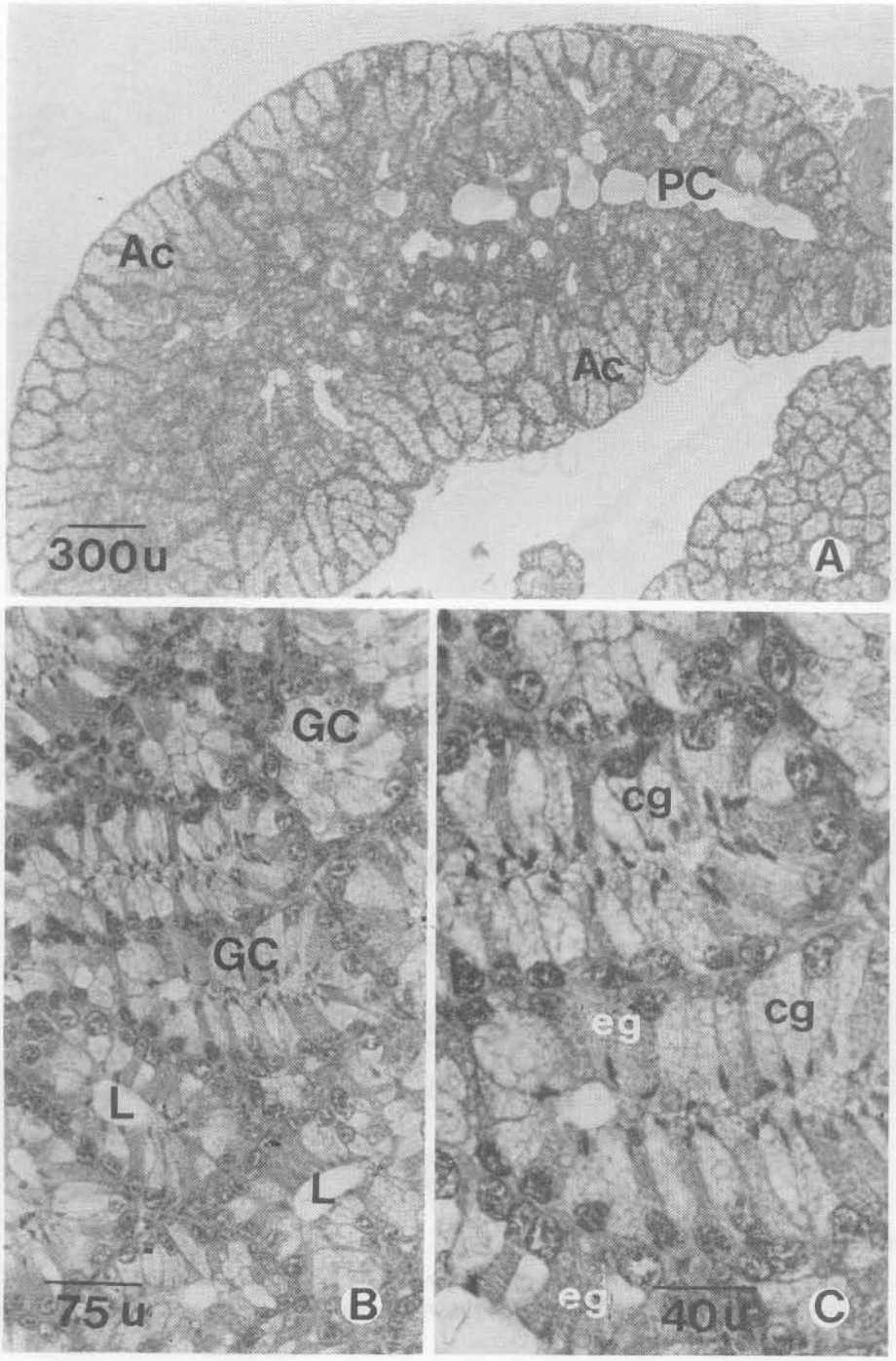

Fig. 7. Prostate gland of control snails.

A : Low magnification, showing numerous prostate acini (Ac). Note the presence of colloid in the prostate canal (PC).

B : Medium mangification, showing prostate acini with gland cells (GC) arranged around a central lumen (L).

C : High magnification, showing gland cells which consist of two types : gland cells containing eosinophilic granules (eg), and those containing clear granules (cg).

