β-GLUCURONIDASE ACTIVITY IS ELEVATED IN THE RAT HARDERIAN GLAND AFTER BROMOCRIPTINE BUT DECREASED AFTER CYPROTERONE ACETATE TREATMENT OR CASTRATION. POSTCASTRATIONAL EFFECTS ON THE ULTRASTRUCTURE OF THE GLAND

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Objective. To investigate the effect of castration, bromocriptine and cyproterone acetate treatment on lysosomal hydrolytic enzymes and ultrastructure of the rat Harderian gland.

Methods. Groups of rats were subjected to the treatment by bromocriptine, cyproterone acetate and castration. Harderian glands were dissected from each experimental animal, cut into small pieces and immediately stored in liquid nitrogen. Only those from castrated animals were fixed in Karnovsky fluid and processed for electron microscopy. The activity of acid phosphatase and β-glucuronidase were estimated by kits from Sigma (St. Louis, Mo, USA). Semi-thin sections were stained with 1% toluidine blue and ultra-thin sections with uranyl acetate and counter-stained with lead citrate. The examination was performed by Joel JEM-1200 EX II electron microscope..

Results. The treatment of male rats with the prolactin release inhibitor bromocriptine induced a significant increase in β-glucuronidase activity. On the contrary, such activity was significantly decreased after treatment with the testosterone receptor antagonist cyproterone acetate as well as after castration. However, these treatments did not alter the activity of lysosomal acid phosphatase.

Castration induced dramatic changes mostly in type A cells such as the appearance of lipid vacuoles with irregular forms and the predomination of smooth endoplasmic reticulum (SER) throughout the cytoplasm. The most dramatic postcastrational change was the degeneration of the mitochondria. These changes in type A cells might be due to the uneven distribution of the testosterone receptors in the rat Harderian gland which are more numerous in type A cells.

Conclusions. the gland physiology is responsive to alteration in circulating prolactin and testosterone levels.

Key words: Harderian glands – Rat – Bromocriptine – Cyproterone acetate – Castration – Lysosomal enzymes – Ultrastructure

The Harderian gland is a compound tubulo-alveolar gland located around the posterior part of the eyeball in the nasal side of the orbit of the majority of the land vertebrates and it is often surprisingly large, in some cases larger than the eye itself (Djeridane 1996). The gland's anatomy and biochemistry is altered by gonadal steroids and several pituitary hormones (Dioria 1984; McMasters and Hoffman 1984; Buzzell 1991) as well as by environmental factors (Menendez-Pelaez et al. 1987; Buzzell 1990). Lysosomal enzymes are known to play an important role in the mediation of hormonal signals, in the disposal of excess hormone vesicles, and in the enzymatic degradation of the intracellular macromolecules whose byproducts are recycled for metabolic use or export (Farquhar 1969; Szego 1975). The present study was

carried out to investigate the effect of decreasing prolactin or testosterone levels on lysosomal hydrolytic enzymes, an area that has not been well studied. The ultrastructure of the rodent Harderian gland has been studied (Bucana and Nadakavukaren 1973; Brownscheidle and Niewenhuis 1978; Djeridane 1996; Sabry et al. in press). In rat, the gland contains two different types of secretory epithelial cells, termed type A and type B (Brownscheidle and Niewenhuis 1978). The Harderian gland may be a target organ for gonadal steroids. Most of the information available on this topic came from studies on the golden hamster, where the Harderian gland shows a high degree of sexual dimorphism (Bucana and Nadakavukaren 1973). Castration of the adult male hamster converts all gland characteristics (both morphological and biochemical) to the female pattern within a few weeks (Payne et al. 1977). The effect of castration on the lysosomal enzymes as well as on the ultrastructure of the rat Harderian gland is also investigated.

Materials and Methods

In the present study, male albino rats (160-200 g body weight) were used. Animals were raised in the facilities of the Faculty of Science, Kuwait University. They were housed in plastic cages (4 per cage) under constant environmental conditions of temperature (22+2 ^{0}C), relative humidity (45-50 %) and a light:dark cycle of 12:12. Food and drinking water were available *ad libitum*. Animals were divided into 3 experimental groups treated as follows:

Group I (bromocriptine treatment). Sixteen male rats were divided into two subgroups: The first subgroup (eight animals) received twice daily a subcutaneous (sc) injection (1 mg/kg body weight) of bromocriptine mesylate (2-bromo-α-ergocryptine methane sulfonate, Sigma, St. Louis, Mo, USA) dissolved in 100 µl of physiological saline for four days. The second subgroup (eight animals) received twice daily a sc injection of physiological saline (100 µl/animal) for four days and served as saline treated control. In both subgroups, the first injection was carried out at 9:00 a.m. and the second injection at 2:00 p.m.

