THE HAMSTER HARDERIAN GLAND: REGULATION OF MORPHOLOGY AND PORPHYRIN BIOSYNTHESIS BY GONADAL STEROIDS

by

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SUMMARY

The Harderian gland is an orbital structure found in most classes of terrestrial vertebrates. The gland is particularly well developed in rodents, where in addition to the normal mammalian lipid secretion, porphyrins (precursors of haem) are also produced and stored, normally as solid intraluminal deposits. In this thesis, the golden hamster has been used exclusively since the female hamster Harderian gland is possibly the richest source of mammalian porphyrins known, while the male gland contains very little porphyrin. Sex differences are also found in the activities of five of the enzymes in the biosynthetic pathway, with levels being greater in the female. A number of morphological sexual dichotomies also exist; briefly, the female gland has only one epithelial cell type (Type I), while the male gland has two cell types (Type I and Type II). Both male cell types contain unique ultrastructural features, the polytubular complexes, which are not found in the normal female gland. The female gland has approximately 40x more interstitial and capsular mast cells than the male gland. Gonadal hormones are responsible for these dichotomies since castration of males results in loss of Type II cells and polytubular complexes, the appearance of large intraluminal porphyrin accretions, a rise in porphyrin content and enzyme activity and an increase in mast cells. Androgen administration to females results in virilization of the gland.

The aims of this thesis were:

1. To investigate the control of porphyrin biosynthesis within the Harderian gland by exogenous and endogenous steroid manipulation, primarily in the female hamster.
2. To investigate the relationship between porphyrin biosynthesis in the Harderian gland and the rest of the body.
3. As a prerequisite to the histological investigations of 1, to investigate the effects of various fixatives in preserving the different forms of porphyrin deposits within the gland.

Porphyrin is normally deposited as solid intraluminal accretions in the female gland, but ovariectomised and post-reproductive senescent females also exhibit interstitial porphyrin deposits, either surrounded by macrophages forming foreign body giant cells or within individual macrophages. For quantitative evaluation of changes resulting from hormone manipulations it is therefore important to use a fixative which preserves these various forms of porphyrins, which are soluble in a wide range of solvents. Four commonly used fixatives were used and porphyrin loss was assessed spectrofluorimetrically in each fixative, the subsequent dehydration and clearing steps in each routine, and the residue left at the end of the processing. These accumulated values were compared with a similar amount of tissue from the same animals placed directly into methanol. Good approximations were obtained. The results indicated that both Bouin's fixative and Acid/formal/alcohol resulted in unacceptably high losses of porphyrin since neither of these

fixatives fix lipids to which porphyrins are thought to be complexed. While Dichromate fixation resulted in less than 2% porphyrin loss it produced tissue which was difficult to section. Both glutaraldehyde routines produced approximately 8% porphyrin loss but the use of amyl acetate as the clearing agent, rather than chloroform, produced better results in terms of tissue preservation especially if the tissue was to be re-embedded for T.E.M. or S.E.M.

Quantitative assessments of the effects of ovariectomy on the morphology, porphyrin content and porphyrin synthesising enzyme activity of the Harderian gland of the female hamster confirms the link between gonadal hormones and gland structure and activity. The results indicate that ovarian hormones are necessary to maintain the morphology and activity of the normal female Harderian gland since ovariectomy results in a series of degenerative changes in gland morphology, and a decrease in enzyme activity. Furthermore, a study investigating the time course of change following ovariectomy demonstrates that this is a progressive phenomenon. Similar, albeit less marked, changes occur in post-reproductive senescent females. Pregnancy and lactation also affected porphyrin biosynthesis in multiparous hamsters, again suggesting a link between gland activity and circulating ovarian hormones. Androgen administration to ovariectomised females resulted in morphological virilization of gland features and a significant decrease in porphyrin content, while enzyme levels fell to normal male values. Castration of male hamsters produced an increase in porphyrin content

and enzyme activity which was modified by oestrogen or progesterone administration but inhibited by testosterone. Circulating levels of testosterone maintain the male gland characteristics even in senescent animals.

HPLC profiles of Harderian gland extracts from intact female hamsters show that protoporphyrin forms more than 90% of the total porphyrin content. Ovariectomy does not alter this profile. In intact males protoporphyrin forms only 72% of the total porphyrin content. Males castrated for six weeks had protoporphyrin levels intermediate between male and female values.

Liver enzyme activity does not alter in response to decreasing levels of ovarian hormones, unlike the Harderian gland. Also in marked contrast to the gland, the liver shows little response to the effects of castration, nor to pregnancy and lactation.

Harderianectomy does not result in an increase in the porphyrin content or enzyme activity of other porphyrin synthesising organs such as liver or kidney, which might be expected due to the increased availability of precursors.

In conclusion, the Harderian gland of the golden hamster is a useful model in which to study the effects of endogenous or exogenous steroid manipulations on porphyrin biosynthesis. The sexual dichotomy of both porphyrin content and enzyme activities suggests that sex hormones control porphyrin synthesis in the gland. This is confirmed by experiments involving castration or ovariectomy which alter both these parameters as well as gland morphology. These changes are

progressive with increasing time post-operatively. The lability of the gland to these various manipulations highlights its usefulness as a tissue in which to investigate porphyrin biosynthesis and its control.

The gland is unusual in that it stores porphyrins, due to low levels of the final enzyme in the haem biosynthetic pathway. A similar accumulation occurs in human erthyropoietic porphyria, a metabolic disorder. Furthermore, the characteristics of the gland controls mimic human acute porphyria, which is more prevalent in females after puberty and is exacerbated by changing hormone levels as in the menstrual cycle or during pregnancy. In the Harderian gland, porphyrin synthesis also changes during puberty, and the female gland shows cyclical and seasonal variations in activity.

ABBREVIATIONS

AChE - acetylcholinesterase

GER - Granular endoplasmic reticulum

SER - Smooth endoplasmic reticulum

5-ALA-S - 5-aminolaevulinic acid synthase

5-ALA - 5-aminolaevulinic acid

PBG - porphobilinogen

PBG-D - porphobilinogen deaminase

URO-D - uroporphyrinogen decarboxylase

COPRO-O - coproporphyrinogen oxidase

PROTO-O - protoporphyrinogen oxidase

AIP - Acute Intermittent Porphyria

OsO_4 - osmium tetroxide

HPLC - high performance liquid chromatography

dH_2O - distilled water

NaOH - sodium hydroxide

CO_2 - carbon dioxide

TEM - transmission electron microscopy

SEM - scanning electron microscopy

UV - ultra-violet

GENERAL INTRODUCTION

The Harderian gland was first described in the orbit of the red deer (Cervus elaphus L) and the fallow deer (Dama dama L) by Johann Jakob Harder, the celebrated seventeenth century physician and naturalist, who wrote of a 'nova glandula lacrimalis' in the Acta eruditorium Lipsiae (= Leipzig) of 1694/95. These orbital glands have subsequently been described in most classes of terrestrial vertebrates.

The Harderian gland is a major orbital gland of ectodermal origin. It is normally situated on the medial side of the orbit and is usually associated with the nictitating membrane at the inner canthus of the eye. It is one of two glands connected with this membrane: the nictitans or superficial gland lies within the nictitating membrane adjacent to the cartilage and consists of small clumps of glandular tissue opening by several ducts onto the surface of the membrane; conversely, the Harderian or deep gland is a separate structure located in a retrobulbar position, but possessing one or more ducts which traverse the nictitating membrane to open on its surface. The presence of both these glands is unusual, the pig being one of the exceptions (Sisson & Grossman, 1975); of the two, the Harderian gland is more widespread in occurrence. Although the Harderian gland is normally present where a nictitating membrane occurs, this relationship is not absolute. Thus, the gland is present in amphibians which have a false "nictitating membrane", a retractable, transparent portion of the lower lid. The transparent brille, formed from closed lids, occurs in snakes which have particularly large Harderian glands but no true nictitating membrane (Walls, 1942), while

in mammals the gland may be present in species which have a vestigial or poorly developed nictitating membrane, such as the anteater and marsupials (Walls, 1942; Prince et al., 1960; Wolff, 1968; Brownscheidle, 1974; Sisson & Grossman, 1975; Sakai, 1981).

It is always assumed that the primary role of the Harderian gland is to provide lubrication for the nictitating membrane (where present) and the anterior surface of the eye, and so prevent the cornea becoming opaque. Thus, the gland is absent in fish and totally aquatic amphibians but is found in semi-aquatic and terrestrial amphibians and is well developed in reptiles, birds and most mammals. In lower vertebrates, the secretory product of the gland may be classified as serous, mucous or seromucous (Paule & Hayes, 1958). In reptiles, the Harderian gland is large, although the nictitating membrane and/or the lacrimal gland are absent in some orders. The pathway for the Harderian gland secretion demonstrates considerable species variation in reptiles, but eventually enters the nasolacrimal duct which empties into the vomeronasal organ in the nasal cavity. From here the secretion enters the oral cavity where it contributes to the saliva. In snakes with rudimentary eyes, the ducts of the Harderian gland open directly into the oral cavity independently of the vomeronasal organ.

In crocodiles, terrestrial vertebrates which have secondarily returned to the water, the Harderian gland produces an oily secretion to protect the eye (Walls, 1942). However, according to Duke-Elder (1958) since oily secretions are

also produced in the snake gland, this may be of more importance in lubricating the enormous mouthfuls of food and assisting swallowing. Thus, in addition to lubricating the eye, the gland secretion may also maintain moisture in the nasal cavity and the olfactory vomeronasal organ or act as an accessory salivary gland. The Harderian gland of Caecilians, legless, wormlike amphibians living mainly underground, fills most of the orbit and lubricates a sensory retractile tentacle instead of the eye (Walls, 1942; Smith & Bellairs, 1947; Bellairs & Boyd, 1947; Wolff, 1968; Cowan, 1969; Kennedy, 1970; Brownscheidle, 1974).

The avian orbital glands are of great importance in providing a secretion which prevents the desiccation of the eye due to the constant exposure to a steady flow of air during flight. The orbital glands and nictitating membrane are therefore well developed in birds, with the Harderian gland usually being larger than the serous lacrimal gland. According to Burns (1975) the avian Harderian glands can be classified into three distinct types depending on their histological characteristics. Type I glands are compound tubuloacinar, as occur in fowl; Type II glands are compound tubular, as found in the duck (Brobby, 1972) while Type III glands are a mixture of Type I and Type II glands, as in the rook. The avian Harderian gland secretes mucus (Paule & Hayes, 1958) but marine birds have particularly large glands which produce copious amounts of a thick oily emulsion to protect the eye from the chemical and osmotic effects of seawater (Slonaker, 1918; Walls, 1942; Ballantyne & Fourman, 1967; Kennedy, 1970; Brownscheidle, 1974; Burns, 1976).

The mammalian Harderian gland is situated at the inner canthus of the eye and is characterised by its histological structure which has been variously described in rodents as compound tubulo-alveolar (Cohn, 1955; Norvell & Clabough, 1972; Bucana & Nadakavukaren, 1972a), implying that it contains a branched duct system and tubular alveoli (Bloom & Fawcett, 1975) or tubulo-alveolar (Brownscheidle & Niewenhuis, 1978; Sakai, 1981; Strum & Shear, 1982). The pig gland has a morphologically distinct duct system within itself (Loewenthal, 1896) while intralobular and interlobular ducts have been described in the compound acinar gland of the nine banded armadillo, which produces mucus and lipid (Weaker, 1981). The mammalian gland is normally lipid secreting, consisting of branching tubules with wide lumina which drain into one main duct while the lacrimal gland, located at the outer canthus of the eye, is a lobulated structure containing acini with narrow lumina, a branched duct system, and produces a serous secretion (Sakai, 1981).

The Harderian gland is present in monotremes, marsupials and placental mammals with the exception of chiroptera, perissodactyls, large terrestrial carnivores and higher primates (Sakai, 1981). According to Giacomini (1887), the Harderian glands are rudimental in monkeys, and Loewenthal (1910) wrote that they are represented in anthropoids and man by a transitory foetal structure in the infero-lateral fornix (Kennedy, 1970). In cases of malformation, the gland has been found to persist in adult life, as has the cartilage of the nictitating membrane

and the presence of a retractor bulbi muscle (Giacomini, 1887; Fleischer, 1907). The gland is well developed in rodents and lagomorphs, and in aquatic marine mammals; the Sirenian gland produces copious amounts of mucus while the secretion is lipid in Pinnepeds and Cetaceans to protect the cornea from seawater (Walls, 1942).

Ocular glands are first found in those vertebrates which have reached a semi-aquatic stage and therefore require some mechanism for the maintenance of corneal moisture. The lacrimal and Harderian glands appear to have evolved about the same time with openings in the lower lid leading to the nasal cavity (Kennedy, 1970). According to Wolff (1968) both have a common origin in a single gland situated in the lower lid; growth of the medial portion produces Harder's gland while the lacrimal gland tends to move to the lateral canthus, then to the upper (Fig. 1). In terrestrial urodeles a row of small glands is present in the lower lid: these may be best developed nasally and temporally and the intervening glands may be lacking so that the two masses of glands are isolated, becoming the forerunners of the Harderian and lacrimal glands (Walls, 1942). It is possible that the secretion of these glands also assists in the maintenance of moisture in the nasal apertures in addition to lubricating the eye (Davis, 1929; Sakai, 1981). Removal of the Harderian gland results in an increase in mucous secretion in the lacrimal duct (Burns, 1979).

In mammals, however, the Harderian gland may no longer

have such an important role in lubrication since a) the Harderian gland is always accompanied by a well developed lacrimal gland, b) the sizes of the Harderian gland, the eye and the nictitating membrane may not be positively correlated, e.g. in the mole (Walls, 1942), big clawed shrew (Sakai, 1981), the mole rat and the "eye-less" mouse (Balemans et al., 1980) where the eye is vestigial but the Harderian gland is relatively large, and c) in rodents, the excretory duct may open on the nasal surface of the nictitating membrane rather than the bulbar surface (Sakai, 1981). Moreover, whilst in some mammals the gland continues to produce a serous or mucous secretion (Paule & Hayes, 1958) others produce a lipid secretion as in rodents (Hais et al., 1968; Bucana & Nadakavukaren, 1972a; Yamazaki et al., 1981) and lagomorphs (Wooding, 1980). Lipids may prevent the evaporation of tears or prevent them falling over the lower lid (Cohn, 1955; Sisson & Grossman, 1975). Furthermore, several alternative functions have been suggested for the Harderian gland. These may conveniently be considered before the structure of the rodent Harderian gland is dealt with in detail.

When rodents groom, they begin at the head end and work caudally in a regular sequence ending with the genitalia and the tail. Exceptions to this pattern are -

1. Grooming during pregnancy/lactation where most grooming involves the nipple line.
2. Grooming during sexual activity which may be restricted to the genitalia.

3. "Social grooming" during interaction with conspecifics where extremely abbreviated grooming of the head may be all that occurs.

Historically this is thought of as 'displacement behaviour' (Rosenblatt & Lehrman, 1963; Steiner, 1973; 1974).

In normal grooming, the forepaws pick up both saliva and secretions from the Harderian gland which exit onto the face at the external nares. The latter can be confirmed because the secretion (containing porphyrin) fluoresces red under ultraviolet light and can be seen to be spread onto the pelage (Thiessen et al., 1976). It has been suggested that the spread of lipid-borne Harderian secretion into the pelage during normal grooming may have a thermoregulatory function, while spread during "social grooming" may have a pheromonal role.

1. Thermoregulatory role of the Harderian gland.

The spread of the Harderian gland products, lipids and porphyrins, may insulate the body from cold and wetness (Thiessen & Kittrell, 1980).

Harderianectomised and sham-Harderianectomised gerbils were briefly immersed in ice cold water and left for 15 minutes before being dried. During this period core body temperatures were recorded from surgically implanted thermistors. The Harderianectomised animals showed a significantly greater body temperature decline than the shams whose body temperatures tended to level off and stabilize. The Harderianectomised animals were less able to control body temperature under cold and damp conditions. To demonstrate that this deficiency was due to reduction in fur lipids, gerbils were shampooed

and subjected to the same test. This group was also significantly more deficient in regulating their body temperature than the control, lipid-intact, group. A further experiment demonstrated that replacing fur lipid would restore thermoregulatory ability; exogenously applied petroleum jelly on shaved animals simulated the lipid barrier and made subjects more efficient in controlling body temperature than an untreated group. However, the thermoregulatory role of Harderian gland lipids appears to be specific to the desert dwelling gerbil, since Harderian-ectomy in mice, rats and hamsters produces only a minimal decrease in the lipid content of the pelage (Thiessen & Kittrell, 1980). The only other example of the spread of Harderian lipids over the pelage retarding heat loss during immersion in water occurs in the semi-aquatic muskrat (Harlow, 1984).

The major effects are due to peripheral insulation by the gland secretion (which may contribute up to 40% of pelage lipids) rather than any general systemic effects since Harderianectomy does not a) alter blood testosterone levels, which affect the sebaceous activity of the gerbil (Thiessen & Rice, 1976), b) affect food and water intake at different environmental temperatures, nor the weight of various organs such as the testes, seminal vesicles or adrenal glands, and c) alter oxygen consumption or evaporative water loss at different environmental temperatures (Thiessen et al., 1977).

The gland secretion also darkens the pelage colour sufficiently to affect body temperature responses to radiant energy (Thiessen et al., 1982). The removal of these gland exudates by sandbathing changes both the colour and character of the pelage and may contribute to the water balance by lowering absorption of heat in the body with a resultant decrease in evaporative loss (Harriman & Thiessen, 1983).

2. Pheromonal role of the Harderian gland.

Mammalian scent glands include specialised skin structures which produce semiochemicals (compounds carrying information or otherwise mediating interactions between organisms). The secretions produced by these glandular structures are usually of a lipid nature (Steiner, 1973; Thiessen & Rice, 1976; Adams, 1980; Albone, 1984).

Since the Harderian gland is a lipid-secreting organ of ectodermal origin whose secretion empties into the nasolacrimal duct and onto the surface of the face, it is possible that this secretion has a pheromonal role.

When intact male golden hamsters were daubed with an homogenate of female Harderian glands, the aggressive responses from male opponents were inhibited. Additional male gland homogenate was neither attack-promoting nor aversive (Payne, 1977). Sexually experienced and inexperienced male golden hamsters were attracted to female Harderian gland smears more than male gland smears and more than muscle homogenates, but the males were unable to differentiate between oestrous and midcycle gland smears. It has been suggested that cephalic olfactory cues may be of importance to tunnel dwelling rodents (Payne, 1979).

During social interactions in gerbils, secretion from the Harderian glands is released during abbreviated bouts of facial grooming. The material (mixed with saliva) is spread over the nose, chin and cheeks. In response to autonomic arousal the skin of these areas becomes vasodilated which in turn may increase the volatility of the pheromone containing secretions of the gland. This gland secretion attracts attention and investigation by conspecifics and causes them to groom also (Thiessen et al., 1976; Thiessen & Yahr, 1977). Part of this secretion is airborne since shock avoidance conditioning indicates that the animals can smell the gland extract, which has a distinct floral odour to humans. Gerbils also respond to gustatory cues as demonstrated by taste-aversion tests (Thiessen et al., 1976). The secretory status of an individual can be determined by examining the body surface under ultraviolet light and rating the intensity of the red fluorescence, caused by the presence of porphyrins. The gland secretion, and thus the fluorescence, is denatured by saliva, having a half life of two minutes (Thiessen & Kittrell, 1980). The frequency of investigation parallels the duration and intensity of the fluorescence (Thiessen et al., 1976). This short duration ensures precise information about the source animal and its state of arousal. Harderianectomised males assume an inferior social position and are sexually rejected by females, even in the absence of intact males. Dominant males more actively spread their Harderian secretion than others (Thiessen, 1977; Thiessen & Yahr, 1977). Thiessen regards the Harderian pheromone as a generalised primer which

presets the conditions for more specific social interactions and without which these interactions are inhibited.

3. Defensive role of the Harderian gland.

a) Bactericidal role

In the rabbit, more than 50% of the total lipid in the pink lobe is a diacyl glycerol ether which usually represents about 1-3% of the total lipid content in most tissues (Jost, 1974). Antibactericidal effects have been attributed to this class of lipids (Emmerie & Engel, 1962), which according to Bodman & Maisin (1958) contributes to wound healing. Since the duct of the gland ends at the conjunctival sac, the secretion is spread across the cornea and may protect it from bacterial infection (Jost, 1974). Kuhnel (1971) also suggested that this class of lipids may have an antibiotic effect.

b) Immune role

The avian Harderian gland is relatively large compared to the lacrimal gland, and is thought to have an immune role in protecting the front of the eye and the conjunctiva against infection. Bang & Bang (1968) initially reported the occurrence of large numbers of plasma cells in the Harderian gland of the fowl, which increase with age (Wight et al., 1971a). Plasma cells have subsequently been described in a wide range of avian species (Burns, 1975). Mueller et al., (1971) suggested that the immune responses of the gland were primarily to local antigenic stimulation of the eye rather than to systemic antigens, although Burns (1976) later showed that the gland responds to both systemic and topical antigenic stimulation. The presence of plasma cells containing

Russell bodies, indicative of activity (Wight et al., 1971a & b; Rothwell et al., 1972), as well as marked lymphocytic invasions (including even germinal centres) which increase in number with age (Mueller et al., 1971) were also noted. The inability of the lymphoid cells of the gland to mount graft-versus-host reaction suggests that it may be a good source of peripheral B lymphocytes, relatively free of T lymphocytes (Sundick et al., 1973). The plasma cells carry immunoglobulin surface determinants with different classes being predominant at different ages (Albini et al., 1974). The gland is also thought to have a special role as an accessory lymphoid organ in Newcastle disease (Neuman & Kaleta, 1977). Lymphocytes are found in both the Harderian and lacrimal gland ducts in birds, either as diffuse lymphoid tissue or as discrete germinal centres (Burns & Maxwell, 1979). The importance of the Harderian gland as a site of immunocompetent cells is shown by the fact that surgical removal of the Harderian gland in fowl resulted in plasma cell infiltration of the lacrimal gland (where they are normally few in number) and antibody production to antigen stimulation in this gland (Burns, 1979).

While the immune role of the gland is well documented in birds, few references exist for mammals. In guinea pigs, immune exophthalmos due to homologous Harderian gland antigen has been observed, with sensitised animals showing different degrees of lymphocyte invasion and cellular damage, and low concentrations of circulating antibody (Pisarev et al., 1968).

4. The Harderian gland as a link in the retinal-pineal-gonadal chain.

Certain parts of the forebrain, notably the pineal gland and the suprachiasmatic nucleus of the hypothalamus, respond to environmental change, such as lighting. As such, they, together with the retina, may form a neural or neurohumoral basis for circadian or circannual rhythmicity within the organism as shown in such phenomena as sleep cycles and annual cycles of coat colour change and reproductive tract activity. Detailed consideration of this topic is beyond the scope of this thesis, but recent references include Pevet et al., (1980), Hoffman (1981), Kappers (1981), Mess et al., (1981), Reiter (1983) and Oksche (1984). The postulated involvement of the Harderian gland in such a retinal-pineal axis is based on the following observations:

1. The Harderian gland may act as an extraretinal photosensitive organ influencing circadian rhythms of pineal indoleamine metabolism in neonatal rats. Removal of these glands abolished the ability to change circadian rhythms of pineal serotonin and hydroxy-indole-O-methyltransferase (HIOMT) activity in blinded 12 day old rats when light: dark cycles were altered (Wetterberg et al., 1970a & b; 1972a & b). Adult rats do not exhibit this phenomenon (Reiter & Klein, 1971).
2. In the golden hamster, a species which shows marked sexual differences in porphyrin content of the Harderian gland with the female containing significantly greater amounts than the male, castration results in increased gland porphyrin content. However, if animals are blinded at the time of castration, the male type gland

is maintained. The pineal inhibits the formation of porphyrin in these blinded castrates, since pinealectomy at the time of blinding and castration permits the appearance of porphyrin (Clabough & Norvell,1973). Pinealectomy also prevents morphological "masculinization" of the Harderian gland which normally occur in blinded females (Clabough & Norvell,1974). Ovariectomy in combination with blinding inhibited the appearance of male morphological characteristics and the decrease in porphyrin following blinding. According to McMasters & Hoffman (1984) ovarian androgen production induces the female Harderian gland masculinization in the blinded female. They further suggest that exposure to androgens during or just prior to a period of blinding-induced pineal activation can induce and maintain the male-type Harderian gland.

3. The possible role of the Harderian glands in mediating the changes in the reproductive organs associated with constant light were investigated by Reiter & Klein (1971). Removal of the glands caused hypertrophy of the uteri in female rats. In the female hamster, continuous light produces enlargement of the uteri while porphyrin concentration in the Harderian gland is significantly increased (Wetterberg et al., 1972c). The wave length of light can affect gonadal size and porphyrin synthesis in the Harderian gland; red light exposure results in low testicular and uterine weights in controls, and low porphyrins in castrates and females (Formgren & Wetterberg,

1976). Gonadal regression occurs in hamsters kept in short photo periods, which also produces morphological changes in the hamster Harderian gland with males assuming female characteristics and vice versa (Nadakavukaren & Lin, 1983). These changes in both the gland and the reproductive organs can be prevented by pinealectomy (Diiora & Nadakavukaren, 1984).

4. The Harderian gland, retina, and pineal gland have a shared biochemistry of indoleamine synthesis in rodents (Cardinali & Wurtman, 1972; Bubenik et al., 1974; 1978; Pang et al., 1977; Balemans et al., 1980). Both melatonin and N-acetyl transferase show diurnal variation in concentration in these extra pineal organs which, as in the pineal, is dependent on light/dark cycles (Bubenik et al., 1978; Feria Velasco et al., 1983; White et al., 1984; Hoffman et al., 1985). In male hamsters an annual cycle is demonstrated in all three organs since the amounts of the methoxyindoles and the diurnal rhythms in their synthesis differed in the various months in natural conditions, during which the tests were performed (Balemans et al., 1983). The extra-pineal synthesis of melatonin and 5-methoxytryptophol is higher than that in the pineal, except at the end of the dark period (Pevet et al., 1980).

Wetterberg et al.,(1970a) have postulated that the Harderian gland may act as a reflector and transducer of light information as the visual elements of the mammalian retina face towards the gland.

Recent investigations on the mammalian Harderian gland have centred mainly on rodents and lagomorphs. These animals are commonly used in laboratories, and have a well developed Harderian gland whose morphology and biochemical properties have been investigated and are well documented. In this thesis, the golden hamster has been used exclusively.

The following sections of the Introduction describe the morphology and the biochemistry of the rodent Harderian gland.

THE RODENT HARDERIAN GLAND

In addition to the Meibomian glands, the glands of Moll & Zeiss in the eyelids, and the conjunctival glands, there are three glands associated with the orbit, namely the exorbital lacrimal gland, the infra-orbital lacrimal gland and the Harderian gland (Fig.2&3), (Davis, 1929; Wolff , 1968; Prince et al., 1960; Sisson & Grossman, 1975). Harder's gland is normally found in those animals with a well-developed nictitating membrane. Mammals which do not possess a Harderian gland have glands of the nictitating membrane, but all rodents examined so far have a Harderian gland. It is the largest of the orbital glands in rodents, occupying a considerable part of the orbit, and is usually horse-shoe-shaped. It surrounds the superior, medial and inferior sections of the posterior half of the eyeball, often interdigitating with orbital structures such as the extraocular muscles and optic nerve (Venable & Grafflin, 1940). The surface of the gland is smooth and covered by a delicate connective tissue capsule which (on the deep surfaces of the gland) appears to also serve as the wall of a retro-orbital venous sinus (Davis, 1929; Sakai, 1981). The gland is convex on the outer surface and concave on the inner to conform to the shape of the walls of the bony orbit and the eyeball (Davis, 1929).

The Harderian gland is intimately associated in its development with the nictitating membrane (Wolff , 1968) and is attached to the membrane base by loose connective tissues in which is a glandular pedicle. The gland can be easily pulled from the orbit by catching this pedicle in the inner canthus (Sakai & Yohro, 1981). A small semilunar shelf of

cartilage projects back under Harder's gland, from the cartilage of the nictitating membrane, and the hilar region of the gland rests on this and is firmly attached to it (Davis, 1929; Venable & Grafflin, 1940). The single extraglandular excretory duct originates at the hilus and passes through loose connective tissue to open on the outer (nasal) side of the nictitating membrane (Davis, 1929; Sakai & Yohro, 1981).

The gland is bilobed, and the smaller superior and larger inferior lobes are separated laterally by an appreciable gap. In the rat, there are impressions on the gland surface indicating the close relationship between the gland and the extra-ocular muscles. The superior and inferior oblique muscles pass in front of the gland in approaching their bulbar attachments. The lateral edge of the gland may also be indented by three clefts, through which pass the optic nerve, the levator palpebrae superioris and the superior, medial and lateral rectus muscles (Venable & Grafflin, 1940).

In general, the rodent Harderian gland is pale in colour but there are exceptions which reflect special characteristics of some species. For example, in gerbils the gland is dark grey (depending on the pelage and eye colour of the strain) due to the existence of large numbers of melanocytes in the interstices (Sakai & Yohro, 1981; Johnston et al., 1983). In the rat, the gland is white in appearance (Kanwar, 1960) while in mice it is speckled with dark brown pigment (Woodhouse & Rhodin, 1963) due to the existence of porphyrin in the alveolar lumina (Cohn, 1955).

Histologically the gland consists of branching tubules separated by small amounts of loose interstitial connective tissue. The tubule walls are formed by a single layer of columnar epithelial cells containing lipid vacuoles, bearing apical microvilli and having myoepithelial cells within their basal laminae. Porphyrin, which is synthesised by the epithelial cells, is normally deposited as large intraluminal accretions.

Epithelial cells

These are either pyramidal or columnar in shape with basally located round or oval nuclei containing distinctive nucleoli: binucleate cells and mitotic figures are rarely observed (Brownscheidle & Niewenhuis, 1978; Watanabe, 1980) except in the gerbil (Sakai & Yohro, 1981; Johnston et al., 1983). Microvilli project from the apical surfaces of the alveolar cells into the lumen which may contain lipid materials, porphyrin accretions or cellular debris (Bucana & Nadakavukaren, 1972; Watanabe, 1980; Johnston et al., 1985a). Junctional complexes (including gap junctions) are present between the cells at the luminal aspect of their lateral surfaces (Brownscheidle & Niewenhuis, 1978; Strum & Shear, 1982) while interdigitations occur between the lateral cell membranes of adjacent cells (Brownscheidle & Niewenhuis, 1978). The basal surface of the epithelium is smooth with few basal infoldings (Watanabe, 1980). Rough endoplasmic reticulum, smooth endoplasmic reticulum, ribosomes and mitochondria are present within the cytoplasm, but Golgi complexes tend to be indistinct (Brownscheidle & Niewenhuis, 1978) and atypical (Kaiho & Ichikawa, 1982; Johnston et al., 1983; 1985a).

The number of distinctive epithelial cell types is species specific: the Plains mouse (Pseudomys australis) exhibits three cell types (Johnston et al., 1985a), the rat and mouse two (Grafflin, 1942; Strum & Shear, 1982), the gerbil only one (Sakai & Yohro, 1981; Johnston et al., 1983). In the golden hamster sex also determines the number of cell types, the female having only one while the male possesses two (Woolley & Worley, 1954; Bucana & Nadakavukaren, 1972a; Clabough & Norvell,1973; Payne, 1980).

All these cell types contain numerous lipid vacuoles. In the mouse and rat the morphological appearance of these droplets defines the cell type, with one cell form containing vacuoles which are either clear or contain fibrogranular material, and which fluoresce red under ultraviolet light (Grafflin, 1942; Strum & Shear, 1982), while the other form contains sudanophilic vacuoles with distinctive myelin-like laminations on their boundaries which are birefringent (Kelenyi & Orban, 1965; Brownscheidle & Niewenhuis, 1978; Watanabe, 1980). Both types of secretory vacuoles fuse with the apical cell membrane suggesting a merocrine secretion (Watanabe, 1980; Brownscheidle & Niewenhuis, 1978). The size of the secretory vacuoles may also be distinctive as is the appearance, distribution and number of mitochondria (Watanabe, 1980; Johnston et al., 1985a). The genesis of the secretory lipid vacuoles is unclear. Distinctive cytoplasmic slashes or curved slits, which are often associated with dense membranous material and may be lipid vacuole

precursors, occur in cells of Pseudomys australis (Johnston et al., 1985a) and Meriones unguiculatus (Kaiho & Ichikawa, 1982; Johnston et al., 1983) and in the cells of the white (non-porphyrin-forming) portion of the rabbit Harderian gland (Bjorkman et al., 1960).

Another specialisation of note is the well developed network of tubular profiles which have been described in the male golden hamster (Bucana & Nadakavukaren, 1972a; Payne et al., 1978) and the wood mouse, Apodemus (Johnston et al., 1985b). In the gerbil well-developed tubular smooth endoplasmic reticulum is observed (Sakai & Yohro, 1981; Kaiho & Ichikawa, 1982) and in Apodemus the tubules are continuous with the smooth endoplasmic reticulum (Johnston, personal communication).

Myoepithelial cells

These are situated between the secretory epithelium and the basement membrane, as in other glands, e.g. salivary, mammary and lacrimal. The cells are stellate, the central portion containing the oval nucleus which has one nucleolus, while the fine cytoplasmic extensions form a basket-like network around the epithelial cells, within the basal lamina (Chiquoine, 1958; Leeson, 1960; Watanabe, 1980). The nucleus lies in the region of the greatest width of the cell body, parallel to and close against the basement membrane, and frequently located adjacent to the apposition of two secretory cells. The upper surface of the cell membrane is often irregular but the lower surface is usually smooth: small villus-like extensions may occur in the nuclear region

which interdigitate with complementary invaginations in the secretory cells (Chiquoine, 1958; Woodhouse & Rhodin, 1963; Johnston et al., 1985a). Furthermore, hemidesmosome junctions occur between the myoepithelial cells and the secretory cells while desmosomes exist between adjacent myoepithelial cells (Brownscheidle & Niewenhuis, 1978; Strum & Shear, 1982; Johnston et al., 1985a). No myoneural junctions have been observed between the myoepithelial cells and the adjacent non-myelinated nerves in the interstitium (Bucana & Nadakavukaren, 1972 a & b; Brownscheidle & Niewenhuis, 1978). The cytoplasm of these myoepithelial cells is distinctive in that 1. the organelles are concentrated only at the poles or the supranuclear region, and 2. fine contractile fibrils which branch and anastomose occur throughout (Chiquoine, 1958; Woodhouse & Rhodin, 1963; Johnston et al., 1985a). These fibrils consist of thick (myosin) filaments, 12-17nm diameter, and thin (actin) filaments, 6-7nm diameter (Strum & Shear, 1982). The myoepithelial cells contract to expel the secretory products of the epithelial cells, and the scalloping of the basement membrane may be due to the contractions of these cells (Chiquoine, 1958; Leeson, 1960).

Connective Tissue

The interstitial connective tissue consists of fibroblasts, collagen fibres and ground substance, and is highly vascular. Most of the capillaries are lined by an attenuated, non-fenestrated endothelium, but in some species, such as the rat, mouse and Apodemus sylvaticus fenestrated capillaries also occur (Brownscheidle & Niewenhuis, 1978; Strum & Shear, 1982; Johnston et al., 1985b). Mast cells are commonly found in the interstitium and the connective tissue capsule which surrounds

the gland (Payne et al., 1982a; Johnston et al., 1983; 1985a). Plasma cells, lymphocytes, neutrophils and eosinophils may also be present (Brownscheidle, 1974; Brownscheidle & Niewenhuis, 1978; Johnston et al., 1983; Payne et al., 1985); free macrophages containing porphyrin and macrophages forming giant cell bodies around large interstitial deposits of porphyrin sometimes occur (Payne et al., 1985; Johnston et al., 1985a). Melanocytes are present in the interstitium of some species (e.g. the gerbil , Sakai & Yohro, 1981; Johnston et al., 1983). Myelinated and unmyelinated nerves occur (Brownscheidle & Niewenhuis, 1978; Sakai & Yohro, 1981; Johnston et al., 1983).

Innervation

The innervation of the Harderian gland has been studied using histochemical methods for catecholamines and acetylcholinesterase (AChE). Huhtala et al., (1977) have shown that in the rat, AChE-positive nerves run as thick bundles in the interstitial tissue and send fine branches around the tubules. Electron-microscopy showed these fibres terminating near the myoepithelial cells which underlie the secretory epithelium. The blood vessels within the interstitium were also surrounded by AChE-containing fibres, and electron microscopy showed these fibres to be in contact with the blood vessels. Formaldehyde-induced fluorescence techniques have also disclosed adrenergic nerves surrounding the blood vessels, and similar fibres were also observed in the interstitial tissue. Selective denervations showed that AChE containing nerves arose from the zygomatic nerve, and were parasympathetic cholinergic fibres. Tashiro et al., (1940) reported that injection of rats with acetylcholine

results in secretion of "blood tears" (porphyrin) from the Harderian gland. These cholinergic fibres would seem to be genuinely secretomotor and may innervate the myoepithelial cells whose contractions might cause the expulsion of the secretory products from the cells or the propulsion of secreted material along the gland system. The vascular cholinergic fibres also appear to have a parasympathetic origin, while the fluorescent adrenergic vascular nerves arise from the superior cervical ganglion and reach the rat Harderian gland via branches of the trigeminal nerve. An account of the nerve supply to the gerbil Harderian gland is described by Sakai & Yohro (1981). This dual control of the blood vessels might affect the supply of precursors for lipid or porphyrin synthesis, or even the re-uptake of products (such as porphyrin) into the circulation (see Chapter 3).

The mouse Harderian gland is also under dual control since unmyelinated nerve endings containing clear vesicles (characteristic of cholinergic nerve terminals) and nerve endings with dense cored vesicles (typical of adrenergic terminals) have been described in the interstitium apposed to blood vessels, secretory cells and myoepithelial cells. Nerve terminals were not observed intervening between the basal lamina and the plasma membrane of the secretory cells or myoepithelial cells (Watanabe, 1980). In the rat gland, Brownscheidle & Niewenhuis (1978) described unmyelinated fibres containing both clear and dense-cored vesicles in close association with blood vessels, while the unmyelinated fibres near the basal lamina of the secretory cells contained

only clear vesicles. The Mongolian gerbil also exhibits dual innervation, again with dense cored adrenergic vesicles in the fibres surrounding blood vessels, while cholinergic fibres with clear vesicles were associated with melanocytes in the interstitium. Again, no nerve terminals were seen within the basal lamina of this species (Sakai & Yohro, 1981). However, autonomic nerve varicosities containing dense cored synaptic vesicles, mitochondria and neurotubules have been described within the basal lamina of the tubules of the rabbit Harderian gland (Kuhnel, 1971).

Blood Supply

Like much of the orbital contents, the arterial supply of the gland is said to arise from the external ophthalmic branch of the (internal) maxillary artery, (Davis, 1929). The venous outflow from the gland and the orbit drains into the orbital sinus from which there are several outlet channels; the posterior ophthalamic vein, the internal ophthalamic vein, the inferior ophthalmic vein, the supra-orbital vein and a small branch of the internal maxillary vein (Davis, 1929; Sakai & Yohro, 1981).

Lipid Content

A prominent feature of the rodent Harderian gland is the presence of numerous sudanophilic granules, indicative of lipid storage vacuoles. These lipid droplets were found to be non-osmiophilic (Schneir & Hayes, 1951) indicating the presence of saturated lipid components. The exact lipid composition in the Harderian glands is species-specific, but the main component in the rat gland is a mixture of wax esters (Murawski & Jost, 1974) while glyceryl ether diesters are the major component in the mouse gland

(Watanabe, 1980), the red (porphyrin-forming) part of the rabbit gland (Rock & Snyder, 1975), and the guinea pig gland (Yamazaki et al., 1981). Fatty acids, cholesterol, triglycerides, phospholipids and glycolipids also occur in the rat and mouse glands. Differences not only occur between the species but also between the sexes of the same species, and with age (Bareggi et al., 1979a,b & c).

These secretory vacuoles are surrounded by unit membranes in mice (Woodhouse & Rhodin, 1963), hamsters (Bucana & Nadakavukaren, 1972a) and rabbits (Kuhnel, 1971; Wooding 1980), and are secreted by exocytosis (merocrine secretion). The Harderian gland is the sole example in which secretory vacuoles of lipid type are secreted by exocytosis: in the mammary gland, the secretory droplets are not surrounded by unit membranes and are secreted by an apocrine mechanism while sebaceous glands exhibit a holocrine mechanism (Bloom & Fawcett, 1975). However, references to holocrine and apocrine secretion are made in many papers (Cohn, 1955; Kanwar, 1960; Tsutsumi et al., 1966; Muller, 1969; Johnston et al., 1983; 1985a; Carriere, 1985).

Porphyrin Content

Rodents are the only known mammals (with the exception of the miniature pig, McCafferty & Pinkstaff, 1970) in which porphyrins, precursors of haem, have been found within the Harderian gland. At first, the gland was thought to be a passive storage organ for blood borne porphyrins (Derrien & Turchini, 1924; Grafflin, 1942) but all the enzymes involved in porphyrin biosynthesis have now been shown to be active

within the gland (Thompson et al., 1984). The porphyrin (synthesised by the epithelial cells) is normally deposited as dense intraluminal accretions which vary in size and colouration (Grafflin, 1942; Brownscheidle & Niewenhuis, 1978) and exhibit red fluorescence under ultraviolet light (Figs. 3, 4 & 5) (Kennedy, 1970), or as intracellular fibrogranular material (Brownscheidle & Niewenhuis, 1978) which also fluoresces red (Strum & Shear, 1982). In some species with low porphyrin concentrations, the gland contains no intraluminal accretions, e.g. some strains of mice (Shirama et al., 1981) and the woodmouse Apodemus sylvaticus (Johnston et al., 1985b). In some rodent species the amount of porphyrin synthesized by the two sexes is comparable, but in others the female produces more than the male as in Meriones unguiculatus (Johnston et al., 1983), Pseudomys australis (Johnston et al., 1985a) and certain strains of the mouse (Strong, 1942; Shirama et al., 1981; Strum & Shear, 1982). The most extreme sex difference occurs in the golden hamster (Bucana & Nadakavukaren, 1972a; Payne et al., 1979). Porphyrin content varies with age in the rat (Rohonyi & Kelenyi, 1962) and mouse (Strong, 1942).