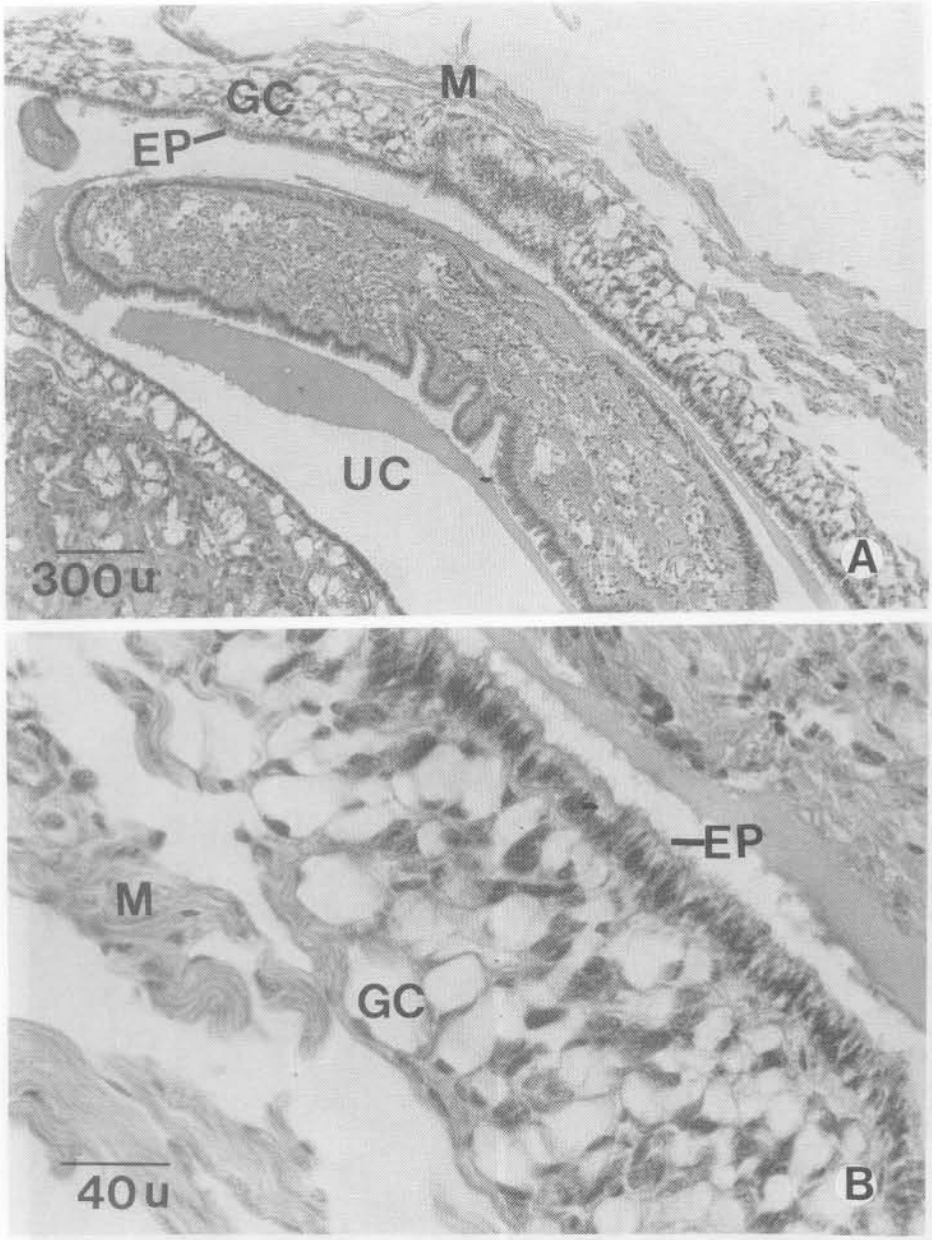

Fig. 8. Uterus of control snails.

A : Low magnification, showing general organization of the uterine wall which is composed of three layers. Note the presence of colloid in the uterine canal (UC). M = muscle, GC = gland cell, EP = simple ciliated columnar epithelium.

B : High magnification, showing three layers of the uterine wall. The inner layer is a simple ciliated columnar epithelium (EP). The middle layer is composed of highly vacuolated gland cells (GC). The outer layer is the muscle layer (M).