Group II (cyproterone acetate treatment). Sixteen male rats were divided into two subgroups: The first subgroup (eight animals) received a daily oral dose (12.5 µg/kg body weight) of cyproterone acetate (Sigma, St. Louis, Mo, USA) dissolved in 100 µl of corn oil for four days. The second subgroup (eight animals) received a daily oral dose (100 µl/animal) of the vehicle only (corn oil) for four days and served as corn oil treated control. In both subgroups, treatment was carried out at 9:00 a.m.

Bromocriptine treated animals and cyproterone acetate treated animals, along with there respective controls, were decapitated between 9:00 – 10:00 a.m. on the morning of the fifth day after initiation of treatment.

Group III (castration). Twenty four male rats were divided into two subgroups. The first subgroup (ten male rats) were castrated under thiopentone sodium (May & Baker) anesthesia (50 mg/kg body weight, ip). After castration, povidone-iodine solution (antiseptic) and neomycine (bactericidal antibiotic) were applied at the site of surgery. The second subgroup (fourteen male rats) were left untreated and served as controls. After two weeks, castrated animals along with there untreated controls were decapitated between 9:00 – 10:00 a.m.

Specimen collection and fixation. Following decapitation, both right and left Harderian glands were removed from both orbits of each experimental animal. The glands of each animal were cut into small pieces, placed in microcentrifuge vials and immediately stored in liquid nitrogen until use for enzyme assays. Only for animals from group III, small pieces of the gland were fixed by immersion in Karnovsky (pH 7.4 at 4 ^{0}C for 24 h) and processed for electron microscopy.

Preparation of homogenates. Tissues were removed from liquid nitrogen, thawed and 10-15 mg pieces were used. Each piece was homogenized in 1 ml cold distilled water using Ultra-Turrax T8 (IKA Labortechnik) homogenizer. Homogenates were centrifuged (10 min, 6500 rpm) to remove cellular debris. The resulting supernatants were individually assayed for lysosomal enzymes activity.

Enzyme assays. The activity of acid phosphatase and β-glucuronidase were carried out using kits and protocols (104 AL and 325-B, respectively) supplied by Sigma (St. Louis, Mo, USA). Enzyme activities were expressed as Sigma units relative to mg tissue wet weight.

Electron microscopy. After fixation, specimens were washed in 0.2 M sodium cacodylate buffer (pH 6.2) and post fixed in 2% osmium tetroxide for 2 h. Specimens were then dehydrated, cleared in propy-