Interstitial porphyrin deposits have also been reported in normal adult rats (Grafflin, 1942), the plains mouse Pseudomys australis (Johnston et al., 1985a) and in female golden hamsters in post-reproductive life or after hormone manipulation. In the latter species interstitial porphyrins may occur within individual macrophages or, if sufficiently large, surrounded by macrophages forming foreign body giant

cells (Payne et al., 1982b; 1985; Spike et al., 1983; 1985). Electron microscopy shows the intraluminal porphyrin deposits to be composed of fine needle-shaped crystals, while porphyrin within the macrophages can be seen in various stages of solubilization (Payne et al., 1985).

Protoporphyrin is the main form within the rodent Harderian gland, but coproporphyrin, and in some species Harderoporphyrin, are also detectable (Kennedy, 1970).

Porphyrin is also seen in the duct of the gland from where it is secreted into the conjunctival sac or possibly directly into the naso-lacrimal duct. In gerbils, red fluorescent secretions leave the external nares and after mixing with saliva are spread over the pelage during grooming (Thiessen et al., 1976). The secretion traverses the naso-lacrimal duct to the naso-pharynx, and some may reach the stomach by way of the oesophagus (Kennedy, 1970).

The Duct System

In rodents, a single excretory duct originates at the gland hilus, passes through loose pedicular connective tissue and generally opens on the outer (nasal) side of the nictitating membrane (Sakai, 1981; Sakai & Yohro, 1981). In no rodent species has a morphologically distinct intra-glandular duct system been reported, as occurs in the armadillo (Weaker, 1981). The extraglandular duct usually has an enlarged lumen or ampulla proximal to the opening at which squamous epithelial cells and mucous cells may be located (Sakai & Yohro, 1981; Johnston et al., 1983; 1985a). In some species the extraglandular duct has a simple structure

(Johnston et al., 1985a) while in others a complex of clefts and crypts has been described (Sakai & Yohro, 1981; Johnston et al., 1983).

The Harderian gland of the golden hamster

The Harderian gland of the golden hamster conforms to the basic rodent pattern, but a number of morphological and biochemical sexual dimorphisms exist (Fig. 6). The first macroscopic difference was noted by Christensen & Dam (1953), the male glands being yellow/white in colour while in females black granules were visible on a brown background. They also noted that the lumen of the tubules of the female glands contained a dark or golden brown pigment which was not seen in male glands. This is now known to be porphyrin and the content is up to 120 times greater in female glands (Thompson et al., 1984). The activity of the enzymes in porphyrin biosynthesis are also generally higher in the female gland (Lin & Nadakavukaren, 1982; Thompson et al., 1984). Woolley & Worley (1954) observed that gonadectomised males and females had granules similar to those seen in intact females. They also noted a second sex difference in that female tubules were composed of only one epithelial cell type (Type I) filled with small vacuoles, while males exhibited Type I cells and a second type (Type II) filled with large vacuoles. Males and females gonadectomised for 30 days tended to be similar to the female type, while Type II cells regressed in males treated with oestrogen (Woolley & Worley, 1954) and appeared in females given androgens (Sun & Nadakavukaren, 1980; Spike et al., 1983; 1985).

Ultrastructural differences also occur: both male cell type contain unique structures, the polytubular complexes (PTCs)

which are cylindrical tubules, frequently grouped together in "logpile" configurations (Fig. 6) and randomly distributed throughout the cytoplasm. These tubules are approximately 30nm in diameter (Bucana & Nadakavukaren, 1972a; Payne et al., 1978). The Type II cells in males have greater numbers of mitochondria, and are also characterised by dilated SER in females. The female Type I cells have prolific membrane structures arranged in concentric lamellar formations or in the form of Golgi profiles with proliferations of cisternae. The membranes are smooth and often associated with vacuoles (Bucana & Nadakavukaren, 1972a). Golgi, GER and a prominent SER are present in both cell types. The vacuoles are bounded by a single membrane.

The female Harderian gland contains some forty times more interstitial mast cells than male glands, and there is a similar sex difference within the connective tissue capsule. Mast cell numbers increase to female levels in castrate males but this change is prevented by androgen administration. There is, however, no apparent relationship between Harderian gland porphyrin content and mast cell numbers (Payne et al., 1982a).

The vacuoles in the Type I and II cells are filled with lipid material in both males and females as shown by Paule et al (1955) who demonstrated their sudanophilic nature. Lin & Nadakavukaren (1981) showed that the major component of both male and female hamster glands is alkyldiacyl glycerol, but cholesterol, fatty acid, phosphotidyl choline, phosphotidyl ethanolamine and an unidentified phospholipid were also

separated by thin layer chromatography. A sex difference in lipid composition was noted since the chain lengths of the predominant fatty acids in female glands ranged from C16-C20 while those in male glands ranged from C10-C20; a prevalence of C16 and C18 occurred in the female gland. No triglycerides, the usual form of lipid storage, were detected which indicates a secretory function.

Whole gland homogenates revealed several differences in protein composition with male glands containing at least three major proteins which are missing from the female gland. Two of these proteins are associated with the tubular clusters of the male gland, while the third may be a structural component associated with the large vacuoles of the male Type II cells (Hoh et al., 1984).

Differences occur in concentrations of metals in the Harderian glands, with males possessing lower concentrations of sodium, manganese and calcium and higher concentrations of iron and molybdenum than females. The data suggests that molybdenum is associated with Type II cells in the male and manganese with Type I cells in both males and females (Hoffman & Jones, 1981).

The gland of the hamster probably also demonstrates dual innervation since fluorescent adrenergic nerves are present in the adventitia of blood vessels, with branches forming a plexus around the tunica media, while AChE fibres occur in the interstitium terminating in bulbous endings adjacent to myoepithelial cells. Small bundles of AChE fibres also occur along ducts and in the connective tissue surrounding the gland (Norvell & Clabough, 1972). Norvell & Clabough also

found more cholinergic endings in males than females, but no sex difference in adrenergic endings. Conversely, Bucana & Nadakavukaren (1972b) found only adrenergic fibres in junctions with myoepithelial cells and secretory epithelial cells, and adjacent to blood vessels. The nerve endings adjacent to the myoepithelial cells also have myoneural junctions.

Immunoreactive melatonin concentrations show sexual dimorphisms, with female levels being up to ten times greater than those in males. In the female gland these levels also vary during the day while male concentrations remain constant (Hoffman et al., 1985).

The effects of sex steroids on the hamster Harderian gland

It has been known for some time that androgens control hamster Harderian gland morphology and porphyrin content. Thus castration of males results in the disappearance of Type II cells, the appearance of intraluminal porphyrin accretions and a marked increase in porphyrin content. Changes were progressive with time after castration (Hoffman, 1971; Payne et al., 1977a). The ultrastructural features of the male gland are also affected by castration with the polytubular complexes disappearing after 2 weeks while membranous structures normally associated with the female gland appeared (Lin & Nadakavukaren, 1979). Furthermore, castrates given diverse androgens either maintained, or reverted to, the male type of gland (Hoffman, 1971; Payne et al., 1977a).

Similarly, the female gland can be altered by testosterone

administration to produce a male-type gland, with the appearance of polytubular complexes and a decrease in lamellar structures after four days of treatment, as well as an increase in Type II cells (Sun & Nadakavukaren, 1980). However, it is not clear how this affects porphyrin content or porphyrin synthesising enzyme activities.

The presence or absence of androgens clearly represents a "coarse tuning" of gland morphology and productivity. Of equal interest is the "fine tuning" of porphyrin production within the female gland, for which little information is available.

Changes occur in the activity of the gland in the female hamster during the oestrous cycle with the porphyrin content and the number of intraluminal porphyrin accretions being highest on day 1 and then decreasing (Payne et al., 1977b). The activity of the rate-limiting enzyme in porphyrin production also shows cyclical activity with the highest value at pro-oestrous (Moore et al., 1977). Porphyrin content also increases during pregnancy and lactation, and the pattern of porphyrin deposition is altered (Payne et al., 1979).

The effects of environmental cues on the rodent Harderian gland

Light has been shown to affect both the morphology and porphyrin synthesis of the Harderian gland in different ways in various rodent species. In the golden hamster, a seasonal breeder requiring a critical lighting regime of more than 12.5 hours of light per day to maintain gonadal integrity and function (Elliot, 1981), continuous light for 72 days produced

a 4-fold increase in porphyrin content, paralleled histologically by a higher amount of intraluminal accretions, than in females kept in 12L : 12D regime, or females kept in constant darkness for the same period. Moreover, Type II cells (male characteristics) occurred in females kept in total darkness, occasionally in animals on a 12L : 12D regime, but never in females exposed to continuous light (Wetterberg et al., 1972c). In contrast, female albino rats kept in continuous light showed regression of the Harderian glands (Reiter, 1973) and produced very low porphyrin levels (Ulrich et al., 1974). In castrate male hamsters, the porphyrin concentration also increased 4-fold in animals kept in continuous light compared to those on 12L : 12D, while no increase was observed in castrates kept in total darkness (Wetterberg, 1972). In addition to light exposure, visual function of the eye is necessary for the increase in porphyrin content in the Harderian gland of castrates, since blinded castrates kept in continuous light had the same low porphyrin concentration as castrates kept in constant darkness (Wetterberg, 1972). The wavelengths of light also affects the porphyrin content in both females and castrate males, with blue light producing the highest concentration while red light exposure resulted in the lowest porphyrin content as well as regression of testicular weight and uterine size (Formgren & Wetterberg, 1976).

Short photoperiods (IL:23D) alter the ultrastructure of the hamster Harderian gland. After 4-10 weeks, the males

show a decrease in the number of polytubular complexes and Type II cells while females show an increase in these parameters and a decrease in the number of membranous whorls. Gonadal regression (and thus altered sex hormone levels) occurred after 4-8 weeks of exposure to short photoperiods (Nadakavukaren & Lin, 1983). These changes can be prevented by pinealectomy, supporting the idea that there are close links between the gland and the retinal-pineal-gonadal axis (Diioria & Nadakavukaren, 1984).

The mouse Harderian gland produces an accumulation of porphyrin after heat exposure: this increase is not observed in the rat gland (Collins, 1957).

PORPHYRINS

All porphyrins are planar tetrapyrrole structures in which 4 pyrrole rings are joined together by 4 methine bridges. A very large number of different porphyrins can be made by attaching different side chains to the 8 β positions of the 4 pyrrole nitrogens and to the 4 methine carbon atoms. Protoporphyrin, the most abundant of the biologically occurring porphyrins, has 4 methyl, 2 vinyl and 2 propionyl groups attached to the 8 β positions making it a dicarboxylic acid, while uroporphyrin which has 4 acetic acid groups and 4 propionic acid groups is an octacarboxylic acid (Fig. 7). The different porphyrins vary widely in their solubility properties and in their electrical charge under physiological conditions, depending on their particular array of substituents.

Free porphyrins are highly coloured and are widely distributed throughout the animal and plant kingdom. Protoporphyrin IX occurs in the pigmented areas of shells of many species of birds while uroporphyrin is found in the shells of molluscs. Porphyrins are excreted into the growth medium of many microorganisms, and protoporphyrin IX and coproporphyrin occur in high concentrations in the root nodules of leguminous plants.

However, the metal complexes of the porphyrins are of more importance: the arrangement of the 4 pyrrole nitrogen atoms in the porphyrin molecule allows the combination with metal atoms, such as iron, copper, zinc and magnesium. The most important of these is protohaem, the iron complex of protoporphyrin IX, which is the prosthetic

group of both haemoglobin and myoglobin which bind oxygen reversibly. Protohaem also combines with various specific proteins to form the enzymes catalase, peroxidase, the cytochromes and tryptophan pyrrolase, all haemoproteins. Protohaem is therefore central to all biological oxidation reactions. The chlorophylls, magnesium porphyrins, are essential for the utilization of biological solar energy and carbohydrate synthesis in most higher plants, algae and photosynthetic bacteria. The co-enzyme vitamin B12 has a central cobalt atom.

The wave lengths absorbed by an organic molecule depend on its structure, the longer the conjugated system the longer the maximum wavelengths that will be absorbed. Thus the conjugated double bonds and alternating series of single and double bonds in the cyclic tetrapyrroles gives these compounds a high degree of resonance resulting in a series of well-marked absorption bands in the visible spectrum and a characteristic red colour. The intense absorption band at approximately 400nm, known as the Soret band, is used in spectrophotometric determinations and can be measured at concentrations as low as 10^{-9}M. The high resonance of the porphyrin ring also confers a fluorescent property to these structures; light is absorbed at wavelengths within the main absorption band and is re-emitted at longer wavelengths, around 600nm as a characteristic red fluorescence by the excited structure as a result of the first radiative decay of the singlet and triplet excited states. Fluorometric determination of porphyrin concentration can be measured with a high degree of specificity and with greater

sensitivity than is possible with spectrophotometry. Fluorometric determinations have been used in all porphyrin assays in this thesis. (Marks, 1969; Smith, 1975; Spikes, 1984; Rimington, 1965; 1985).

HAEM BIOSYNTHESIS

Although haem biosynthesis can occur in all body tissues, it takes place predominantly in immature red blood cells (for the synthesis of haemoglobin) and in the liver, to produce haem for a number of haemoproteins (Battersby et al., 1980; Brooker et al., 1982). Eight enzymes are involved in the haem biosynthetic pathway (Figs. 8 and 9). The first of these enzymes 5-amino-laevulinic acid synthase (5-ALA-S), is mainly located in the mitochondrion where it is loosely bound to the inner membrane, although it may also be found free within the mitochondrial matrix (McKay et al., 1969; Scotto et al., 1983). This enzyme condenses the precursors glycine and succinyl Co-A to form 5-aminolaevulinic acid (5-ALA). Succinyl Co-A is formed from acetate in the tricarboxylic acid cycle, while glycine becomes the activated pyridoxal-phosphate-Schiff Base in this reaction (Granick, 1966; Moore et al., 1979). It is a relatively unstable enzyme with a short half life in vivo of approximately 1 hour in mammalian species (Moore & Disler, 1985). It normally has a low basal activity and is present in very low concentrations (McColl et al., 1981) but is grossly elevated in porphyria or by porphyrinogenic inducers (Granick, 1966). This enzyme is strongly inhibited by protohaem and protohaemin, the end products of the pathway, and this feedback may allow an organism to control porphyrin synthesis and avoid generating unnecessary amounts of biosynthetic intermediates (Sinclair & Granick, 1976). The mechanism of this negative feedback

is unknown, but 5-ALA-S is thought to be the rate-limiting enzyme in porphyrin biosynthesis (Moore & Disler, 1985).

The next four enzymic steps take place in the cytoplasm. The first of these is the combination of 2 moles of 5-ALA to form the monopyrrole porphobilinogen (PBG) by the action of the enzyme 5-aminolaevulinic acid dehydratase, in a series of stages (Shemin, 1976). PBG is the unique precursor of porphyrins.

The next stage in the pathway involves the combined action of two enzymes, porphobilinogen deaminase, PBG-D) (hydroxymethylbilane synthase is the synonym of this) and uroporphyrinogen III co-synthase (synonym porphobilinogenase). These two enzymes condense four molecules of PBG to form the linear tetrapyrrole hydroxymethylbilane and cyclize them to form the first of the porphyrins, uroporphyrinogen III. Four isomers of uroporphyrinogen can be formed, but the important porphyrin derivatives such as protohaem , chlorophylls and cytochromes, are all based on the asymmetrical type III isomer, since only this isomer may proceed past the stage of coproporphyrinogen in the biosynthetic sequence that will form haem. If the co-synthetase activity is reduced, as in congenital porphyria, large amounts of the type I isomers are formed which are excreted in the urine (Moore et al., 1979; Battersby et al., 1980; Moore & Disler, 1985; Rimington, 1985). Types II and IV isomers have not yet been found in living systems. Porphobilinogen deaminase has been defined as the secondary control point in the pathway, since it has the second lowest activity (Moore & Disler, 1985).

Uroporphyrinogen III is then decarboxylated sequentially by uroporphyrinogen decarboxylase (URO-D) to give heptacarboxylic, hexacarboxylic and pentacarboxylic intermediates prior to the formation of coproporphyrinogen III, a tetracarboxylic structure. Uroporphyrinogen I is decarboxylated more slowly than the type III isomer (Moore & Disler, 1985).

The pathway then re-enters the mitochondrion where the propionyl groups in positions 2 and 4 of coproporphyrinogen III are decarboxylated and oxidised to yield the two vinyl groups of protoporphyrinogen IX, by coproporphyrinogen oxidase (COPRO-O) (Smith, 1975). This enzyme is located in the intermitochondrial space and is not membrane bound (Elder & Evans, 1978). The monovinyl Harderoporphyrinogen is the intermediate in this reaction, with the propionyl group in position 2 of coproporphyrinogen being decarboxylated first (Kennedy et al., 1970; Moore & Disler, 1985).

Protoporphyrinogen IX is then oxidised by the removal of 6H atoms to form protoporphyrin IX, by protoporphyrinogen oxidase (PROTO-O) (Poulson & Polglaze, 1975).

The final step in the pathway involves the insertion of ferrous iron into the protoporphyrin molecule by ferrochelatase to form protohaem, the end product of the pathway (Goldberg et al., 1956). This mitochondrial enzyme is firmly attached to the inner mitochondrial enzyme (McKay et al., 1969).

PORPHYRIA

Although type I and III isomers of coproporphyrin and uroporphyrin and protoporphyrin IX are often found in relatively small amounts in tissue fluids and urine, and protoporphyrin and deutero-porphyrin occur in the faeces of carnivores as a result of bacterial decomposition of haem compounds ingested as meat or due to intestinal haemorrhage (Rimington, 1965; Smith, 1975), large amounts of porphyrins in body tissues usually only occur as a result of porphyria, a metabolic disorder with overproduction of porphyrins and 5-ALA leading to clinical abnormalities (Goldberg, 1959; Rimington, 1965; 1985; Moore et al., 1979). Porphyria is usually hereditary in origin but sporadic and toxic porphyrias also occur. The six classified forms of porphyrias are each characterised by derangement and partial blockage of specific stages in the haem biosynthetic pathway, leading to a build up of the precursor prec eding the blockage and its excretion. In addition, all have raised 5-ALA-S activities. A number of common drugs may induce a porphyric attack in patients. Steroids have been demonstrated to be porphyrinogenic; oral contraceptives or hormonal changes during the menstrual cycle or pregnancy may trigger an attack in porphyrics. Attacks are more common in women and rarely occur before puberty. (McColl & Fairbairn, 1979; Moore et al., 1973; 1979; Rimington, 1985; Moore & Disler, 1985).

PORPHYRIN SYNTHESIS IN THE RODENT HARDERIAN GLAND

Rodents are the main mammalian group in which porphyrins have been located within the Harderian gland. Porphyrin pigment was first described in the gland of the white mouse (Loewenthal, 1892). The gland was originally thought to be a passive storage organ of porphyrin formed elsewhere in the body (Derrien & Turchini, 1924; Grafflin, 1942) or to act as an excretory organ for porphyrins. However, later experiments in the mouse comparing the capacity of various homogenised tissues to produce porphyrin by adding a solution of 5-ALA showed that the Harderian gland had the greatest porphyrin-producing enzyme activity and the authors, Davidheiser & Figge (1955), concluded that the mouse Harderian gland was a porphyrin-forming structure. It was also demonstrated that the porphyrinogenic enzyme activity in the mouse gland varies with age and strain (Figge & Davidheiser, 1957), and between rodent species (Davidheiser & Figge, 1958). Porphyrin content in the rat gland was shown to increase with age (Kennedy et al., 1970).

Tomio & Grinstein (1968) provided experimental evidence for the de novo biosynthesis of protoporphyrin XI from glycine and succinyl-Co-A by the rat Harderian gland. The presence of 5-ALA-S in the mitochondrial fraction was demonstrated by the formation of ^{14}C-5-ALA. The presence of 5-aminolaevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen decarboxylase and coproporphyrinogen oxidase systems and the similarity of the biosynthetic

pathway with other living cells was also shown by these authors. The entire enzymatic complement required to synthesize porphyrins from the precursors was also demonstrated in the mouse: however, compounds which induced hepatic porphyria were without effect on the porphyrin content of the mouse Harderian gland. This possibly reflects a different form of regulation of the porphyrin pathway in mouse Harderian gland, and genetic studies showed that the locus regulating porphyrin content in the gland of inbred mice is distinct from the liver locus (Margolis, 1971).

Further differences in the effect of porphyrinogenic drugs on the liver and the Harderian gland were illustrated when the liver 5-ALA-S activity increased 7 fold compared to a 2 fold increase in the gland enzyme activity following a single injection of allylisopropylacetamide (Wetterberg, 1971). Differences in the time taken to reach peak enzyme activity also occurred. The injection resulted in an increase in the total porphyrin content of the gland which returned to normal values in 48 hours: excess porphyrin, which fluoresced red under ultra-violet illumination, was excreted onto the nose and whiskers (Wetterberg et al., 1971). Distinct mechanisms may also exist in the hamster since the presence of pyridoxal 5' phosphate is required to detect 5-ALA-S in the hamster Harderian gland (Lin & Nadakavukaren, 1981) but hepatic 5-ALA-S activity occurs independently of this additive (Gross & Hutton, 1971).

The golden hamster exhibits sex differences in porphyrin content (Christensen & Dam, 1953) with the female gland

containing considerably more porphyrin than the male (Payne et al., 1979). The porphyrin content of the gland is influenced by hormones (Hoffman, 1971; Payne et al., 1977a & b; 1979; Lin & Nadakavukaren, 1979) and lighting (Wetterberg, 1972; Wetterberg et al., 1972b & c; Nadakavukaren & Lin, 1983). Differences in enzyme activities also exist. Formgren & Wetterberg (1973) demonstrated that uroporphyrinogen-III-cosynthetase activity is higher in females, increases in castrated males and is also dependent on visual function. The lack of porphyrin in the male gland may be due to an androgen-dependent enzyme system within male cells which breaks down or prevents the formation of porphyrin stores (Bucana & Nadakavukaren, 1973). Jones & Hoffman (1976) demonstrated in vitro synthesis of porphyrins from precursors in both male and female hamster glands and suggested that the low concentration of porphyrin in males was due to a deficiency of 5-ALA. However, 5-ALA-S activity does occur in the male gland but the activity of this enzyme is higher in the female (Lin & Nadakavukaren, 1981; Thompson et al., 1984). The activity of 5-ALA-S and porphyrin content of the female gland show marked seasonal variation with maxima in both during the summer (Moore et al., 1980), cyclic oscillations during the oestrous cycle (Moore et al., 1977; Payne et al., 1977b), and changes in porphyrin content during pregnancy and lactation (Payne et al., 1979).

All the enzymes in the haem biosynthetic pathway are present in the hamster Harderian gland, and five of these enzymes (ALA-S, PBG-D, URO-D, COPRO-O & PROTO-O) have

significantly higher activities in the female gland than the male, in animals kept under similar temperature and lighting regimes. The accumulation of porphyrins within the gland is due to the low level of ferrochelatase activity. No sex differences were observed in liver porphyrin content, but female blood porphyrin levels were significantly higher (Thompson et al., 1984).

The rodent Harderian gland contains large amounts of porphyrin mainly in the form of protoporphyrin IX (Payne et al., 1982) but coproporphyrin is also present, and in some species Harderoporphyrin has been detected (Kennedy, 1970) (Fig. 10). The gland is therefore a useful mammalian organ in which to study porphyrin biosynthesis since its enzyme activities and porphyrin content are considerably greater than other major porphyrin synthesising organs such as the liver and kidney (Table 1) (Wetterberg et al., 1971; Thompson et al., 1984). Unlike these other organs, the Harderian gland can be removed without any detrimental effect to the animal, in order to examine the gland's contribution and effect on porphyrin synthesis in the rest of the body. The sexual dimorphisms occurring in the gland of the golden hamster facilitate the study of the influence of steroids on the regulation of haem biosynthesis by endogenous or exogenous manipulation of sex steroids.

AIMS OF THIS PRESENT STUDY

1. To investigate the control of porphyrin biosynthesis within the Harderian gland by exogenous and endogenous steroid manipulation, primarily in the female hamster.
2. To investigate the relationship between porphyrin biosynthesis in the Harderian gland and in the rest of the body.
3. As a prerequisite to the histological investigations of 1, to investigate the effects of various fixatives in preserving the different forms of porphyrin deposits within the gland.

The following quantitative experiments were undertaken to elucidate these aims:

1A. The role of ovarian hormones in maintaining porphyrin biosynthesis and gland morphology were examined in a number of experiments using a number of different experimental groups.

a) by comparing ovariectomised, androgen-treated and control females

b) by examining a time sequence of changes after ovariectomy

c) by examining a time sequence of changes from post puberty to post-reproductive senescence in females

d) by comparing pregnant, lactating and control multiparous females

e) by comparing the effects of oestrogen, progesterone (or testosterone) treatment in castrated males, with castrates, intact males and intact females

1B. The role of androgens in maintaining a biochemical and morphological male type gland was assessed

a) by comparing a time sequence of changes from post puberty until senescence in males

b) by comparing the effects of testosterone administration to castrates, with untreated castrates, intact males and intact females (as in 1Ae).

2. The relationship between porphyrin biosynthesis in the Harderian gland and the rest of the body was examined in two ways.

a) the effect on porphyrin levels and/or enzyme activities in the liver, kidney or blood was assessed in relation to changes in the Harderian gland, as a result of changes in hormone levels.

b) the effect of the removal of the Harderian gland on liver and kidney porphyrin content and enzyme activities and blood porphyrin levels was studied in (i) both males and females after 5 months, (ii) during a time course in females, and (iii) in Harderianectomised and/or ovariectomised females.

3. In order to preserve the various forms of porphyrin deposits within the gland at both light and electron microscope levels, the first experiment examines the effects of several commonly used fixatives on porphyrin solubility during fixation and dehydration of Harderian gland tissue.

GENERAL MATERIALS AND METHODS

INTRODUCTION

Seven experiments were performed in this study involving a total of 605 animals. Although each experiment was different, a common methodology was employed: the maximal use of one animal involved:-

1. Histological processing of the Harderian gland for both light microscopy and transmission electron microscopy.
2. Estimation of the total porphyrin content of the Harderian gland, liver and kidney and porphyrin profiles of these organs by high performance liquid chromatography.
3. Estimation of the activity of the rate limiting enzyme in porphyrin production in the Harderian gland, liver and kidney.
4. Estimation of the blood porphyrin content.

The specific methods involved in each particular experiment are listed in subsequent sections, while the present section will describe the general methods employed in detail.

ANIMALS

All the golden hamsters (Mesocricetus auratus, Waterhouse) used in this study came from a closed colony, of approximately 700 animals with a breeding success rate of 75%, established in the Anatomy Department of Glasgow University in 1968. The animals were housed in polypropylene cages with stainless steel wire mesh tops: up to three hamsters were housed in the smaller cages (27.5 x 20.6 x 10cm, plus 5cm for the wire top), and a maximum of eight hamsters were kept in the larger cages (40.6 x 25.6 x 15cm plus 5cm for the wire top), with woodshavings and shredded paper bedding (Arthur Woollcott Co.). The animals were given water and CRN diet (Labsure) ad libitum and maintained at a temperature of 21^{o}C under a 15L:9D lighting regime throughout the year. This lighting regime was chosen since hamsters are seasonal breeders and variations in photoperiod regulate the annual growth and atrophy of the sex organs: studies have shown that hamsters normally require long photoperiods (i.e. $\geq$ 12.5h light/24h) for the maintenance of testicular size and function (Hoffman et al., 1965; Gaston & Menaker, 1967; Reiter, 1972; Elliot, 1981).

OPERATIVE PROCEDURES

In experiments where the gonads and/or the Harderian glands were removed, the following procedures were carried out. The animals were weighed and given the appropriate dose of sodium pentobarbitone B.P. (60 mg/ml), (Sagatal - May & Baker Ltd.), by intra-peritoneal injection with a 1ml syringe and a 26G 3/8 needle. The animals were then left until completely anaesthetised, i.e. when no movement of the vibrissae was detected or limb reflexes to paw pinching.

Ovariectomy of Females

Fur was removed from the lower back with scissors and the area was swabbed with diluted Hibitane antiseptic (Chlorhexidene gluconate 5% w/v, ICI). A single midline dorsal incision, approximately 4cm in length, was made in the skin, then lateral incis ions made in the abdominal musculature on either side, in turn, approximately 1½cm from the midline. The ovary and uterine horn were exteriorised on each side in turn, the ovary freed from fat and the ovary and fallopian tube excised (no ligature was required as little bleeding occurred), the uterine horn was then replaced and the muscular incision stitched with 25mm Mersilk needle and suture thread (Ethicon 4/0). The midline incision was then closed with 12mm Michelle clips.

Castration of Males

The scrotum was swabbed with antiseptic and a transverse midline skin incision made between the penis and anus. The wall of each scrotal sac was cut in turn and the testis squeezed out. The testicular blood vessels were ligatured and the testis

and epididymis removed. The skin incision was then stitched.

Harderianectomy

Harderianectomy was performed as follows: firstly, horizontal incisions were made above and below the eye into the fornices of the conjunctiva; secondly, vertical incisions were made on the medial (nasal) and lateral sides of the pigmented nictitating membrane and the latter freed; thirdly, by pulling the nictitating membrane gently but firmly, the Harderian gland (which is attached to it) can be drawn out of the orbit. Care must be taken during the third stage to close the lids over the eye and press inwards so that removal of the gland (which is of irregular shape and surrounds the optic nerve and ocular muscles, e.g. Venable & Grafflin, 1940) does not damage the optic nerve or compromise its vascular supply.

Steroid administration

Steroids were given to some groups of gonadectomised hamsters by subcutaneous injections of steroids dissolved in Arachis oil, in a 0.1ml dose from a 1ml syringe with a Microlance 23G 1¼ needle, three times per week for the duration of the treatment. The following concentrations of steroids were administered: 15mg testosterone/ml Arachis oil; 10mg progesterone/ml Arachis oil; and 0.2mg 17 β-oestradiol/ml Arachis oil.

These doses of steroids were chosen as being at least the dose required to maintain reproductive tract weight or sexual behaviour (Powers & Valenstein, 1972; Gandelman, 1973; Payne & Bennett, 1976).

TISSUE COLLECTION

A total of 605 animals was used in these studies. All animals were killed with an overdose of sodium pentobarbitone B.P., 60mg/ml, (Sagatal - May & Baker Ltd) by intra-peritoneal injection of 0.5ml from a 1ml syringe with a 26G $^{3}/_{8}$ needle. Blood was immediately withdrawn, using a previously heparinised 5ml syringe and a 23G 1¼ needle, by heart puncture for the later determination of blood porphyrin levels using a haematofluorimeter. The eyes were then removed, the skull cut open, and the Harderian glands excised from the orbit with forceps, after freeing the nictitating membrane. Harderian glands were used for either:-

i) Histological examination by light and transmission electron microscopy.

ii) Determination of porphyrin content.

iii) Determination of the activity of the porphyrinogenic enzyme 5-aminolaevulinic acid synthase (5-ALA-S, the rate limiting enzyme for porphyrin production).

When required, the liver was also removed, half of each lobe being retained for 5-ALA-S activity, while the remainder was kept for porphyrin estimations. In some instances the enzyme activity and porphyrin content of the kidneys were also investigated: in this case the kidneys were halved longitudinally, excess fat and connective tissue removed, and one half of each kidney used in each assay.

HISTOLOGICAL PROCESSING AND EXAMINATION

Preparation of Harderian gland material for light microscopy

1. The glands were excised with forceps. After the connective

tissue was trimmed off, they were fixed by immersion in 20ml of 3% glutaraldehyde in phosphate buffer (pH 7.4) for 48 hours. Perfusion of the whole animal with glutaraldehyde was impossible in view of the need to measure porphyrin content and enzyme activity in the other gland and other tissues such as liver and kidney, as well as blood porphyrin content (see above).

2. The tissue was transferred to phosphate buffer (pH 7.4) for 7 days, to facilitate cutting.
3. The tissue was dehydrated through an alcohol series in a Histokinette, receiving 2 hours in each of the following solvents:- 70%, 90%, 1st Absolute, 2nd Absolute, 3rd Absolute, 1st amyl acetate, 2nd amyl acetate, 3rd amyl acetate. In order to soften the tissue, 10% celloidin was added to all alcohols.
4. The tissue was embedded in wax, receiving 4 hours in the 1st change and 2 hours in the 2nd change.
5. 5 μm sections were cut on either a Jung or Spencer microtome until half the gland was cut.
6. An interrupted series of 1 in 25 was mounted, giving approximately 8 sections per slide.
7. Slides were routinely stained with 1. Haematoxylin & Eosin (Harrison's haematoxylin), a general stain; 2. Masson's, a sufficiently dark stain which facilitated the counting of Type II epithelial cells, and 3.0.1% Toluidine blue lightly counterstained with eosin, used to identify mast cells which stain metachromatically, after being dewaxed in xylene (May & Baker) and

hydrated to water through a graded alcohol series. The sections were stained in accordance with the procedures outlined in Bancroft & Stevens (1982), Theory and Practice of Histological Techniques. The sections were then dehydrated through a graded alcohol series, carboxylene and xylene and left to clear for five minutes before coverslips were mounted with Histomount (Hughes & Hughes Ltd.).

Preparation of Harderian gland material for transmission electron microscopy.

Where tissue from light microscopy proved of interest, the tissue residue was reprocessed for electron microscopy by the following procedure. The times given are approximations for tissue measuring 6mm x 2mm x 2mm, longer times being required when the tissue block was larger.

1. The half gland was dewaxed in 3 changes of xylol (2-3 hours per change).
2. Once completely dewaxed, the tissue was transferred to the 1st change of propylene oxide for 30 minutes, and to the 2nd change for 1 hour.
3. The tissue was transferred to 50:50 propylene oxide: araldite mixture for 1-3 hours, then into 25:75 propylene oxide:araldite for 24 hours, and finally into pure araldite mixture for $\pm$ 24 hours.
4. The tissue was embedded in fresh araldite in polypropylene capsules and placed in an oven at 40^{o}C for 12 hours, 50^{o}C for 12 hours then 60^{o}C until cured.

5. The araldite was trimmed and semithin sections (1-2 μm) cut to localise the area of interest. The tissue was then retrimmed and thin sections (60-80 nm) cut and stained by the following method:-
   a) 5 minutes in uranyl acetate (50% alcohol saturated with uranyl acetate),
   b) 3 washes in d H_2O,
   c) dried on filter paper (Whatman 's No. 1),
   d) stained with lead citrate for 5 minutes on dental wax (with NaOH pellets present in the petri dish to absorb any excess CO_2) using a modification of Reynold's method (1963),
   e) washed in 3 changes of d H_2O,
   f) dried on filter paper (Whatman 's No. 1).

Histological Quantification

The maximum number of parameters which could potentially be measured in an individual animal are detailed below:

1. Mast cells were counted in every section, in both the interstitium of the tissue and in the connective tissue capsule surrounding the gland.
2. Intraluminal porphyrin accretions were normally counted in two sections (4 and 8) except in experiment 2 where they were counted in every section.
3. Large interstitial porphyrin deposits were normally counted in two sections (4 and 8), except in experiment 2 where they were counted in every section.
4. Small interstitial porphyrin deposits, such as occur within free macrophages, were counted in two sections (4 and 8).

5. The number of tubules containing neutrophils within the lumen was counted in two sections (4 and 8).
6. The number of mitotic figures occurring was counted in one section (8).

   The area and perimeter of each relevant section were measured using a MOP-AMO_2 planimeter (Kontron Messgerate GMBH) to give derived values in terms of frequency per mm^2 of section, or per mm of capsule.
7. Type II cells were counted in two sections (4 and 8) using the midline intersection of a graticule. Only those tubules cut transversely and showing a definite lumen were counted; the number of profiles containing Type II cells was expressed as a percentage of the total number of tubule profiles counted (approximately 50 profiles per animal).
8. In one experiment (experiment 2) the presence of polytubular complexes were determined by re-embedding three tissue blocks from each of the four groups of animals for electron microscopy. Fifty cells from each animal were examined for the presence of these ultrastructural features.
9. The percentage area of tubule degeneration.

Photomicrography

In experiments where the percentage area of tubule degeneration in the Harderian gland was determined, the following procedure was adopted: two sections (4 & 8) from each animal were photographed at low power and the areas of degeneration were then outlined on the photograph (printed

to give a final magnification of approximately x100), while re-examining the sections at high power. A MOP-AMO_2 planimeter was then used to measure the total area of each section and the area of degenerating tubules within that section, resulting in a percentage degeneration score for each animal. Degeneration was defined as extreme reduction in epithelial height involving all or part of a tubule profile, usually accompanied by a marked reduction in visible nuclei.

For this and all light photomicrography in this thesis, a Leitz vario-orthomat and 35mm film was used.

ESTIMATIONS OF PORPHYRIN CONTENT IN TISSUES

The analytical techniques in Total Porphyrin Assays, Blood Porphyrin estimations and High Pressure Liquid Chromatography determination employ fluorescence detectors since porphyrins fluoresce red when excited with ultraviolet light.

Details of the techniques used are as follows:-

Blood Porphyrin Estimation

1. Blood was withdrawn from the ventricles following heart puncture with an heparinised syringe, placed in a universal container and kept at room temperature, until required. Standing times of up to 20 hours will not result in abnormal readings.
2. The container was shaken and 20 µl of blood placed on a glass coverslip in the slide carrier of a calibrated (625nm emission, 397nm excitation) Buchler Tm Zp Haemafluor haematofluorimeter, using Child & Ep settings. The haematofluorimeter detects "free erythrocyte protoporphyrin" since protoporphyrin in these erythrocytes fluoresces in the red region of the spectrum when excited with ultra-violet light.
3. The blood was mixed with a pipette tip to oxygenate it and readings were noted until a constant reading was obtained.
4. The sample reading computed by the Hemafluor ZP is based on average hematocrit values (packed cell volumes (PCV), 35 for children) and this reading is converted to give results in nmol protoporphyrin/l by the following

equation:- $X = (\text{reading} \times 16.95) \times \frac{40}{35}$. Actual data obtained for the hamster suggest a comparable PCV (Thompson, Hordovatzi , Moore, McGadey & Payne, 1984).

Total Porphyrin Extraction from Harderian glands

1. The gland was excised from the orbit with forceps and tissue for assay was weighed and then placed in 5mls of methanol (Analar) in a universal container, which could be stored in a fridge at $4^{o}C$ until required for extraction.
2. The methanol was decanted into a measuring cylinder.
3. The gland was homogenised (Polytron) in 5ml of 95:5 methanol:conc. HCl. The blade was then rinsed with a small volume of this mixture and added to the homogenate. This was centrifuged for 5mins at 2000g in an M.S.E. Scientific Instruments (Fisons Gray) centrifuge (GF6). The resulting supernatant was examined under ultraviolet light for the presence of fluorescence and then decanted into the measuring cylinder containing the methanol.
4. Step 3 was repeated until no further fluorescence was visible under ultra violet light.
5. The total volume of the supernatant was noted, then mixed well. 1ml of supernatant was filtered with Whatman's No. 1 filter paper into a conical flask and diluted with 9ml of 1.5 M HCl.
6. The filtrate was then decanted into a glass fluorimetry cell and the reading noted on a spectrofluorometer (Perkin Elmer 3000 Fluorescence Spectrometer) standardised with coproporphyrin (COP-1-5, 5 µg per vial, Sigma Chemical Co.) giving a reading of 50 with settings of 396 nm for

excitation and 594 nm for emission, falling in the Soret range.

7. The protoporphyrin concentration in the Harderian gland was calculated using the following equation:-

$$\text{Protoporphyrin} = \frac{F \times A \times K}{(O \times 562)} \text{ nmol/g tissue}$$

where F = Fluorimeter reading

A = Acid volume in ml

K = Volume of supernatant in ml

O = Weight of the gland in grams

562 = molecular weight of protoporphyrin

Since protoporphyrin constitutes 95% of the Total Porphyrin Content of the hamster Harderian gland (as will be confirmed by preliminary HPLC studies in these present experiments) all further quantitiative data are expressed simply as total porphyrin content (nmol/g tissue).

Total Porphyrin Extractions from Liver and Kidney

1. Half of each liver lobe was placed on polystyrene weighing boats and weighed. Each kidney was halved longitudinally and one half of each kidney placed together and weighed.
2. Tissues were placed in 10mls of 4:1 ethyl acetate:glacial acetic acid and homogenised (Polytron), then stored in a freezer (- 20^{o}C) until required.
3. When required, the homogenates were allowed to reach room temperature, sonicated, placed in a Buchner funnel and washed with 20ml ethyl acetate under vacuum.
4. The filtrate was placed in separating funnels and washed with 1.5M HCl to extract any porphyrin present. The acid

volume forming the lower fraction was decanted and examined under ultra violet light for fluorescence.

5. Step 4 was repeated until no further fluorescence was visible. The total volume of acid added was noted and the total volume of the fractions collected. These fractions were well mixed and then filtered with Whatman's No. 1 filter paper into a conical flask.
6. The filtrate was then decanted into a glass fluorimetry cell and the reading noted on the fluorimeter (using the same setting, as in the Harderian gland porphyrin extractions - see above).
7. The porphyrin content was calculated as before and expressed in nmol protoporphyrin/g tissue.

Preparation of samples for High Performance Liquid Chromatography (HPLC)

Esterification of a) Total porphyrin extractions from Harderian glands and b) Total porphyrin extractions from liver and kidney.

1. The remaining supernatant from Harderian gland porphyrin extractions in 95:5 methanol:conc. HCl, or the remaining acid washing fractions from kidney or liver porphyrin extractions were adjusted to pH 5 by the addition of either saturated sodium acetate or concentrated acetic acid.
2. A small amount of talc was added to absorb any porphyrin present, and the samples were left in the dark for 15-60 minutes.
3. The samples were then poured into a Buchner funnel, under

vacuum and rinsed through with a small volume of water to remove any acetate. The filtrate was then discarded.