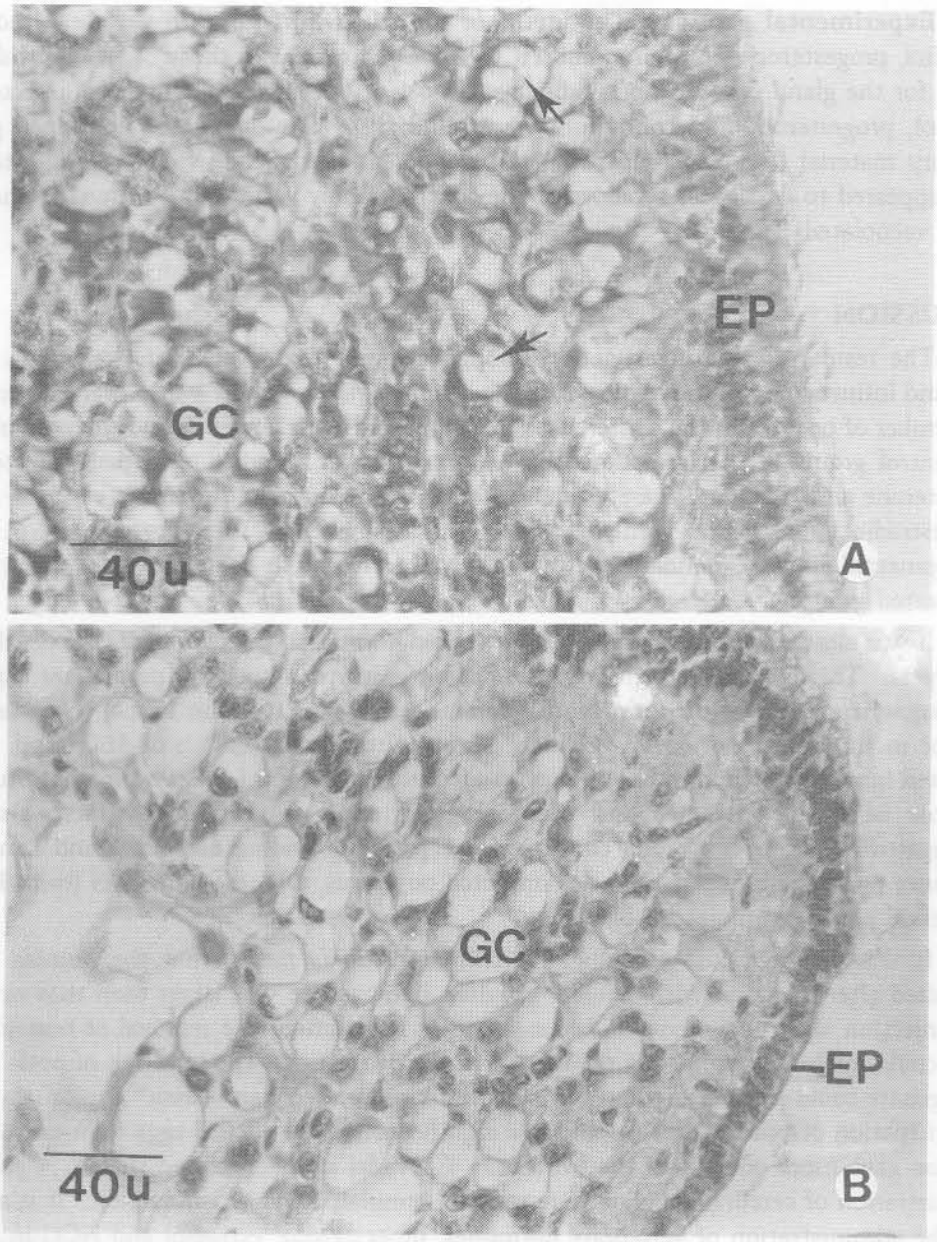

Fig. 9. Uterus of snails injected with vertebrate hormones.

A : Uterus of snails injected with estradiol, progesterone and testoviron. Most of the gland cells (GC) contain globular secretory material (arrow).

B : Uterus of snails injected with HCG. Gland cells (GC) are highly vacuolated, similar to those in the uterus of control snails.

EP = simple ciliated columnar epithelium.

Experimental groups : The uterus of the snails injected with vertebrate hormones (estradiol, progesterone, testoviron and HCG) appeared similar to those of the control groups except for the gland cells in the middle layer. Most of the gland cells of snails injected with estradiol, progesterone and testoviron were round to oval in shape and contained globular secretory material (Fig. 9A). However, the gland cells in the uterus of snails injected with HCG appeared to be similar to those of the control. They were round to oval in shape and highly vacuolated (Fig. 9B).

DISCUSSION

The results of this investigation strongly suggest that vertebrate hormones exert a profound influence upon the ovotestis of the snail, *A. fulica*. There was a marked increase in the number of oocytes in the ovotestis of snails injected with hormones when compared with the control groups. The largest number of oocytes was found in the snails injected with progesterone and HCG and there was an increase in the number of oocytes of snails injected with estradiol. Testoviron seemed to exert the least effect. The oocytes were in various developmental stages. Abundant spermatozoa were present in the ovotestis of both control and treated groups.

It was clearly demonstrated, here, that steroid hormones could stimulate the development of oocytes. This is in agreement with studies on gastropods such as pulmonate slug, *Arion subfucus*, where removal of optic tentacles or the injection of brain homogenate led to an increase in the number of eggs[32]. Pelluet[33] reported that the ovotestis of *Milax* spp. showed a marked increase in the number of oocytes after the injection of brain hormone. Studies on the role of optic tentacles and cerebral ganglia on gametogenesis have also been investigated[13,16,34,35]. The results confirmed the previous finding of Pelluet and Lane[32] that hormones produced by optic tentacles inhibited oogenesis, whereas hormones from the brain or cerebral ganglia stimulated oogenesis.