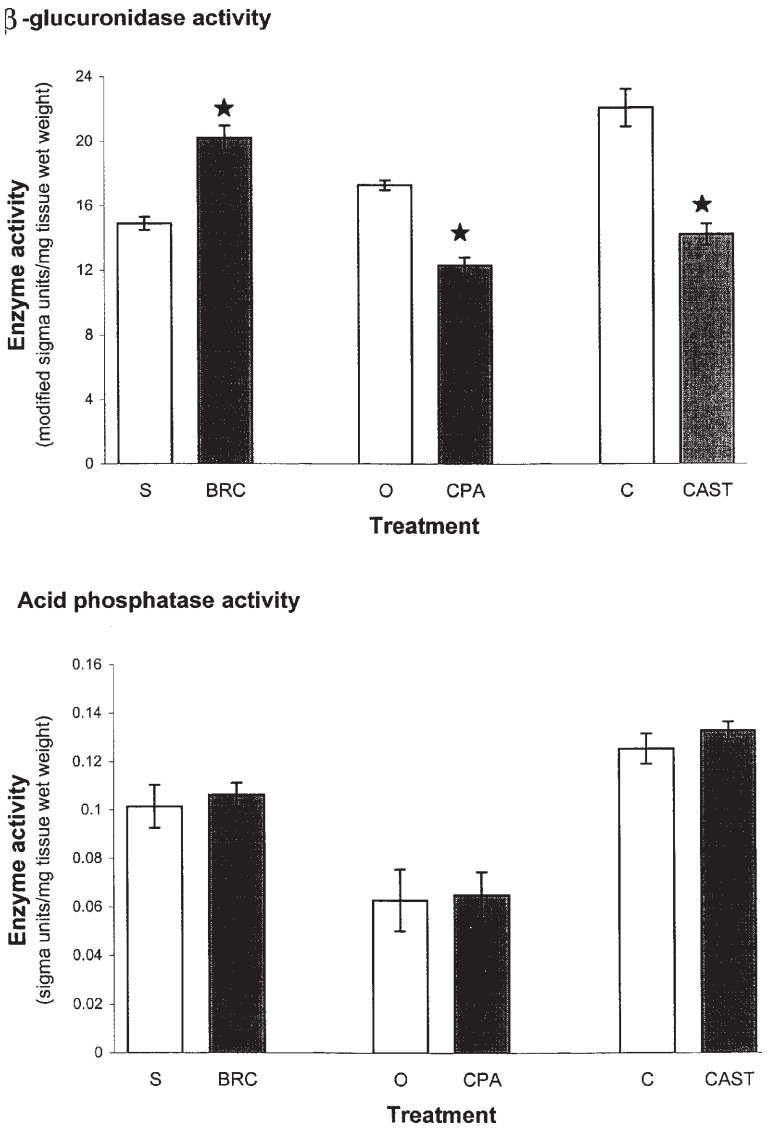


Fig. 1 Lysosomal enzyme activity in the rat Harderian gland of male albino rats after various treatments. Upper panel: β-glucuronidase activity. Lower panel: acid phosphatase activity. BRC: bromocriptine treated rats, S: saline treated control rats, CPA: cyproterone acetate treated rats, O: corn oil treated rats, CAST: castrated rats, C: control untreated rats. *:P<0.001 vs. corresponding control.

lene oxide for 10 min and embedded in araldite. Semi-thin sections (1 μm) were stained with 1% toluidine blue. Ultra-thin sections were stained with uranyl acetate for 30 min, washed with double distilled water, counter-stained with lead citrate and washed once more. Examination was performed in a Joel JEM-1200 EX II electron microscope.

Statistical analysis. Data are represented as means ± SEM and were statistically analyzed using Student's t-test in Statistical Package for Social Science (SPSS) software (Version 7.5, SPSS Inc.).

Results

Treatment of the male rats with bromocriptine for 4 days induced an increase in the activity of β-glucuronidase in the Harderian gland (P<0.001) while the activity of acid phosphatase was unaltered (Fig. 1). On