4. The talc which had collected on the filter was then washed with 95:5 methanol:sulphuric acid. This extract was left overnight in the dark to esterify and collected in a boiling tube.
5. Twenty ml of chloroform was added to the filter to remove any adherent porphyrin esters and this was collected in the boiling tube along with the previously collected porphyrin esters.
6. Distilled water was added to the filtrate, forming two layers. The uppermost was water which was removed with a suction pipette. This was repeated three times to wash the filtrate.
7. The filtrate was then washed with 3% sodium hydrogen carbonate, to remove any acid present and this top layer was also removed.
8. The filtrate was then washed twice with water, as before (6).
9. Sodium sulphate (anhydrous), which absorbs any adherent water, was added until a crystal clear solution was obtained.
10. The remaining solution was filtered with Whatman's No. 1 filter paper into a boiling tube and then washed with chlorofrom to remove any porphyrin esters adhering to the filter paper or sodium sulphate crystals.
11. The chloroform was evaporated from the filtrate at $60^{o}C$ in a water bath under a stream of air leaving the

porphyrin esters adhering to the glass wall of the boiling tube. The boiling tube was then sealed with clingfilm and stored in a freezer (-20°C) until required.

12. Prior to HPLC determination, the sample was reconstituted with 1ml chloroform and filtered (Whatman's No. 1) before being loaded into a syringe and 20-100 µl injected into the column.
13. The HPLC column was calibrated with a standard (Porphyrin ester chromatographic marker kit - Porphyrin Prod.) using a fluorescence detector set at 405 nm excitation, 625 nm emission.

Free Porphyrin Acids

This method minimises porphyrin loss during the preparation of the samples and is important in some studies (e.g. Experiment 1) where only small volumes of solvents (some containing very low porphyrin concentrations) are available.

1. Solvents were poured into boiling tubes, placed in a water bath at 60°C and blown down. When all the solvent had evaporated the boiling tubes were stored in a freezer (-80°C) until required.
2. The samples were then reconstituted with 1-4 mls of 1.5M HCl and a reading taken on the spectrofluorimeter to estimate the amount of porphyrin present. This permitted one to gauge the volume of the sample to be injected and the expansion required (max 100 µl at x 100 expansion) on the integrator.

3. The sample was then injected into a reverse-phase HPLC column (Micro Bonderpack C18 (Waters)), previously calibrated with a free porphyrin acid standard containing penta-, hexa- and heptacarboxylic, uro-, copro- and meso-porphyrins (Porphyrin Products, Logan, Utah). The sample was run in an acetonitrite, phosphate buffer system. The solvents were run as a gradient of 0 to 50% acetonitrite (methyl cyanide) in 0.1mM sodium phosphate buffer pH 7.5 over 30 minutes at a flow rate of 1ml/ minute. A fluorescence detector with settings of 396nm excitation and 594nm emission (Perkin Elmer, 3000) was employed.

DETERMINATION OF THE ACTIVITY OF 5-AMINOLAEVULINIC ACID SYNTHASE (EC2.3.1.37) IN THE HARDERIAN GLAND, LIVER OR KIDNEYS

Reference: Thompson, Hordovatzi, Moore, McGadey & Payne, 1984.

1. After removal (see above) tissues were then homogenised (Polytron)with 1ml saline. 100 µl of the homogenate was pipetted into a 5ml plastic tube containing 200 µl of reaction mixture (see Appendix 1) and 100 µl of incubation buffer (see Appendix 1). The remaining 900 µl of homogenate was retained for protein estimation (Lowry et al., 1951).
2. The above mixture was incubated for one hour at 37°C in a water bath.
3. To stop the reaction, 200 µl of the incubate was added to a small round bottomed polystyrene tube (LP3) containing 40 µl of a solution of 5-aminolaevulinic acid (5-ALA, Sigma Chemical Co.) in trichloroacetic acid (TCA), (10mg 5-ALA per 0.5ml 2N TCA).
4. This solution was then sonicated to remove any protein adhering to the ALA, and could then be frozen until the electrophoresis could be performed.
5. The precipitate was removed by centrifugation (10 min at 3500 rpm) and 10 µl of the clear supernatant spotted onto Whatman's 3MM paper for electrophoresis, using a 10 µl capillary tube.
6. The 5-ALA and glycine were separated by high voltage electrophoresis at 2000 volts for 45 minutes, at 4°C

in potassium hydrogen phthalate buffer (0.05M).

7. Following electrophoresis, the paper was dried in an oven at 90^{o}C, the two amino acids identified by spraying with Ninhydrin , cut out and combusted in a sample oxidiser (Packard Tri-Carb B306).
8. The collected scintillant (22ml) was then placed in a scintillation counter (Packard Tri-Carb Liquid Scintillation Spectrometer 3320) and beta particle emission was counted over one hundred minutes.
9. A standard computer programme was used to express the results as nmol 5-ALA formed/g protein/hr.

PROTEIN ESTIMATION

Reference: Lowry, Rosebrough, Farr & Randall, 1951.

This method of colorimetric determination of protein concentrations is dependent on two distinct steps which lead to the final colour: a) the reaction of protein with copper in an alkaline solution, and b) the reduction of the Folin (phosphomolybdic-phosphotungstic) reagent by the copper-treated protein. It is suitable for analyses of large numbers of similar protein samples when measuring very small amounts of protein or highly diluted protein, and measurement of mixed tissue proteins.

Harderian gland homogenates were diluted with distilled water by 1:200, liver and kidney homogenates by 1:1000 and 1ml pippetted into each of two test tubes. The stock protein standard, bovine serum albumin (200mg/l), was also pippetted in 1ml aliquot in duplicate, as were the three dilutions of this standard (1:1, 1:3, 1:7) and distilled water, the latter being the reagent blank. Five ml of the protein reagent (see Appendix 2) was added to each sample of blanks, standards and diluted homogenates, mixed and left for ten minutes. To each of these samples 0.5ml of Folin phenol reagent (diluted 1:1 distilled water) was added, the contents mixed immediately and left for thirty minutes to colour up. The samples were then read on a Spectrometer (previously zeroed with the two blank samples) with the wave length setting at the absorption peak of 750nm.

A linear regression computer programme was then used

to estimate the protein concentrations of the diluted Harderian gland, liver or kidney samples from the known standard protein concentrations and their readings. The correlation coefficient of the known protein concentrations (X reading) and their corresponding readings (Y) had to be a minimum of 0.97 to be acceptable. Liver and kidney samples were diluted by 1:1000 and the programme therefore gave a direct result in g/l; since Harderian glands were diluted by 1:200 the result from the programme had to be divided by five to give the final concentration in g/l. The mean concentration of the two results of an unknown protein had to be within 10% of the upper reading to be acceptable; if greater than this the estimation procedure was repeated.

EXPERIMENT 1: THE QUANTIFICATION OF PORPHYRIN LOSS FROM A PORPHYRIN SYNTHESISING TISSUE - THE HARDERIAN GLAND - DURING ROUTINE HISTOLOGICAL PROCESSING.

INTRODUCTION

The rodent Harderian gland manufactures and stores protoporphyrin and is an increasingly-used model of porphyrin biosynthesis. For example, in the Harderian gland of the female golden hamster, both porphyrin content and the activity of 5-aminolaevulinate synthase (the rate-limiting enzyme for porphyrin synthesis) are substantially higher than in other porphyrin producing tissues such as liver and kidney (Thompson et al., 1984; Spike et al., 1985; 1986). (Table 1).

Normally, porphyrin is deposited in the form of large solid intraluminal accretions in the hamster (Bucana & Nadakavukaren, 1972a),mouse (Woodhouse and Rhodin, 1963), rat (Kanwar, 1960; Brownscheidle & Niewenhuis, 1978) and gerbil (Johnston et al., 1983). In addition, porphyrins may also occur as large interstitial accretions where tubule walls have degenerated and, subsequently, as small accretions within individual interstitial macrophages (Payne et al., 1982b; 1985; Spike et al., 1985; 1986). These forms give insight into the state of gland integrity and all may be seen at the light microscope (as well as electron microscope) level (Figs. 11 - 14). However, since porphyrins are soluble in a wide range of organic solvents, histological routines must be employed which will not only give good tissue preservation

but also minimise porphyrin loss during processing. In this experiment, a range of commonly used fixatives were employed and the loss of porphyrin from the Harderian gland of the female golden hamster measured at each stage in the fixation and dehydration series for each routine.

MATERIALS AND METHODS

Five routines were examined in order to quantify, spectrofluorimetrically, the % loss of porphyrin at different stages in the fixation and dehydration of the Harderian glands from female golden hamsters. Four of these routines involved fixation for 48 hours in 1) AFA (alcohol/formaldehyde/acetic acid); 2) Bouin's fixative; 3) Zenker's dichromate fixative, and 4) 3% buffered glutaraldehyde (see Table 2 for composition and pH of each fixative). Forty-eight hours was chosen as a standard fixation time since this is a) the minimum time necessary to fix hamster Harderian gland in glutaraldehyde if double-embedding techniques (an important feature of many studies, Payne et al., 1985; Spike et al., 1985) are to be employed and b) compatible with fixation times for hamster Harderian gland in Bouin's fixative (Payne et al., 1982a). The tissue was then dehydrated through a graded alcohol series of 70%, 90% and 3 x absolute, followed by three changes of chloroform and finally transferred to methanol so that the remaining porphyrin could be determined. A standard 20 ml was used for all solutions, with an immersion time of 2 hours in each. The fifth routine again used 3% buffered glutaraldehyde as the fixative, but amyl

acetate was substituted as the clearing agent in place of chloroform. The porphyrin loss at each stage and the residue left in the tissue was compared with the porphyrin content of a similar amount of tissue from the same animal placed directly into methanol, thus providing an internal verification. Initially, two female hamsters were used in each routine, the four glands being excised then halved longitudinally. Half of each gland was placed in fixative while the remaining half was placed in 5 ml methanol (Analar). The experiment was repeated once more for each glutaraldehyde routine and the dichromate routine, while the AFA was repeated a further 3 times and the Bouin's four times.

Spectrofluorimetry

Preparation for spectrofluorimetry differed slightly according to the solvent being used.

1. The glands placed directly into methanol (and the processed glands placed finally in methanol for residue determination) were homogenised (Polytron) with 95:5 methanol: conc. HCl and centrifuged several times (see Payne et al., 1977b)until no further fluorescence was detected in the supernatant under UV light. The total volume of supernatant collected was noted, mixed and 1 ml filtered with Whatman's No. 1 filter paper into a conical flask and diluted with 9 ml 1.5M HCl. This filtrate was then decanted into a glass fluorimetry cell and the reading noted on the fluorimeter (Perkin Elmer 3000 Fluorescence Spectrometer) standardised with coproporphyrin (COP-1-5, 5 µg per vial, from Sigma

Chemical Company) giving a reading of 50, and zeroed with 1.5M HCl. Fluorimeter settings were 396 nm for excitation and 594 nm for emission.

2. The alcohols from the dehydration series were well mixed, and 1 ml from each solution diluted with 9 ml 1.5M HCl, filtered and a reading taken on the fluorimeter, as above.

3. The chloroforms, amyl acetates, and AFA fixatives were evaporated in a water bath. Concentrated HCl and a few ml of chloroform were then added and the solution was sonicated and centrifuged to extract any porphyrin present. The supernatant was then washed several times with conc. HCl, then diluted several times to give a final concentration of 1.5M. This was filtered and decanted into a glass fluorimetry cell, and the reading noted.

4. The remaining fixatives, Bouin's, Dichromate and glutaraldehyde, were adjusted to pH 4.00 by the addition of saturated sodium acetate or glacial acetic acid, and any porphyrin present was extracted with ether in a separating funnel. The ether was then washed with 1.5M HCl, which was filtered and decanted into a fluorimetry cell and the reading recorded.

The porphyrin concentration present in each fixative and solvent were then determined.

<u>High Performance Liquid Chromatography</u>

The solvents and fixatives were prepared for High Performance Liquid Chromatography to determine which porphyrin free acids were being lost at each stage in the

routines. The solutions were poured into boiling tubes, placed in a water bath at 60°c and blown down until all the liquid had evaporated, leaving any porphyrin present adhering to the glass wall of the boiling tube. The samples were stored in a freezer (-80°C) and later reconstituted with 1 ml of 1.5M HCl: 100 µl of this solution was then injected into the HPLC column, previously calibrated with a free porphyrin acid standard containing penta-, hexa- and heptacarboxylic, uro-, copro- and meso-porphyrins (Porphyrin Products, Logan Utah), using a fluorescence detector with settings of 396 nm excitation and 594 nm emission (Perkin Elmer 3000).

RESULTS

Spectrofluorimetry (see Figs. 15-19)

Using the AFA routine, a substantial proportion (18%) of the tissue porphyrin was lost in the fixative itself, any subsequent losses occurring in absolute alcohols, leaving a residue of 71% in the fixed and dehydrated glands. In the Bouin's routine, there was a negligible loss in the fixative, but a considerable loss occurred in the 90% and the absolute alcohols. As in the AFA routine, the porphyrin loss in the chloroforms was very low, but as little as 10% of the total porphyrin (average loss 65%)can be left in the processed tissue.

The Dichromate routine minimised total porphyrin loss (less than 2%) with the absolute alcohols accounting for any leaching. The glutaraldehyde routines both resulted

in less than 10% loss of porphyrin which occurred in the alcohols. For each pair of glands, the total porphyrin loss and the residue within the fixed tissue gave an excellent approximation to the porphyrin content of the other gland from the same animal placed directly into methanol, regardless of the histological routine employed.

HPLC (see Table 3)

Only three porphyrin free acids were quantifiable; hexacarboxylic porphyrin, coproporphyrin and protoporphyrin, the latter being the most abundant (protoporphyrin constitutes 95% of the total porphyrin concentration in the Harderian glands of female golden hamsters). Only trace amounts of uroporphyrin, hepta- and pentacarboxylic porphyrins were observed.

In the AFA routine, the only fractions which were detected occurred in the fixative, which showed 40% coproporphyrin and 60% protoporphyrin.

In the Bouin's routine, the fixative could not be processed as the picric acid becomes explosive when dry. However, the pooled 70% and 90% alcohols revealed 6.72% hexacarboxylic porphyrin and 93.28% protoporphyrin, while the pooled absolute alcohols and chloroforms both contained 100% protoporphyrin.

The Dichromate fixative was also too difficult to process for HPLC, and neither the pooled alcohols, or chloroforms contained any porphyrin free acids.

In the glutaraldehyde routine, only the pooled 70% and 90% alcohols contained any acid - protoporphyrin.

These results correlate well with those obtained from the spectrofluorimetric assay.

SUMMARY

In tissue which is used as a model of porphyrin biosynthesis it is important to use a fixative which both minimizes porphyrin loss and which gives good fixation for light microscopy, transmission electron microscopy and scanning electron microscopy. In the present investigations, these requirements were achieved optimally with fixation in 3% buffered glutaraldehyde and the use of amyl acetate as a clearing agent.

EXPERIMENT 2: QUANTITATIVE STUDIES ON THE EFFECTS OF HORMONES ON STRUCTURE AND PORPHYRIN BIOSYNTHESIS IN THE HARDERIAN GLAND OF THE FEMALE GOLDEN HAMSTER. I. THE EFFECTS OF OVARIECTOMY AND ANDROGEN ADMINISTRATION.

INTRODUCTION

A number of morphological and biochemical sex differences occur in the Harderian gland of the golden hamster (see "GENERAL INTRODUCTION"). In the male gland, the role of the testis in regulating these characteristics is well known since castration results in the gland assuming the female pattern after a few months while androgen administration prevents these changes (Hoffman, 1971; Payne et al.,1977a; 1982a). In the female gland, the links between gonadal hormones and gland morphology and activity are less well documented. Although porphyrin content and porphyrin-synthesising enzyme activity alters over various reproductive states (Payne et al., 1978) and with the season (Moore et al., 1980), the exact physiological basis of these observations is unclear, nor have they been correlated with morphological changes. Ovariectomy results in a series of degenerative changes in gland structure (Payne et al., 1982b,1985), while androgen administration (Sun & Nadakavukaren, 1980) or exposure to extremely short daylength (Nadakavukaren & Lin, 1983) may produce morphological virilization of the female gland. However, these changes have not been quantified, nor have they been correlated with changes in porphyrin

content or enzyme activity.

This experiment was undertaken to quantify the effects of ovariectomy and/or androgen administration on the morphology, porphyrin content and enzyme activity of the Harderian gland of the female hamster by comparing the glands of intact males, intact females, females which had been ovariectomised for 5 months and females which had been ovariectomised for 5 months but had received androgen administration during that period.

MATERIALS AND METHODS

The animals used in this study were a) intact males, n = 11, b) intact females, n = 20, c) females bilaterally ovariectomised for 5 months, n = 22 and d) females bilaterally ovariectomised for 5 months during which time they received androgen administration, n = 11. All animals were one year old at termination and were killed with an overdose of sodium pentobarbitone (Sagatal).

Both Harderian glands were removed, one gland being fixed and processed for light microscopy. The other gland was halved longitudinally, one half being weighed and placed in methanol for the later determination of the total porphyrin content while the other half was immediately assayed for 5-ALA-S activity. The protein concentration of the gland was estimated by Lowry's method (1951), and enzyme activity expressed as nmol ALA formed/g protein/h .

The following histological parameters were measured in each animal:-

1. Mast cells mm^2/ section and mast cells mm/capsule.
2. Intraluminal porphyrin accretions mm^2/section.
3. Large interstitial porphyrin deposits mm^2/section.
4. Small interstitial porphyrin deposits mm^2/section.
5. The number of tubules containing neutrophils mm^2/section.
6. The percentage area of tubule degeneration.
7. The % of tubules with Type II cells.
8. The percentage of polytubular complexes. Three tissue blocks from each group, chosen at random, were re-embedded for transmission electron microscopy, and 50 cells from each animal were examined for the presence of polytubular complexes.

Detailed procedures of the methods used in this experiment are described in "GENERAL MATERIALS AND METHODS".

Statistical analyses: For each parameter, the three groups of females were compared using one-way analysis of variance (F). Where this proved significant, individual inter-group comparisons were made using Dunnett's test (Dunnett, 1964). Individual female groups were compared with males using Student's t test.

RESULTS

Two broad patterns of change were observed when the experimental groups were compared with control animals.

a) A pattern of masculinization changes occurring predominantly in the testosterone-injected ovariectomised females.

b) A pattern of degenerative changes occurring predominantly in the ovariectomised females.

Masculinization changes (see Table 4)

i) Total porphyrin content of Harderian glands (Fig. 23): Fluorometric assay revealed that the porphyrin content of the Harderian glands of ovariectomised females was similar to that of intact ones. Conversely, testosterone administration resulted in a highly significant (-80%) reduction in porphyrin content to some 700 nmol/g, although this was still significantly higher than male values (some 40 nmol/g) ($t = 8.04$, $p < 0.001$). These data were supported by counts of the number of solid intraluminal porphyrin accretions (Fig. 25) which were similar in control and ovariectomised females, but which were significantly reduced (-60%) in androgen-treated females. Nevertheless, the low levels found in androgen-treated females were still significantly higher than the zero levels found in control males ($t = 6.47, p < 0.001$).

ii) 5 -aminolaevulinic acid synthetase (ALA-S) activity in Harderian glands: The activity of this rate-limiting enzyme was significantly reduced from control levels both in ovariectomised (-65%) and androgenised (-92%) females. The low level of enzyme activity in androgenised females did not differ significantly from the level of activity in male glands (Fig. 24).

iii) Type II Cells: Tubule epithelial cells containing very large lipid vacuoles (Type II cells) are

considered characteristic of male Harderian glands (Fig. 20). In this present study, 99% of tubule profiles in male glands contained Type II cells while less than 1% of tubule profiles in female glands did so. Compared with intact control females, the frequency of Type II cells was raised significantly in both ovariectomised (8.7%) and androgen-treated (38%) females, although the latter still differed significantly from male values ($t = 12.19, p < 0.001$)(Fig.2

iv) Polytubular complexes: Further evidence of gland masculinisation was seen at the ultrastructural level, since all the androgen-treated females examined possessed polytubular complexes identical to those in male cells (Fig.22). They occurred in 93% of the cells of androgen-treated females, and in 100% of the cells of intact control males. They were not seen in either control or ovariectomised females.

Degenerative changes (see Table 5)

Normal female hamster Harderian gland tubules consist of a single layer of tall columnar epithelial cells, possess small lipid vacuoles, and surround a lumen which may contain solid porphyrin accretions. These epithelial cells are supported by myoepithelial cells located within the basal lamina.

In ovariectomised females, a number of degenerative events have been described (Payne et al., 1982b;1985) which may form a sequence. Firstly, the epithelium becomes

attenuated and the enlarged lumen is invaded by neutrophils. As the tubules degenerate further, the large intraluminal porphyrin accretions are found within the interstitium where they often become surrounded by investments of macrophages (including foreign body giant cells). These large interstitial accretions would appear to be broken down into progressively smaller deposits occurring within individual free macrophages (Figs. 13 & 14).

i) Tubule degeneration (Table 5): Degeneration is here defined as tubule profiles whose walls are (wholly or in part) highly attenuated; these tubules are often dilated but this feature was not used as a criterion. The area of tubule degeneration was similar in all three groups of females (see Table 5). However, this measure presumably reflects current change in the gland epithelium. Other measures which may forecast degeneration (invasion by neutrophils) or which may be the result of previous degeneration (interstitial porphyrin deposits) showed considerable changes in the hormonally manipulated females compared with controls. Specifically:-

ii) Tubules containing neutrophils (Table 5; Fig. 26): The number of tubules containing neutrophils was significantly raised in ovariectomised (but not androgenised) females compared with controls. Male glands do not exhibit this tubule invasion.

iii) Interstitial porphyrin accretions (Table 5 ; Figs. 27 & 28 The total porphyrin content (and the number of visible

intraluminal accretions) were similar in intact and ovariectomised females, but both were greatly reduced in androgen-treated females (see Table 4). However, compared with intact control females, the number of large interstitial accretions was raised in ovariectomised and androgen-treated females, significantly so in the latter group ($t = 3.60$, $p < 0.01$). Similarly, the number of small (phagocytosed) interstitial porphyrin accretions were raised in ovariectomised and androgen-treated females, significantly so in the former group ($t = 4.06$, $p < 0.01$). Male glands possess neither intraluminal nor interstitial porphyrin stores.

iv) Mast cells (Table 5): As previously reported (Payne et al., 1982a)there is a large sex difference in mast cell numbers in the hamster Harderian gland, both in the interstitial tissue ($t = 3.29$, $p < 0.01$) and in the connective tissue capsule ($t = 4.58$, $p < 0.001$) with females possessing significantly higher numbers than males. Ovariectomy resulted in a further rise in mast cell numbers, both in the interstitium ($t = 4.26$, $p < 0.01$) and capsule ($t = 4.60$, $p < 0.01$). Mast cell numbers in androgen-treated females were intermediate between intact and ovariectomised females, differing significantly from the latter (interstitium, $t = 2.82$, $p < 0.05$; capsule $t = 4.79$, $p < 0.01$) (Figs. 29 & 30). (HPLC analyses of Harderian gland extracts from intact females and females ovariectomised for 5 months are given in Appendix 3).

SUMMARY

This quantitative assessment of the effects of ovariectomy on the morphology, porphyrin content and porphyrin synthesising enzyme activity of the Harderian gland of the female golden hamster confirms the link between gonadal hormones and gland structure and activity. The results indicate that ovarian hormones are necessary to maintain the morphology and activity of the normal female Harderian gland in this species, since ovariectomy results in a series of degenerative changes which may form a sequence. Firstly, the tubule walls become attenuated and neutrophils invade the enlarged lumen. The subsequent elimination of the tubule walls results in previously intraluminal porphyrin stores coming to lie within the interstitium where they may be surrounded by macrophages (often forming foreign body giant cells). The large interstitial deposits are progressively broken down and individual macrophages containing small porphyrin stores are numerous within the interstitium. Mast cell numbers rise in the Harderian glands of ovariectomised females. When ovariectomy is accompanied by androgen administration, virilization of the gland occurs, with decreased porphyrin content and porphyrin synthesising activity and the appearance of male morphological features such as Type II cells and polytubular complexes.

EXPERIMENT 3: QUANTITATIVE STUDIES ON THE EFFECTS OF HORMONES ON STRUCTURE AND PORPHYRIN BIOSYNTHESIS IN THE HARDERIAN GLAND OF THE FEMALE GOLDEN HAMSTER. II. THE TIME COURSE OF CHANGE FOLLOWING OVARIECTOMY.

INTRODUCTION

Experiment 2 demonstrated that ovarian hormones are necessary to maintain the morphology and biochemical features characteristic of the female Harderian gland, since ovariectomy resulted in a series of degenerative morphological changes and a decrease in the activity of the rate-limiting enzyme for porphyrin production. However, it is also known that gland degeneration may occur in aged female hamsters (Payne et al., 1985) and in female mice exposed to extreme lighting conditions (Strum & Shear, 1982). In order to confirm that ovariectomy per se can result in changes in Harderian gland morphology and porphyrin biosynthesis, and to determine whether degeneration is progressive, this study reports quantitative data on the time course of changes which take place in the Harderian gland of ovariectomised female hamsters when age and seasonal factors are controlled.

MATERIALS AND METHODS

The animals used in this study were adult female golden hamsters. They included intact control females (n = 10) and females ovariectomised for 10 weeks (n = 10), 20 weeks (n = 10), 30 weeks (n = 11), 40 weeks (n = 8) and 50 weeks (n = 8). All the animals were killed when one year old and at the same time

of year, by an overdose of sodium pentobarbitone (Sagatal).

Blood was withdrawn for the determination of blood porphyrin levels. Both Harderian glands were removed, one gland being fixed and processed for light microscopy. The remaining gland was halved longitudinally, one half being assayed immediately for 5-ALA-S activity while the other half was weighed and placed in methanol for the determination of the total porphyrin content. The activity of 5-ALA-S in the liver was also determined, and protein concentrations of liver and Harderian glands were estimated.

The following histological parameters were measured:-

1. Mast cells mm^2/ section and mast cells mm/capsule.
2. Intraluminal porphyrin accretions mm^2/section.
3. Large interstitial porphyrin accretions mm^2/section.
4. Small interstitial porphyrin accretions mm^2/section.
5. The number of tubules containing intraluminal neutrophils mm^2/section.
6. The percentage area of tubule degeneration.
7. The number of epithelial cells showing mitotic figures/ mm^2 section.

Detailed procedures of the methods employed in this experiment are described in "GENERAL MATERIALS AND METHODS".

Statistical Analyses

For each parameter, the five groups were compared by a one-way analysis of variance (F). Where this proved significant, comparisons of control values with those obtained from particular groups of ovariectomised females were made using Dunnetts' test (Dunnett, 1964).

RESULTS

A. Changes in porphyrin content and biosynthesis

i) Blood porphyrin levels

Blood porphyrin levels showed significant variance between the groups ($F = 5.79, p < 0.001$), being significantly elevated at 20 ($t = 2.98, p < 0.05$), and 40 ($t = 2.81, p < 0.05$) weeks following ovariectomy (Fig. 33).

ii) Porphyrin content of the Harderian gland

There was no significant variance between the groups with regard to the porphyrin content of the Harderian gland ($F = 0.55$, n.s.). Figure 31 shows a slight progressive fall in porphyrin content after ovariectomy (which totalled -15% by 30 weeks post-op), followed by a rise at 40 and 50 weeks post-op.

iii) 5 -aminolaevulinic acid synthetase activity in the Harderian gland.

In contrast to the porphyrin content, the activity of the rate-limiting enzyme for porphyrin biosynthesis showed a significant variance over the groups ($F = 4.27$, $p < 0.01$). Compared with controls, the enzyme activity in the Harderian glands of ovariectomised females showed a progressive fall (significant for females ovariectomised for 40 weeks $t = 3.23, p < 0.01$ and 50 weeks $t = 3.46$, $p < 0.01$) with a transient rise at 30 weeks post-op (Fig. 32).

iv) 5-aminolaevulinic acid synthetase activity in the liver

The activity of this enzyme in the liver (another porphyrin

synthesising tissue) also showed a significant variance between the groups ($F = 12.75, p < 0.001$). However, this was due to markedly elevated levels at 10 weeks post-ovariectomy ($t = 4.54, p < 0.01$); enzyme activity fell thereafter (Fig. 34).

B. Degenerative changes in gland histology

Normal female hamster Harderian glands consist of a single layer of columnar epithelial cells possessing numerous lipid vacuoles and surrounding a lumen in which solid porphyrin accretions may be stored. These epithelial cells are supported by myoepithelial cells located within the basal lamina. After ovariectomy a number of degenerative changes occur (Payne et al., 1982b: 1985; Spike et al., 1985) which may form a sequence as shown in Figure 44.

i) Tubule degeneration

Degeneration is here defined as tubule profiles whose walls have become grossly attenuated, either in whole or in part. Measurements of the area of degeneration per unit area of a 5 μm thick section reveal a significant variance between the groups ($F = 6.47, p < 0.001$). Figure 35 shows that, in general, degeneration was greater in ovariectomised females than in controls, and this reached significance for females ovariectomised for 20, 40 and 50 weeks ($t = 3.21$, 3.89 and 5.10 respectively, $p < 0.01$).

ii) Tubules containing neutrophils

There was a significant variance between the groups in the number of tubules showing neutrophilic invasions per unit area of section ($F = 4.93, p < 0.001$). Figure 36 shows that there was a general increase in this measure

with time after ovariectomy, and this difference became highly significant for females ovariectomised for 40 weeks compared with intact controls ($t = 4.58, p < 0.01$).

iii) Mitotic figures

The frequency of mitotic figures per unit area of section showed a significant variance between the groups ($F = 6.65$, $p < 0.001$). This was due to rises in the frequency of mitotic figures at 30 and 40 weeks post-ovariectomy (see Fig. 37). In the case of the latter, mitotic figures were nearly 5x more frequent than in intact control females ($t = 4.19$, $p < 0.01$).

iv) Mast cells

The number of mast cells/mm^2 tissue section showed a significant variance between the groups ($F = 20.49$, $p < 0.001$) with numbers rising markedly with time after ovariectomy to reach a seven-fold increase by 40 weeks post-op. By 50 weeks post-op mast cell numbers had begun to decrease. Forty and fifty week ovariectomised females differed significantly from intact controls ($t = 8.23$, $p < 0.01$ and $t = 4.92$, $p < 0.01$ respectively) (Fig. 39). Similarly, the number of mast cells/mm of the gland capsule also showed a significant variance ($F = 7.09$, $p < 0.001$) with numbers being significantly elevated at 30 weeks ($t = 5.02$, $p < 0.01$) and 40 weeks ($t = 3.32$, $p < 0.01$) following ovariectomy (Figure 40).

v) Interstitial porphyrin accretions

Large interstitial porphyrin deposits (believed to be intraluminal accretions whose surrounding tubule has degenerated) show a progressive rise in frequency with

time after ovariectomy (Fig. 41). There is a significant variance between the groups ($F = 8.28$, $p < 0.001$) and 40 week ovariectomised females differ significantly from controls ($t = 5.92$, $p < 0.01$). Similarly, small interstitial porphyrin deposits (i.e. those within interstitial macrophages) also show a marked and progressive rise with time after ovariectomy although by 50 weeks post-op, numbers are beginning to decrease (Fig. 42). Again, there is a significant variance between the groups ($F = 12.28$, $p < 0.001$) and 40 week and 50 week ovariectomised females differed significantly from controls ($t = 6.70$, $p < 0.01$ and $t = 4.50$, $p < 0.01$ respectively).

In a previous experiment involving male and female hamsters, sex differences were evident with the females containing considerably more mast cells and porphyrin than the males. However, within the female group there was no apparent quantitative relationship between the two parameters (Payne et al, 1982a). In this present experiment, where animals were killed at the same age having been ovariectomised for varying time intervals, there was a strong positive correlation between interstitial mast cell numbers and the numbers of small interstitial porphyrin deposits. Using a basic statistical test for two variables, a coefficient of correlation $r = 0.38$ was obtained. This was statistically significant ($t = 3.08$, $p < 0.01$, df 56). The data was

entered into a statistical curve fitting programme; assuming a linear relationship the equation $y = 11.36 + 6.07x$ was obtained (Fig. 43).

A strong positive correlation occurred between the number of small interstitial porphyrin deposits and the degenerative features, and also between these features (Table 6).

It was of interest that most of these degenerative features showed a significant inverse correlation to 5-ALA-S activity, which is an index of the dynamic state of porphyrin synthesis in the gland (Table 6).

vi) Intraluminal porphyrin accretions

Although the frequency of intraluminal porphyrin accretions/mm^2 section was slightly lower in ovariectomised females than in intact controls, this only reached statistical significance in the 50 week group ($t = 3.88$, $p < 0.01$) and significant variance was found between the groups ($F = 3.68$, $p < 0.01$) (Fig. 38).

The basic statistical test for two variables showed that there was no obvious relationship between the number of intraluminal accretions and 5-ALA-S activity. Likewise, there was no significant correlation between interstitial mast cell numbers and the number of intraluminal accretions (Table 6).

SUMMARY

This experiment provides quantitative confirmation of the effects of ovariectomy on the morphology and activity of the hamster Harderian gland (Payne et al., 1982b; 1985), namely that ovariectomised females exhibit degeneration of gland tubules with neutrophilic invasion (Figs. 45-48). It also demonstrates that this is a progressive phenomenon, increasing with time after ovariectomy when age and seasonal factors are controlled. Strong positive correlations exist between the various degenerative parameters while there is a significant inverse relation between most of these features and the gland enzyme activity. The data show that at 30 weeks post-operatively there is a reversal of the increasing occurrence of tubule degeneration accompanied by the beginnings of a rise in the frequency of mitotic figures within epithelial cells. Furthermore, at 30 weeks post-op there is a transient reversal of the pattern of progressive decrease in gland 5-ALA-S activity. This apparent recovery is limited since enzyme activity is markedly decreased and tubule degeneration increased at 40 and 50 weeks after ovariectomy, even though the incidence of mitotic figures continues to rise until 40 weeks.

Although the porphyrin content of the gland as determined by biochemical assay does not alter significantly over the time course, by 50 weeks post-op there is a significant decrease in the number of intraluminal accretions, and there is a progressive increase in the number of both large and small interstitial porphyrin deposits which reach

a peak at 40 weeks and thereafter begin to decrease in number. Hence there is a clear redistribution of porphyrin stores. The increase in blood porphyrin levels in 20, 30 and 40 week post-op animals may be due to the blood borne dispersal of these small accretions within free interstitial macrophages since by 50 weeks post-op both blood porphyrin levels and the number of interstitial deposits has increased. The porphyrin-containing macrophages are frequently surrounded by clusters of plasma cells (Fig. 49) and lymphocytic invasions may be prolific (Fig. 50). Tubule degeneration shows a marked and progressive increase as the post-operative interval increases, while the remaining histological parameters peak at 40 weeks post-op.

It is understood that the 50-week group may not be comparable because of the young (prepubertal) age at which operation took place. This group has been omitted from the published account (Spike et al., 1986).

EXPERIMENT 4: PORPHYRIN BIOSYNTHESIS AND EXCRETION DURING PREGNANCY AND LACTATION IN MULTIPAROUS FEMALE GOLDEN HAMSTERS.

INTRODUCTION

In the porphyrias, a group of metabolic disorders resulting in the over production of porphyrin and its precursors, steroid hormones play an important role in precipitating attacks. These disorders are 1) rare before puberty (Goldberg et al., 1969) 2) occur more frequently in women than in men (Goldberg et al., 1969) and 3) are often precipatated by hormonal changes such as occur in the menstrual cycle (Zimmerman et al., 1966; Lamon et al., 1978; McColl et al., 1982) or when taking the contraceptive pill (Koskelo et al., 1966; Goldberg et al., 1983). Pregnancy is also known to trigger porphyric attacks (Neilson et al., 1958; Zimmerman et al., 1966; Brodie et al., 1977).

The Harderian gland of the female golden hamster is a useful model in which to study the effects of pregnancy and lactation on porphyrin biosynthesis since the characteristics of the gland and its activity (Fig. 6) mimic many aspects of the human disorder. In addition, the female Harderian gland shows both cyclical (Payne et al., 1977b; Moore et al., 1977) and seasonal (Moore et al., 1980) variation in porphyrin content and 5-ALA-S activity, as well as changes in the size and number of porphyrin accretions during pregnancy (Payne et al., 1979).

This experiment examines porphyrin content and 5-ALA-S activity in the Harderian gland, the liver (a close association between female hormones and liver metabolism has been established, Lyberatos et al., 1972; De Klerk, 1975) and the kidney, and blood porphyrin levels in pregnant and lactating hamsters. In addition, urinary and faecal porphyrin levels were determined since increased urinary excretion of porphyrins and precursors occurs in normal women during pregnancy (Lyberatos et al., 1972; De Klerk et al., 1975; Brodie et al., 1977).

MATERIALS AND METHODS

A total of 69 adult female hamsters aged 9-11 months was used. These consisted of stock which had had several pregnancies as part of a routine animal house breeding programme. All animals were housed under a reversed lighting regime of 12 hours dull red and 12 hours white light. This enabled oestrous to be determined by behavioural as well as vaginal criteria (Orsini, 1961) so that the day of mating was known precisely. These animals were then mated and killed at intervals to give the following groups.

1. Control female hamsters (n = 14)
2. Pregnancy day 8 (n = 13)
3. Pregnancy day 14/15 (n = 13)
4. Lactation day 1/2 (n = 6)
5. Lactation day 6/12 (n = 9)

(Birth is normally on day 16 of pregnancy in the hamster).

All animals were killed during the summer with an overdose of sodium pentobarbitone and blood withdrawn by ventricular puncture for porphyrin determination. Both Harderian glands were removed, one being placed in 5ml methanol (Analar) for porphyrin determination while the remaining gland was immediately assayed for its 5-ALA-S activity. The liver was removed and each lobe divided into two, with one half being assayed for its enzyme activity and the other homogenised and stored in 4:1 ethyl acetate: glacial acetic acid at -20°C until its porphyrin content could be estimated. During the course of the experiment it was decided in addition to examine the porphyrin content of the kidney, faeces and urine, and the 5-ALA-S enzyme activity in the kidney. The kidneys were halved longitudinally, one half of each kidney being assayed for enzyme activity while the remaining halves were homogenised and stored in 4:1 ethyl acetate: acetic acid at -20°C for the later quantification of the porphyrin content. An additional 41 animals were examined for kidney porphyrins and enzyme activity and 28 of these were kept in individual metabolic cages for 24 hours prior to culling in order to collect urine and faeces sample.

Determination of urinary porphyrin content

The volume of the 24 hour urine sample was measured. A known volume of urine not exceeding 1ml was run through a resin ion exchange column (Bio-rad Laboratories Gmbh, Munich). The porphyrin was extracted from the column using 2x 2ml aliquots of 10.5% HCl solution. Total porphyrin was measured on a spectrofluorimeter (Perkin Elmer 3000

Fluorescence Spectrometer) (396nm excitation, 594nm emission) against a coproporphyrin standard (COP-1-5 5 µg per vial, Sigma Diagnostics, St. Louis). The results were expressed an nmol/24 hours.

Determination of faecal porphyrin

The 24 hour sample was divided into two. One half was weighed then placed in an oven for 4 hours at 100°C. The dry weight was recorded. This enabled the wet weight; dry weight ratio to be established. The other half was weighed and the porphyrin extracted with 1:6 ether: glacial acetic acid mixture until no fluorescence could be detected in the extracting solvent under ultraviolet illumination. The extract was washed twice with 3% sodium acetate solution to remove extraneous material, once with 0.005% iodine solution to convert porphyrinogens to porphyrins, and once with water to remove the iodine solution. Total porphyrin was then extracted from the solvent with a 1.5M HCl solution until no fluorescence was detected under ultraviolet illumination. The volume of acid used was noted. Total porphyrin content was measured on a spectrofluorimeter (396nm excitation, 594 nm emission) against a coproporphyrin standard. Results were expressed as nmol porphyrin/g faeces (dry). Other procedures are described in detail in "GENERAL MATERIALS AND METHODS".

Statistics

Analyses of Variance (F) was used to compare the various parameters measured: where this proved significant individual inter group comparisons were made using Dunnett's test (Dunnett, 1964).

RESULTS

1. Harderian gland porphyrin content (nmol/g) (Fig. 52)
   Porphyrin content in the Harderian gland at day 8 of pregnancy was similar to the control value then decreased at day 15 of pregnancy, but showed a subsequent rise during the two lactation periods examined. However, variation within each group was such that there was no overall significant variance ($F = 2.03$, ns).
2. Harderian gland 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 53)
   The gland enzyme activity at day 8 of pregnancy was also similar to control values but increased to its maximum value prior to partuition. The enzyme activity had decreased by days 1-2 of lactation and by days 6-12 had reached control levels. There was no significant variance ($F = 1.19$, ns).
3. Liver porphyrin (nmol/g) (Fig. 51)
   There was no significant difference in liver porphyrin content although the concentration reached a maximum value at day 15 of pregnancy and then decreased to below control levels during lactation ($F = 0.88$, ns).
4. Liver 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 53)
   There was no significant difference in the liver enzyme activity ($F = 0.97$, ns). The greatest value was found in the controls; levels then dropped progressively during pregnancy and lactation.

5. Kidney porphyrin (nmol/g) (Fig. 51)

A significant variance occurred in kidney porphyrin content ($F = 3.72$, $p < 0.05$). This was due to the sudden increase at day 8 of pregnancy ($t = 2.82$, $p < 0.05$, and $t = 2.74$, $p < 0.05$ compared to control and day 6-12 of lactation respectively). Levels then fell before partuition and remained at control levels during lactation.

6. Kidney 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 53)

The kidney 5-ALA-S activity also reached a maximum value at day 8 of pregnancy, then decreased to control levels before birth and during lactation. There was no significant variance due to the variation within each group ($F = 0.41$, ns).

7. Blood porphyrin (nmol/l) (Fig. 52)

Significant variance occurred in blood porphyrin levels ($F = 2.57$, $p < 0.05$). At day 8 of pregnancy there was a considerable decrease from control levels ($t = 2.55$, $p < 0.05$). Levels then rose gradually reaching a maximum in the early lactation period before returning to control levels during the later lactation period.

8. Faecal porphyrin (nmol/g) (Fig. 52)

Faecal porphyrin levels altered significantly over the time period ($F = 4.07$, $p < 0.05$). The content rose at day 8 of pregnancy, returned to control levels by day 15, then increased significantly at day 6 of lactation ($t = 2.94$, $p < 0.05$) compared to control levels.