It was reported for the slugs *A. reticulatus* and *L. flavus*, that the number of eggs oviposited after injection with cerebral ganglion homogenate was larger than that oviposited after injection with optic tentacle homogenate[13]. In addition, the removal of tentacles in *L. flavus* could inhibit spermatozoa production in the ovotestis[34]. The injection of optic tentacle homogenate could restore spermatogenesis[34]. In *A. fulica*, Berry and Chan[35] demonstrated that the extirpation of optic tentacles produced significantly more shelled eggs than normal snails and also gave more oocytes in the ovotestis. Chavadej *et al.*[16] reported that in *A. fulica*, the administration of cerebral ganglion homogenate stimulated oocyte production. It is apparent that the administration of vertebrate hormones, progesterone, estradiol and HCG in *A. fulica* could increase the oocyte production similar to that produced by the removal of optic tentacles or the injection of cerebral ganglion homogenate.

In *A. fulica*, there was a remarkable change in histology of the albumen glands in snails injected with estradiol, progesterone and HCG. The albumen canal of these snails contained some secretory material probably galactogen indicating secretion of gland cells or secretory cells. The latter appeared to be in an active state due to their increased size and due to the fact that their cytoplasm was filled with globular secretory material. Their nuclei were small because they were pushed to the periphery at the cell base. These changes coincided with the increase in production of oocytes in the ovotestis.

In pulmonate molluscs, the albumen gland secretes perivitelline fluid around the eggs following fertilization[36]. As mentioned earlier, primary constituent of this fluid is

galactogen[37-40] which provides a major energy source for the developing embryo[41]. The amount of galactogen in the albumen gland is related to the stage of the reproductive cycle[36].

Several studies have established that there is endocrine regulation of the synthesis of galactogen in the albumen gland either by the dorsal bodies alone or by the dorsal bodies and the brain combined[7,14,27,42-44]. In the basommatophoran, *L. stagnalis*, the caudodorsal cell hormone produced by a group of neurosecretory cells in the cerebral ganglia was also found to stimulate galactogen synthesis[45]. In the stylommatophoran, *H. pomatia*, a brain factor termed galactogenin stimulates galactogen synthesis[46], whereas in *L. maximus*, the galactogen stimulating factor was derived from the endocrine dorsal bodies[47]. In *Helisoma duryi*, both the dorsal bodies and the cerebral ganglia could independently stimulate polysaccharide synthesis in the albumen gland. The product from the dorsal bodies that stimulated galactogen synthesis might be a steroid, whereas that from the cerebral ganglia was a peptide[44]. Hence, it may be concluded that galactogen synthesis in the albumen gland of *A. fulica* is stimulated by the hormones, estradiol, progesterone and HCG.

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บทคัดย่อ

การศึกษาอิทธิพลของฮอร์โมนของสัตว์มีกระดูกสันหลังต่อระบบสืบพันธุ์ของหอยทากยักษ์ (*Achatina fulica*) กระทำโดยการฉีดฮอร์โมนเอสทราไดอัล เทสโทไวรอน โพรเจสเทโรน และฮิวแมนโคริโอนิกโกนาโดโทรฟิน (HCG) เข้าไปในหอยทากยักษ์ ส่วนการศึกษาการเปลี่ยนแปลงของเนื้อเยื่อในอวัยวะต่าง ๆ ของระบบสืบพันธุ์ ได้แก่ โอโวเทสทิส ต่อมแอลบิวเมน ต่อมโพรสเทต และยูเทรัสนั้น กระทำโดยกระบวนการพาราฟินเพื่อศึกษาด้วยกล้องจุลทรรศน์

จากการศึกษาดังกล่าวข้างต้นพบว่า ในโอโวเทสทิสของหอยที่ฉีดด้วยฮอร์โมนโดยเฉพาะโพรเจสเทโรน HCG และเอสทราไดอัลมีจำนวนโอโอไซต์เพิ่มขึ้นมากเมื่อเปรียบเทียบกับกลุ่มควบคุม และกลุ่มที่ฉีดด้วยเทสโทไวรอน การเปลี่ยนแปลงของเนื้อเยื่อที่เห็นเด่นชัดจะพบในต่อมแอลบิวเมน หอยที่ฉีดด้วยฮอร์โมนเอสทราไดอัล โพรเจสเทโรนและ HCG จะมีต่อมแอลบิวเมนขนาดใหญ่ ในช่องแอลบิวเมนจะมีสารเซครีทอรีบรรจุอยู่ ส่วนเซลล์ต่อมนั้นมีขนาดใหญ่ขึ้นและมีไซโทพลาสซึมที่บรรจุด้วยสารเซครีทอรีที่มีลักษณะเป็นเม็ดกลม