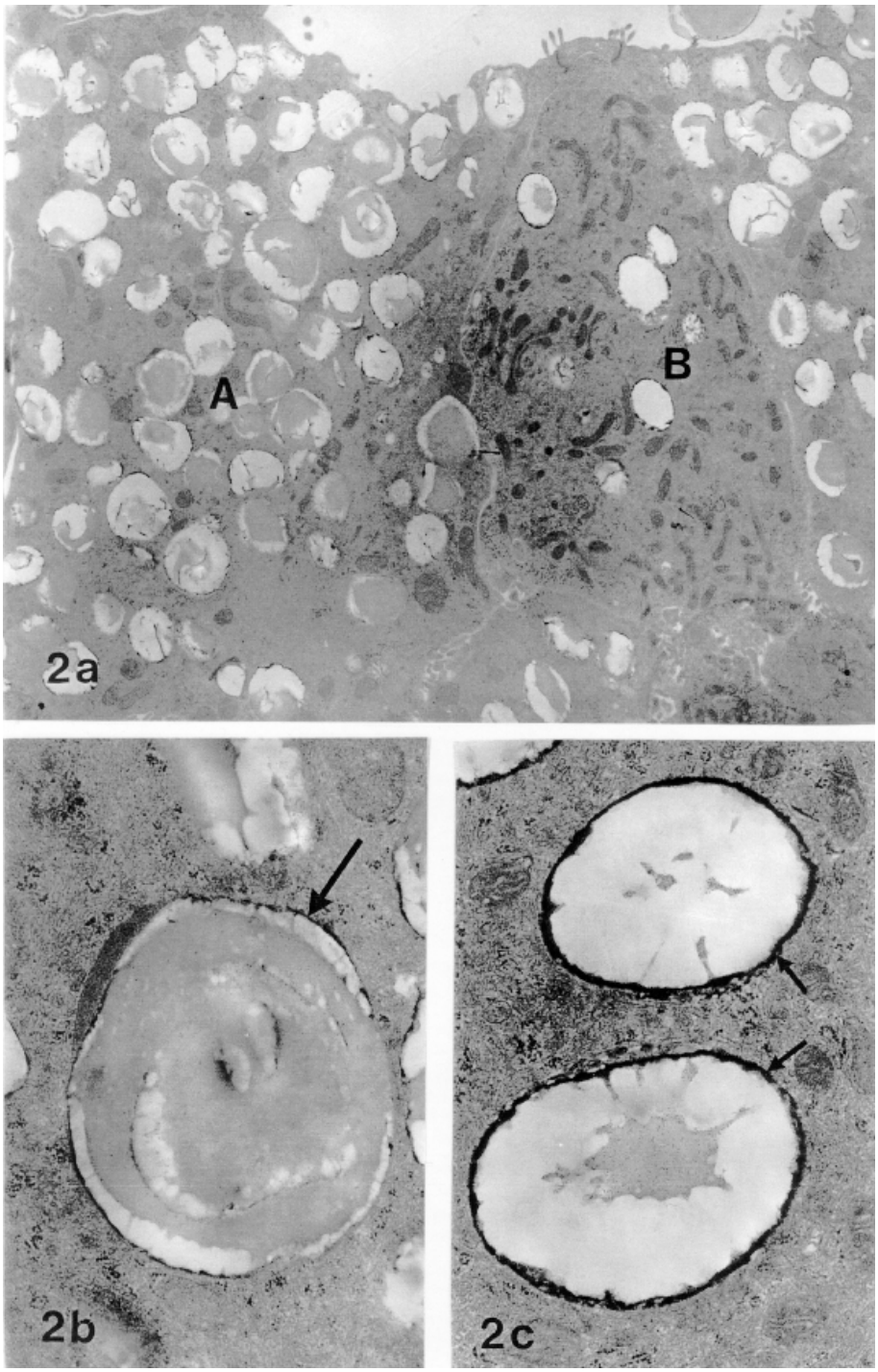
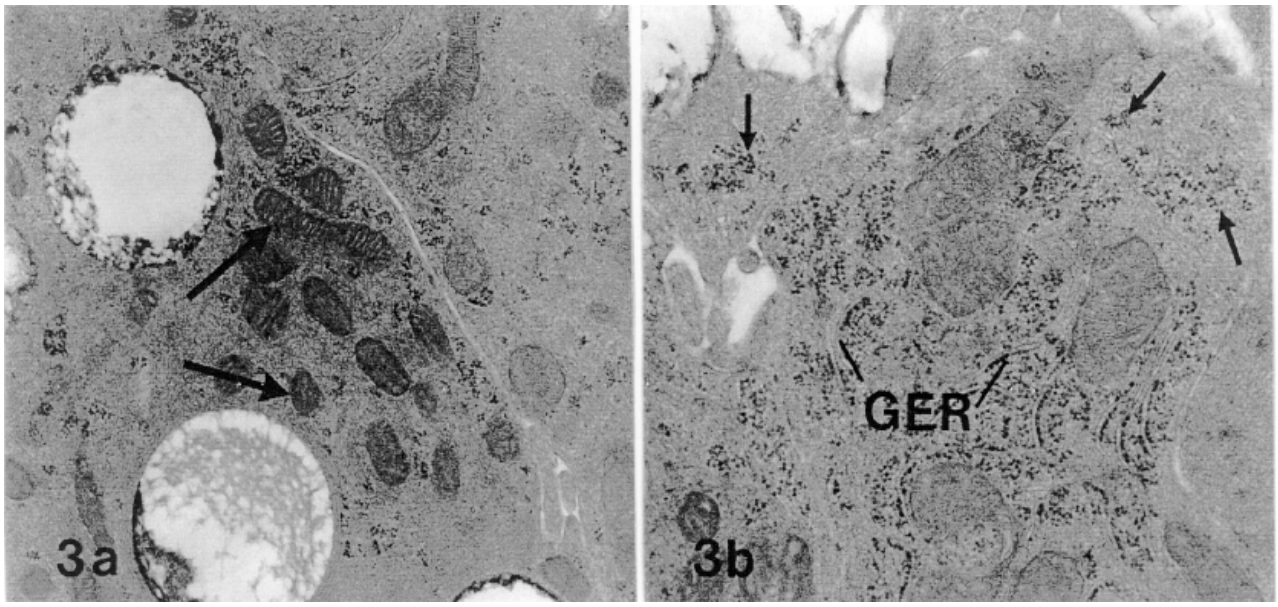