9. Urinary porphyrin (nmol/24 hours) (Fig. 51)

Urinary porphyrin levels were extremely low and did not alter significantly over the time period ($F = 2.36$, ns).

SUMMARY

This experiment examined porphyrin biosynthesis during pregnancy and lactation in multiparous female hamsters. Clinical data suggests that pregnancy often triggers attacks in human porphyrics. The results indicate that the porphyrin content and enzyme activity of the Harderian gland and liver did not alter significantly over the period. However, the porphyrin content of the kidneys showed a significant change with levels raised during mid pregnancy (kidney enzyme activity paralleled this profile, but the increase was not significant) while blood porphyrin levels were significantly reduced at mid pregnancy. Porphyrin values in urine were extremely low, but the higher values found in faeces altered significantly over the period being high at mid pregnancy and significantly raised during lactation. This suggests that multiparous females may respond to increased porphyrin biosynthesis in pregnancy by increased excretion.

EXPERIMENT 5: MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN THE HARDERIAN GLAND, AND CHANGES IN BLOOD PORPHYRIN LEVELS AND LIVER 5-ALA-S ACTIVITY IN MALE AND FEMALE GOLDEN HAMSTERS FROM POST-PUBERTY UNTIL SENESCENCE.

INTRODUCTION

Sex differences in male and female hamster Harderian glands were first observed by Christensen & Dam (1953) who noted that these dimorphisms were only apparent from 3 weeks after birth. Further developmental studies showed that mitotic division occurred in both sexes during the first three weeks of life and was not observed thereafter. At 2 weeks of age, male and female Harderian glands are similar and both resemble the adult female type gland but by 4 weeks the sexual dimorphisms were evident; in females, the intraluminal porphyrin pigment increased until 4 weeks while in males no pigment was seen after 4 weeks, by which time 2 cell types were recognisable (Bucana & Nadakavukaren, 1972c; 1973). Payne et al (1978) were able to measure intracellular porphyrin until 6 weeks after birth in males. The porphyrin content of the Harderian gland varies with age in some rodent species, increasing with age in rats (Rohonyi & Kelenyi, 1962) while in mice it decreases after 75 days (Strong, 1942). Morphological studies on post reproductive female hamsters showed degenerative changes occurring in an escalating pattern (Payne et al., 1985) (Fig. 35).

Although morphological studies have been made during the first few months and in old age in females, there has been no comparative study through the life span of either male or female hamsters. This experiment examines various morphological and biochemical parameters in male and female hamsters from post puberty until senescence.

MATERIALS AND METHODS

The female hamsters used in this experiment consisted of 5 groups of animals of the following ages a) 2 months (n = 10), b) 6 months (n = 10), c) 12 months (n = 10), d) 18 months (n = 10) and e) 24 months (n = 9). Six groups of male hamsters were used: i) 2 months (n = 10), ii) 6 months (n = 10), iii) 12 months (n = 11), iv) 18 months (n = 9), v) 24 months (n = 10) and vi) 30 months+ (n = 9). Animals were killed throughout the year, as the age groups became available. Although the members of some groups were killed in different months, a lighting regime of 15L:9D was used to minimise the effects of seasonal variation.

The animals were killed with an overdose of Sagatal, and blood withdrawn for the determination of blood porphyrin levels. The liver was removed and half of each lobe was assayed for 5-ALA-S activity. Both Harderian glands were excised: one gland was halved longitudinally, one half being assayed for its 5-ALA-S activity, while the total porphyrin content was estimated in the remaining half. The other gland was placed in 3% buffered glutaraldehyde (pH7.4) and processed for light microscopy. The following histological

parameters were measured in each gland:

a) interstitial mast cells/mm^2

b) peripheral mast cells/mm

c) intraluminal porphyrin deposits/mm^2

d) large interstitial porphyrin deposits/mm^2

e) small interstitial porphyrin deposits/mm^2

f) mitotic figures/mm^2

g) tubules containing neutrophils/mm^2

h) % area of tubule degeneration

i) % Type II cells

Detailed procedures are described in "GENERAL MATERIALS AND METHODS".

Statistical analyses

Analysis of variance was used to compare the various parameters measured in each group: where this was significant, intergroup comparisons were made using Dunnett's test (1964).

RESULTS

BIOCHEMICAL CHANGES

A. Female hamsters

1. Blood porphyrin levels (nmol/l) (Fig. 56)

Significant variance occurred between the five age groups ($F = 4.01$, $p < 0.01$) with the 18 month levels being significantly raised compared with the 2 month ($t = 4.18$, $p < 0.01$), 6 month ($t = 2.53$, $p < 0.05$) and 12 month ($t = 2.67$, $p < 0.05$). At 24 months, blood porphyrin levels were also raised compared with these three groups, but this was not significant.

2. Harderian gland porphyrin (nmol/g) (Fig. 54)

There was very significant variance between the different

age groups ($F = 16.07$, $p < 0.001$), with levels being highest at 2 months and gradually decreasing as the age increased. The two month values were almost double that of the twelve month females ($t = 3.43$, $p < 0.01$) and more than three times that of the 18 month ($t = 6.08$, $p < 0.01$) and 24 month ($t = 6.15$, $p < 0.01$) levels. The 6 month values were also three times greater than the 18 month ($t = 5.12$, $p < 0.01$) and 24 month ($t = 5.26$, $p < 0.01$) animals, and these two latter groups were both half the value of the twelve month females ($t = 2.60$, $p < 0.05$ and $t = 2.80$, $p < 0.05$ respectively).

3. Harderian gland 5-ALA-S activity (nmol ALA formed/g protein /h) (Fig. 55)

Significant variance occurred between the five groups ($F = 4.60$, $p < 0.01$) with levels being lowest at 2 months, peaking at 6 months and gradually decreasing thereafter. Compared to the 6 month values, the 2 month ($t = 3.83$, $p < 0.01$), 18 month($t = 3.32$, $p < 0.01$) and 24 month ($t = 2.77$, $p < 0.05$) levels were significantly reduced.

4. Liver 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 57)

There was no significant variance in liver enzyme activities between the various age groups.

B. Male hamsters

1. Blood porphyrin levels (nmol/l) (Fig. 60)

Significant variance occurred between the six age groups ($F = 2.54$, $p < 0.05$) with levels decreasing between 2 and 12 months and then gradually rising, reaching highest

values at 30 months. The 12 month group was significantly less than the 30 month group ($t = 2.97$, $p < 0.05$).

2. Harderian gland porphyrin (nmol/g) (Fig. 58)

There was no significant variance between the groups, with levels remaining very low throughout.

3. Harderian gland 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 59)

No significant variance occurred between the age groups although levels rose in the 18, 24 and 30 month groups.

4. Liver 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 61)

Significant variance occurred in liver enzyme activity ($F = 2.83$, $p < 0.05$), with the lowest activities in the two youngest age groups. The 12 month levels were raised and activities gradually decreased until 30 months when they rose to a maximum value. Compared to the 30 month animals, the 2 month ($t = 3.18$, $p < 0.05$), 6 month ($t = 2.96$, $p < 0.05$) and 24 month ($t = 2.62$, $p < 0.05$) were significantly less.

MORPHOLOGICAL CHANGES

A. Female hamsters

a) Interstitial mast cells/mm^2 (Table 7)

There was significant variance in the number of interstitial mast cells between the five age groups ($F = 6.66$, $p < 0.001$). The 2 month females had the highest number and were significantly greater than the 6 month ($t = 4.11$, $p < 0.01$), 18 month ($t = 3.85$, $p < 0.01$) and 24 month ($t = 3.39$, $p < 0.01$) groups. The 12 month females had the second

highest number and this was significantly greater than the 6 month group ($t = 3.03$, $p < 0.05$) which has the lowest figure.

b) Peripheral mast cells/mm (Table 7)

Peripheral mast cells also showed significant variance ($F = 5.63$, $p < 0.001$) with the 2 month females again having the greatest number: this was significantly higher than the 6 month ($t = 4.78$, $p < 0.01$), 18 month ($t = 4.05$, $p < 0.01$) and 24 month ($t = 3.72$, $p < 0.01$) groups. The 12 month females again had the second highest number which was significantly greater than the 6 month group ($t = 2.88$, $p < 0.05$) which was the lowest of the groups.

c) Intraluminal porphyrin accretions/mm^2 (Table 7)

There was a highly significant difference between the various age groups in the number of intraluminal deposits ($F = 53.06$, $p < 0.001$). The greatest number occurred in the 2 month group and this was significantly greater than the 6 month ($t = 12.89$, $p < 0.01$) 12 month ($t = 6.29$, $p < 0.01$), 18 month ($t = 10.83$, $p < 0.01$) and 24 month ($t = 10.92$, $p < 0.01$) groups. The 12 month females had a value of more than half that of the 2 month females, and this was also significantly higher than the other three age groups: 6 months ($t = 6.79$, $p < 0.01$), 18 months ($t = 4.67$, $p < 0.01$) and 24 months ($t = 4.76$, $p < 0.01$).

In a previous study (Payne et al., 1982a), involving male and female hamsters, sex differences were observed with both mast cell numbers and porphyrin content being high in

females, but there was no obvious relationship between the two parameters. In this experiment where intraluminal porphyrin accretions and interstitial mast cells were counted and both measurements appeared to be positively related in the female groups it was decided to compare the two parameters statistically to see if there was a significant correlation. Individual pairs of mast cell numbers and intraluminal porphyrin accretions were plotted (Fig. 62) and the correlation coefficient determined using basic statistics for two variables. A t-test was then performed on this data. A correlation coefficient of $r = 0.43$ ($t = 3.25$, $p < 0.01$) was obtained. The data was then entered into a statistical curve fitting programme: assuming a linear relationship $y = -0.28 + 0.63X$.

d) Large interstitial porphyrin deposits/mm^2 (Table 7)

The number of large interstitial porphyrin deposits increased with age and this variance is significant ($F = 7.84$, $p < 0.01$). The number of large interstitial deposits increased 5 fold at 24 months compared to 12 month and 18 month values and is significantly greater than the 2 month ($t = 4.63$, $p < 0.01$), 6 month (4.54, $p < 0.01$) 12 months ($t = 3.81$, $p < 0.01$) and 18 month ($t = 4.00$, $p < 0.01$) groups.

e) Small interstitial porphyrin deposits/mm^2 (Table 7)

The pattern for the number of small interstitial porphyrin deposits parallels that of the large interstitial deposits, and the variance is also significant ($F = 8.96$, $p < 0.001$). The 24 month females have significantly higher numbers of small interstitial deposits than the 2 month ($t = 4.95$, $p < 0.01$), 6 month ($t = 4.94$, $p < 0.01$), 12 month ($t = 3.88$, $p < 0.01$) and 18 month ($t = 3.96$, $p < 0.01$) females.

f) Mitotic figures/mm^2 (Table 7)

There was significant variance in the number of mitotic figures ($F = 2.27$, $p < 0.05$) with the 2 month and 12 month females having the highest numbers. The lowest number occurred in the 6 month group which was significantly less than the 2 month group ($t = 2.53$, $p < 0.05$).

g) Tubules containing neutrophils/mm^2 (Table 7)

Significant variance occurred in this parameter, with the numbers increasing as the age increased. The 24 month females had the greatest number of tubules containing neutrophils and this was significantly greater than the 2 month ($t = 5.90$, $p < 0.01$) 6 month ($t = 5.86$, $p < 0.01$), 12 month ($t = 4.03$, $p < 0.01$) and 18 month ($t = 4.62$, $p < 0.01$) groups ($F = 12.19$, $p < 0.001$).

h) % Area of tubule degeneration (Table 7)

The % area of tubule degeneration increased as the age of the females increased and was significant ($F = 5.18$, $p < 0.01$). The 18 month group had a greater % of degeneration than the 2 month group ($t = 2.56$, $p < 0.05$) while the 24 month group had a greater % than the 2 month (3.55, $p < 0.01$), 6 month ($t = 3.39$, $p < 0.01$) and 12

month ($t = 3.13$, $p < 0.05$) groups.

i) % Type II cells (Table 7)

There was considerable variance between the age groups in the % of Type II cells which was highly significant ($F = 22.89$, $p < 0.001$). The 2 month and 12 month females had the lowest %, while the 18 and 24 month females had the highest %, with the 6 month figure being intermediate. The 2 month value was significantly less than the 6 month ($t = 3.45$, $p < 0.01$), 18 month ($t = 5.78$, $p < 0.01$) and 24 month ($t = 6.42$, $p < 0.01$) females, while the 12 month group was also significantly less than the 6 month ($t = 3.92$, $p < 0.01$), 18 month ($t = 6.69$, $p < 0.01$) and 24 month ($t = 7.44$, $p < 0.01$) groups.

B. Male Hamsters

a) Interstitial mast cells/mm^2 (Table 8)

Although the group averages were very low compared to female values, there was still a significant difference between the six age groups in the number of interstitial mast cells ($F = 3.94$, $p < 0.01$). The 2 month males had the highest number and this was significantly greater than 6 month ($t = 3.66$, $p < 0.01$) 12 month ($t = 3.0$, $p < 0.05$), 18 month ($t = 3.5$, $p < 0.01$) and 24 month ($t = 3.66$, $p < 0.01$) groups. The 30 month group also had raised values but there was no significant difference between this group and the others.

b) Peripheral mast cells/mm (Table 8)

There was also a significant difference in the number of

peripheral mast cells ($F = 6.07$, $p < 0.01$). The 2 month group had significantly higher values than the 6 month ($t = 4.76$, $p < 0.01$), 12 month ($t = 4.81$, $p < 0.01$), 18 month ($t = 4.64$, $p < 0.01$), 24 month ($t = 4.81$, $p < 0.01$) and 30 month ($t = 4.28$, $p < 0.01$) animals.

c) Intraluminal porphyrin accretions/mm^2 (Table 8)

The 2 month males had intraluminal porphyrin accretions which fluoresced red under ultraviolet light, but none were present in the 6 or 12 month males. Although intraluminal deposits were present in the 18, 24 and 30 month groups (Figs. 63 & 64) the exact nature of these accretions is not known since fluorescence was not detected under ultraviolet light; these may, therefore, be lipid deposits. Since the composition of these intraluminal deposits is not consistent, analysis of variance was not performed on this parameter.

d) Large interstitial porphyrin deposits /mm^2 (Table 8)

There were no large interstitial porphyrin deposits in any of the age groups examined.

e) Small interstitial porphyrin deposits/mm^2 (Table 8)

There were no small interstitial porphyrin deposits in any of the age groups examined.

f) Mitotic figures/mm^2 (Table 8)

There was significant difference in the number of mitotic figures occurring in the various age groups ($F = 9.41$, $p < 0.01$). The 2 month males had significantly greater numbers than the 6 month ($t = 3.04$, $p < 0.05$), 12 month ($t = 4.56$, $p < 0.01$), 18 month ($t = 3.91$, $p < 0.01$)

24 month ($t = 4.13$, $p < 0.01$) and 30 month ($t = 4.34$, $p < 0.01$) males.

g) Tubules containing neutrophils/mm^2 (Table 8)

Neutrophils were present in small numbers in the tubules of the older males but the increase was not significant.

h) % Area of Tubule Degeneration (Table 8)

Tubule degeneration was not detectable in any of the age groups studied.

i) % Type II cells (Table 8)

Some variation occurred in the % of Type II cells with the 12 month and 18 month males having the highest values, the 2 month males having the lowest value, while the 6 month, 24 month and 30 month values were intermediate, but the difference was not significant.

SUMMARY

BIOCHEMICAL CHANGES

Harderian gland porphyrin content in females varied considerably. The highest levels occurred in 2 month animals and thereafter decreased as age increased. There was no variation in gland porphyrin content in the male age groups, which remained very low.

Harderian gland enzyme activities also varied significantly in females, reaching a peak at 6 months and then decreasing as age increased. Although male gland enzyme activities did not show significant variance, levels increased from 12 months as age increased. Compared with previous reports, male enzyme

activities were unusually high in all the age groups examined.

Liver enzyme activities did not show significant variance in females, but did in males.

Blood porphyrin levels were highest in the oldest age groups in both males and females.

MORPHOLOGICAL CHANGES

Peripheral and interstitial mast cells occurred most frequently in the 2 month females and in 2 month males for each respective age group. The 2 month females also had the greatest number of intraluminal porphyrin deposits, and, taken over all ages, there was a strong correlation between the number of these deposits and the number of interstitial mast cells in females. Intraluminal porphyrin deposits occurred only in 2 month males but intraluminal deposits of an indeterminate nature occurred in 18, 24 and 30 month males. Mitotic figures also featured more prominently in the 2 month animals in each age group. In males, there was no significant difference in the % of Type II cells while in females the 6 month group and the two post-reproductive groups had a significantly greater % of these cell types.

The degenerative features such as the number of tubules containing neutrophils, the percentage area of tubule degeneration and the amount of small and large interstitial porphyrin deposits all increased as the females grew older and were greatest in the post reproductive animals. The male age groups showed few of these degenerative features.

EXPERIMENT 6 : THE EFFECTS OF CASTRATION AND STEROID ADMINISTRATION ON PORPHYRIN BIOSYNTHESIS IN THE HARDERIAN GLAND OF THE MALE GOLDEN HAMSTER.

INTRODUCTION

The male type of Harderian gland is expressed whenever significant androgen levels are present (McMasters & Hoffman, 1984). Thus, testosterone administration to females results in a reduction of porphyrin deposits which coincides with an increase in polytubular complexes (Sun & Nadakavukaren, 1980) and Type II cells, while 5-aminolaevulinic acid synthase levels fall to male levels (Spike et al., 1983; 1985).

Conversely, castration of male hamsters is known to alter the morphology of the male type gland, leading to a loss of Type II cells (Hoffman, 1971) and polytubular complexes (Lin & Nadakavukaren, 1979). The resulting female type gland begins to accumulate porphyrin deposits by four weeks post operatively. These changes are more marked with time after castration (Clabough & Norvell,1973; Payne et al., 1977a). Treatment with various androgens maintains the typical male appearance of cell types and suppresses the formation of porphyrin accretions (Hoffman, 1971; Payne et al., 1977a).

This experiment examined the possible effects of oestrogen and progesterone in modifying the increase in the activity of 5-ALA-S and the subsequent appearance of porphyrin in castrates, which would normally be expected shortly after castration.

MATERIALS AND METHODS

Two groups of control animals and four experimental groups were used in this experiment:

a) normal male hamsters (n = 10)

b) castrated males (n = 37)

c) castrates given androgens (4.5mg testosterone/week)(n = 22)

d) castrates given oestrogen (0.06mg 17 β oestradiol/week) (n = 36)

e) castrates given progesterone (0.3mg progesterone/week) (n = 31)

f) normal female hamsters (n = 21)

Where animals received steroid injection, these began from the time of castration. Approximately equal numbers of control and experimental animals were killed at 2, 4 and 6 week intervals after the time of castration. The animals were 6-12 months old when killed during the winter months, with an overdose of sodium pentobarbitone (Sagatal).

Blood was withdrawn by ventricular puncture for the determination of blood porphyrin levels. Both Harderian glands were excised, one assayed to measure the 5-ALA-S activity, the other weighed and stored in 5ml methanol (Analar) for the later determination of its porphyrin content. Half of each liver lobe was removed, and the 5-ALA-S activity of this organ estimated.

Detailed procedures of the methods used are to be found in "GENERAL MATERIALS AND METHODS". Harderian gland extracts from intact males and castrates were prepared for HPLC analyses - see Appendix 3.

Statistical Analyses: For each parameter each treatment group of castrates (± steroids) was compared separately with both the control males and the control females, using one way analysis of variance (F). Because normal male and female values are so dichotomous, a single analysis of variance involving both would automatically be significant, thus greatly reducing the sensitivity of the analysis. Where variance proved significant, individual inter-group comparisons were made using Dunnett's test (Dunnett, 1964).

RESULTS

1. Castrates

i) Harderian gland porphyrin content (Fig. 65)

Harderian gland porphyrin content showed significant variance between the control males and the three treatment groups ($F = 18.3$, $p < 0.001$). By four weeks post operatively, Harderian gland porphyrin levels had increased, and were significantly raised by 6 weeks compared to intact males ($t = 6.17$, $p < 0.01$).

When compared with control females, there was also considerable variance ($F = 46.25$, $p < 0.001$), with the 2 week ($t = 8.86$, $p < 0.01$), 4 week ($t = 10.04$,

$p < 0.01$) and 6 week ($t = 6.49$, $p < 0.01$) castrates all possessing significantly less porphyrin than female values.

ii) Harderian gland 5-ALA-S activity (Fig. 66)

The gland enzyme activity showed significant variance between experimental and control males ($F = 3.87$, $p < 0.05$). Only the four week treatment group was significantly greater than male values ($t = 3.05$, $p < 0.05$) although six week values were also raised.

The gland enzyme activity also showed significant variance to control females ($F = 3.12$, $p < 0.05$). In this comparison, only the 2 week treatment group was significantly less than female values ($t = 2.79$, $p < 0.05$).

iii) Liver 5-ALA-S activity (Fig. 67)

There was no significant variance in liver enzyme activity between the treatment and control groups.

iv) Blood porphyrin levels (Fig. 68)

Blood porphyrin levels showed significant variance between the treatment groups and intact males ($F = 7.34$, $p < 0.001$), being significantly elevated at 2 weeks ($t = 4.89$, $p < 0.01$). There was a similar significant variance when castrates were compared with intact females ($F = 6.30$, $p < 0.01$), with the 2 week treatment group's level being significantly raised ($t = 2.78$, $p < 0.05$). Blood porphyrin levels fell to normal levels by four and six weeks.

2. Castrates given testosterone

i) Harderian gland porphyrin content (Fig. 65)

There was a significant variance in Harderian gland porphyrin content between intact males and treatment groups ($F = 3.95$, $p < 0.05$). Testosterone injected castrates showed significantly lower porphyrin levels than castrates at 2 weeks ($t = 2.65$, $p < 0.05$) and six weeks ($t = 3.13$, $p < 0.05$), suggesting a supra normal dose.

A highly significant variance was found between control females and the treatment groups ($F = 39.94$, $p < 0.001$), with all three groups having significantly less porphyrin at 2 weeks ($t = 8.04$, $p < 0.01$) 4 weeks ($t = 6.18$, $p < 0.01$) and 6 weeks ($t = 8.04$, $p < 0.01$).

ii) Harderian gland 5-ALA-S activity (Fig. 66)

The gland enzyme activity also showed significant variance between intact males and experimental groups ($F = 5.75$, $p < 0.01$), with two week ($t = 2.93$, $p < 0.05$) and four week ($t = 3.02$, $p < 0.05$) testosterone treated castrates having significantly lower activity than controls. There was also a significant variance between the female group and the treatment groups ($F = 8.14$, $p < 0.01$) with all three groups of testosterone treated castrates having significantly lower enzyme activity at 2 weeks ($t = 3.73$, $p < 0.01$), 4 weeks ($t = 3.37$, $p < 0.01$) and 6 weeks ($t = 3.43$, $p < 0.01$).

iii) Liver 5-ALA-S activity (Fig. 67)

There was no significant variance in liver enzyme activity between control males and testosterone injected castrates,

despite a decrease in activity at four weeks post-op. There was also no significant variance between control females and the experimental group.

iv) Blood porphyrin levels (Fig. 68)

There was no significant variance between experimental groups and intact males in blood porphyrin levels, although levels were raised at two weeks post-op. Again, no significant variance existed between control females and the treated castrates.

3. Castrates given oestrogen

i) Harderian gland porphyrin content (Fig. 65)

There was a highly significant variance between the experimental groups and the control males in Harderian gland porphyrin content ($F = 97.87$, $p < 0.001$). Although by 2 weeks of treatment porphyrin levels had not altered from control male values, the levels were raised by four weeks and significantly elevated by six weeks ($t = 14.2$, $p < 0.01$).

There was also a highly significant variance between the three treatment groups, and the control females ($F = 58.03$, $p < 0.001$), with the two week ($t = 11.23$, $p < 0.01$), four week ($t = 9.66$, $p < 0.01$) and six week ($t = 8.24$, $p < 0.01$) groups all being significantly less than control females values.

ii) Harderian gland 5-ALA-S activity (Fig. 66)

The gland enzyme levels also showed significant variance between the experimental groups and the control males ($F = 9.80$, $p < 0.001$). Levels were raised by 2 weeks of treatment and increased over the next four weeks, being significantly elevated by six weeks ($t = 4.89$,

$p < 0.01$).

Significant variance also occurred between the experimental groups and the control females ($F = 4.23$, $p < 0.01$). Two week ($t = 2.99$, $p < 0.05$) and four week ($t = 2.61$, $p < 0.05$) castrate males showed significantly less activity than control females but the value of the six week group was not significantly different from female levels.

iii) Liver 5-ALA-S activity (Fig. 67)

Liver enzyme activity also showed considerable variance compared to male values ($F = 8.90$, $p < 0.001$) but this was due to the transient increase at 2 weeks ($t = 4.58$, $p < 0.01$) in treated males. Liver enzyme activity was also significantly different from female values ($F = 10.27$, $p < 0.001$), again due to elevated levels at 2 weeks ($t = 5.20$, $p < 0.01$).

iv) Blood porphyrin levels (Fig. 68)

Compared to normal males, there was significant variance between the groups ($F = 10.61$, $p < 0.001$). The blood porphyrin levels were significantly raised by two weeks ($t = 3.68$, $p < 0.01$), while levels decreased progressively by four and six weeks.

Compared to females, there was also significant variance between the groups ($F = 7.35$, $p < 0.001$) with the six week group being significantly less than control females ($t = 4.26$, $p < 0.01$).

4. Castrates given progesterone

i) Harderian gland porphyrin content (Fig. 65)

The gland porphyrin content showed significant variance compared to control males ($F = 10.08$, $p < 0.001$). Two week castrates showed levels similar to control male values, by four weeks they had increased ten fold and by six weeks were significantly elevated ($t = 4.75$, $p < 0.01$).

The gland porphyrin content also showed a highly significant variance compared to control females ($F = 41.3$, $p < 0.001$), with 2 week ($t = 8.97$, $p < 0.01$), 4 week ($t = 8.18$, $p < 0.01$) and 6 week ($t = 7.23$, $p < 0.01$) groups all showing significantly less porphyrin content than female values.

ii) Harderian gland 5-ALA-S activity (Fig. 66)

The gland enzyme levels showed considerable variance compared to control males ($F = 8.14$, $p < 0.001$). This was due to the significant increase at six weeks ($t = 4.10$, $p < 0.01$) in experimental males. Two and four week levels were barely above control male values. The gland enzyme activities also showed considerable variance in comparison to control females ($F = 6.02$, $p < 0.01$). Although levels at six weeks were within the female range, those at two weeks ($t = 3.40$, $p < 0.01$) and four weeks ($t = 3.33$, $p < 0.01$) were significantly less in the treated males.

iii) Liver 5-ALA-S activity (Fig. 67)

There was no significant variance in liver enzyme activity between the experimental and the control groups.

iv) Blood porphyrin levels (Fig. 68)

No significant variance occurred between the castrates given progesterone and intact males in blood porphyrin levels and the levels showed very little variation at 2, 4 and 6 week intervals. When compared to female hamsters, however, variance did occur ($F = 3.00$, $p < 0.05$) with the 2 week ($t = 2.44$, $p < 0.05$) and 6 week ($t = 2.47$, $p < 0.05$) treatment groups having significantly reduced levels.

SUMMARY

Castration of male hamsters resulted in the following changes:-

1. Progressive increases in Harderian gland porphyrin content.
2. Increases in 5-ALA-S activity in the Harderian gland.
3. Initial increase in blood porphyrin levels.

The liver 5-ALA-S activity was not affected.

Testosterone administration to castrates prevents these changes.

The administration of oestrogen or progesterone to castrates did not markedly alter these changes, with the following exceptions: oestrogen administration resulted in elevated liver 5-ALA-S activity two weeks post operatively, while progesterone administration prevented elevated blood porphyrin levels at two weeks, and gland 5-ALA-S activities rose more slowly.

EXPERIMENT 7: THE EFFECT OF HARDERIANECTOMY ON OTHER PORPHYRIN-SYNTHESISING ORGANS:

A) IN MALES AND FEMALES AFTER 5 MONTHS

B) DURING A TIME COURSE IN FEMALES

C) IN OVARIECTOMISED FEMALES

INTRODUCTION

The Harderian gland of the female hamster is considerably more active in synthesising porphyrins than other porphyrin producing organs (Thompson et al., 1984) (Table 1). However, it is not known what contribution the gland makes to the porphyrin economy of the body either in terms of depleting available precursors or releasing porphyrin stores. Porphyrin synthesis in the liver and kidney, both in terms of porphyrin content and 5-ALA-S activity, together with blood porphyrin levels were therefore compared in control females and males, and females and males Harderianectomised for five months, (Experiment 7A). A second experiment (Experiment 7B) examined any changes occurring after Harderianectomy over a time period of five months. Since ovariectomy is known to affect Harderian gland and liver 5-ALA-S activity and blood porphyrin (Spike et al., 1983; 1985), a third experiment examined possible interactive effects of Harderianectomy and ovariectomy by measuring porphyrin levels and enzyme activities in four groups of females; sham Harderianectomised + sham ovariectomised: sham Harderianectomised + ovariectomised: Harderianectomised + ovariectomised and Harderianectomised + sham ovariectomised, to see if porphyrin synthesis was affected in other organs (Experiment 7C).

MATERIALS AND METHODS

The three experiments described in the Introduction were undertaken with treatment as follows:

Experiment 7A:
a) Sham Harderianectomised females (n = 21)
b) Harderianectomised females (n = 24)
c) Sham Harderianectomised males (n = 8)
d) Harderianectomised males (n = 10)

These animals were culled 5 months after the operations had been performed.

Experiment 7B:
a) Sham Harderianectomised females (n = 31)
b) Females Harderianectomised for 1 month (n = 10)
c) Females Harderianectomised for 2 months (n = 10)
d) Females Harderianectomised for 5 months (n = 24)

Experiment 7C:
a) Sham Harderianectomised + sham ovariectomised females (n = 21)
b) Sham Harderianectomised + ovariectomised females (n = 18)
c) Harderianectomised + ovariectomised females (n = 15)
d) Harderianectomised + sham ovariectomised females (n = 24)

These groups were killed 5 months after the operation date.

All animals were killed in February, when approximately one year old, with an overdose of sodium pentobarbitone. Blood was withdrawn by ventricular puncture and retained for the determination of blood porphyrin levels. The liver was removed, half of each lobe being assayed together for ALA-S activity while the remaining halves were homogenised and stored in 10ml 4:1 ethyl acetate:glacial acetic acid at -20°C until required for porphyrin content determination. The kidneys were removed, halved longitudinally, one half from each kidney being assayed together for enzyme activity, while the other halves were homogenised and stored in 5ml 4:1 ethyl acetate:glacial acetic acid and stored at -20°C until porphyrin content could be determined. The orbits of Harderianectomised animals were examined for the presence of any gland remnants but none were detected. Several kidney and liver samples from each group in Experiment 7C were prepared for HPLC. Detailed procedures are described in "GENERAL MATERIALS AND METHODS".

Statistical Analyses

In the first experiment, the control and experimental groups were compared using Students' t-test. In experiments 7B and C Analysis of Variance was performed: where this proved significant, intergroup comparisons were made using Dunnett's test (1964).

RESULTS

Experiment 7A

Liver porphyrin (nmol/g) (Fig. 69)

The 5 month Harderianectomised females showed a significant decrease in liver porphyrin ($t = 3.25$, $p < 0.01$), compared with sham operated (control) females. Males Harderianectomised for this time also showed a significant decrease ($t = 2.44$, $p < 0.05$) compared with controls.

Liver 5-ALA-S activity (nmol ALA formed/g protein/h)(Fig. 70)

Harderianectomised females showed a significant decrease in enzyme activity ($t = 3.21$, $p < 0.01$), but experimental male values were not significantly different from control males.

Kidney porphyrin (nmol/g) (Fig. 71)

Neither experimental females nor males were significantly different from controls.

Kidney 5-ALA-S activity (nmol ALA formed/g protein/h)(Fig. 72)

Harderianectomised female and male values were not significantly different from controls.

Blood porphyrin (nmol/l) (Fig. 73)

Both female ($t = 1.77$, $p < 0.10$) and male ($t = 1.91$, $p < 0.10$) Harderianectomised groups exhibited slightly raised blood porphyrin levels compared to control animals.

Experiment 7B

Liver porphyrin (nmol/g) (Fig. 74)

There was significant variance in liver porphyrin content ($F = 6.92$, $p < 0.001$). One and two month figures were similar

to control values, but a significant decrease was found in five month Harderianectomised females ($t = 3.59$, $p < 0.05$).

Liver 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 75)

Significant variance occurred in liver enzyme activity over the time course ($F = 5.90$, $p < 0.01$). Two month Harderianectomised females had similar values to the controls, but one month ($t = 2.92$, $p < 0.05$) and five month figures ($t = 3.49$, $p < 0.01$) were significantly lower.

Kidney porphyrin (nmol/g) (Fig. 76)

Significant variance occurred in kidney porphyrin content ($F = 4.88$, $p < 0.01$) due to the increase at one month ($t = 3.65$, $p < 0.01$). Values returned to control levels by two and five months.

Kidney 5-ALA-S (nmol ALA formed/g protein/h) (Fig. 77)

There was no significant difference over the time course in kidney enzyme activity although the five month values were lower than the control and the one and two month values.

Blood porphyrin (nmol/l) (Fig. 78)

Blood porphyrin levels showed a steady rise after Harderianectomy but the increase was not significant.

Experiment 7C

Liver porphyrin (nmol/g) (Fig. 79)

There was significant variance between the four groups ($F = 3.33$, $p < 0.05$), with the ovariectomised + sham Harderianectomised females being significantly less than the controls ($t = 2.97$, $p < 0.05$). The Harderianectomised + sham-ovariectomised group and the Harderianectomised + ovariectomised females also had lower liver porphyrin than the controls, but this was not significant.

Liver 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 80)
Significant variance occurred between the groups ($F = 5.83$, $p < 0.01$). This was due to the low activities of Harderianectomised + sham ovariectomised females ($t = 4.07$, $p < 0.01$) and Harderianectomised + ovariectomised females ($t = 2.88$, $p < 0.05$) compared with the ovariectomised + sham Harderianectomised group whose enzyme activity was nearly double that of the control.

Kidney porphyrin (nmol/g) (Fig. 81)
There was no significant variance in porphyrin content between the groups ($F = 2.33$, ns). Although the ovariectomised + sham Harderianectomised group had raised kidney porphyrin levels, it was within the normal range.

Kidney 5-ALA-S activity (nmol ALA formed/g protein/h) (Fig. 82)
No significant differences were observed between the groups in enzyme activity ($F = 0.86$, ns)

Blood porphyrin (nmol/l) (Fig. 83)
There was no significant difference between the groups ($F = 1.96$, ns) although levels were raised in ovariectomised + sham Harderianectomised females and in sham-ovariectomised + Harderianectomised females compared with controls.

High Performance Liquid Chromatography

Liver samples

Coproporphyrin formed 100% of the total porphyrin concentration in the liver of control females and in sham Harderianectomised + ovariectomised females. The three livers from Harderianectomised + ovariectomised females consisted of 35.57% coproporphyrin (range 0-75.34), 8.22% uroporphyrin (range 0-24.65), 45.47% penta-carboxylic-porphyrin (range 0-83.5) and 10.70% hexacarboxylic-

porphyrin (range 0-16.4). Traces were too low for satisfactory chromatographic identification in the two samples from the Harderianectomised + ovariectomised group.

Kidney samples

Coproporphyrin formed 100% of the total porphyrin concentration of kidney samples from control females, and Harderianectomised + sham ovariectomised females. Protoporphyrin formed 100% of the porphyrin concentration in the one trace obtained from the sham-Harderianectomised + ovariectomised group. The trace obtained for the Harderianectomised + ovariectomised sample was too low to allow satisfactory chromatographic identification.

SUMMARY

Experiment 7A

Female hamsters Harderianectomised for five months showed significant decreases in both liver porphyrin content and 5-ALA-S activity. Blood porphyrin levels rose slightly, but neither kidney porphyrin content nor enzyme activity were altered.

Male hamsters Harderianectomised for five months showed the same trends as females, with the exception that liver enzyme activity did not decrease.

Experiment 7B

During a time course of changes following Harderianectomy in females, liver enzyme activity and porphyrin content were

significantly reduced by five months. After one month liver enzyme activity had decreased, while kidney porphyrin content had increased. Kidney enzyme activity showed no significant changes, and while blood porphyrin levels rose gradually, this was not significant.

Experiment 7C

There was no significant difference in kidney porphyrin content, enzyme activity nor blood porphyrin levels between the four groups. Liver porphyrin content varied considerably between the groups, and ovariectomy resulted in a significant decrease from control levels. There was also considerable variance in liver enzyme activities with both the Harderianectomised groups ($\pm$ ovariectomy) being significantly less than the ovariectomised group, whose activity was nearly twice that of control females. HPLC analyses of liver samples showed that ovariectomy did not alter the porphyrin profile whereas Harderianectomy accompanied by ovariectomy changed the components of the porphyrin profile. HPLC analyses of kidney samples showed that Harderianectomy did not affect the porphyrin profile but ovariectomy altered the profile from 100% coproporphyrin to 100% protoporphyrin, thereby removing two carboxyl groups.

DISCUSSION

The Discussion will consider the following topics, which may include 1 or more of the experiments described above:

1. The prevention of porphyrin loss from the Harderian gland during routine histological processing (Experiment 1).
2. The role of ovarian hormones in regulating and/or maintaining a) porphyrin biosynthesis and b) morphology, in the Harderian gland of the female hamster. Data from the following experiments will be included in this section: Quantitative studies on the effects of hormones on structure and porphyrin biosynthesis in the Harderian gland of the female golden hamster. 1. The effects of ovariectomy and androgen administration (Experiment 2); 2. The time course of change following ovariectomy (Experiment 3); Porphyrin biosynthesis and excretion during pregnancy and lactation in multiparous female golden hamsters (Experiment 4); Morphological and biochemical changes in the Harderian gland of female golden hamsters from post puberty until senescence (Experiment 5); and the effects of oestrogen or progesterone treatment on porphyrin biosynthesis in castrate males (Experiment 6).
3. The role of androgens in maintaining a morphologically and biochemically masculine gland. Data considered here will include results from Experiment 5; Morphological and biochemical changes in the Harderian gland of male hamsters from post puberty until senescence, and Experiment 6, the effect of testosterone administration on porphyrin biosynthesis in castrate males.
4. The relationship between porphyrin biosynthesis in the

gland and the rest of the body a) as a result of exogenous or endogenous steroid manipulation (Experiments 2 - 6) and b) as a result of Harderianectomy (Experiment 7).

1. THE PREVENTION OF PORPHYRIN LOSS FROM THE HARDERIAN GLAND DURING ROUTINE HISTOLOGICAL PROCESSING.

As may be seen, visible porphyrin occurs as large intraluminal, large interstitial or small interstitial (within macrophages) deposits. The preservation of as much porphyrin as possible must therefore be a major consideration in the choice of fixatives and dehydrating agents.

The most prominent feature of Harderian gland epithelial cells in rodents is the large number of lipid vacuoles (Kanwar, 1960; Bucana & Nadakavukaren, 1972a; Watanabe, 1980). Histochemical analyses and biochemical determination of rodent Harderian glands have revealed the presence of neutral lipids, phospholipids, glycolipids, triglycerides, cholesterol, fatty acids, glyceryl ether diesters and fatty acid synthetase (Cohn, 1955; Paule et al., 1955; Kanwar, 1960; Hais et al., 1968; Rock, 1977; Bareggi et al., 1979a,b,c; Watanabe, 1980; Lin and Nadakavukaren, 1981; Kitamoto et al., 1985). In the albino mouse, the intraluminal porphyrin accretions react strongly with Sudan black and acid haematein and these reactions are abolished by prior use of the pyridine extraction test: this indicates that the material stained is partly composed of lipids and phospholipids which may be attached to the porphyrin as a lipoprotein complex (Cohn, 1955). Watanabe (1980) provided further evidence for this in the mouse, since densely stained fibrillar material (porphyrin compounds) was seen both in the lumen and within the secretory lipid vacuoles of B cells; both regions fluoresce red in UV light (Tsutsumi et al., 1966). This fibrogranular component is also seen in the rat Harderian gland in both the lumen and the cytoplasmic

lipid vacuoles of secretory A cells (Brownscheidle & Niewenhuis, 1978) and is similar to descriptions of aggregates of protoporphyrin crystals within the lipid droplets and cytoplasm of hepatocytes in some cases of porphyria (Matilla & Molland, 1974; James et al., 1980). The association of porphyrin with cytoplasmic lipid droplets in the rat Harderian gland has been suggested by Rohonyi & Kelenyi (1962), and Woodhouse & Rhodin (1963), while Tsutsumi et al (1966) suggest that the porphyrin is mixed with the lipid in the gland lumina after secretion.

The behaviour of fixatives towards lipids may, therefore, determine porphyrin losses during routine histological processing. In Experiment 1, the fixatives which resulted in the least % loss of porphyrin from the Harderian gland were, firstly, dichromate which fixes both lipids and phospholipids, and secondly, glutaraldehyde, which fixes most lipids but has a slower penetration time than dichromate. Both AFA and Bouin's routine resulted in unacceptably high losses of porphyrin from the tissue. AFA is a quick penetrating fixative for mucopolysaccharides and glycogen. The greatest porphyrin loss with this routine occurred into the fixative, which correlates with Kanwar's (1960) findings that the pigment (porphyrin) is not fixed by formaldehyde alone. Bouin's (another quick penetrating fixative used routinely) also does not fix lipids or phospholipids which are later dissolved by alcohols and chloroforms; porphyrin losses were considerable and occurred chiefly at these stages. These results are in agreement

with those of Grafflin (1942), Cohn (1955) and Kanwar (1960) who all found that dichromate gave the best pigment preservation.