Fig. 2 (a) Electron micrograph of the Harderian gland of a control male albino rat showing the two epithelial cell types (A and B) (x 4000). (b) Lipid vacuole of type A cell is limited by a unit membrane (arrow) and contain electron-dense material (x 15000). (c) Lipid vacuoles of type B cell are characterized by having peripheral rims of high electron density (arrows) (X15000).



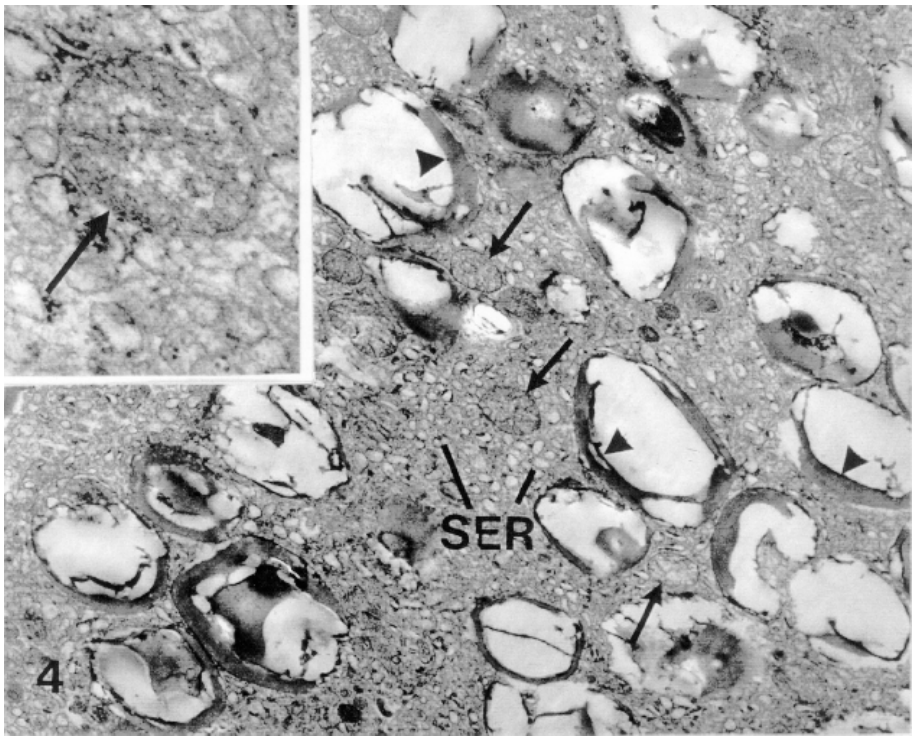


Fig. 3 Electron micrographs of the Harderian gland of a control male albino rat. (a) Pleomorphic mitochondria (arrows) (x 15000), (b) GER and free ribosomes in the form of polysomes (arrows) (x 20000) are extensively present throughout the cytoplasm of type B cells.

Fig. 4 Electron micrograph of a type A cell in the Harderian gland of a castrated male albino rat showing numerous irregular shapes of lipid vacuoles containing electron-dense material coating the inside of the vacuoles' membrane (arrow heads). Degenerated mitochondria (arrows) and SER are evident (x 7500). Inset: A higher magnification of a degenerated mitochondria (arrow) (x 30000).

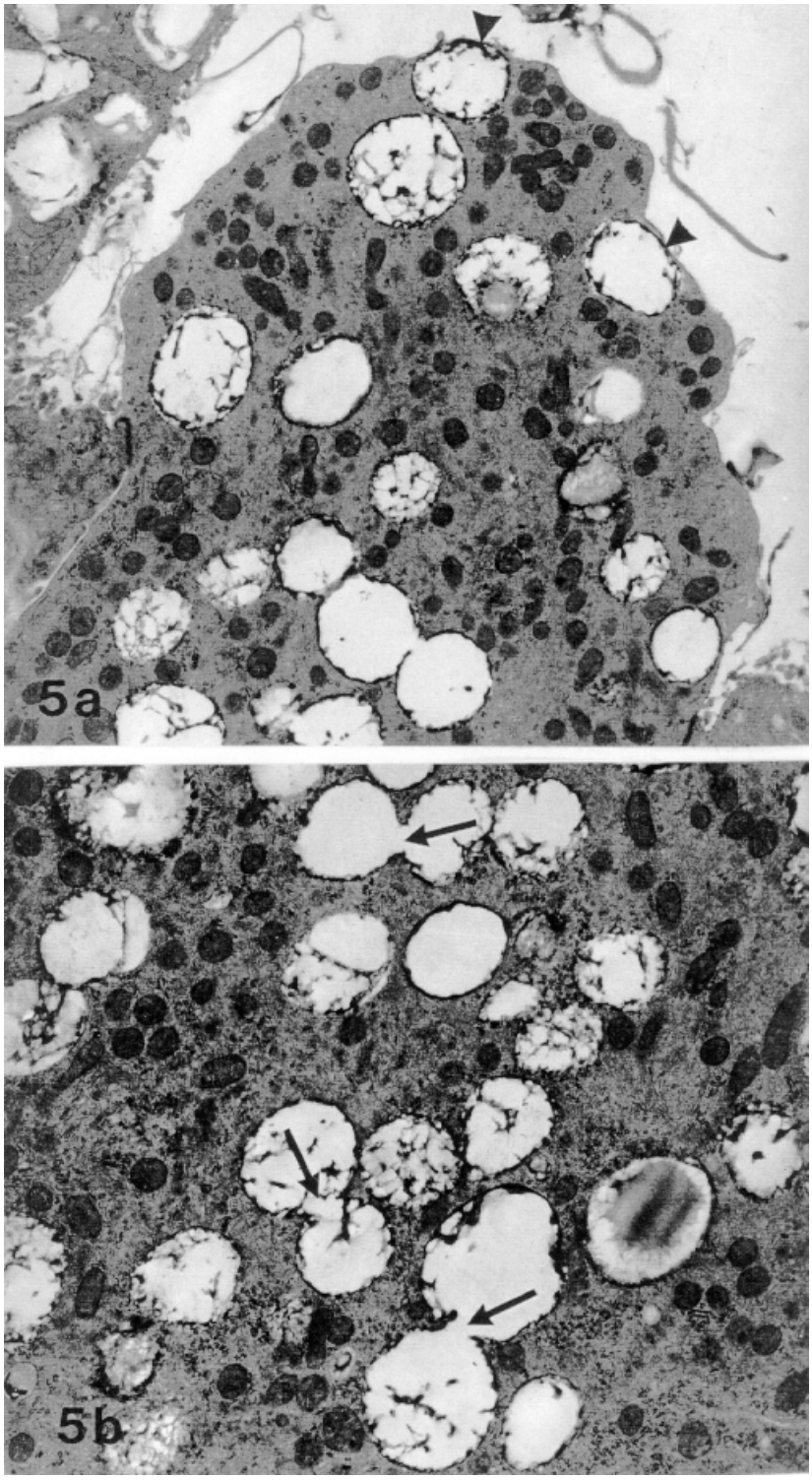


Fig. 5. Electron micrographs of the Harderian gland of a castrated male albino rat showing type B cells. (a) The unit membrane of lipid vacuoles are seen fusing with the apical cell membrane (arrow heads) as a first step of exocytotic release (x 6000). (b) Some of the lipid vacuoles are fusing (arrows).

the contrary, treatment with cyproterone acetate for 4 days induced an adverse effect where a decrease in β-glucuronidase activity ($P<0.001$) was detected but still the acid phosphatase activity was not changed (Fig. 1). Similar to cyproterone acetate treatment, a significant decrease in β-glucuronidase activity ($P<0.001$) was noted in the Harderian glands of castrated rats as compared to the untreated controls (Fig. 1).