Glutaraldehyde and dichromate, as well as resulting in substantially lower porphyrin losses than AFA or Bouin's fixative, also allow the use of tissue for transmission electron microscopy. In the hamster Harderian gland it has proved particularly important to employ double-embedding routines (Payne et al., 1985; Spike et al., 1985). Under these circumstances, supra-normal fixation times (e.g. 48 hours or more) are desirable from previous experience. However, under such long fixation, dichromate may render tissues extremely hard for routine sectioning, due to phospholipids being chromated. Furthermore, previous experience with hamster Harderian gland prepared directly for transmission electron microscopy using more routine fixation times in a glutaraldehyde/dichromate mixture (H.S. Johnston, unpublished data) suggests that, while mitochondrial preservation is improved by dichromate addition, cytoplasmic definition shows less contrast than with glutaraldehyde fixation alone and often results in shrinkage artifacts.

Glutaraldehyde fixation resulted in a slightly higher percentage loss of porphyrin from the tissue than dichromate but produced better tissue preservation. It also has the potential disadvantage of making the tissue hard, and this is enhanced when chloroform is used as a clearing agent. However, the use of amyl acetate as a clearing agent prevented additional hardening (without increasing porphyrin

losses) and so makes sectioning easier. If the tissue has to be re-embedded for either transmission electron microscopy or scanning electron microscopy, glutaraldehyde fixation is preferable (even although there is no secondary fixation with OsO_4) as it gives better cytoplasmic definition and produces least shrinkage in this tissue: amyl acetate produces better results than chloroform as a clearing agent.

In Bouin's and AFA when the porphyrin concentration in the tissue was high (above 5000 nmol/g), the percentage loss of porphyrins was lower. This may be due to a solubility factor, in that when only 20 ml of solvent was used at each stage in dehydration, the solvents become saturated with porphyrin and no more would dissolve at each stage. If so, increasing the volume of solvent would result in greater porphyrin loss at each stage. Alternatively, when porphyrin is present in higher concentrations it may form larger accretions which take longer to dissolve, or may be bound to proteins, and these bonds may be less easily broken by the solvents. The exact composition of these laminar intraluminal accretions is unknown at present. During routine histological processing it was noted that glands containing high porphyrin concentrations were poorly preserved with either AFA or Bouin's fixation. This is believed to be due to the inward penetration of the fixative being impeded by the outward movement of the interstitial porphyrin dissolved by these non-lipid preserving fixatives, resulting in leaching of porphyrin from the tissue.

In conclusion, in tissue which is used as a model of porphyrin biosynthesis it is important to use a fixative which both minimizes porphyrin loss and which gives good fixation for light microscopy, transmission electron microscopy and scanning electron microscopy. In the present investigations these requirements were achieved optimally with fixation in 3% buffered glutaraldehyde and the use of amyl acetate as a clearing agent. The results of this study may be applicable to other tissues with visible porphyrin stores such as liver in cases of porphyria cutanea tarda (Cortes et al., 1980; James et al., 1980).

2. THE ROLE OF OVARIAN HORMONES IN REGULATING PORPHYRIN BIOSYNTHESIS AND MORPHOLOGY IN THE HARDERIAN GLAND.

The quantitative assessment of the effects of ovariectomy (alone, or coupled with androgen administration) on the morphology, porphyrin content and porphyrin synthesising enzyme activity of the Harderian gland of the female golden hamster confirms the link between gonadal hormones and gland structure and activity. The results indicate, firstly, that ovarian hormones are necessary to maintain the structure and activity of the normal female gland in this species and, secondly, that androgen administration results in the masculinisation of gland characteristics. Further quantitative evidence for the involvement of ovarian hormones is provided in the study of the age series of females, where decreasing ovarian activity during senescence produces similar (but less pronounced) effects to ovariectomy on both gland activity and morphology. Furthermore, during pregnancy and lactation (periods of changing ovarian hormone levels) there are alterations in gland enzyme activity and porphyrin content, while oestradiol or progesterone administration to castrate males results in more sustained enzyme activity than in castrates.

a) Porphyrin biosynthesis

The activity of 5-ALA-S had decreased significantly by 20 weeks post-operatively in ovariectomised females compared to control animals of the same age, although porphyrin content was similar in both groups. It is not known what proportion of the total porphyrin content of the

gland, as determined by biochemical assay, is accounted for by intraluminal, interstitial and the newly synthesised intraepithelial porphyrins. Nor is it known what are the likely rates of addition to, or depletion of, solid porphyrin stores in the normal female gland. That such stores can change is indicated by firstly their dramatic increase in males following castration (Hoffman, 1971; Payne et al.,1977a), and secondly their marked decrease in ovariectomised females treated with androgens. In the experiment involving the time course of changes following ovariectomy, the total porphyrin content of the Harderian glands remained relatively static following ovariectomy. However, the synthesis of new porphyrin must have decreased since ovariectomised females showed a progressive decrease in 5-ALA-S activity with increasing time post-operatively, apart from a transient increase at 30 weeks which resulted in raised porphyrin content at 40 weeks post-operatively. This suggests that the bulk of the porphyrin detected biochemically is intraluminal stores. Indeed there is little quantitative correlation between 5-ALA-S activity and the number of intraluminal porphyrin accretions, whose numbers show a gradual decrease with time after ovariectomy, which does not become significant until 50 weeks post-op. However, ovariectomy alters the pattern of porphyrin stores, with considerable increases in both large and small interstitial deposits as a result of the breakdown of tubules containing porphyrin accretions. Both types of interstitial deposits reach a maximum at 40 weeks post-

operatively and then decrease. Interestingly, blood porphyrin levels show a similar profile, and this may be due to the small interstitial porphyrin stores (i.e. those within macrophages) leaving the gland by a vascular or lymphatic route, although there is no direct evidence of this, nor of the mechanism by which it might occur. Alternatively, decreased porphyrin synthesis by the Harderian gland may result in increased availability of precursors (glycine and succinate) to other porphyrin synthesising tissues. Ovariectomy accompanied by androgen administration produces a more rapid decrease in 5-ALA-S activity, with levels falling to male values by 20 weeks, whereas this takes 40-50 weeks in ovariectomised females. Because of this, androgen administration produces a more dramatic decrease in total porphyrin content and the number of visible porphyrin accretions in 20 weeks which is not approached by ovariectomise females, even after 50 weeks. It is of interest that in females ovariectomised for 20 weeks the significant increase is in the number of small (phagocytosed) interstitial porphyrin deposits, suggesting that degeneration proceeds further in ovariectomised than in androgen-treated females where the process stops with the sequestration of large interstitial porphyrin deposits. Again it is of interest that androgen-treated females do not display elevated blood porphyrin levels by 20 weeks post-operatively (unpublished results).

HPLC analyses of total porphyrin extracts from the Harderian glands of intact females confirm a previous report

(Payne et al., 1982a)that protoporphyrin IX forms more than 90% of the total porphyrin content. Ovariectomy does not alter this porphyrin profile, presumably because most of the intraluminal porphyrin stores and the interstitial deposits formed by tubule degeneration following ovariectomy (Payne et al., 1985; Spike et al., 1985) had been synthesised prior to ovariectomy. Although porphyrin synthesis is significantly decreased following ovariectomy, the stepwise decarboxylation to protoporphyrin IX continues, since there is no increase in hexa- or hepta-carboxylic porphyrin or uroporphyrin. In normal adult male hamsters, this stepwise decarboxylation does not proceed as completely as in the female since hexa- and penta-carboxylic porphyrins and coproporphyrin are present in greater amounts than in the female gland while the proportion of protoporphyrin IX is approximately 20% less than in the female. Circulating androgens may be responsible for decreased enzyme activities at these decarboxylation steps since castration for 6 weeks results in protoporphyrin percentages which are intermediate between male and female levels; decarboxylation proceeds further since coproporphyrin is the only other major porphyrin present. It would be interesting to see if castration for longer periods would produce a porphyrin profile even more similar to the female pattern.

Chromatographic analyses of rat Harderian glands has shown the presence of a tricarboxylic porphyrin, termed Harderoporphyrin (Kennedy, 1970). In the HPLC analyses of porphyrin methylesters performed in this present experiment,

the standard does not contain Harderoporphyrin, but in samples containing Harderoporphyrin this component appears as a shoulder on the protoporphyrin peak (Fig. 10). Where this shoulder appeared, it was not any greater in height than the coproporphyrin peak and therefore forms less than 4% of the total porphyrin content. This is considerably less than the percentage found in the rat Harderian gland (protoporphyrin 64%, Harderoporphyrin 29%, coproporphyrin 9%) (Kennedy, 1970). This may reflect species differences, since protoporphyrin occurs almost exclusively in the gerbil Harderian gland (Johnston et al., 1983).

Further evidence for the role of ovarian hormones in maintaining gland enzyme activity, and thus porphyrin content, is seen in the age series (Experiment 5). By 2 months, the hamsters are sexually mature and porphyrin content - which varied significantly in the female age groups - was at its highest level, and thereafter decreased. (Intraluminal porphyrin accretions were also highest at 2 months). The 5-ALA-S activity showed a similar pattern, peaking at 6 months and then decreasing. These parameters appear to be dependent on the levels of circulating ovarian hormones since they are significantly reduced in the post reproductive senescent females. Although not reflecting porphyrin

formation, the pattern of porphyrin stores within the gland also alters as the animals age: intraluminal deposits show a greater decrease in number compared to ovariectomised females but this is because 2 month females have nearly twice as many deposits as 12 month females which was the age of the control groups in both experiments involving ovariectomised females. Interstitial porphyrin deposits show a progressive increase as the females age but the increase is not as pronounced as in the ovariectomised group: by 24 months the intact females have a similar number of small interstitial deposits to females ovariectomised for 30 weeks. Blood porphyrin levels are raised in the 18 month groups, which may be due to the increase in interstitial deposits.

Comparative studies in the porphyrin content of the Harderian gland in other rodent species show that in the rat visible porphyrin appears on day 13 after birth (Rapin, 1952) coinciding with the eyes opening (Wetterberg et al., 1972b), and in the albino rat porphyrin content increases as the weight increases (Rohonyi & Kelenyi, 1962). In gerbils, porphyrin is rarely seen at birth but is abundant when the eyes open and increases up to 3 weeks of age (Arvy & de Lerma, 1961). As mice become sexually mature the porphyrin producing enzymes appear and increase in concentration. From this time on there is a relatively rapid increase in porphyrin-producing enzyme activity which reaches a maximum at 8 weeks and remains constant until at least 52 weeks (Figge & Davidheiser, 1957). Conversely, in C_3H mice the porphyrin content is greater in females at 75 days than at 250 days, while in males it

remains constant (Strong, 1942). Visible porphyrin is absent in neonatal hamsters but appears in both males and females at 2 weeks. By 4 weeks, the number of intraluminal deposits have increased in females but have almost disappeared in males. The disappearance of porphyrin in males coincides with the appearance of polytubular complexes, which are thought to contain enzymes which breakdown porphyrin (Bucana & Nadakavukaren, 1973). Since porphyrin synthesis in the Harderian glands of mice (Davidheiser & Figge, 1957) and hamsters (Bucana & Nadakavukaren, 1973) appears to be dependent on developing gonadal hormones, it would be interesting to see if the enzymes in the haem pathway in the gland are sequentially induced (as occurs in perinatal liver, (Woods & Dixon, 1970; Moore et al., 1972)) as hormone levels rise, by measuring enzyme levels and HPLC profiles.

Pregnancy and lactation also affect porphyrin levels and enzyme activity in the Harderian gland, although not significantly in this particular study. The enzyme activity increased during late pregnancy and early lactation, resulting in increased porphyrin levels during lactation. These results differ from a previous study (Payne et al., 1979) in which porphyrin levels were significantly raised during pregnancy and lactation compared to controls. The porphyrin levels in the two experiments are similar for the pregnant and lactating females, the difference occurring in control levels, which were very low in the earlier experiment. The porphyrin content of the female Harderian gland shows considerable variability even in animals of the same age,

and this may account for these discrepancies. A previous study showed that breeding female mice had more porphyrin in their Harderian glands than virgins, suggesting a relationship between hormone levels and porphyrin synthesis (Bittner & Watson, 1946).

Experiment 6 examined the effect of castration on the Harderian gland, in the immediate post-operative period. At 2 weeks post-operatively there was no difference between control males and any of the castrates or castrates treated with steroids. However, by 4 weeks the castrates had the highest 5-ALA-S activity which were within the female range, but by 6 weeks the enzyme activity had decreased although porphyrin levels continued to rise. In contrast, animals given either oestrogen or progesterone showed a more gradual rise in both enzyme activity and porphyrin content, both peaking at 6 weeks post-operatively. The administration of female hormones to castrates would therefore appear to sustain 5-ALA-S activity at female levels, compared to castration alone where the removal of male hormones results in a transitory increase in enzyme activity.

b) Morphology

The intact female hamster Harderian gland consists of tubules lined by columnar epithelial cells containing small lipid vacuoles (Type I cells). Epithelial cells containing large lipid vacuoles (Type II cells) are characteristic of the male hamster and are rarely observed in sexually mature females until senescence. These epithelial cells surround a lumen which may contain a solid porphyrin accretion. Myoepithelial cells lie within the basal lamina. As a result of ovariectomy or senescence a sequence of degenerative events

occur in which porphyrin stores become localised within the gland interstitium (Figs. 11, 13 & 14).

Experiments 2, 3 & 5 in this thesis provide quantitative confirmation of the previously qualitative description of gland degeneration, and demonstrate that this is a progressive phenomenon. As the epithelial cells become greatly attenuated, the tubules are invaded by neutrophils which may be phagocytosin necrotic epithelial debris in general (Vethamanay, Vethamanay & Bessis, 1975) or lipid droplets to which neutrophils may be specifically attracted (Turner et al., 1975; Tainer et al., 1975) and which are produced in quantity by the epithelial cells (Lin & Nadakavukaren, 1981). The interstitial neutrophils exhibit a normal appearance with multilobed nuclei and small cytoplasmic granules (Fig. 47) whereas those in the lumen of the tubules show a marked and consistent alteration in appearance, with the loss of most of the cytoplasmic granules and the presence of numerous large cytoplasmic vacuoles (Fig. 48).

Although neutrophils have been observed in close proximity to intraluminal porphyrin deposits, porphyrin crystals have not been observed within the neutrophils (Payne et al., 1985). Strum & Shear (1982) reported leucocytes phagocytosing lipid droplets produced by epithelial cells of the mouse Harderian gland.

Following the disappearance of the epithelial and myoepithelial cells, the intraluminal porphyrin accretions became stranded within the interstitium of the gland. Interstitial porphyrin is rarely observed in normal female hamsters but has been reported in the rat (Grafflin, 1942) and the Australian murid Pseudomys australis (Johnston et al.,

1985a). Rarely, these deposits were invested by a basal lamina (presumably all that remained of a gland tubule) but more commonly these large porphyrin accretions are surrounded by an investing layer of macrophages (Payne et al., 1985). While these may be free macrophages held together by interdigitations, more frequently the lateral cell membranes have disappeared resulting in a foreign body giant cell capable of sequestering particulate matter (Sutton & Weiss, 1966). The surface in contact with the porphyrin appeared to be fused on with no visible membrane; a normal cell membrane was present on the aspect facing away from the accretion (Fig. 13)(Payne et al., 1985).

Individual free macrophages containing characteristic needle-like porphyrin crystals, either densely compacted or loosely arrayed are also observed in the interstitium of the gland. These may represent stages in the digestion of porphyrin, as may pale areas which have been described within the cytoplasm of these macrophages and which may represent solubilised porphyrin (Fig. 14) (Payne et al., 1985). It is unclear as to whether these macrophages are secondarily derived from the foreign body giant cell. Blood porphyrin levels are elevated in ovariectomised females (increasing progressively with time post operatively) as are the numbers of small interstitial deposits; these increases also occur, to a lesser extent, in post-reproductive senescent females. Elevated blood porphyrin levels might therefore be due to the small interstitial porphyrin deposits entering the general circulation via a vascular or lymphatic route from the gland, but there is no direct evidence of this, nor of

the mechanism by which it might occur.

Similar responses to abnormal porphyrin synthesis and accumulation has been described in the liver in cases of porphyria cutanea tarda. These include focal hepatocyte necrosis associated with groups of pigment-laden macrophages, marked hepatocyte hyperplasia and the presence of periductal lymphocyte aggregations (Cortes et al., 1980). Granulomas with central acicular inclusions have also been observed (Moreno et al., 1977). Liver biopsies from cases of human erythrohepatic protoporphyria also report porphyrin-containing macrophages and lymphocytes in portal tracts, and porphyrin-containing Kupffer cells, some of which formed multi-nucleate cells. The cytoplasm of the Kupffer cells had many phagolysosomes containing lipid and crystals at the periphery (Matilla and Molland, 1974). The appearance of the liver in griseofulvin-induced porphyria in mice is similar (Matilla & Molland, 1974).

Macrophages breakdown red blood cells, with bilirubin being partly a product of this metabolism. The haemoglobin in the red blood cells represents the final stage of haem synthesis with protoporphyrin IX being the penultimate step in the pathway. The macrophages may recognize the cyclic tetrapyrrole configuration of protoporphyrin IX in the interstitium, and therefore degrade it, possibly to the linear tetrapyrrole bilirubin.

Macrophages are also involved in lipid metabolism, having the necessary enzymes to hydrolyse and esterify both triglycerides and cholesterol (Day, 1964). The uptake of lipids and lipoproteins *in vitro* has been demonstrated (Casley-Smith & Day, 1966) and cholesterol has been shown

to produce giant cell granuloma in atherosclerosis (Adams et al., 1963). Since porphyrin is thought to be complexed to either lipids (Rohonyi & Kelenyi, 1962; Woodhouse & Rhodin, 1963; Tsutsumi et al., 1966; Matilla & Molland, 1974) or lipoproteins (Cohn, 1955), this may account for the association of macrophages with interstitial porphyrin deposits. Alternatively, the abnormal localisation of porphyrin within the interstitium may result in a general antigenic response from the macrophages.

One of the major sex differences in the hamster Harderian glands is the porphyrin content. A previous study showed that mast cell numbers exhibit the same sex difference, and that castration results in an increase in both porphyrin content and mast cells. However, no significant correlation was found between mast cell numbers and total porphyrin nor mast cell numbers and the number of intraluminal porphyrin accretions in either intact females or castrated males (Payne et al., 1982a). In Experiment 5, however, a significant positive correlation occurred between mast cell numbers and the number of intraluminal accretions, both these parameters being greatest in the youngest age group of females examined and thereafter decreasing (Fig. 62). This result was unexpected since the studies on ovariectomy had shown that mast cell numbers increased progressively with time post operatively reaching a maximum at 40 weeks. The number of small interstitial porphyrin deposits in ovariectomised females shows a similar rise post-operatively and there was a strong positive correlation between these two parameters.

These animals were all killed at the same age but had been ovariectomised for varying time intervals. Minor sex differences in mast cell numbers have been reported in rat and human tissues (Hellstrom & Holgen, 1950; Sundberg, 1955) and mast cell numbers fluctuate in the female reproductive tract during the oestrous cycle or pregnancy (Harvey, 1964; Seyle, 1965). However, a marked sex difference in mast cell numbers in the hamster Harderian gland is unprecedented and suggests a more powerful relationship between mast cell numbers and hormonal status than has previously been reported (Payne et al., 1982a).

The functional relationship between mast cell numbers and the hormonal status of the gland is unclear. Mast cells contain heparin which may have a lipid clearing action (Shoulders & Meng, 1957) and a quantitative sex difference has been reported in the lipid content of the male and female gland (Lin & Nadakavukaren, 1981). Mast cells have also been shown to produce a weak reaction with the ferrous ion (Lillie et al., 1978). Both the female and castrated male Harderian glands manufacture large amounts of porphyrin but mast cell numbers are highest in ovariectomised females in which porphyrin synthesis has significantly decreased. Mast cells are prevalent in hibernating species, such as the hamster, where their heparin content may prevent clotting during prolonged periods of reduced circulation (Smith, 1963). The greater number of mast cells in the ovariectomised females may be involved in preventing the increased number of interstitial porphyrin stores from occluding small blood vessels, but does not explain the presence of so many mast

cells in intact females and castrate males in which interstitial deposits are rarely seen, nor the sex difference.

Payne et al (1982b; 1985) have postulated that many aspects of gland degeneration suggest an immune response. The abnormal presence of porphyrins in the interstitium of ovariectomised and post-reproductive females possibly complexed with lipids or lipoproteins, may induce such a response. Thus, the porphyrin-containing macrophages are frequently surrounded by clusters of plasma cells (Fig. 49) and lymphocyte invasions are marked (Fig. 50). Macrophages are thought to act as non-specific accessory cells involved in such functions as antigen uptake, catabolism and presentation to T and B cells in the initiation of an immune response (Pierce & Kapp, 1976; Mosier, 1976). Macrophages may alter antigens (or make them more immunogenic) and hold antigens on the surface in order to focus cell interactions with lymphocytes (Nelson, 1976). Mast cells may also be involved in this immune response since they increase progressively in numbers with time post-operatively and may be T-lymphocyte dependent (Burnet, 1977; Askenase & van Loveren, 1983).

The mast cell was thought to be derived from undifferentiated mesenchymal cells due to its perivascular location and the observation that restoration of mast cells appeared to occur through differentiation of small mesenchymal cells following extensive mast cell destruction (Fawcett, 1955; Hunt & Hunt, 1956).

More recently, data suggests that a portion of the mast cell population in rodents may be of T-lymphocyte origin since thymus cells in the rat can differentiate into mast cells under a variety of conditions. Furthermore, tissue

culture of rat or mouse thymus glands leads to the appearance of mast cells in the culture (Metcalfe et al., 1981). However mast cells are present in the subcutaneous tissue of athymic mice , devoid of T-cells (Dexter et al., 1981). Bone marrow derived cells may also be mast cell precursors since bone marrow transplants from mice with Chediak-Higashi syndrome to irradiated recipients resulted in the appearance of mast cells with characteristic giant cytoplasmic granules (Kitamura et al., 1979). A number of differences exist between mucosal and connective tissue mast cells suggesting sub-populations from different lineages (Jarrett & Haig, 1984).

The mast cell is involved in the immune response since it has specific receptors for Ig E; aggregation of the receptors induces the biochemical events leading to the degranulation process (Metcalfe et al., 1981). IgG surface receptors have also been reported in mouse cells (Tigelaar et al., 1971).

Mast cells contain a number of enzymes including peroxidase (Henderson et al., 1979). In neutrophils and macrophages, a similar peroxidase contributes to the microbiocidal activity of these cells (Klebanoff, 1968). Mast cells have been shown to be phagocytic to a number of particulates (including ferritin) which differ in size, chemical nature and surface charge, but appear to be deficient in several hydrolytic enzymes found in conventional lysosomes present in macrophages (Padawer, 1971). Erthyrophagocytosis by neoplastic mast cells has been described in tissues from a domestic cat with severe anaemia (Madewell et al., 1983).

In the age series examined in Experiment 5, degenerative changes such as neutrophilic invasion of the tubule lumina, tubule degeneration and the occurrence of interstitial porphyrin deposits were all minimal in the youngest females and increased thereafter. These changes became marked in post reproductive senescent females and are therefore in general agreement with the pattern of progressive degeneration within the gland with increasing time after ovariectomy (Experiment 3). However, the degenerative changes observed in the post-reproductive females were never as extreme as those which occur in females which had been ovariectomised for 20 weeks or more.

Ovariectomy alone does not result in masculinization of the female hamster Harderian gland, but when it is accompanied by androgen administration the gland assumes male characteristics within a short time. The total porphyrin content of the glands, both in terms of biochemical assay and the number of visible porphyrin accretions is greatly reduced, and there is an increase in the number of interstitial deposits, particularly in the number of large deposits. Tubule degeneration is less apparent in these androgenised females compared to ovariectomised females, and is not significantly different from the control females. Further evidence of the masculinizing effects on the female Harderian gland is seen by the marked increase in Type II cells and the appearance of polytubular complexes. The only other female groups to have an increased number of Type II cells were the two post reproductive senescent groups in the age series.

These experiments demonstrate that ovariectomy influences both porphyrin biosynthesis and morphology in the female hamster Harderian gland, and that this is a progressive phenomenon, increasing with time after ovariectomy when age and seasonal factors are controlled. Similar but less extreme changes occur during senescence in the female hamster Harderian gland. It had previously been suggested that gonadotrophins (particularly luteinising hormone) act directly on the gland, so that both castration and ovariectomy affect the gland as a consequence of elevated gonadotrophin levels (Hoffman, 1971; Clabough & Norvell, 1973). This seems unlikely, since while castration leads to hugely raised porphyrin levels (Experiment 6) (Payne et al., 1977a) ovariectomy leads to decreased enzyme activities and intraluminal porphyrin content in females. Since the Harderian gland possesses receptors for testosterone (Gustaffson & Poussette, 1975) and oestradiol (Weaker et al., 1983) this further suggests that gonadal hormones (rather than gonadotrophins) control gland structure and activity. Furthermore, cytosol androgen receptors have been demonstrated in the rat lacrimal gland; a sex difference is found in the number of these binding sites, with the male having more than the female. A small number of progesterone binding sites also occurred in both sexes (Ota et al., 1985). The secretory component of the rat lacrimal gland is increased by androgens (Sullivan et al., 1984).

Androgens are thought to be responsible for maintaining the male-type gland (McMasters & Hoffman, 1984). However,

circulating androgen levels are normally higher than oestrogens, even in cycling female hamsters. Plasma testosterone and androstenedione are both detected during the oestrous cycle (Saidapur & Greenwald, 1978) and both these androgens maintain the male type Harderian gland in castrates (Payne et al., 1977a). The female type gland may respond preferentially to oestrogens, and only when oestrogen synthesis is decreased does it respond to androgens. This may account for the appearance of Type II cells in senescent females due to the ovary producing androgens in these age groups. In post-menopausal women the ovary is known to produce androgens (Eckstein, 1977). Androgens are also thought to be the ovarian hormones which mediate conversion to the male type gland in blinded females (McMasters & Hoffman, 1984).

3. THE ROLE OF ANDROGENS IN MAINTAINING THE MALE-TYPE GLAND.

The role of androgens in maintaining the male-type gland is demonstrated in Experiments 5 & 6. In Experiment 6, castrate males have raised 5-ALA-S enzyme activities and porphyrin content by four weeks post-operatively. Previous experiments have shown that the increase in porphyrin content is progressive with time after castration (Hoffman, 1971; Payne et al., 1977a), and this is confirmed in the present experiment. This increase in enzyme activity and porphyrin content is related to the removal of circulating androgens, since castrates given testosterone propionate retain the biochemical characteristics of the male-type gland. Experiment 2, in which ovariectomised females were given testosterone propionate shows that androgenised females have significantly less intraluminal porphyrin accretions and thus porphyrin content than ovariectomised females. The androgenised females also exhibit enzyme activities within the male range, as well as a highly significant increase in Type II cells and the presence of polytubular complexes within the epithelial cells.

In the age series examined in Experiment 5, the gland porphyrin content remained very low in all the male age groups, and neither the enzyme activity nor the porphyrin content showed significant variance. It would appear, therefore, that even during senescence circulating androgen levels are sufficient to inhibit porphyrin synthesis. Intraluminal porphyrin deposits occur in pre-pubertal male glands at 2 weeks but by 4-6 weeks have almost disappeared

(Bucana & Nadakavukaren, 1973; Payne et al., 1977a) and as Experiment 5 has shown, a few still persist at 8 weeks. The disappearance of intraluminal porphyrin accretions coincides with the sexual maturation of the males and the appearance of polytubular complexes within both types of epithelial cells (Bucana & Nadakavukaren, 1973): Bucana & Nadakavukaren (1973) suggested that these complexes may contain an enzyme or enzyme system which breaks down porphyrin. Further evidence for the relationship between these complexes and androgens was demonstrated by their disappearance in castrates which accumulate porphyrin (Lin & Nadakavukaren, 1979) but appear in androgenised females which show decreased porphyrin levels and the appearance of male morphological features (Sun & Nadakavukaren, 1980)(Experiment 2). Testosterone administration to castrates prevents the increase in porphyrin content and enzyme activity (Hoffman, 1971; Payne et al., 1977a) (Experiment 6). Jones & Hoffman (1976) failed to detect any influence of testosterone or oestrogen on porphyrin production by male glands in vitro. They suggested that increased porphyrin synthesis in the male could be produced by the addition of ALA to the incubation medium and that normal production of ALA might be repressed in the male gland. However, Experiments 5 & 6 in this thesis, and those of Lin & Nadakavukaren (1982) and Thompson et al., (1984) indicate that the male gland contains endogenous ALA, and that 5-ALA-S is active in the gland. Furthermore, Jones & Hoffman's experiment is difficult to evaluate because additional ALA resulted in increased porphyrin production in female glands also. Again, it is suggested

that the polytubular complexes may prevent the ALA formed from condensing into PBG (Jones & Hoffman, 1976). However, it is most probable that the rate-limiting enzyme and other enzymes in the pathway are inhibited directly by circulating androgens, since sex differences occur in 5 of the enzymes in the pathway (Thompson et al., 1984).

The values in this thesis for porphyrin content and 5-ALA-S activity in the female gland are in close agreement with those obtained by Thompson et al., (1984). The values for male gland porphyrin content in Experiments 5 & 6 and enzyme activities in Experiment 6 are also similar to those cited by Thompson et al (1984). However, the enzyme activities occurring in males of all ages in Experiment 5 are four to eight-fold higher. The reason for this difference is not clear, especially since liver enzyme activities are comparable between all experiments. It is known that there is marked seasonal variation in 5-ALA-S activity in female Harderian glands, with activity being highest in summer (Moore et al., 1980). A similar pattern in males (although at present uninvestigated) would account for the present findings. Low 5-ALA-S activity in male (as opposed to female) glands is the normal finding (Jones & Hoffman, 1976; Mindegaard, 1976; Thompson et al., 1984). A smaller sex difference (akin to that found in Experiment 5) has also been reported (Lin and Nadakavukaren, 1982). There is clearly high variability and unidentified factors may be operating. Nevertheless, the low porphyrin levels found in the glands of these males demonstrate that porphyrin synthesis is being inhibited *in vivo*. Although there is an overlap in 5-ALA-S activity between the top end of the male range and the bottom end of the female range in Experiment 5, male porphyrin levels remain less than 100nmol/g while female levels are rarely less than 1000nmol/g.

Male gland morphology remains unaltered over the age range examined, with the percentage of Type II cells remaining high even during senescence. Degenerative features which occur in the ageing female gland are not apparent in senescent males. This is probably related to the fact that male hamsters remain reproductively active throughout their entire life span of 2 to 3 years (Hafez, 1970) whereas reproductive senescence may begin about 14 months in the female with decreasing size of litters and increasing gestation periods (Soderwall et al., 1960). However, even by 20 months ovarian involution is not complete and females have been known to raise litters when 27 months old (Whitney, 1963). This would explain why degenerative changes in the Harderian gland are less marked in senescent females than in females which have been ovariectomised for 20 weeks or more.

The only unusual feature in elderly males is the appearance of laminar intraluminal accretions. Since these accretions occurred only in 18, 24 and 30 month males, and did not fluoresce red under ultraviolet light, they may represent some malfunction of the lipid secretory mechanism. Similar retained secretion occurs in the human lacrimal gland (Damato et al., 1984). Transmission electron microscopy revealed the laminar nature of these deposits and the presence of lipid vacuoles of a similar size to those in the epithelial cells of the tubules (Figures 63 & 64).

The appearance of large cytoplasmic tubules arising from endoplasmic reticulum (as is thought to be the case

in the polytubular complexes in the Harderian gland of Apodemus sylvaticus, H.S. Johnston, personal communication) has been described in various tissues including renal interstitial cells of rats (Ledingham & Simpson, 1973), uterine glands of pigs (Crombie, 1972; Perry & Crombie, 1972), the human retina (Matthews and Martin, 1971) and rat testicular interstitial cells (Leeson & Leeson, 1981; 1983). The tubules from interstitial renal and testicular cells are identical in size but approximately four times larger than in the male hamster Harderian gland. Furthermore, they do not occur with the high frequency associated with the tubules in the epithelial cells of the male Harderian gland. The large tubules found in the uterine glands are thought to be related to a synthetic process or type of ionic regulation (Crombie, 1972) while those in the renal interstitial cells may result from the action of antidiuretic hormone in dehydrated rats (Bulger et al., 1966). Leeson & Leeson (1983) suggest that the formation of these tubules by the endoplasmic reticulum is a general response to different stimuli, perhaps initiated by an unrecognised physiopathological state.

How do sex hormones affect other target tissues?

The main changes in the Harderian gland resulting from hormone manipulations are:-

1. Changes in the number of cell types
2. Changes in interstitial features
3. Changes in enzyme activities
4. Changes in the height of the tubule walls

5. Changes in the number of mitotic figures.

The first three features are understandable in view of the fact that a) the gland is possessed by both sexes and b) the gland exhibits sexual dimorphisms. The other changes are less explicable and some insight may be gained by comparing the effect of hormones (or their lack) on other target tissues.

The Harderian gland is not the only organ in which morphology and activity are regulated by gonadal hormones. Androgens maintain the normal structure and function of the male accessory sex organs. Thus, the mucosal folds of guinea pig seminal vesicle are less prominent after castration, the cells become shorter with fewer microvilli, and degeneration of glandular cells occurs. Similar changes occur in old males (Wong, 1983). Castrated male rats show a large decrease in protein synthesis by the seminal vesicles (Ostrawski et al., 1982). A decrease in the size of the epithelium and the glandular acini of the rat ventral prostate lobe is observed following castration (Huttunen et al., 1981). However, androgens also influence the development of other organs, such as stimulating hair growth in laboratory animals. Castrated male mice have significantly shorter vibrissae than control males, while intact males treated with testosterone propionate show a significant increase in the final length (Ibrahim & Wright, 1983). Testosterone propionate treatment promotes cell proliferation in the small intestine and in colonic tumours while castration retards cell division in colonic

tumours in rats (Tutton & Barkla, 1982).

Ovariectomy results in the atrophy of the female reproductive tract (Allen et al., 1937) and a decrease in the size of the mammary glands (Richardson, 1955). Oestrogen administration produces rapid growth of epithelial tissues of the female genital organs and mammary glands due to an increase in the number of mitoses and the thickness of the epithelium (Allen et al., 1937; Martin & Claringbold, 1960). In the uterus of the pregnant mouse changing hormone levels regulate cell division with oestrogen producing an increase in luminal and glandular mitoses but not in the connective tissue stroma on days 2 and 3 of pregnancy, while the increase in progesterone on day 4 results in a reversal of this pattern (Martin & Finn, 1968). Oestrogen stimulates the secretory process in the parathyroid gland related to calcium and phosphorus metabolism. The ultrastructure of this gland in the hamster changes following ovariectomy, showing a decrease in the number of secretory granules (Emura et al., 1984).

Testosterone is one of several factors which influences plasma lipids and lipoproteins. Castrated male rats showed an increase in plasma cholesterol, phospholipids and cholesterylester, compared to intact males and castrates given testosterone propionate (Haug et al., 1984). Decreased levels of ovarian hormones, such as occurs during the menopause or following ovariectomy also results in increased serum cholesterol and triglycerides, and changes in lipoprotein distribution while oestrogen therapy maintains a normal lipid balance (Notelovitz et al., 1983).

Sebaceous secretion is controlled by steroid hormones. Sebum production is stimulated by testosterone or other androgens, and inhibited by oestrogens (Ebling, 1974). Testosterone affects the size of the sebaceous gland, and causes an increase in mitoses and intracellular synthesis (Ebling, 1974). Conversely, castration leads to atrophy of sebaceous glands (Ebling, 1963) and a decrease in sebum production (Thody & Shuster, 1970a & b). The sebaceous glands of golden hamsters possess specific cytosol and nuclear receptors for dihydrotestosterone. Both cytosol and nuclear receptor proteins are rapidly decreased by castration but reactivation by testosterone *in vivo* initiates new protein synthesis (Adachi & Kano, 1972; Adachi, 1974). Ovariectomy had no marked effect on the sebaceous gland activity of rats (Shuster & Thody, 1974). The response to progesterone in the adult rat differs, decreasing sebum secretion in males while producing a slight increase in females. In gonadectomised adults progesterone increased sebum secretion in both sexes (Thody & Shuster, 1978). The flank organ of the golden hamster, which consists of sebaceous glands, hair follicles and melanocytes, is androgen dependent. Castration results in decreased size of organ and decreased RNA, DNA & protein levels (Giegel et al., 1971). The Harderian gland also synthesises lipids, and castration of male golden hamsters results in the disappearance of Type II cells which contain large lipid vacuoles (Hoffman, 1971; Payne et al., 1977a).

4. THE INTER-RELATIONSHIP BETWEEN PORPHYRIN BIOSYNTHESIS IN THE HARDERIAN GLAND AND THE REST OF THE BODY.

These following experiments were designed to see if the Harderian gland responds to the effects of endogenous or exogenous steroid manipulations in the same way as other porphyrin producing organs such as the liver and kidney, and thereby evaluate the usefulness of the Harderian gland as a model for porphyrin biosynthesis. Furthermore, since the Harderian gland can be removed from the animal without any apparent detrimental effects, the role of the gland in the porphyrin economy of the body could be evaluated.

a) as a result of steroid manipulation (Experiments 3 - 6)

During the time course of change following ovariectomy, the activity of liver 5-ALA-S showed a transient increase at 10 weeks post-operatively, but this figure fell within the normal range of enzyme activity for the hamster liver. Nor did liver enzyme activity show any significant variation with age in females. The raised blood porphyrin levels in ovariectomised and post-reproductive females is thought to be related to the blood borne dispersion of interstitial porphyrin deposits in the Harderian gland which increase in both these groups (Payne et al., 1985). Liver enzyme activity does not appear to alter in response to decreasing levels of ovarian hormones, unlike the Harderian gland, which shows decreasing enzyme activity. In the male age series liver enzyme activity was raised in the oldest group examined and these males also had the highest blood porphyrin levels. Since the gland porphyrin levels remained within the normal range, the raised blood porphyrin levels in the 30 month old

males may be due to the distribution of excess liver porphyrins as a result of increased enzyme activity, possibly resulting from a disturbance in haem biosynthesis in these elderly males. In neither sex does the liver respond in a similar manner to the Harderian gland.

Also in marked contrast to the Harderian gland, the liver shows little response to the effects of castration, apart from an initial increase in the castrates given oestrogen. Liver tissue has previously been shown to produce more porphyrins following steroid administration, as in chick embryo liver cell culture (Granick & Kappas, 1967) and the most potent inducers were the 5β-OH neutral steroids of the C19 and C21 series which are <u>in vivo</u> derivatives of the steroid hormones rather than being primary secretions themselves (Granick & Kappas, 1967). This latter group includes aetiocholanolone and dehydro-epiandrosterone (DHA). The latter induces hepatic 5-ALA-S in the rat (Moore et al., 1973). The female sexual cycle has also been shown to ffect the activity of 5-ALA-S in the rat liver, with highest levels occurring in proestrus, and the lowest during dioestrus. Intermediate levels occur during oestrus and metoestrus (Held & Przerwa, 1976). A similar pattern of enzyme activity has been demonstrated in the female hamster Harderian gland (Payne et al., 1977b; Moore et al., 1980).

In Experiment 4, pregnancy and lactation had little effect on liver enzyme activity and porphyrin content which remained within the normal ranges, apart from a slight increase in porphyrin content in late pregnancy. In contrast, both kidney enzyme activity and porphyrin content changed

during this period, being highest in mid pregnancy. Blood porphyrin levels showed a significant decrease at this time. Although urinary porphyrin excretion did not alter during pregnancy and lactation, faecal porphyrin increased at mid pregnancy possibly in response to the excess porphyrins synthesized or sequestered by the kidney. Kidney enzyme and porphyrin levels, as well as blood porphyrin levels, returned to control values during late pregnancy and lactation. Faecal porphyrin however decreased to control levels in late pregnancy and then showed a significant and progressive rise in the lactation period. This may have been due to the decrease in liver porphyrin during late pregnancy and lactation, and/or excess Harderian gland porphyrins.

These results differ from the excretory pattern observed in pregnant women. An increase in urinary porphyrin and precursors has been observed during late pregnancy (Lyberatos et al., 1972), between weeks 29-34 (De Klerk et al., 1975) and up to 28 weeks after which they tailed off (Brodie et al., 1977). Faecal porphyrins did not show any consistent trends during pregnancy but levels fell post-natally (Brodie et al., 1977). Erythrocyte protoporphyrin showed a slight increase during successive months, but this was not significant (Fay et al., 1949). Lyberatos et al (1972) suggest that there is a direct relationship between porphyrin excretion and steroid hormone production in pregnancy, although De Klerk et al (1975) suggest that increased excretion may not be a result of steroid mediated induction of hepatic

haem biosynthesis as there was no correlation between total oestrogen concentration and urinary ALA and coproporphyrin concentration. However, oral contraceptives produce increased ALA excretion in normal patients, and while total coproporphyrin excretion remains the same there is an increase in Type I isomers suggesting altered liver biosynthesis (Koskelo et al., 1966). Oestrogens or combinations of oestrogenic and progestogenic agents cause increased excretion of porphyrin precursors in patients suffering from Acute Intermittent Porphyria and may cause attacks.
A cyclic appearance of symptoms premenstrually also suggests a hormonal relationship (Zimmerman et al., 1966; McColl et al., 1982). Pregnancy precipitates attacks in porphyrics, particularly in the early and late stages, when sudden changes in steroid hormone balance are more likely (Disler & Moore, 1985). One study showed that a high maternal mortality occurred in primigravidae, and those who survived tended to do much better in their first pregnancy than successive ones which were associated with more serious exacerbations of the disease (Neilson et al., 1958). Later studies (Zimmerman et al., 1966; Brodie et al., 1977) showed that although porphyrics showed more symptoms during pregnancy there was a low incidence of maternal death but a high incidence of prematurity and abortions coinciding with acute attacks.

These changes in excretion of porphyrin and its precursors in both normal and porphyric pregnant females would appear to be caused by the effect of increased steroid

hormones on hepatic ALA-synthase. Dehydroepiandrosterone and other porphyrinogenic steroids occur in excess in the urine and plasma of patients with AIP both during attacks and remissions. Dehydroepiandrosterone levels are higher in pregnant than non-pregnant women (Nieschlag et al., 1974). However, in pregnant multiparous hamsters liver 5-ALA-S activity was not significantly altered by changing hormone levels, Harderian gland enzyme activity showed a slight increase in late pregnancy, but only the kidney enzyme activity showed any significant increase.