Ultrastructurally, there are two epithelial cell types (A and B) in the rat Harderian gland (Fig. 2a). The type A cells are characterized by the large number of lipid vacuoles present throughout most of the cytoplasm (Fig. 2a), while the lipid vacuoles of type B cells were less numerous and smaller in size (Fig. 2a). The large lipid vacuoles of type A cells are filled with electron dense material (Fig. 2b). The lipid vacuoles of type B cells, however, often possess border laminations or electron-dense outer rim membranes (Fig. 2c). Type B cells are also characterized by the presence of large numbers of pleomorphic mitochondria (Figs. 2a and 3a), extensive granular endoplasmic reticulum (GER) and free ribosomes in the form of polysomes (Fig. 3b).

After castration, changes were mainly observed in type A cells. Lipid vacuoles possessed irregular shapes and were found to contain electron-dense material coating the inside of the vacuole's membrane (Fig. 4) rather than lying within the vacuole (Fig. 2b). While the GER was still visible, the smooth endoplasmic reticulum (SER) was present extensively throughout the cytoplasm in the central part of the cell (Fig. 4). In addition, mitochondria were mostly degenerating (Fig. 4). The castration effect was less dramatic in type B cells. The normal architecture of the cell was preserved (Fig. 5a). In some instances, the unit membrane of the lipid vacuoles were observed fusing with the apical cell membrane (Fig. 5a) as a first step of exocytosis. Fusion between some lipid vacuoles (coalescence) were frequently observed (Fig. 5b).

Discussion

In the present study, treatment of male rats with bromocriptine for four days induced a significant elevation in β-glucuronidase activity. However, acid phosphatase activity was unaltered. Bromocriptine is an ergot alkaloid whose main pharmacological effect is as a dopamine agonist. As such, it is used clinically and experimentally to reduce prolactin levels, since the secretion of prolactin is normally inhibited by dopamine (Ben-Jonathan 1985). In an earlier study, Vaughan et al. (1988) found that hypophysectomy altered acid phosphatase, α-mannosidase and β-glucuronidase activity in the rat Harderian gland. They have also found that administration of prolactin to hypophysectomized rats had no effect on enzyme activity, whereas injection of bromocriptine into intact rats stimulated acid phosphatase activity.

Alteration of prolactin level in experimental animals affects several important physiological processes in the Harderian gland of various animal species, among these is porphyrin biosynthesis. Studies by Marr et al. (1995) demonstrated that bromocriptine can suppress the expected rise in porphyrin content in castrated male hamsters as well as prevent the postcastrational gland feminization. On the other hand, prolactin administration reverses the suppressive effects of bromocriptine and can increase porphyrin biosynthesis in castrated males when administrated alone (Marr et al 1995). While bromocriptine treatment prevented the castration-induced increase in porphyrin concentration in male hamster Harderian gland, it was not capable of reducing porphyrin levels in female glands (Buzzell et al. 1989b). Likewise, Buzzell et al. (1992) concluded that combining two procedures (castration and hypophysectomy) of male hamster lead to a blunt increase in Harderian porphyrin, suggesting that a pituitary hormone, prolactin, is necessary for low testosterone levels to lead to increased porphyrin.

Melatonin production in the Harderian gland is another important process that has been proven in several rodent species (Djeridane et al. 1998). Bromocriptine does not affect the activities of enzymes involved in melatonin synthesis in male hamsters (Buzzell et al. 1989b). It seems that bromocriptine might act by interfering with the hypothalamo-pituitary axis, where dopaminergic neurons control, not only prolactin release, but also, several neuroendocrine systems (Anderson and Eneroth 1987).

It seems that alteration of prolactin level does not markedly affect the Harderian gland physiology unless this is accompanied by a lowered testosterone level. This could be attributed to the presence of testosterone receptors in the Harderian gland. Androgen receptors were first reported in the Harderian gland of male rat (Gustafsson and Pousette 1975) and sub-

sequently in both sexes of the golden hamster (Vilchis et al. 1987; Vilchis and Perez-Palacios 1989).

In the present study, treating male rats with cyproterone acetate, a testosterone receptor antagonist (Ganten et al. 1989) for four days induced a significant decrease in β-glucuronidase activity. Similar results were obtained in castrated rats. On the other hand, acid phosphatase activity was unaltered in both treatments.