These experiments suggest that the liver and the Harderian gland may have different thresholds for steroid stimulation of 5-ALA-S activity, and that the gland is more responsive than the liver to decreasing levels of ovarian hormones (as in the age series and the ovariectomised females) resulting in decreased enzyme activity and porphyrin content. Conversely the hamster liver is less responsive to increases in the levels of ovarian hormones, (as in castrates given either oestrogen or progesterone, or during pregnancy) than the Harderian gland.

b) <u>as a result of Harderianectomy</u> (Experiment 7)

Harderianectomy of male and female hamsters does not affect either kidney porphyrin content nor enzyme activity, other than a transient increase in female kidney porphyrin at 1 month post operatively. However, the liver porphyrin content of both males and females is significantly reduced by 5 months post-operatively. Female liver enzyme activities are significantly lower at 1 and

5 months compared to controls, whereas female enzyme activities at 2 months and those of males at 5 months post-operatively were similar to controls. Blood porphyrin levels are significantly raised in both males and females at 5 months possibly as a result of the uptake of liver porphyrins. The increased availability of precursors such as glycine and succinate as a result of Harderianectomy does not lead to an increase in hepatic or kidney porphyrin synthesis as might be expected, but may be responsible for the increase in leucocyte porphyrin synthesis resulting in raised blood porphyrins.

5.

IS THE HARDERIAN GLAND A USEFUL MODEL OF PORPHYRIN BIOSYNTHESIS?

The experiments in this thesis were not intended to investigate the function of the Harderian gland but to assess its usefulness as a model for porphyrin synthesis and, in particular, to evaluate the effect of the manipulation of exogenous or endogenous steroids.

(i) The Harderian gland is unusual in porphyrin producing tissues in that it accumulates porphyrins, the function(s) of which is not known. It has been suggested that there is a failure in the regulatory mechanism of the rodent gland porphyrin synthesis, which in Myomorphs leads to the overproduction of protoporphyrin (Tomio & Grinstein, 1968). A similar porphyrin accumulation is observed in human erythropoietic protoporphyria (Moore et al., 1979; Moore & Disler, 1985; Rimington, 1985). The subsequent accumulation of protoporphyrin in the Harderian gland is due to the low activities of ferrochelatase, the final enzyme in the pathway which incorporates iron into the protoporphyrin molecule (Thompson et al., 1984). This may account for the formation of solid intraluminal accretions.

(ii) The rodent Harderian gland 5-ALA-S activities and porphyrin levels do not show the same pattern in response to hormone manipulations as the liver (Davidheiser &

Figge, 1955; Thompson et al., 1984; Experiments 3-6) further suggesting separate control mechanisms for liver and gland haem biosynthesis, as indicated by Margolis (1971).

(iii) The female hamster Harderian gland is possibly the richest source of mammalian porphyrins known, yet the male gland contains little porphyrin. This sexual dichotomy in both porphyrin content and enzyme activities suggests that sex hormones control porphyrin synthesis within the gland. This is borne out by experiments involving castration or ovariectomy which alter both the porphyrin content and enzyme activities of the gland, as well as its morphology; these changes are progressive with increasing time post-operatively. The lability of the gland to various experimental hormonal manipulations demonstrates its usefulness as a model for porphyrin biosynthesis.

(iv) Furthermore, the characteristics of control of the gland mimic human acute porphyria. Porphyrin synthesis in the Harderian gland changes during puberty (Bucana & Nadakavukaren, 1972c,1973; Payne et al., 1979) and pregnancy (Payne et al., 1979) and shows seasonal as well as cyclical variations (Payne et al., 1977b; Moore et al., 1977, 1980). Clinical evidence suggests that female sex hormones influence acute porphyria. Symptoms of the disease appear after puberty, and females are more affected than males (Goldberg et al., 1969; Rimington, 1985). Furthermore,

pregnancy or particular stages of the menstrual cycle can precipitate attacks in some individuals (Brodie et al., 1977; McColl et al., 1982). Both oestrogens and progesterones can induce porphyrin biosynthesis in vitro (Disler & Moore, 1985). Experimental porphyria normally involves the administration of chemicals to animals to produce increased excretion of haem precursors (Sweeney, 1985). While this is of value in understanding the mechanisms of haem synthesis and control, these chemicals may disturb a range of biochemical functions including hepatic drug metabolism.

In conclusion, the hamster Harderian gland is a useful model in which to study the effects of steroids on porphyrin biosynthesis since it responds so readily to both exogenous and endogenous steroid manipulations which can be assessed both biochemically and morphologically.

What experiments might be performed next?

1. Investigation into porphyrin biosynthesis.

a) The turnover time of porphyrin stores within the Harderian gland has not yet been investigated, either in terms of how long the intraluminal stores remain within the gland, nor the rate of addition to or depletion of these stores. The administration of radioactive precursors to the gland, and the examination of the gland by autoradiography at varying time intervals following the initial injection may elucidate this problem. The possible contribution of Harderian gland porphyrins to the rest of the body could be

examined by whole body autoradiography.

b) Further investigations into the extent to which the inhibition of porphyrin synthesis is androgen-dependent are likely to be fruitful, particularly those involving *in vitro* mixing of male and female gland homogenates. Preliminary investigations (G.G. Thompson, personal communication) have shown that male gland homogenates inhibit 5-ALA-S activity in female tissue. Further experiments could examine the inhibitory effect on female enzyme activity of gland homogenates from males at varying time intervals following castration or after the restoration of androgen administration. This could be accompanied by quantitative light and electron microscope studies on changes in the frequency of Type II cells and polytubular complexes and the correlation of these changes with the time course of changes in males' own enzyme activity. Conversely, one could examine the effect of androgen administration to ovariectomised females for varying periods on the ability of their glands to inhibit enzyme activity in normal female glands during *in vitro* mixing of homogenates, and quantify at light and electron microscope levels the degree of histological virilization at each time period. These measurements would elucidate correlations between increasing inhibitory ability and increases in male morphological features. A subsequent important aspect would be the isolation of a cell fraction containing the inhibitory factor, as an essential pre-requisite to the eventual biochemical determination of the molecule(s) contained therein.

2. Investigation into the role of the Harderian gland.

a) The role of the rodent Harderian gland in the immune system has yet to be investigated, although the gland plays an important part in protecting the avian eye from both topical and systemic antigens (Mueller et al., 1971; Burns, 1976), and contains large numbers of plasma cells (Bang & Bang, 1968; Wight et al., 1971a; Burns, 1975) and lymphocytes (Mueller et al., 1971). The rat lacrimal gland is part of the secretory immune system since it contains immunoglobulin producing cells with IgA producing cells predominant. Macrophages and lymphocytes are also observed and these mononuclear cells and the plasma cells can be increased by systemic and/or local immunologic stimulation of the gland (Gudmundson et al., 1984). The abnormal localization of porphyrin within the interstitium of the Harderian gland of ovariectomised hamsters also results in increased numbers of macrophages, lymphocytes and plasma cells and led Payne et al (1985) to suggest that many aspects of gland degeneration suggest an immune response. Experiments involving systemic and/or topical administration of antigens to conventional and germ free hamsters, with or without lacrimal glands, may provide further evidence for this postulated involvement.

b) The Harderian gland is thought to be part of the retinal-pineal system since it is strongly influenced by environmental lighting in addition to changes in hormone

levels. Constant illumination regimes produce degenerative changes within a few days (Strum & Shear, 1982) which can only be replicated by several months of ovariectomy. Furthermore, short day regimes can produce changes in female gland morphology which cannot be replicated at all by ovariectomy (Nadakavukaren & Lin, 1983). The Harderian gland may be involved in photoreception and seasonal breeding. The porphyrins synthesised by the gland are phototransducing molecules which might in some way affect retinal photoreception. Hamsters are seasonal breeders responding markedly to changing day length (Elliot, 1981) and recent studies have shown that the porphyrin content of the female gland shows seasonal variation with levels being lowest in winter and greatest in summer (Payne et al., 1977b). Alternatively, the Harderian gland might act as an accessory pineal, since the retina, pineal and Harderian gland all synthesise melatonin. Seasonal differences occur in the methylating capacities of the 5-hydroxyindoles in male hamsters in the retina, pineal and Harderian gland (Balemans et al.,1983), and diurnal rhythms and sex differences are found in melatonin concentrations with levels being higher in females (Hoffman et al., 1985).

The possible involvement of the Harderian gland as part of the retinal-pineal system could be investigated by examining the inter-relationships between environmental cues and gland morphology and function in several ways.

Firstly, by examining changes in porphyrin biosynthesis in the Harderian gland and other tissues such as liver as a result of changes in lighting and/or temperature regimes. In order to see if such changes are hormonally dependent, experiments would involve both intact and gonadectomised animals.

Secondly, the role of the Harderian gland in influencing the response of the reproductive system to seasonal change could be studied by comparing reproductive tract weights and activities in both Harderianectomised and intact animals in different lighting/temperature conditions. The effects of Harderianectomy and/or pinealectomy could also be examined.

Thirdly, the possible involvement of porphyrin synthesis in controlling the availability of precursors for melatonin formation could be examined by determining possible circadian rhythms in porphyrin content and enzyme activity in the Harderian and pineal glands. Any such changes could be correlated with serotonin levels and the activity of tryptophan pyrrolase within these tissues. Tryptophan pyrrolase is a haemoprotein which catalyses the rate-limiting step of tryptophan degradation and thus controls the availability of tryptophan for serotonin and melatonin synthesis.

TABLE 1

The comparative values of porphyrin content (nmol/g) and 5-ALA-S activity (nmol ALA formed/h/g protein) in the Harderian gland, liver and kidney of the female golden hamster.

Female golden hamster	Harderian gland n = 21	Liver n = 21	Kidney n = 12
Porphyrin content (nmol/g)	3931 ± 365	2.94 ± 1.02	14.01 ± 2.25
Activity of 5-ALAS (nmol ALA formed/h/ g protein)	3793 ± 744	100.42 ± 18.20	92.57± 26.10

All figures are the means ± SEM.

TABLE 2

Composition and pH of fixatives (per 100ml)

A.F.A. (Alcohol/formaldehyde/acetic acid)		pH 4.00
Formaldehyde, 40%	5ml	
90% alcohol	90ml	
Glacial acetic acid	5ml	
Bouin's fixative		pH 2.17
Picric acid, saturated, aqueous	71ml	
Formaldehyde, 40%	24ml	
Glacial acetic acid	5ml	
Zenker's formalin fixative		pH 4.00
Mercuric chloride	5gm	
Potassium dichromate	2.5gm	
Sodium sulphate	1gm	
Distilled water	95ml	
Formaldehyde 40%, before use,	5ml	
Glutaraldehyde fixative		pH 7.20
0.1M phosphate buffer, pH 7.4	88ml	
Glutaraldehyde, 25%	12ml	

TABLE 3

HPLC analyses of Porphyrin Free Acids in fixatives and solvents

% of Porphyrin Free Acids

Routine	Hexacarboxylic -porphyrin	Copro- porphyrin	Proto- porphyrin
1. AFA			
Fixative	ND	39.32	60.68
All Alcohols	ND	ND	ND
3 x Chloroform	ND	ND	ND
2. Bouin's			
Fixative	*		
70 and 90% Alcohols	6.72	ND	93.28
3 x Absolute Alcohol	ND	ND	100.00
3 x Chloroform	ND	ND	100.00
3. Dichromate			
Fixative	*		
All Alcohols	ND	ND	
3 x Chloroform	ND	ND	
4. Glutaraldehyde			
Fixative	ND	ND	ND
70% and 90% Alcohols	ND	ND	100.00
3 x Absolute Alcohol	ND	ND	ND
3 x Chloroform	ND	ND	ND

ND = not detectable

* = sampling impossible (see text)

	Intact females (n = 20)	Ovariectomised Females (n = 22)	Androgen-treated Females (n = 11)	Analysis of variance (F)	Intact males (n = 11)
Intraluminal porphyrin accretions/mm^2 section	8.49 ± 0.92	8.60 ± 1.31	3.52 ± 0.57**	5.01 $p < 0.05$	-
Total porphyrin content	3300 ± 302	2947 ± 236	706 ± 82**	18.63 $p < 0.001$	43 ± 4
5-ALA-S activity	5176 ± 1492	1826 ± 442*	430 ± 112**	9.62 $p < 0.001$	169 ± 20
% Tubules with Type II cells	0.6 ± 0.3	8.7 ± 1.1**	38.0 ± 5.1**	80.21 $p < 0.001$	98.8 ± 1.0
	(n = 3)	(n = 3)	(n = 3)		(n = 3)
Polytubular complexes present					
i) % animals	-	-	100		100
ii) % cells	-	-	93 ± 2		100

Differs significantly from intact (control) females; *$p < 0.05$, ** $p < 0.01$

Table 4 The porphyrin content and porphyrinogenic enzyme activity of the hamster Harderian gland, together with a number of morphological parameters usually associated with the male gland, in intact female hamsters (n = 20), ovariectomised females (n = 22), androgen-treated females (4.5mg testosterone/week, n = 11) and intact males (n = 11). This table shows i) the frequency of intraluminal porphyrin accretions/mm^2 of a 5 μm section, ii) the total porphyrin content (nmol/g tissue), iii) the activity of the porphyrinogenic enzyme 5-aminolaevulinic acid synthetase (ALA-S) (nmol ALA formed/g protein/hour), iv) the % tubules containing Type II cells, and v) the % animals (n = 3 per group) and the % cells (50 per animal) containing polytubular complexes. All figures are means ± SEM.

	Intact females (n = 20)	Ovariectomised females (n = 22)	Androgen-treated females (n = 11)	Analysis of variance (F)	Intact males (n = 11)
% Area of tubule degeneration	3.57 ± 0.77	5.43 ± 0.91	4.51 ± 0.92	1.36 n.s.	-
Tubules with luminal neutrophils/mm^2 section	1.36 ± 0.24	2.30 ± 0.35*	0.76 ± 0.13	6.33 $p < 0.01$	-
Large interstitial porphyrin accretions/ mm^2 section	0.10 ± 0.03	0.42 ± 0.09	0.82 ± 0.32**	6.87 $p < 0.01$	-
Small interstitial porphyrin accretions/ mm^2 section	1.78 ± 0.43	6.93 ± 1.8**	4.67 ± 1.03	8.86 $p < 0.001$	-
Mast cell/mm^2 section	6.71 ± 1.51	25.73 ± 4.30**	11.19 ± 2.75	10.41 $p < 0.001$	0.31 ± 0.14
Mast cells/mm capsule	0.73 ± 0.12	1.82 ± 0.27**	0.49 ± 0.12	15.98 $p < 0.001$	0.06 ± 0.01

Differs significantly from intact (control) females; *$p < 0.05$, **$p < 0.01$

Table 5 A number of morphological parameters usually associated with gland degeneration in the Harderian glands of intact female hamsters (n = 20), ovariectomised females (n = 22), androgen-treated females (4.5 mg testosterone/week, n = 11) and intact males (n = 11). This table shows i) the percentage area of tubule degeneration in 5 μm sections, ii) the frequency of tubules containing luminal neutrophils/mm^2 of a 5 μm section, the frequencies of iii) large and iv) small (or phagocytosed) interstitial porphyrin accretions/ mm^2 of a 5 μm section, v) the frequency of interstitial mast cells/mm^2 of a 5 μm section and vi) the frequency of mast cells/mm of gland capsule. All figures are means ± SEM.

	Interstitial mast cells	Small interstitial porphyrin deposits	Tubules containing neutrophils	% Area of tubule degeneration	Intraluminal porphyrin accretions	5-ALA-S activity
Small interstitial porphyrin deposits	0.38***					
Tubules containing neutrophils	0.25	0.32**				
% Area of tubule degeneration	0.49****	0.45****	0.38***			
Intraluminal porphyrin accretions	-0.07	0.02	-0.05	-0.14		
5-ALA-S activity	-0.33**	-0.24	-0.27*	-0.34***	-0.10	

df 56 $*p < 0.05$, $**p < 0.02$, $***p < 0.01$, $****p < 0.001$.

TABLE 6

The coefficient of correlation (r) obtained from a basic statistical test for 2 variables. The variables are degenerative features occurring during a time course of changes following ovariectomy, and also the 5-ALA-S activity and the number of intraluminal porphyrin accretions.

	Age in months 2	6	12	18	24	Analyses of variance (F)
Interstitial mast cells/mm^2	18.30± 2.73	2.21± 1.41	13.72± 5.38	3.22± 0.85	5.01± 1.29	6.66, $p < 0.001$
Peripheral mast cells/mm	1.00± 0.18	0.14± 0.08	0.66± 0.22	0.27± 0.05	0.33± 0.09	5.63, $p < 0.001$
Intraluminal porphyrin accretions/mm^2	28.58± 1.70	4.72± 0.90	16.94± 1.65	8.54± 1.43	8.37± 1.02	53.06, $p < 0.001$
Large interstitial porphyrin deposits/mm^2	0.03± 0.02	0.04± 0.02	0.12± 0.05	0.10± 0.04	0.54± 0.16	7.84, $p < 0.001$
Small interstitial porphyrin deposits/mm^2	0.02± 0.00	0.13± 0.09	1.04± 0.32	0.88± 0.18	4.30± 1.29	8.96, $p < 0.001$
Mitotic figures/mm^2	0.48± 0.11	0.01± 0.001	0.42± 0.14	0.14± 0.05	0.33± 0.24	2.27, $p < 0.05$
Tubules containing neutrophils/mm^2	0.00± 0.00	0.07± 0.04	0.58± 0.19	0.37± 0.08	1.71± 0.40	12.19, $p < 0.001$
% Area of tubule degeneration	1.03± 0.38	1.33± 0.39	1.63± 0.46	4.13± 1.33	5.33± 1.25	5.18, $p < 0.01$
% Type II cells	0.18± 0.20	9.85± 2.32	0.59± 0.23	16.38± 2.38	18.16± 3.65	22.89, $p < 0.001$

Table 7

Changes occurring in morphological parameters in the Harderian glands of female hamsters from post puberty until senescence (Experiment 5).
All figures are means ± SEM. 't' values and their significance levels are given in the text.

	Age in months 2	6	12	18	24	30	Analyses of variance (F)
Interstitial mast cells/mm^2	0.69± 0.21	0.04± 0.02	0.15± 0.06	0.06± 0.03	0.04± 0.02	0.26± 0.25	3.94, $p < 0.01$
Peripheral mast cells/mm	0.06± 0.02	0.00± 0.00	0.001 ± 0.001	0.005± 0.001	0.006± 0.004	0.01± 0.001	6.02, $p < 0.001$
Intraluminal accretions/mm^2	0.11± 0.03	0.00± 0.00	0.00± 0.00	0.16± 0.06	0.37± 0.08	0.43± 0.12	See Text
Large interstitial porphyrin deposits/mm^2	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00, ns
Small interstitial porphyrin deposits/mm^2	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00, ns
Mitotic figures/mm^2	0.21± 0.05	0.07± 0.04	0.00± 0.00	0.03± 0.02	0.02± 0.01	0.01± 0.001	9.41, $p < 0.001$
Tubules containing neutrophils/mm^2	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.30± 0.29	0.015± 0.008	0.01± 0.01	1.09, ns
% Area of tubule degeneration	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00, ns
% Type II cells	93.14± 2.66	97.65± 1.13	98.81± 0.97	98.37± 0.84	97.17± 0.83	97.04± 0.15	2.35, ns

Table 8

Changes in morphological parameters in the Harderian glands of male hamsters from post puberty until senescence (Experiment 5).
All figures are means ± SEM: 't' values and their significance levels are given in the text.

Figure 1

Diagram showing the common gland in the lower lid (a) from which the Harderian gland and the lacrimal gland are thought to arise (according to Wolff, 1961). Migration of the medial portion of the common gland gives rise to the Harderian gland (d) in the medial (M) canthus of the eye. Migration of the gland laterally gives rise to the lacrimal gland, firstly in the lower lid (b) (where it is located in amphibia, reptiles and birds) and secondly in the upper lid (where it is located in mammals), in the lateral (L) canthus of the eye.

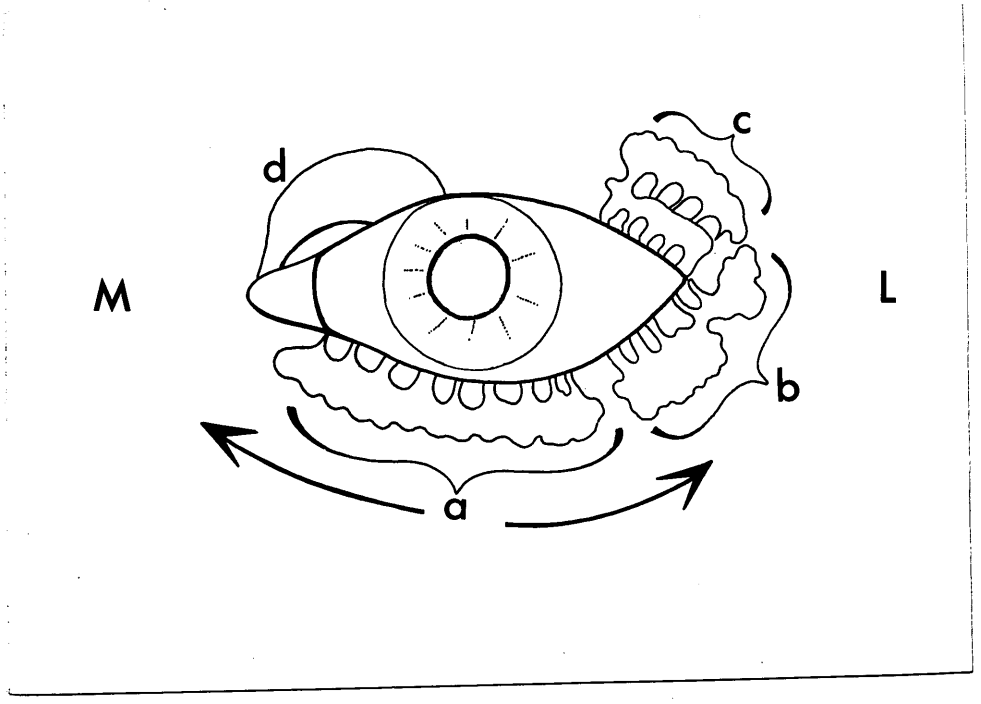
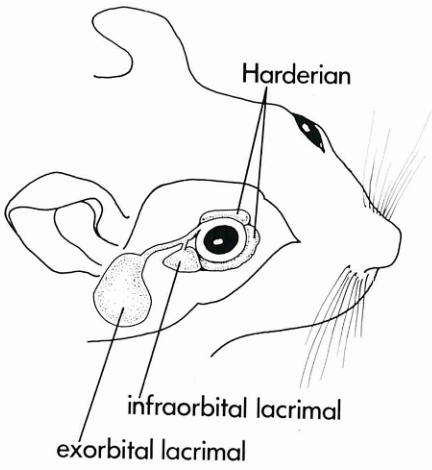

M
L
d
c
b
a

Figure 2

Diagram showing the relative positions of the Harderian, infraorbital lacrimal and exorbital lacrimal glands in the rodent (from Venable & Grafflin, 1940).

Figure 3

Photograph (taken under ultraviolet illumination) showing the head of a female golden hamster in which the eye has been removed to reveal the position of the Harderian gland. The red appearance of the gland is due to the autofluorescence of its high porphyrin content.


Harderian
infraorbital lacrimal
exorbital lacrimal

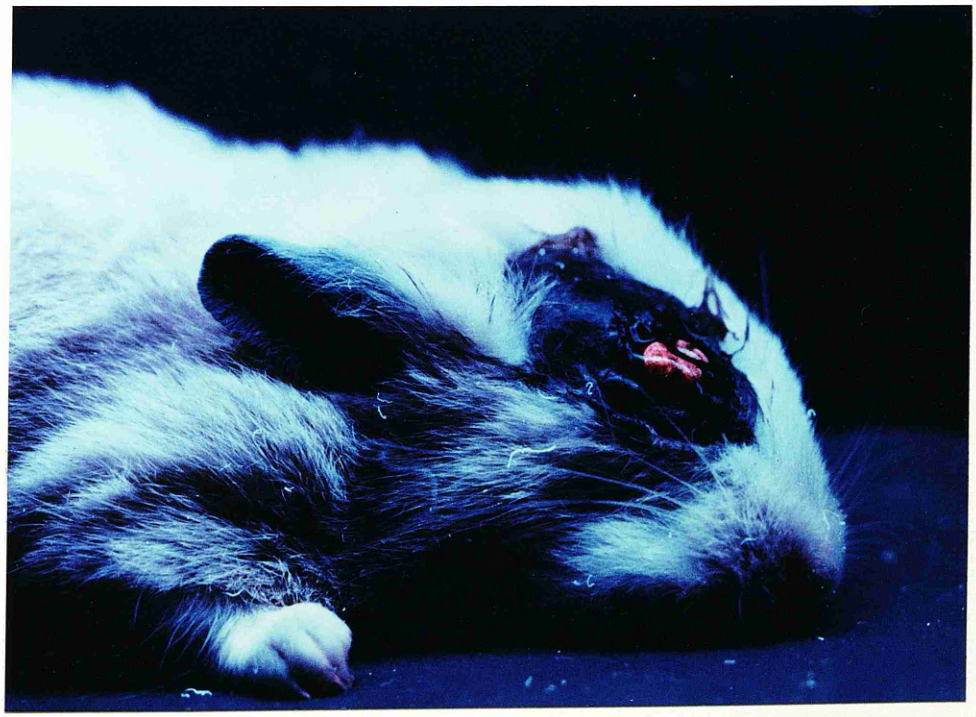
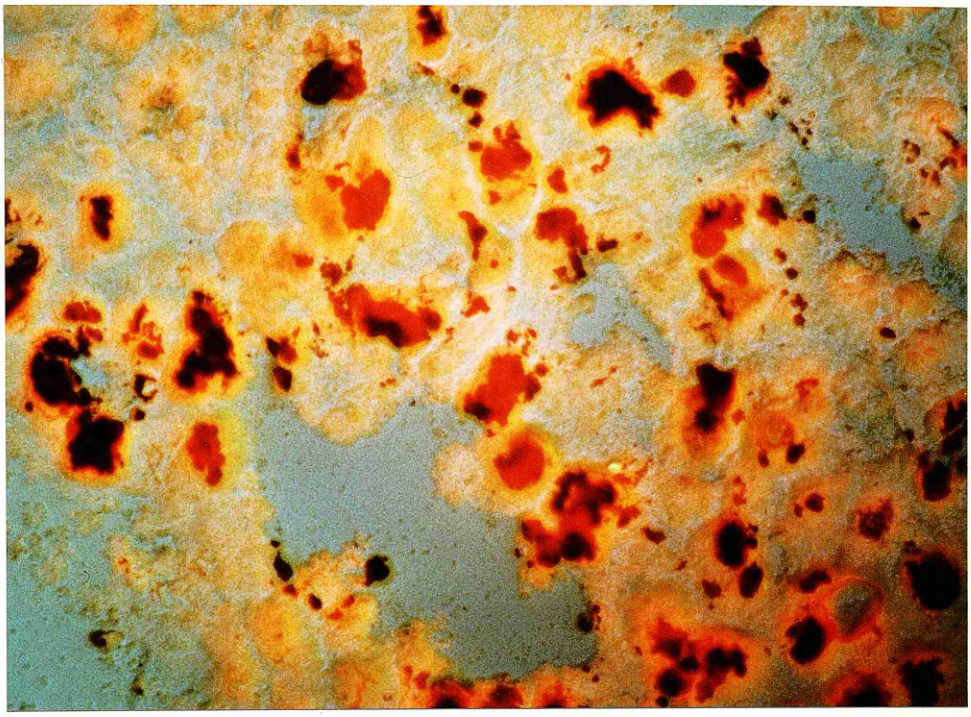


Figure 4

Light micrograph of an unstained frozen section (20 μm) of a female hamster Harderian gland. The gland section was illuminated under ultraviolet light to show the red fluorescence of the solid intraluminal porphyrin accretions. (x 120).

Figure 5

Light micrograph of an H & E stained section (5 μm) of a female hamster Harderian gland. The gland section was illuminated under a mixture of ultraviolet and white/yellow light in order to show both the dark red fluorescence of intraluminal porphyrin accretions and the gland tissue. (x 110)



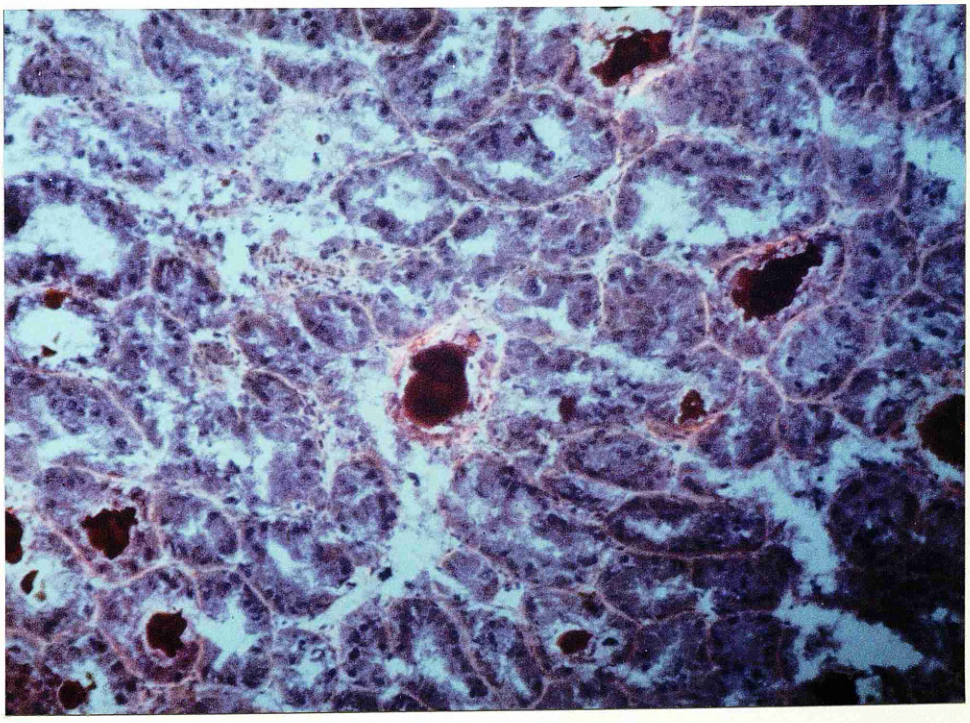
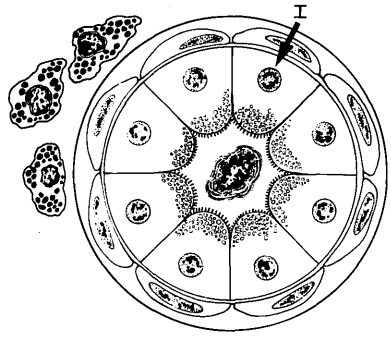


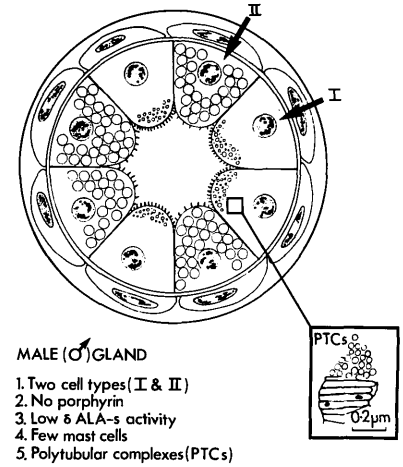
Figure 6

A summary of morphological and biochemical sex differences in the Harderian gland of the golden hamster (for details see text).



FEMALE (♀) GLAND

1. One cell type (I)
2. Makes porphyrin
3. High δ-amino laevulinate synthetase (δ ALA-s) activity
4. Numerous mast cells

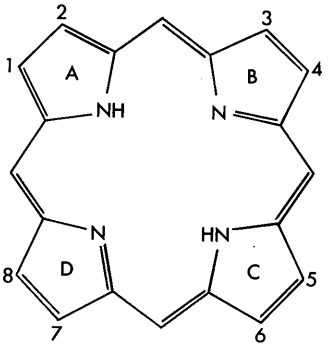


MALE (♂) GLAND

1. Two cell types (I & II)
2. No porphyrin
3. Low δ ALA-s activity
4. Few mast cells
5. Polytubular complexes (PTCs)

Figure 7

The structure of the porphyrin molecule showing the Fischer nomenclature. The types of porphyrin are defined by the substituents of the side chains of positions 1 to 8 in Fischer nomenclature.



PORPHYRIN ($C_{20}H_{14}N_4$)
(FISCHER)

	1	2	3	4	5	6	7	8
Uroporphyrin III	A^H	P^R	A^H	P^R	A^H	P^R	P^R	A^H
Coproporphyrin III	Me	P^R	Me	P^R	Me	P^R	P^R	Me
Harderoporphyrin	Me	V	Me	P^R	Me	P^R	P^R	Me
Iso-Harderoporphyrin	Me	P^R	Me	V	Me	P^R	P^R	Me
Protoporphyrin	Me	V	Me	V	Me	P^R	P^R	Me

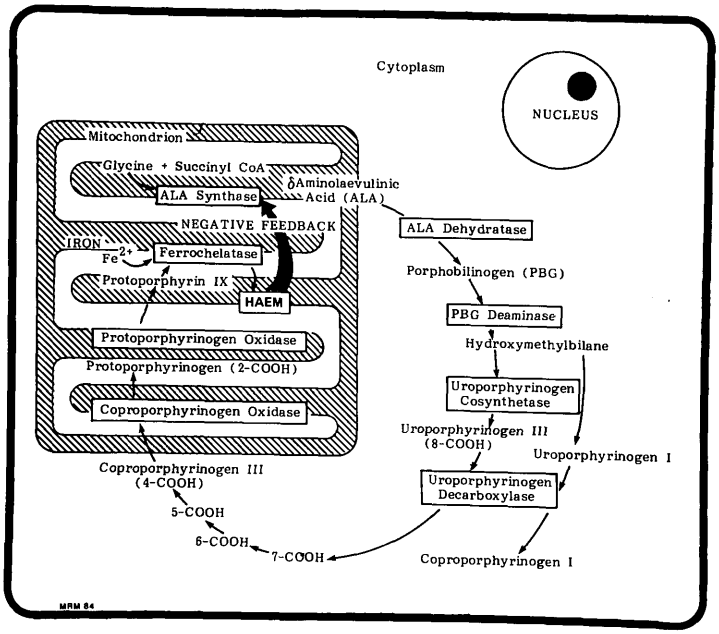
Me = $-CH_3$ (methyl)
A^H = $-CH_2CO_2H$ (acetyl)
P^R = $-CH_2CH_2CO_2H$ (propionyl)
V = $-CH{=}CH_2$ (vinyl)

Figure 8

A schematic diagram of the pathway of haem biosynthesis in the cell showing i) the mitochondrial enzymes and ii) the cytoplasmic enzymes (from Moore & Disler, 1985).

Figure 9

Control of haem biosynthesis. The enzyme ALA synthase and the free haem pool are the central control components. Biosynthesis may be affected by substrate availability and by the presence or absence of the end product, haem. Haem acts on ALA synthase not only by limiting its formation, but also by inhibiting its activity and trans-membrane transfer. Haem concentrations in the cell are maintained by the balance between synthesis and degradation (from Moore & Disler, 1985).


Cytoplasm
NUCLEUS
Mitochondrion
Glycine + Succinyl CoA
ALA Synthase
δAminolaevulinic Acid (ALA)
NEGATIVE FEEDBACK
IRON
Fe2+
Ferrochelatase
Protoporphyrin IX
HAEM
Protoporphyrinogen Oxidase
Protoporphyrinogen (2-COOH)
Coproporphyrinogen Oxidase
Coproporphyrinogen III (4-COOH)
5-COOH
6-COOH
7-COOH
ALA Dehydratase
Porphobilinogen (PBG)
PBG Deaminase
Hydroxymethylbilane
Uroporphyrinogen Cosynthetase
Uroporphyrinogen III (8-COOH)
Uroporphyrinogen I
Uroporphyrinogen Decarboxylase
Coproporphyrinogen I
MRM 84

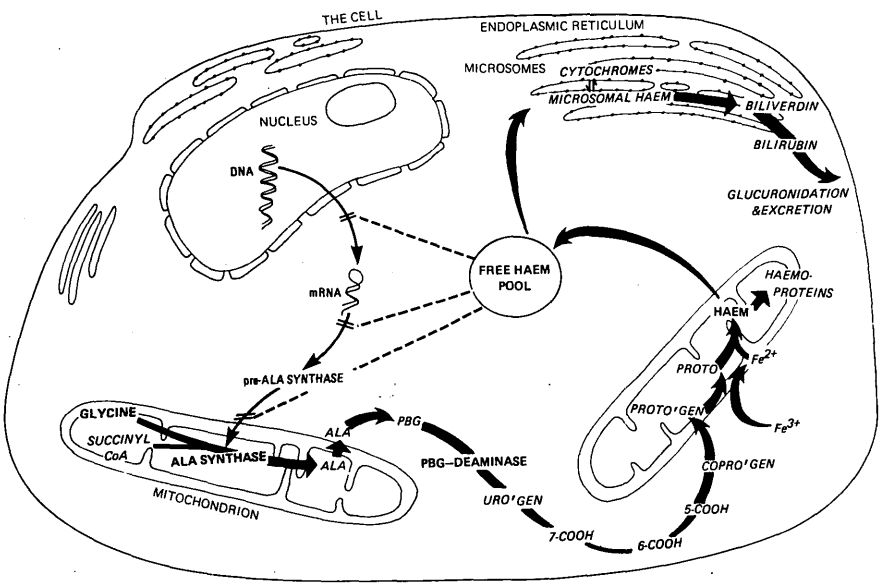
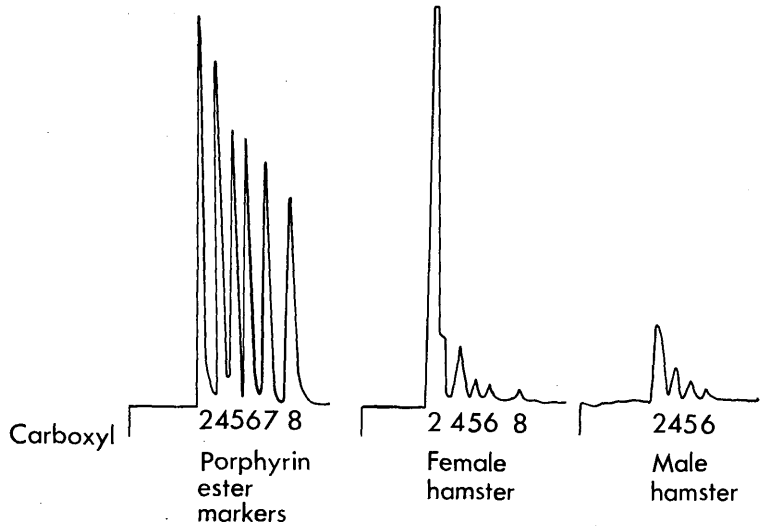

THE CELL
ENDOPLASMIC RETICULUM
MICROSOMES
CYTOCHROMES
MICROSOMAL HAEM
BILIVERDIN
BILIRUBIN
GLUCURONIDATION &EXCRETION
NUCLEUS
DNA
mRNA
FREE HAEM POOL
HAEMO-PROTEINS
HAEM
pre-ALA SYNTHASE
PROTO
Fe2+
PROTO'GEN
Fe3+
GLYCINE
SUCCINYL CoA
ALA SYNTHASE
ALA
ALA
PBG
PBG-DEAMINASE
COPRO'GEN
URO'GEN
5-COOH
7-COOH
6-COOH
MITOCHONDRION

Figure 10

High performance liquid chromatography (HPLC) chromatograms of the carboxyl groups in porphyrin ester markers, female hamster Harderian glands and male hamster Harderian glands. Proto-porphyrin (2 carboxyl groups) occurs predominantly in both female and male Harderian glands. Harderoporphyrin (3 carboxyl groups), when present, appears as a shoulder on the protoporphyrin peak. Coproporphyrin (4 carboxyl groups) is the other main constituent porphyrin of the gland.

Harderian gland HPLC chromatograms



Figures 11-14 are of glutaraldehyde-fixed tissue which was processed for light microscopy (Fig.11), dewaxed and re-embedded for transmission electron microscopy (Figs. 12-14). (For details see Payne et al., 1985).