Concerning the role of gonadal steroids in regulating the Harderian gland structure and function, it is obvious that the Syrian hamster has received the lion's share of interest among the various rodent species (Hoffman 1971; Clabough and Norvell 1973; Payne et al. 1977; Spike et al. 1985; Rodriguez-Colunga et al. 1993; Buzzell 1996). Sexual differences in the Harderian gland melatonin content was demonstrated in the hamster. It was confirmed that there is a sexual dimorphism in melatonin synthesis in the hamster Harderian gland, and melatonin as well as its synthesizing enzymes are affected by castration or androgen manipulations (Menendez-Pelaez et al. 1988, 1989; Buzzell et al. 1989a; Marrufo et al. 1989). Additional evidence that there are receptors for androgens in the rat Harderian gland of both sexes and that the gland is able to metabolize testosterone to its active form, 5-dihydrotestosterone (Menendez-Pelaez et al. 1990) strongly support the hypothesis that androgens, and not gonadotropins, are responsible for the structural and functional maintenance of sexual dimorphism.

The results of the present study shows that bromocriptine increased the activity of β-glucuronidase while cyproterone acetate treatment or gonadectomy decreased the activity of the enzyme in the rat Harderian gland. The exact function of this acid hydrolase in the Harderian gland remains to be elucidated. However, lysosomal enzymes play an important role in the mediation of hormonal signals, in the disposal of excess hormone vesicles and in the enzymatic degradation of macromolecules whose byproducts are recycled for metabolic use or export (Farquhar 1969; Szego 1975). Lysosomal enzymes are also involved in cellular lytic processes characteristic of holocrine secretion (Brands et al. 1965), one of the three (holocrine, merocrine, apocrine) mechanisms described for the rodent Harderian gland (Watanabe 1980; Carriere 1985; Johnston et al. 1985).

It is clear from the present ultrastructural observations, as well as from earlier observations (Brownscheidle and Niewenhuis 1978; Shirama and Hokano 1992; Djeridane 1996) that the Harderian gland of the male albino rat contains two epithelial cell types (A and B). Generally, type A cells are characterized by the presence of a large number of lipid vacuoles present throughout most of the cytoplasm, on the contrary, lipid vacuoles of type B cell are less numerous and smaller in size. Large numbers of pleomorphic mitochondria, extensive GER and free ribosomes (in the form of polysomes) are more numerous within the cytoplasm of type B cells. The two epithelial cell types are also distinguishable by the shape of their lipid vacuoles. While the lipid vacuoles of type A cells are filled with electron dense materials, the lipid vacuoles of type B cells often possess border laminations of electron-dense outer rim membranes. In the present study, the postcastrational morphological changes were mainly observed in type A cells. Lipid vacuoles acquired irregular forms and contained electron-dense material coating the inside of the vacuoles membrane rather than lying within the vacuole. Also, while GER was still visible, the SER was extensively present and predominant throughout the cytoplasm. The third, and most dramatic, postcastrational change was the degeneration of the mitochondria. On the other hand, the castration effect was less dramatic in type B cells. While the normal architecture of the B cells was preserved, in some instances, the unit membrane of the lipid vacuoles were observed fusing with the apical cell membrane as a first step of exocytosis. Fusion between some of the lipid vacuoles (coalescence) was frequently observed. In an earlier study, Brownscheidle and Niewenhuis (1978) reported a slight decrease in the number of pockets of SER in type B cells of castrated male rats. The abundance of the SER in the Harderian gland epithelial cells is characteristic of steroid secreting endocrine cells. The most direct indication that these epithelial secretory cells might play a role in steroid synthesis comes from the work of Brooksbank et al. (1973). They demonstrated that the rat Harderian gland selectively accumulates the precursor of a particular pheromone steroid. Moreover, pheromonal effects have been observed in the hamster (Payne 1977) and gerbils (Thiessen et al. 1976).

After the initial finding of Gustafsson and Pousette (1975) about the presence of androgen receptors in the male rat Harderian gland, Vilchis et al. (1992) reported that androgen receptor in the Harderian glands of rats, guinea-pigs, mice and hamsters share common bind-

ing characteristics. The present results clearly show that type A cells of the rat Harderian gland are more sensitive to the decrease in circulating androgen levels caused by castration. It could be suggested that testosterone receptors in the male rats Harderian gland are unevenly distributed, being more numerous in the type A cells. This suggestion requires further studies on mapping of the testosterone receptors in the rat Harderian gland.

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