Figure 11

Light micrograph of the Harderian gland in an ovariectomised female hamster showing intraluminal porphyrin accretions (*), large interstitial porphyrin deposits (O), and small interstitial porphyrin deposits (arrows), (5 µm thick tissue section, 0.1% Toluidine blue lightly counterstained with eosin). x 300

Figure 12

Electron micrograph showing the crystalline nature of the intraluminal porphyrin accretion surrounded by epithelial cells containing small lipid vacuoles (Type 1). x 5,600

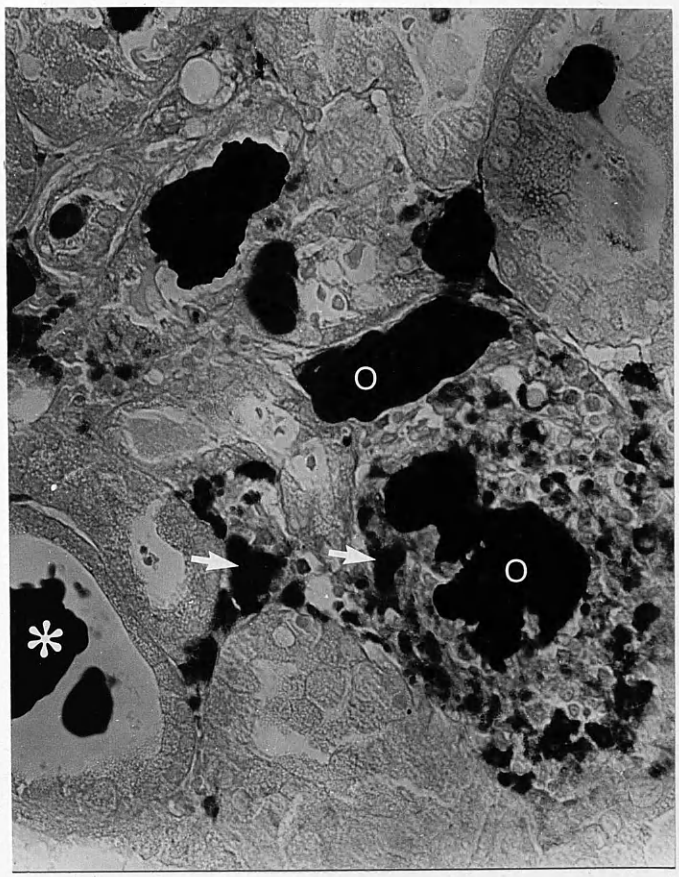
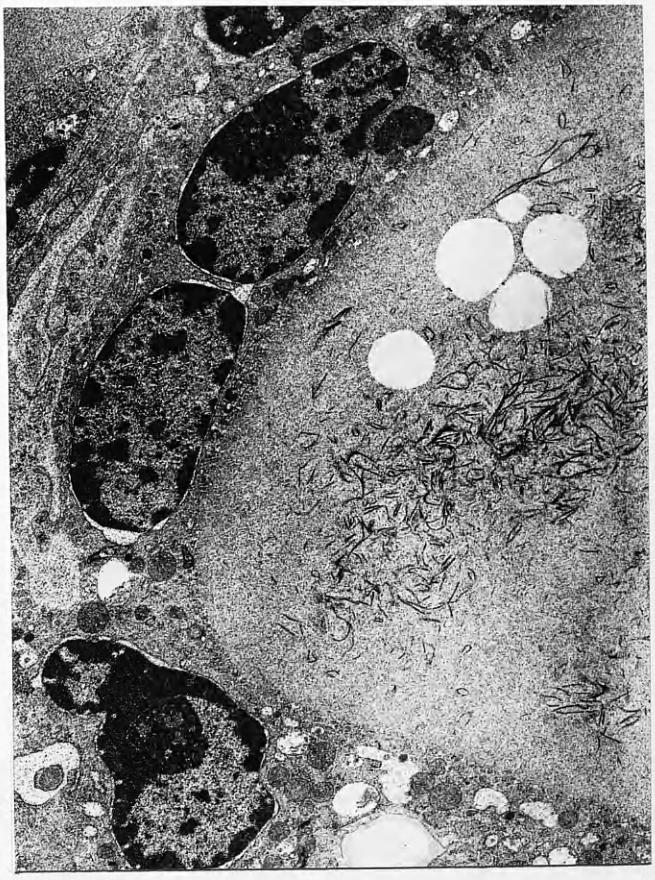

O
O
*

Figure 13

A large interstitial porphyrin deposit surrounded by macrophages whose lateral walls have broken down to form a foreign body giant cell. x 6,000

Figure 14

An interstitial macrophage containing three clumps of porphyrin within the cytoplasm. These porphyrin deposits range from dispersed needle-like crystals (A) to dense aggregates (B). x 12,600



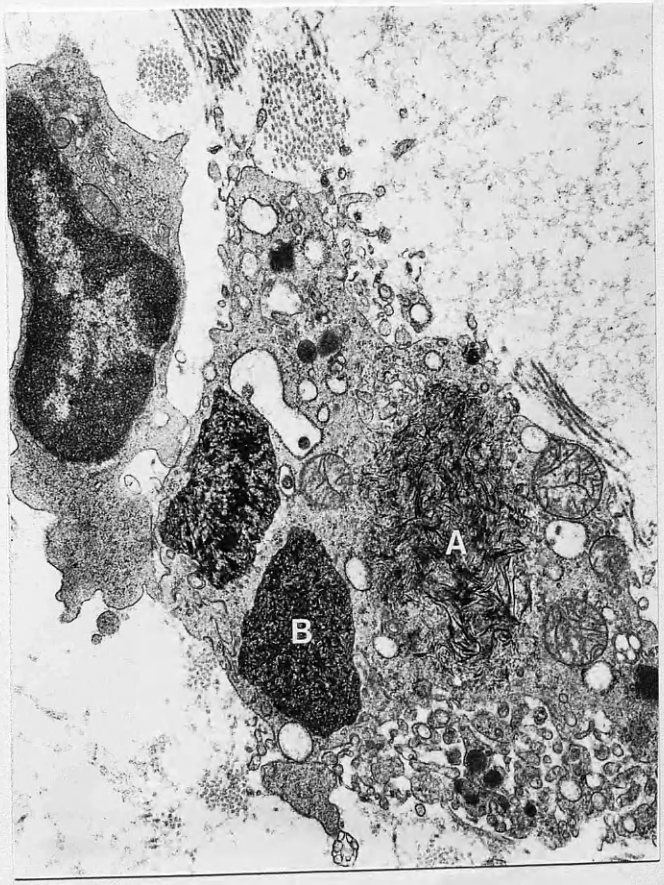
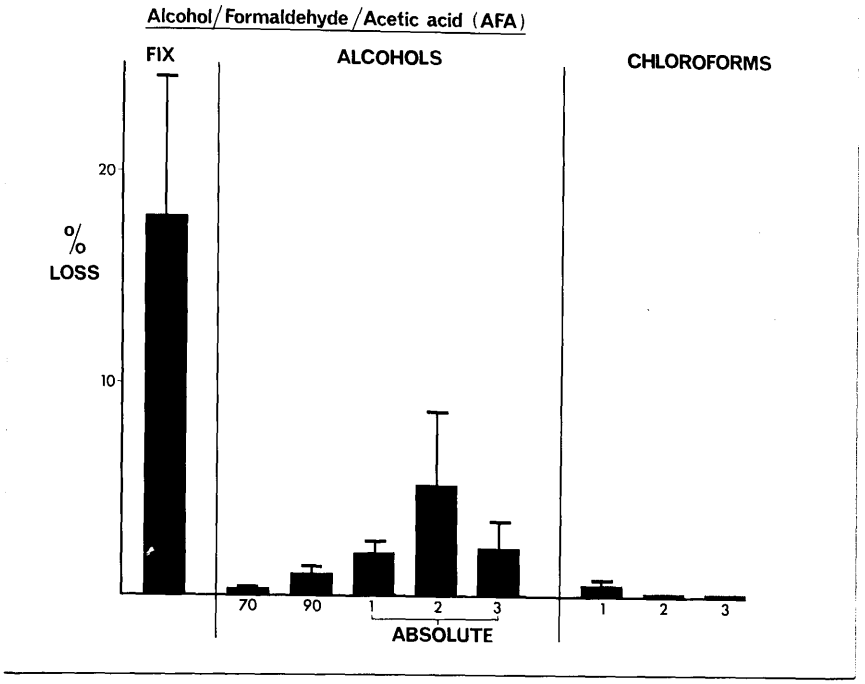

A
B

Figure 15

Histograms showing the percentage loss of porphyrin from female hamster Harderian glands after fixation in A.F.A. (Alcohol/formaldehyde/acetic acid), subsequent dehydration in 70%, 90% and 1st, 2nd and 3rd absolute alcohols and clearing in 1st, 2nd and 3rd chloroforms, as determined by spectrofluorometric assays.

Figure 16

Histograms showing the percentage loss of porphyrin from female hamster Harderian glands after fixation in Bouin's, subsequent dehydration in 70%, 90% and 1st, 2nd and 3rd absolute alcohols and clearing in 1st, 2nd and 3rd chloroforms, as determined by spectrofluorometric assays.


Alcohol/Formaldehyde/Acetic acid (AFA)
FIX
ALCOHOLS
CHLOROFORMS
20
%
LOSS
10
70
90
1
2
3
ABSOLUTE
1
2
3

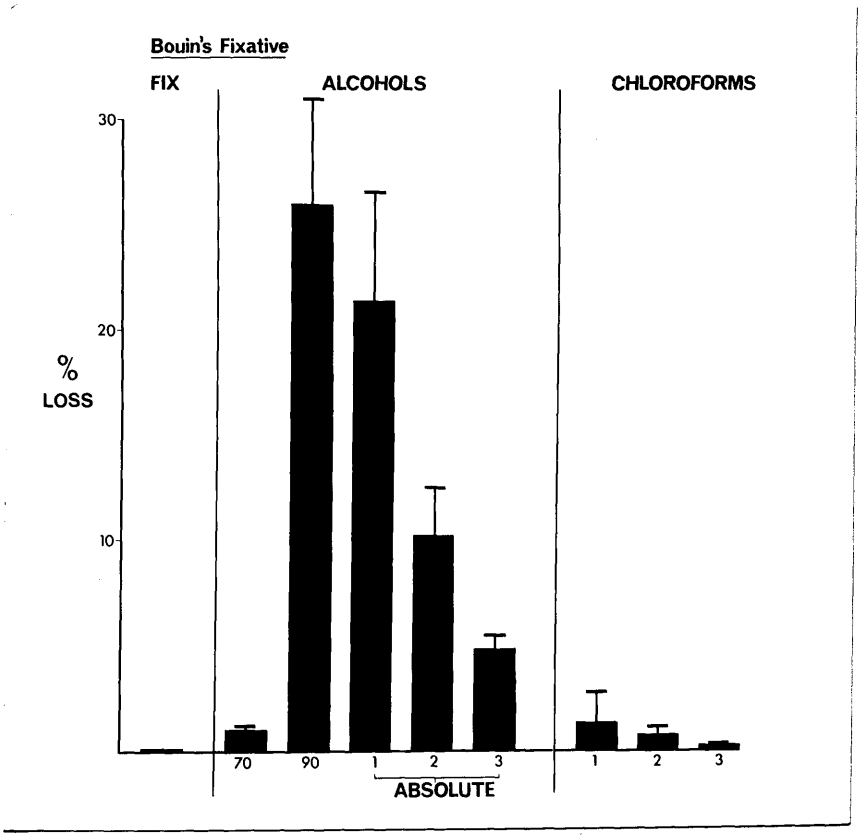

Bouin's Fixative
FIX
ALCOHOLS
CHLOROFORMS
30
20
%
LOSS
10
70
90
1
2
3
ABSOLUTE
1
2
3

Figure 17

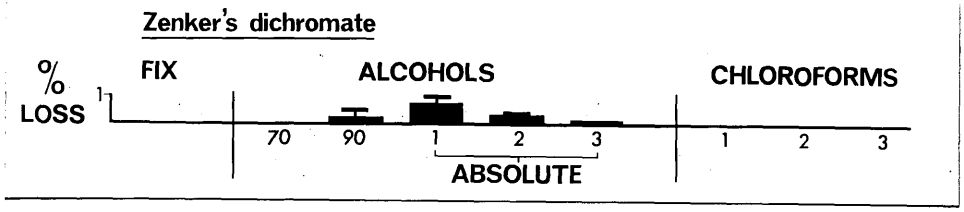
Histograms showing the percentage loss of porphyrins from female hamster Harderian glands fixed in Dichromate, dehydrated in 70%, 90%, 1st, 2nd and 3rd absolute alcohols and cleared in 1st, 2nd and 3rd chloroforms, as determined by spectrofluorometric assays.

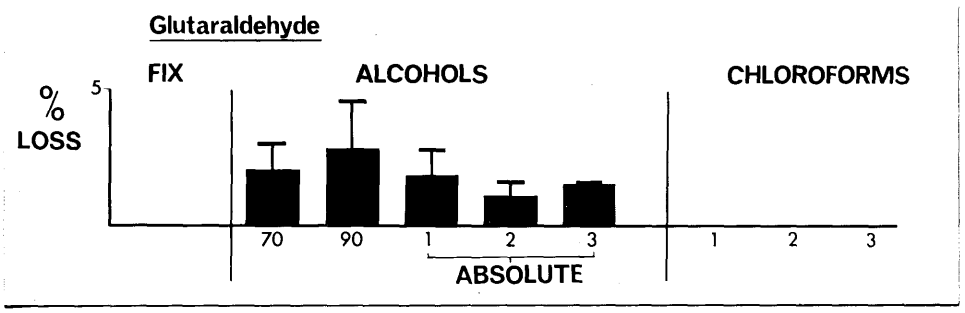
Figure 18

The percentage loss of porphyrins from female hamster Harderian glands fixed in 3% Glutaraldehyde, dehydrated in 70%, 90%, 1st, 2nd and 3rd absolute alcohols and cleared in 1st, 2nd and 3rd chloroforms, as determined by spectrofluorimetric assays.

Figure 19

The percentage loss of porphyrins from female hamster Harderian glands fixed in 3% Glutaraldehyde, dehydrated in 70%, 90%, 1st, 2nd and 3rd absolute alcohols and cleared in 1st, 2nd and 3rd amyl acetates, as determined by spectrofluorometric assays.


Zenker's dichromate
%
LOSS
1
FIX
ALCOHOLS
CHLOROFORMS
70
90
1
2
3
ABSOLUTE
1
2
3


Glutaraldehyde
FIX
ALCOHOLS
CHLOROFORMS
%
LOSS
5
70
90
1
2
3
ABSOLUTE
1
2
3

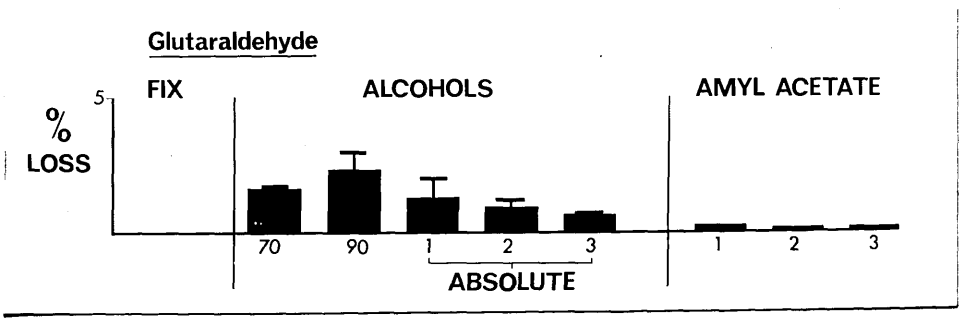

Glutaraldehyde
FIX
ALCOHOLS
AMYL ACETATE
%
LOSS
5
70
90
1
2
3
ABSOLUTE
1
2
3

Figure 20a

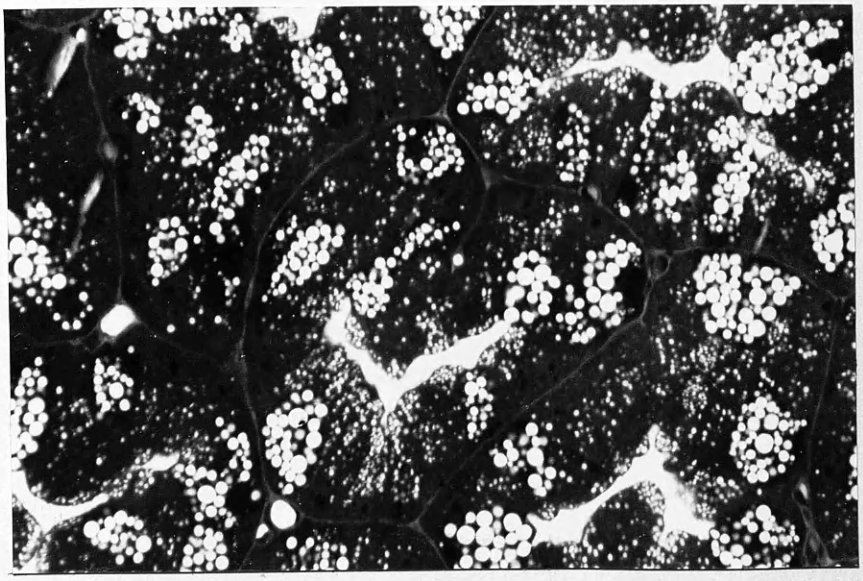
An area of male hamster Harderian gland. The tubule walls are composed of epithelial cells with small lipid vacuoles (Type 1) and cells with extremely large vacuoles (Type II). No porphyrin accretions are present in the lumen (5 μm thick tissue section, H & E). x 450

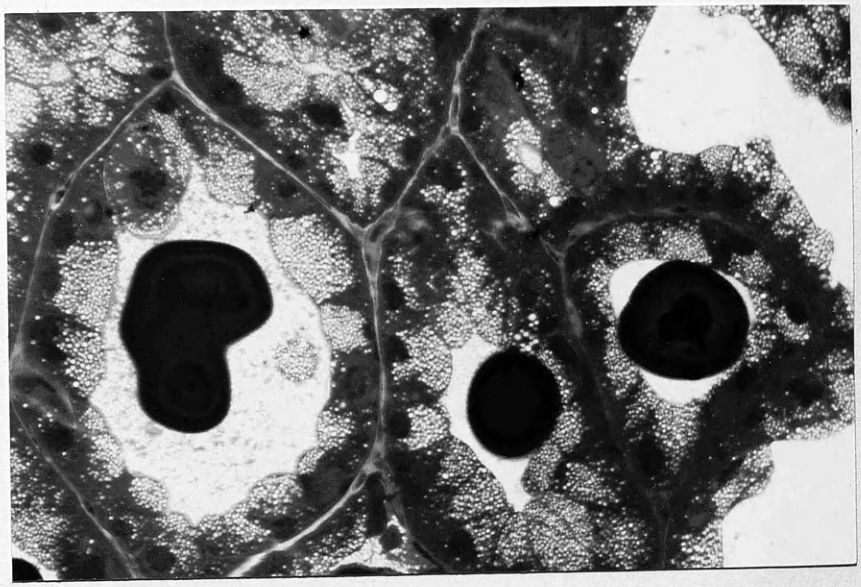
Figure 20b

An area of female hamster Harderian gland. The tubule walls are composed of Type I cells only. Intraluminal porphyrin accretions may be seen (5 μm thick tissue section, H & E). x 360

Figure 20c

An area of Harderian gland from an androgen-treated ovariectomised female hamster. Type II cells with large lipid vacuoles clearly present. Intraluminal porphyrin stores (not seen here) are less frequent than in control females (5 μm thick tissue section, H & E). x 470





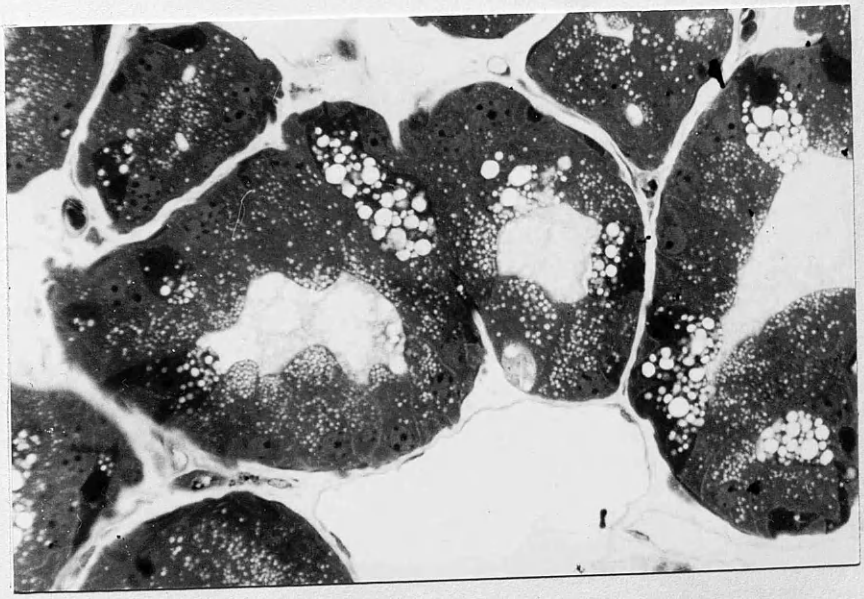
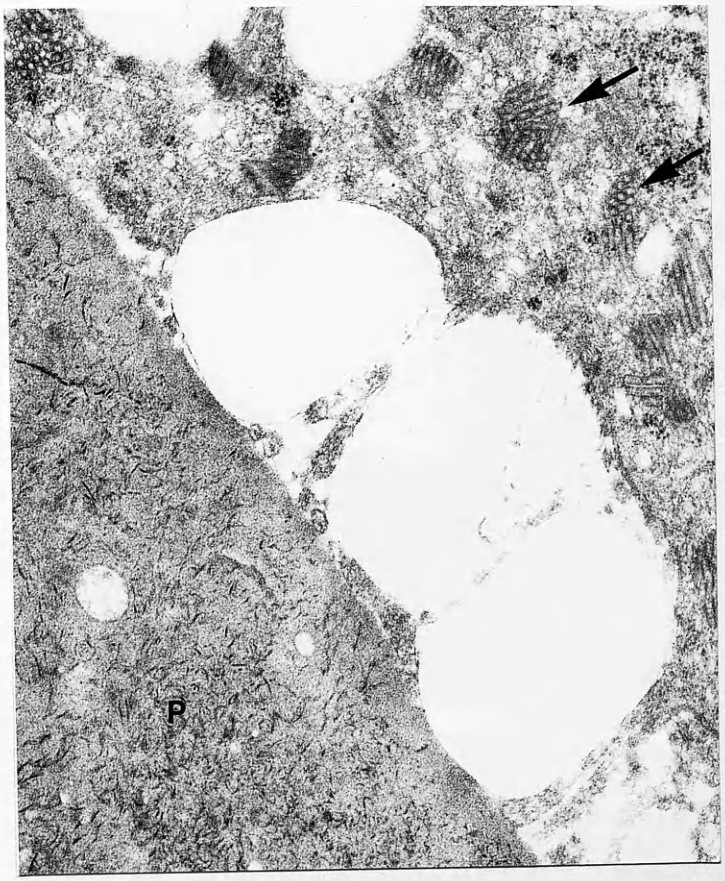


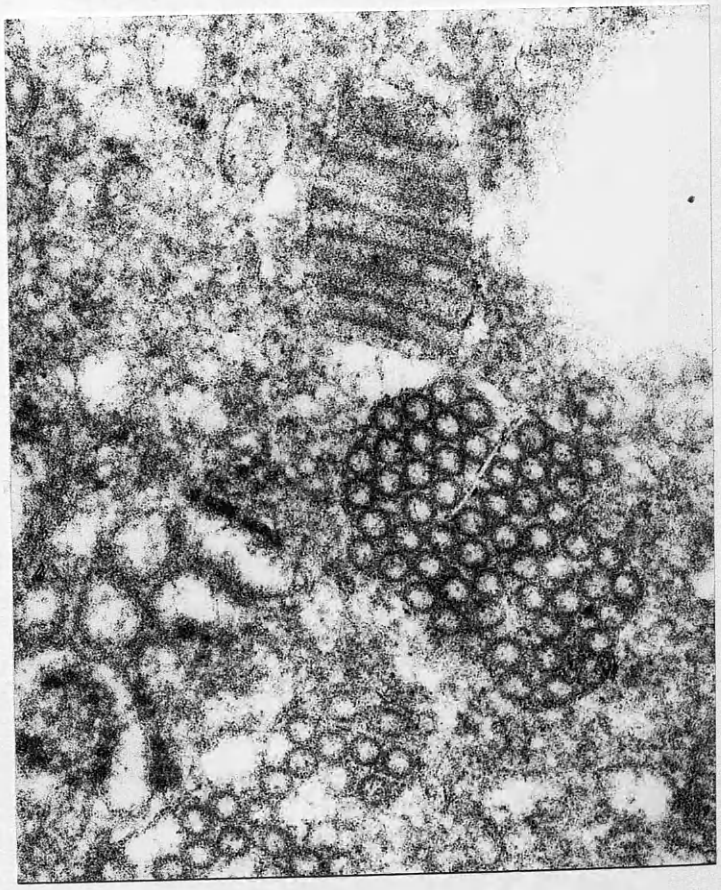
Figure 21

An area of apical cytoplasm from an epithelial cell of the Harderian gland of an androgen-treated ovariectomised female hamster. Several polytubular complexes (arrows), characteristic of male gland cells, can be seen. An intraluminal porphyrin accretion (P) is present.
x 30,000

Figure 22

Polytubular complexes from the Harderian gland of an androgen-treated ovariectomised female hamster.
x 85,000


P



Figures 23 - 30

Changes in biochemical and morphological parameters after ovariectomy for 5 months (♀) and ovariectomy accompanied by androgen administration for 5 months (♀ + AND). Control intact female (♀) and intact male (♂) values are included. The t values are given in the appropriate text in Experiment 2.

Figure 23 - Porphyrin content of the Harderian gland (nmol/g).

Figure 24 - 5-ALA-S activity of the Harderian gland (nmol ALA formed/h/g protein)

Figure 25 - Intraluminal porphyrin accretions/mm^2 section

Figure 26 - Tubules with neutrophils/mm^2 section

Figure 27 - Interstitial porphyrin accretions/mm^2 section

Figure 28 - Small interstitial porphyrin deposits/mm^2 section

Figure 29 - Mast cells/mm^2 section

Figure 30 - Mast cells/mm capsule

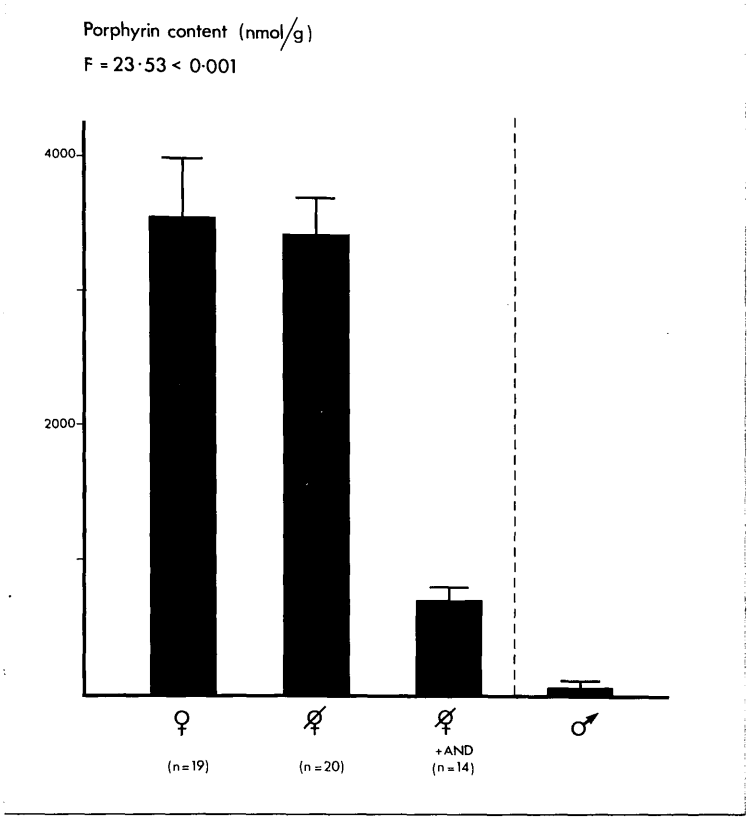


Figure 23

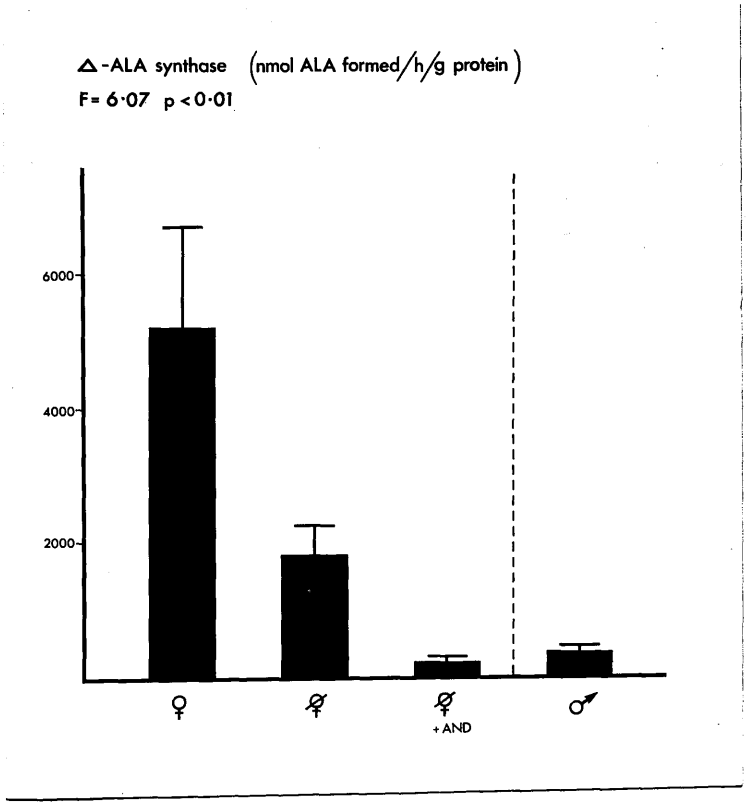


Figure 24

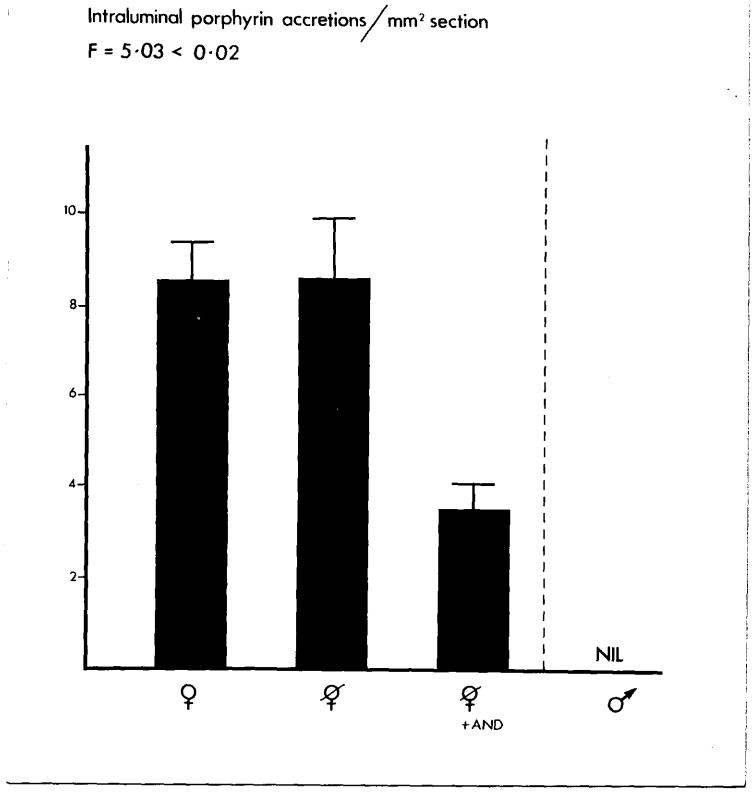


Figure 25

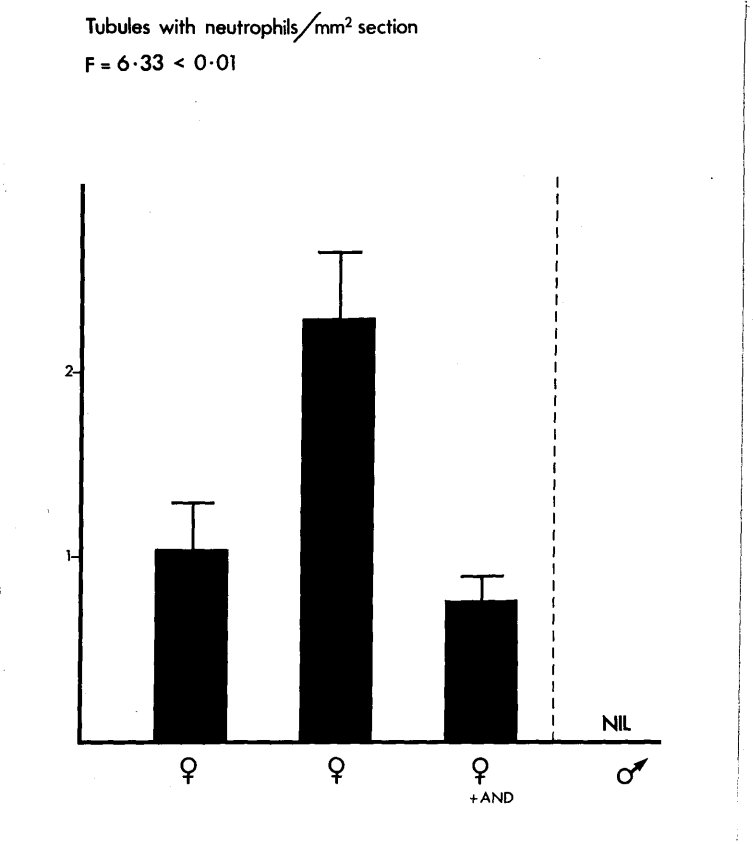


Figure 26

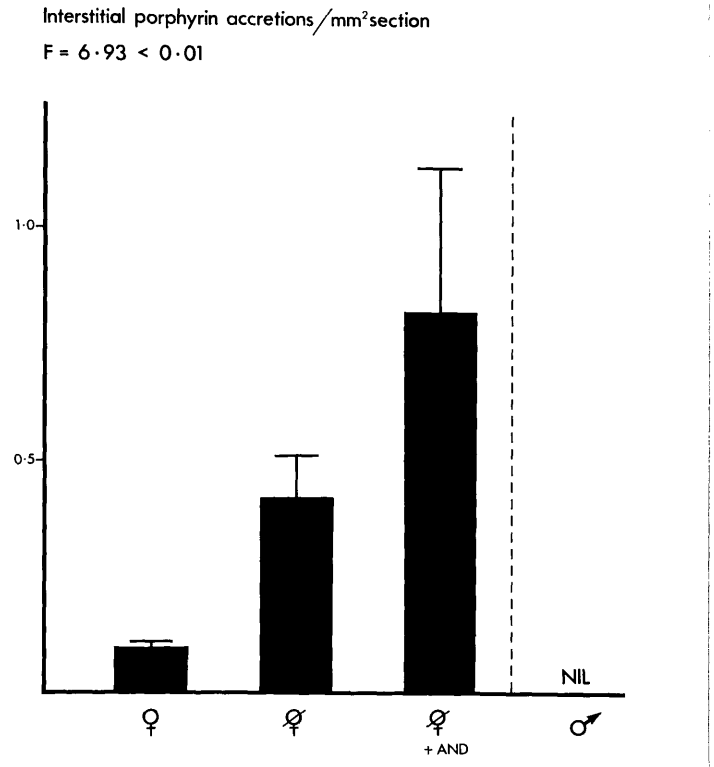


Figure 27

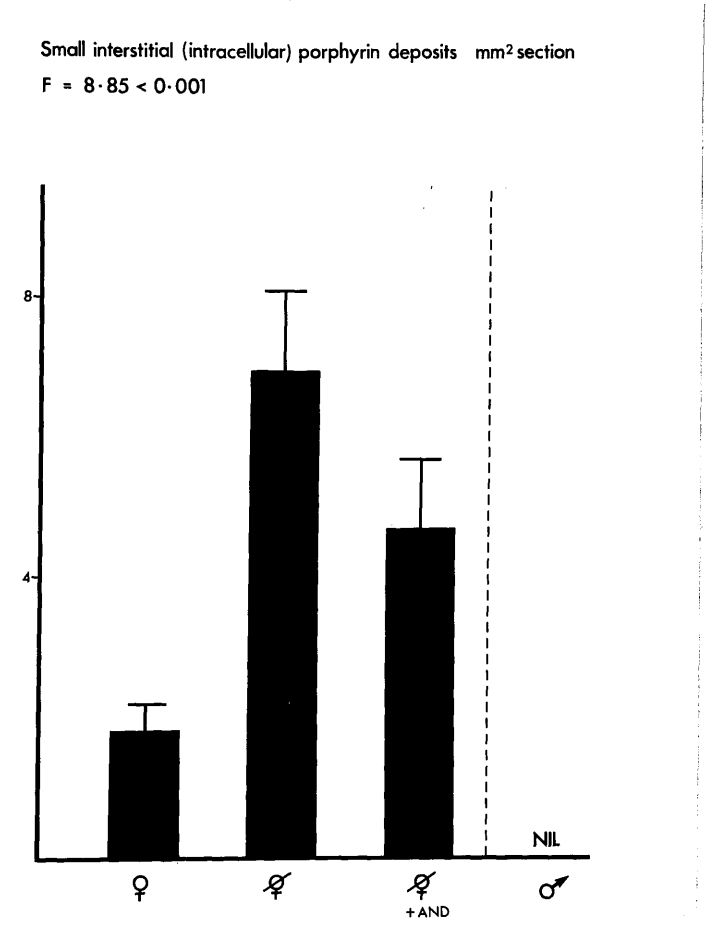


Figure 28

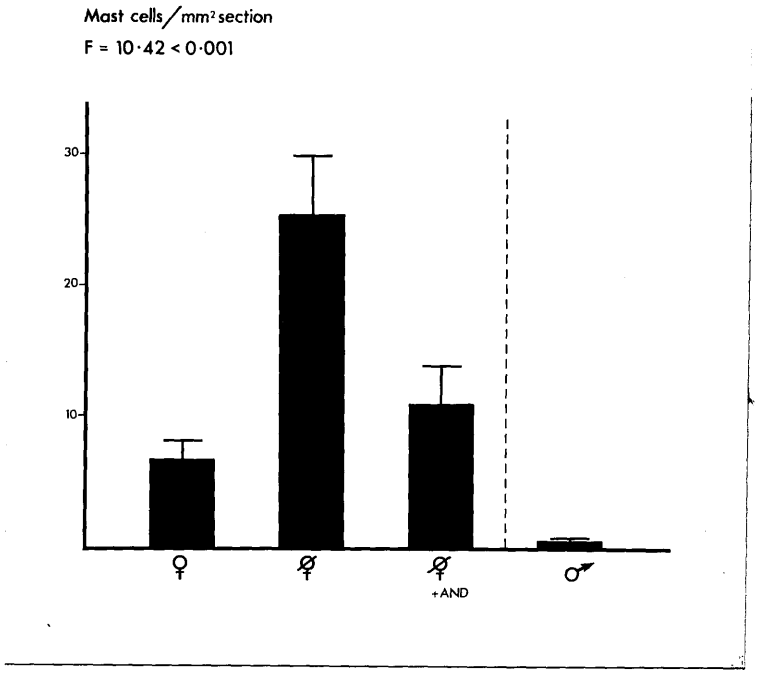


Figure 29

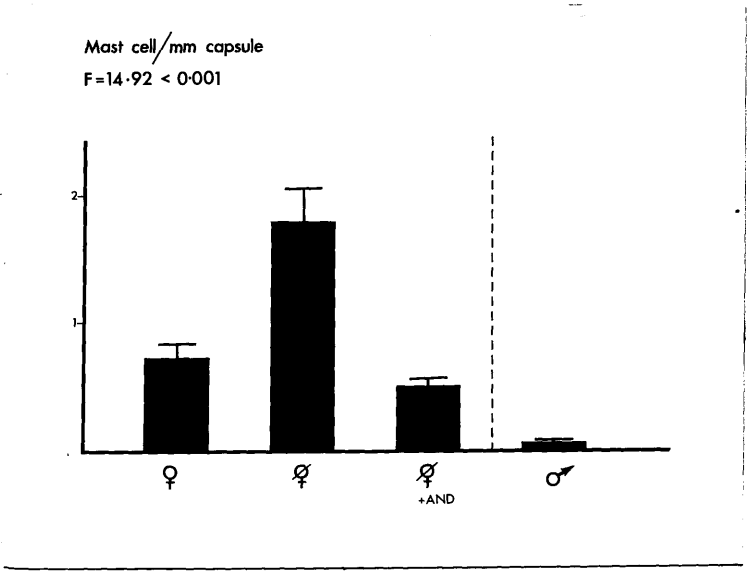


Figure 30

Figures 31-42

Changes occurring in biochemical and morphological parameters in intact control females (♀) and in females ovariectomised (♀) for 10, 20, 30, 40 or 50 weeks. Where appropriate, t values are given in the text of Experiment 3.

*$p < 0.05$ vs control female

**$p < 0.01$ vs control female

Figure 31 - Harderian gland porphyrin content (nmol/g)

Figure 32 - Harderian gland 5-ALA-S activity (nmol ALA formed/g protein/h)

Figure 33 - Blood porphyrin levels (nmol/l)

Figure 34 - Liver 5-ALA-S activity (nmol ALA formed/ g protein/ h)

Figure 35 - % Area of tubule degeneration

Figure 36 - Tubules containing neutrophils/mm^2 section

Figure 37 - Mitotic figures/mm^2 section

Figure 38 - Intraluminal porphyrin accretions/mm^2 section

Figure 39 - Interstitial mast cells/mm^2 section

Figure 40 - Peripheral mast cells/mm capsule

Figure 41 - Large interstitial porphyrin deposits/mm^2 section

Figure 42 - Small interstitial porphyrin deposits/mm^2 section

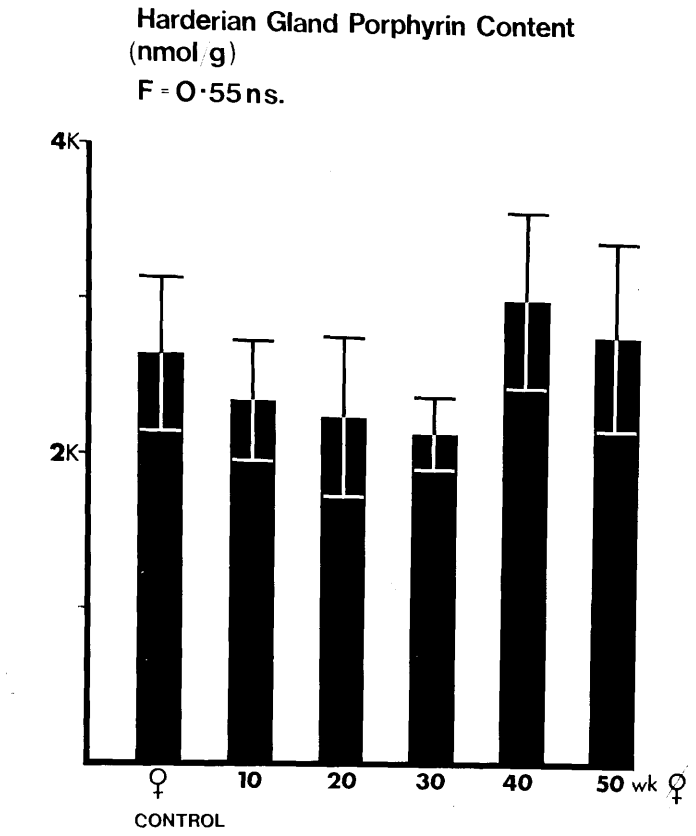


Figure 31

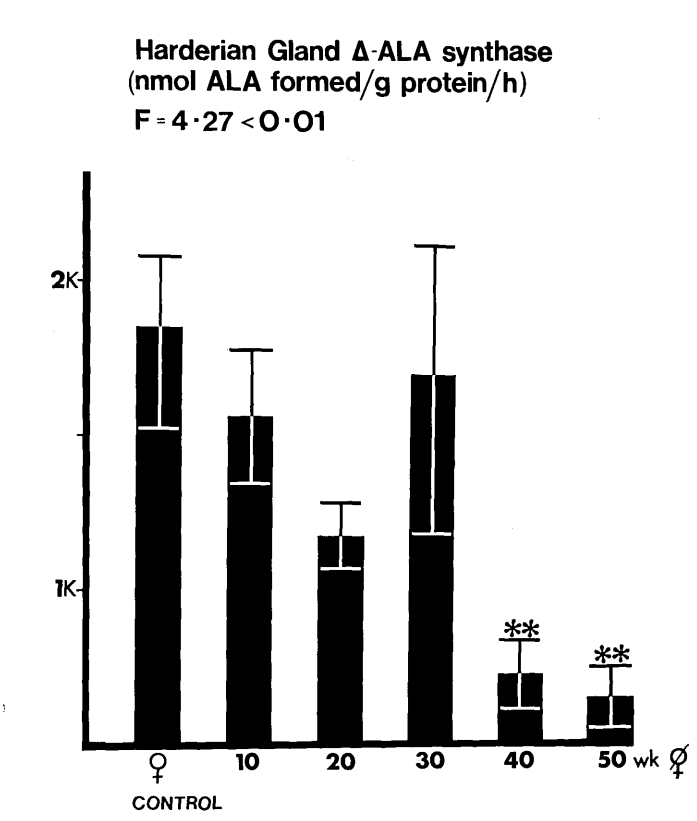
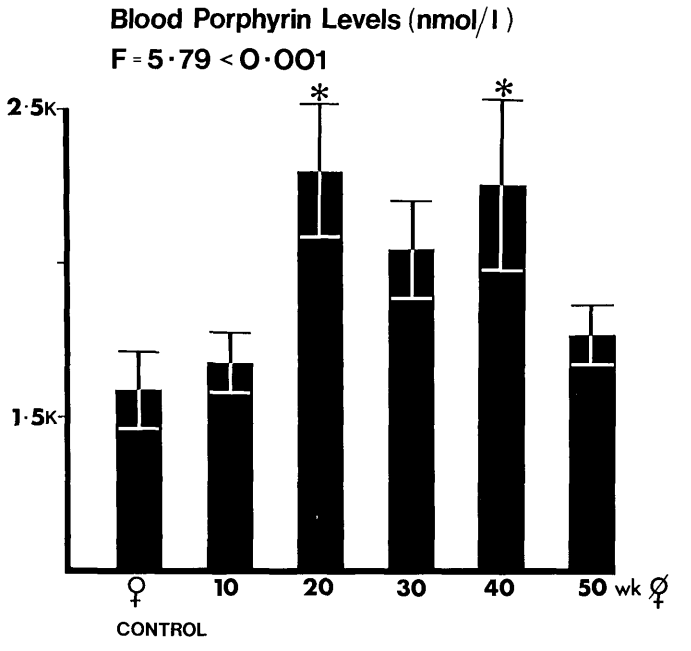


Figure 32



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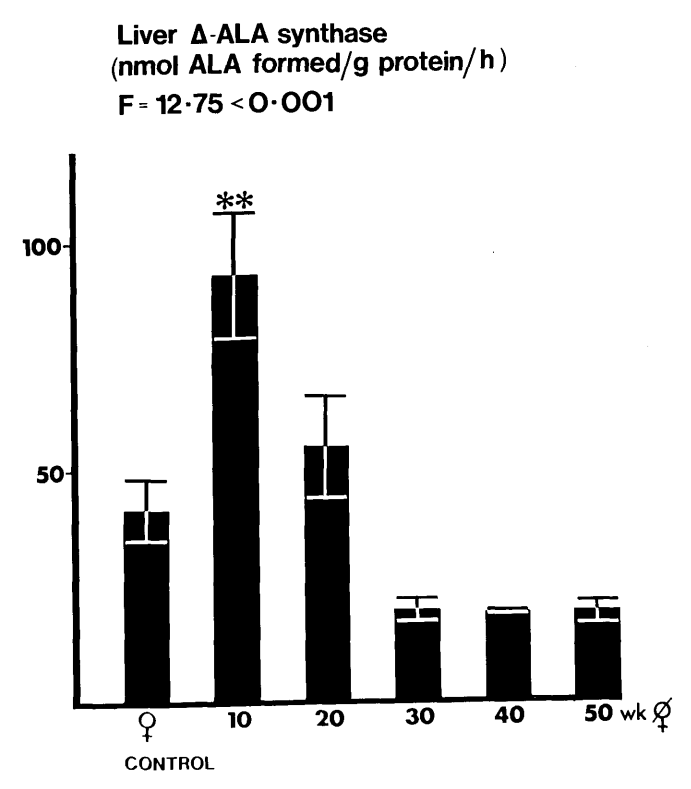


Figure 34

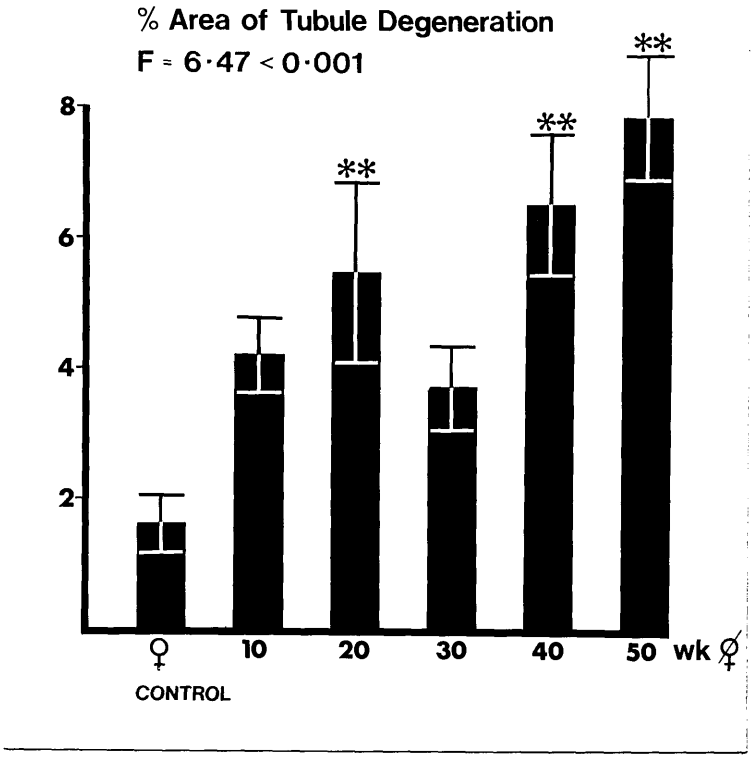


Figure 35

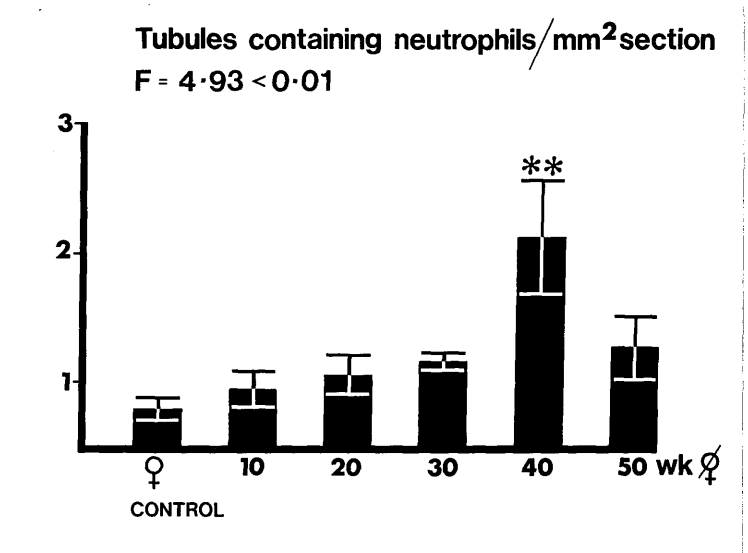


Figure 36

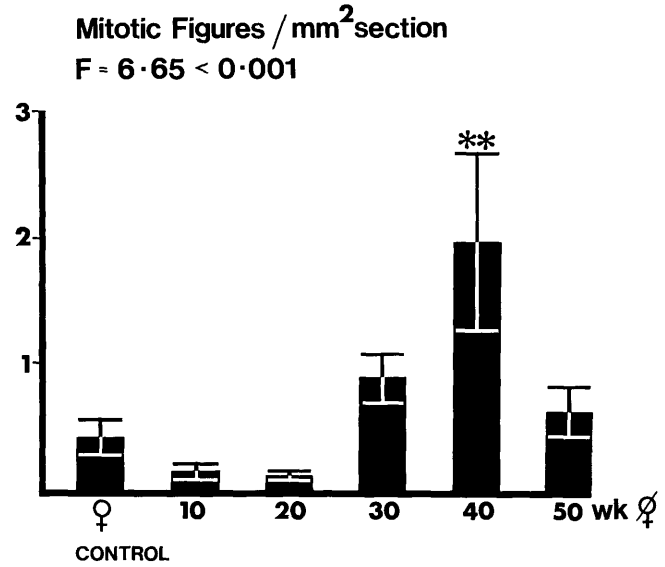


Figure 37

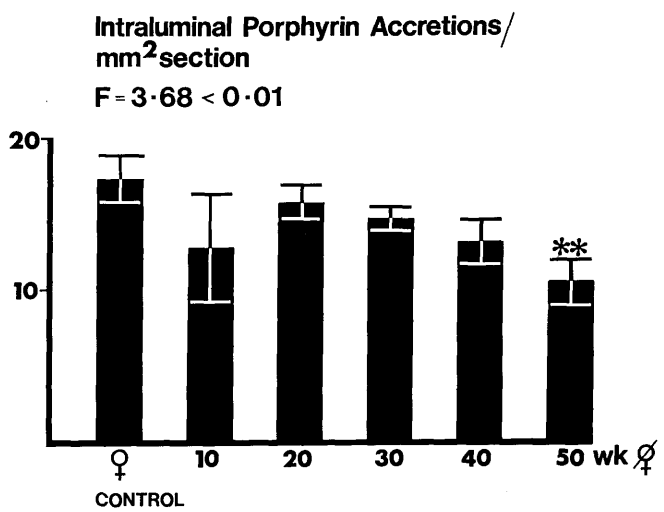


Figure 38

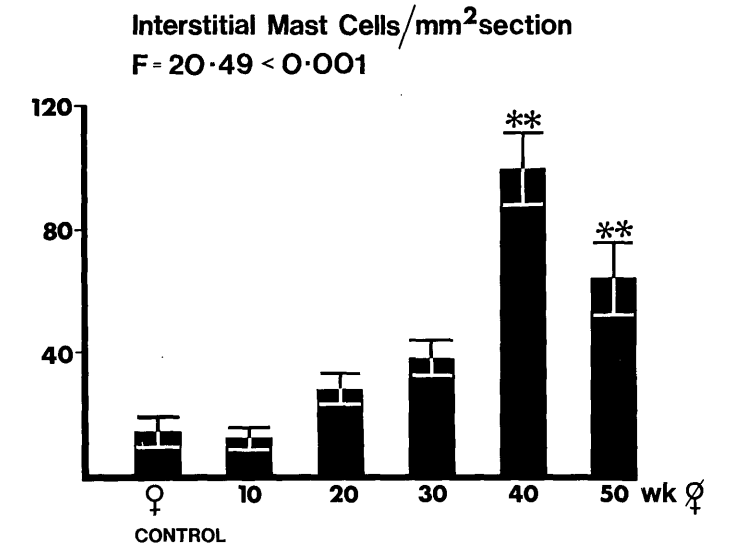


Figure 39

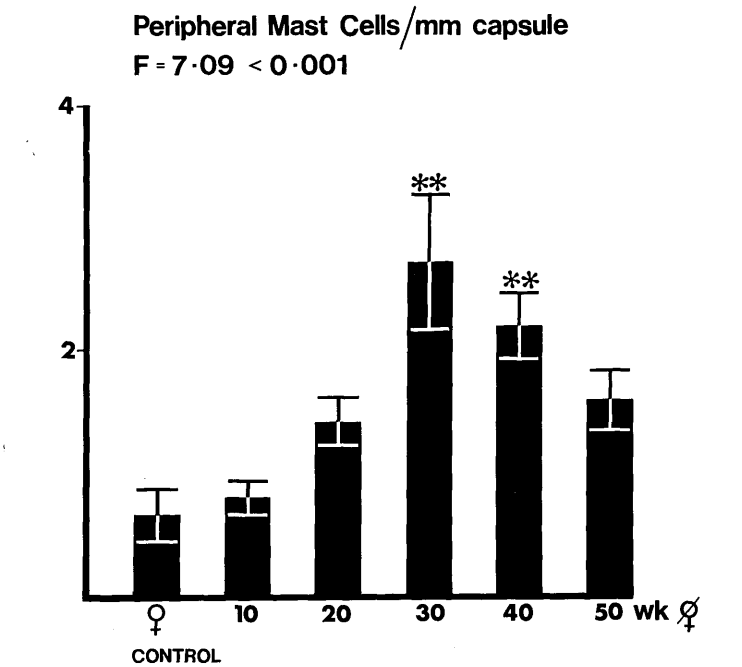


Figure 40

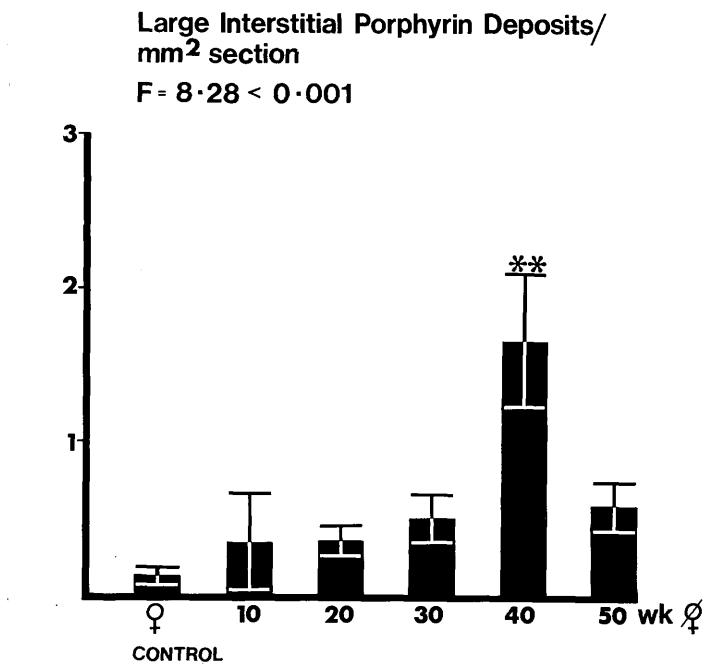


Figure 41

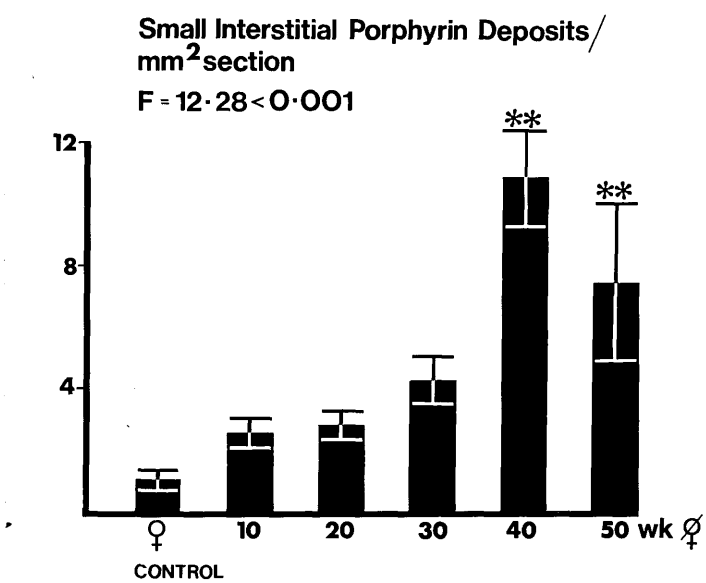


Figure 42

Figure 43

Graph showing the correlation (r = 0.38) between interstitial mast cells/mm^2 of a 5 µm thick tissue section and small interstitial porphyrin deposits/mm^2 in control females and females ovariectomised for 10, 20, 30, 40 or 50 weeks. Assuming a linear relationship, $y = 11.36 + 6.07x$.

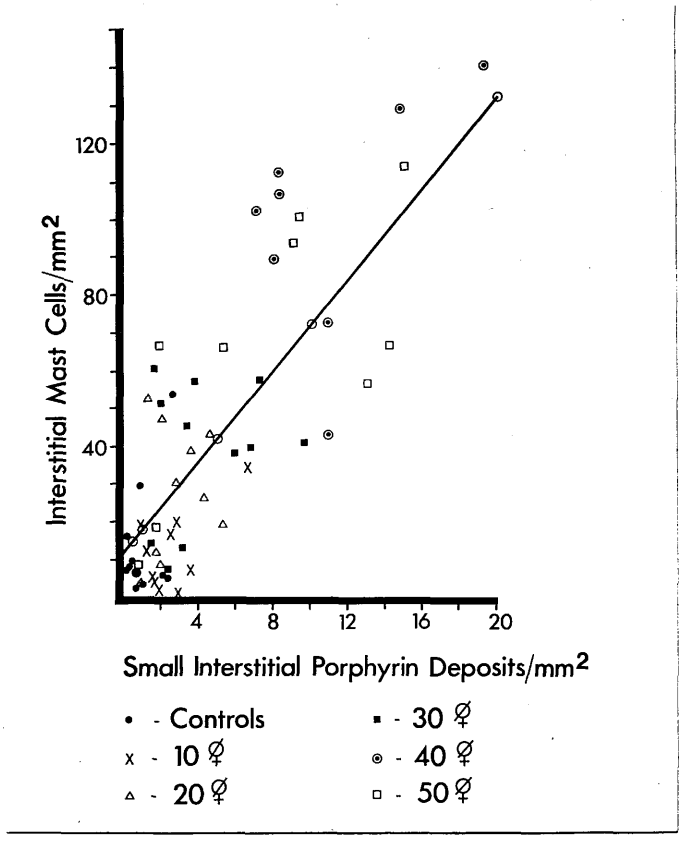
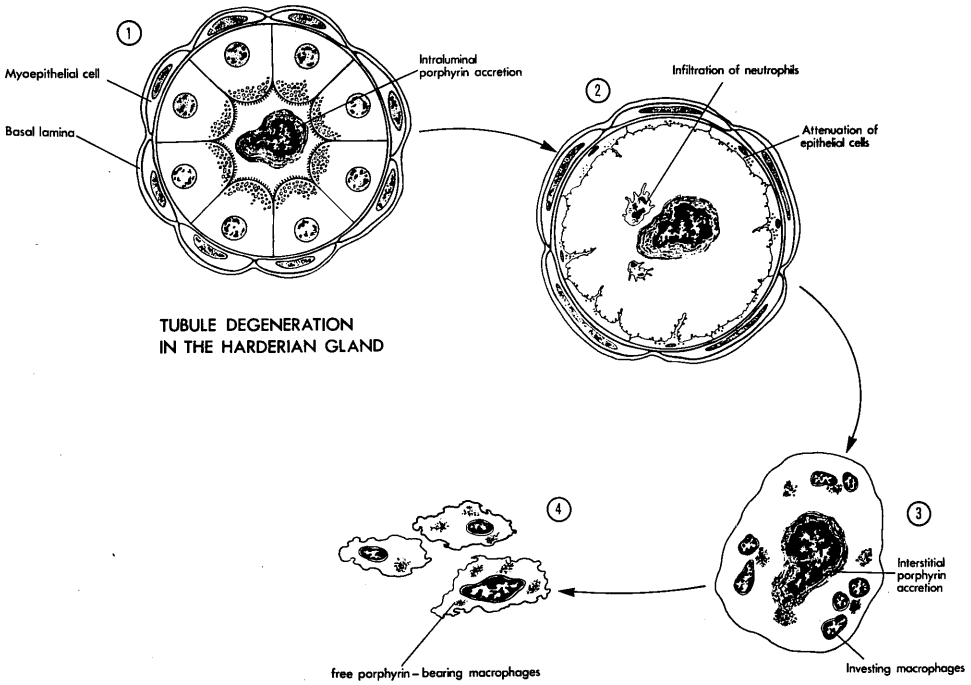

Interstitial Mast Cells/mm2
120
80
40
4
8
12
16
20
Small Interstitial Porphyrin Deposits/mm2
• - Controls
x - 10 ♀
△ - 20 ♀
▪ - 30 ♀
⊙ - 40 ♀
□ - 50 ♀

Figure 44

Schematic diagram showing the proposed pattern of tubule degeneration in the Harderian gland of ovariectomised or senescent post-reproductive female hamsters.



TUBULE DEGENERATION
IN THE HARDERIAN GLAND

Figure 45

A light micrograph of the Harderian gland of an ovariectomised female hamster, showing an area of tubule degeneration. The characteristic features are tubule dilation and neutrophil invasion of these tubules (5 μm thick tissue section, H & E). x 200

The sections in Figures 45-49 came from female hamsters ovariectomised for 5 months.

Figure 46

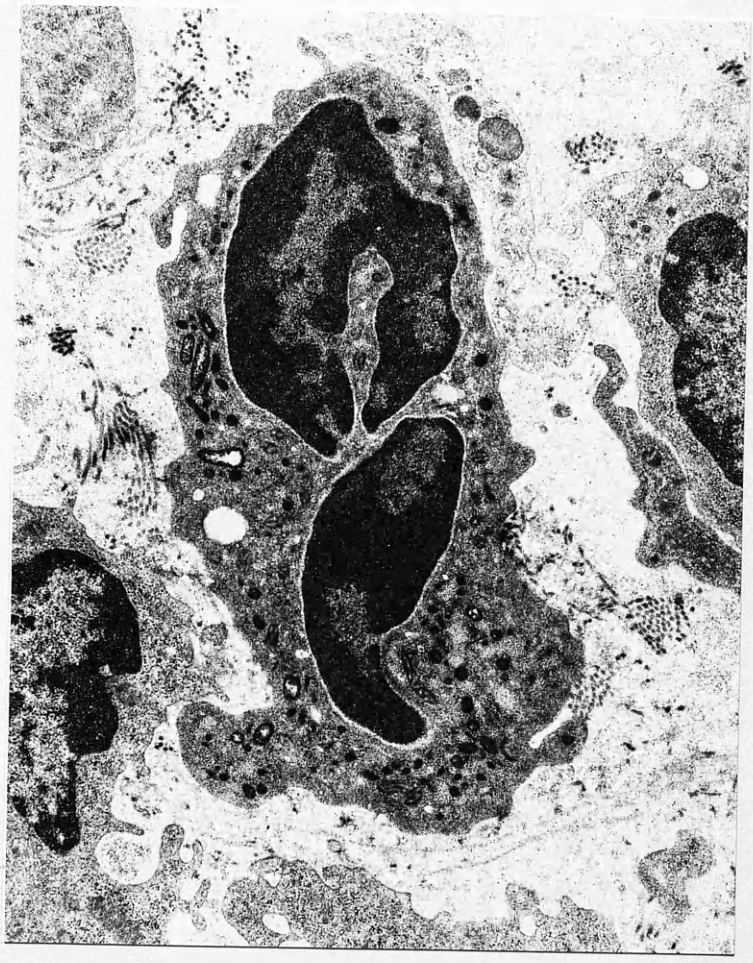
A light micrograph of the Harderian gland of an ovariectomised female hamster showing a gland tubule with abnormal attenuation of its epithelium. Beneath this is a large interstitial porphyrin store near which macrophages (lines) and mast cells (arrows) are observed (5 μm thick tissue section, 0.1% Toluidine blue counterstained with eosin). x 320

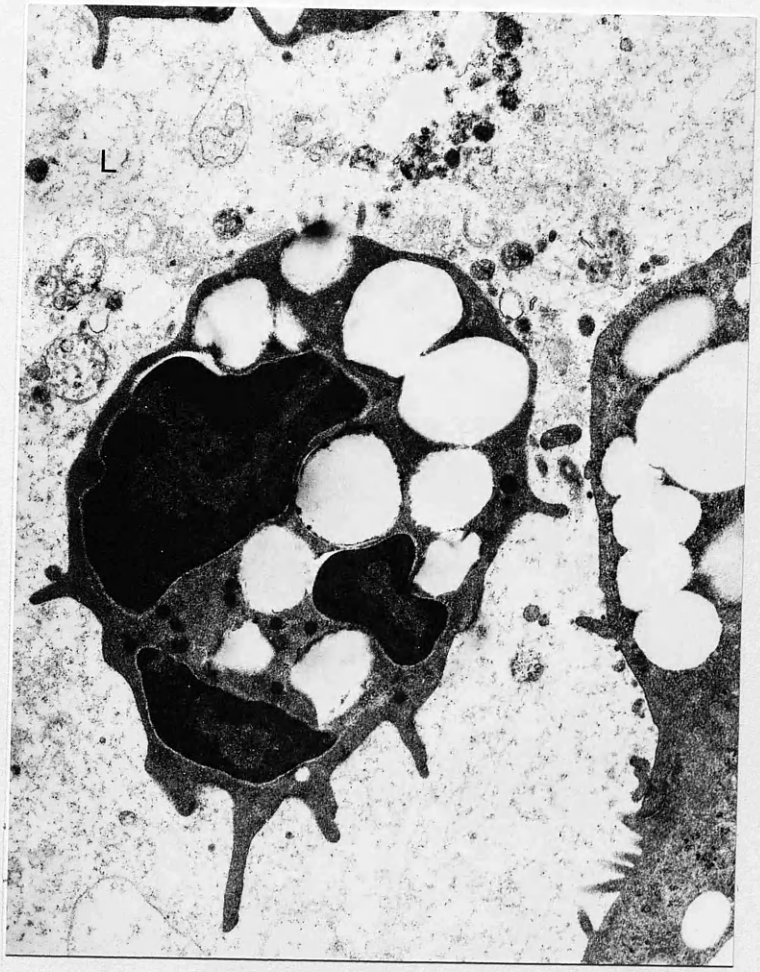
Figure 47

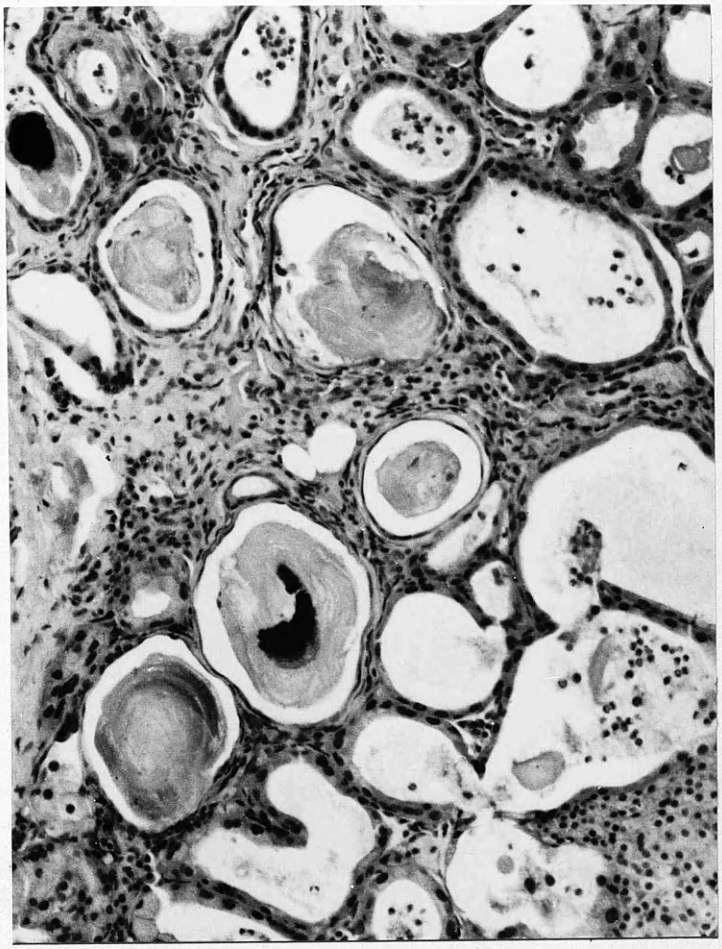
A normal neutrophil in the interstitial connective tissue of an ovariectomised female hamster. The cytoplasm contains numerous small granules, mitochondria and only an occasional vacuole.
x 12,200

Figure 48

A neutrophil in the lumen (L) of the Harderian gland of an ovariectomised female hamster. It bears several filopodial extensions and the cytoplasm contains numerous large vacuoles.
x 12,000




L



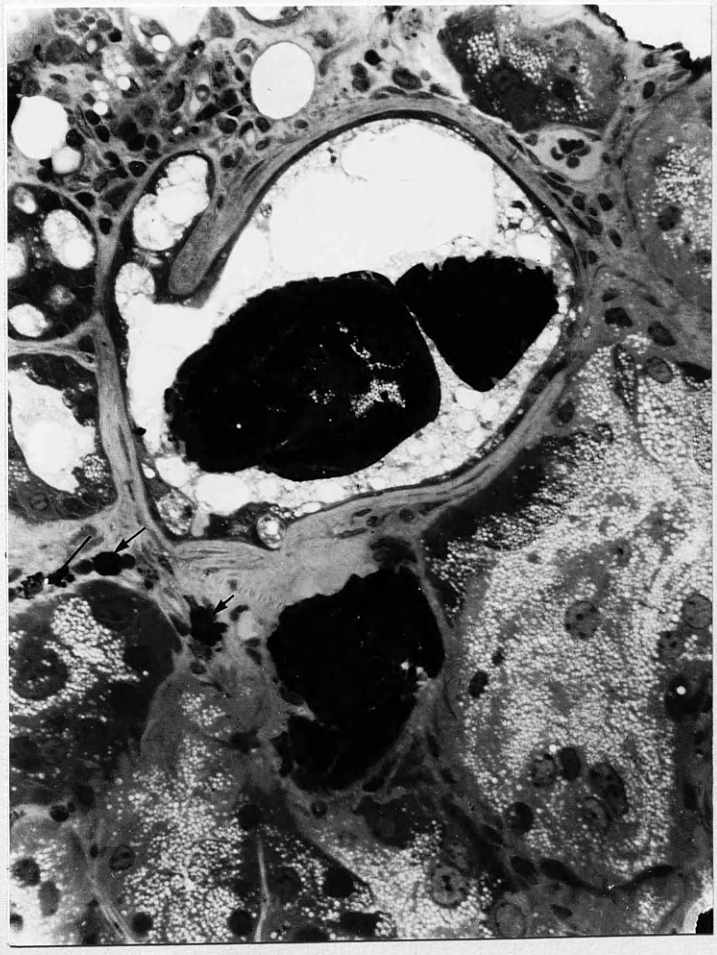
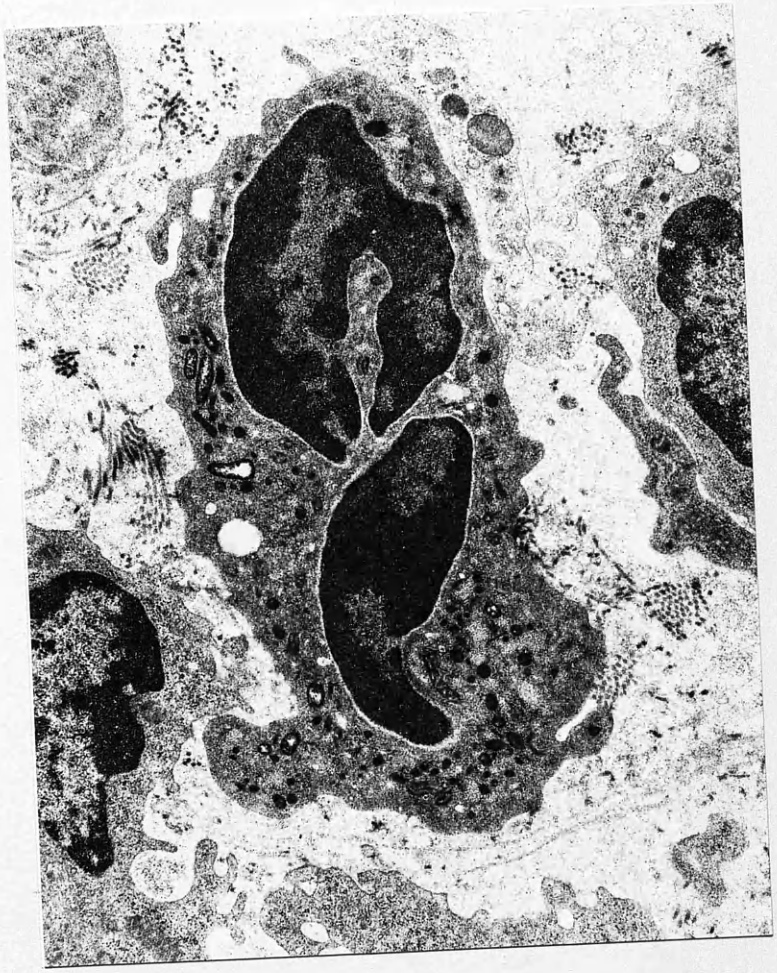


Figure 47

A normal neutrophil in the interstitial connective tissue of an ovariectomised female hamster. The cytoplasm contains numerous small granules, mitochondria and only an occasional vacuole.
x 12,200

Figure 48

A neutrophil in the lumen (L) of the Harderian gland of an ovariectomised female hamster. It bears several filopodial extensions and the cytoplasm contains numerous large vacuoles.
x 12,000



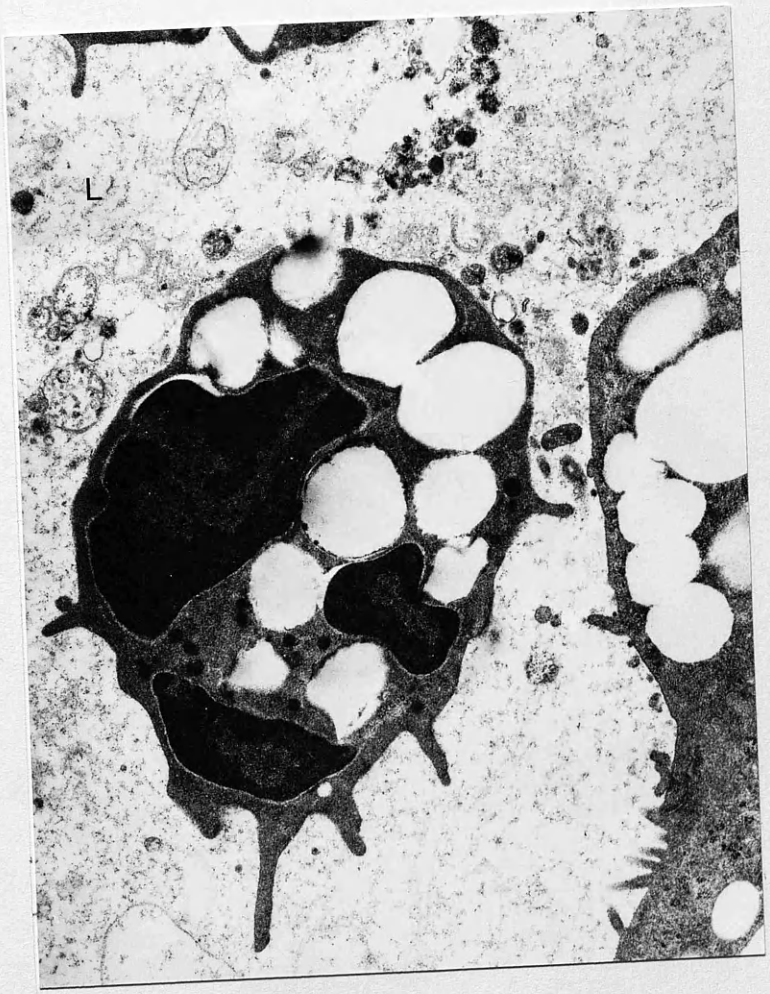
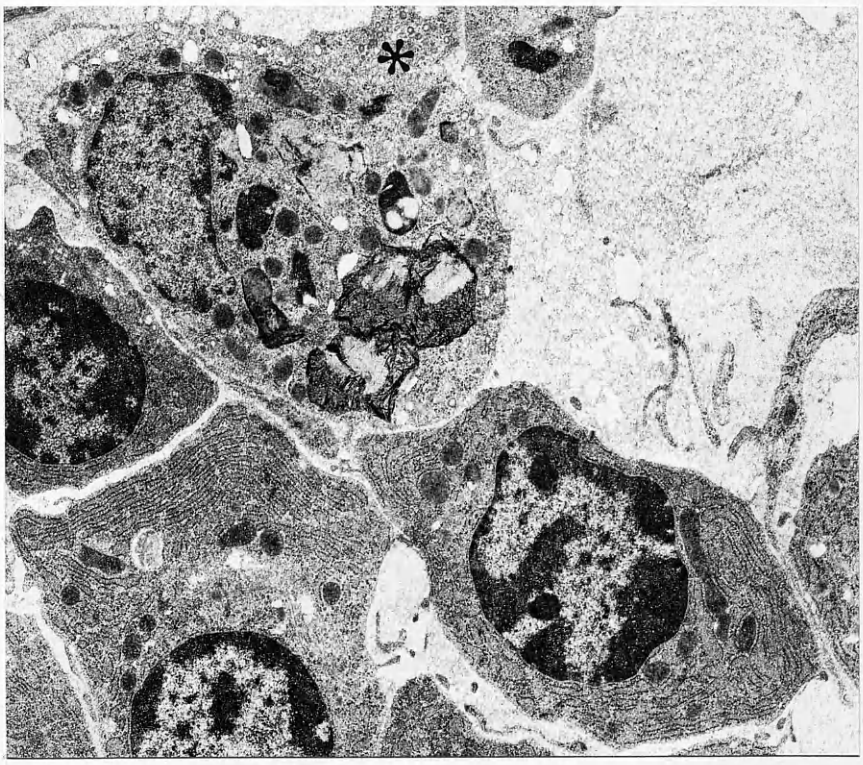

L

Figure 49

Electron micrograph showing a porphyrin-containing interstitial macrophage (*) surrounded by plasma cells in the Harderian gland of an ovariectomised female hamster. x 8,500

Figure 50

Light micrograph of an area of the Harderian gland of an ovariectomised female hamster showing lymphocyte invasion. This is also observed in post-reproductive senescent females (5 µm thick tissue section, 0.1% Toluidine blue lightly counterstained with eosin). x 500



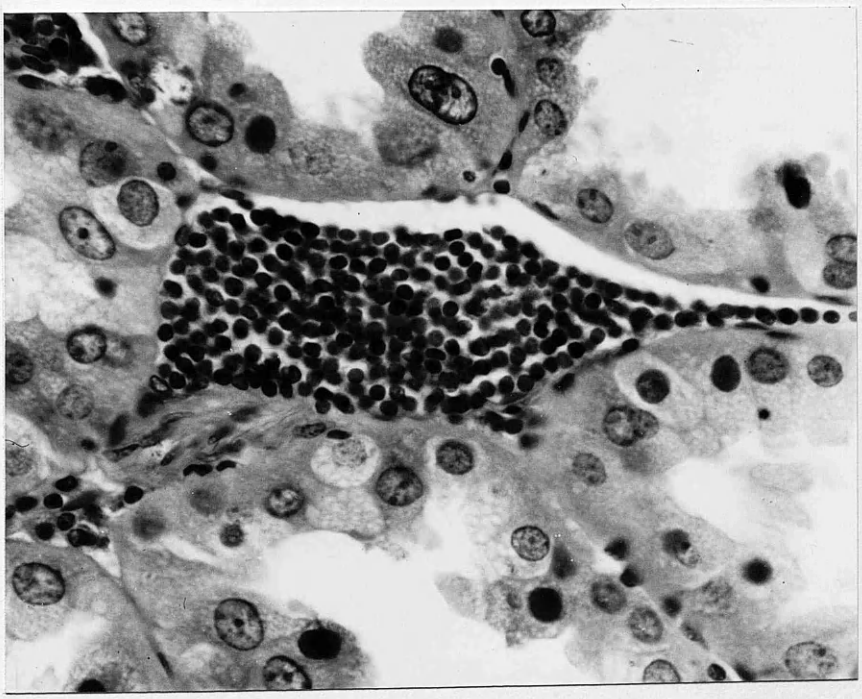


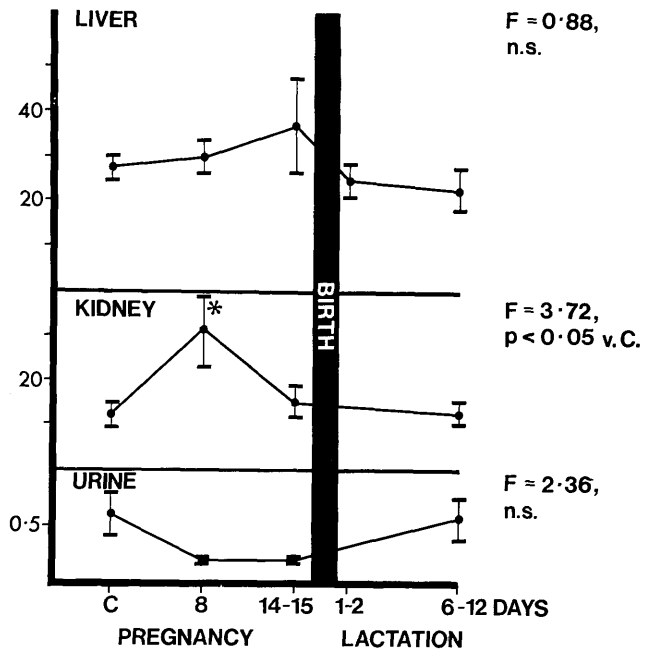
Figure 51

Porphyrin content in control female hamsters (C), hamsters at days 8 and 14/15 of pregnancy, and days 1-2 and 6-12 of lactation in liver and kidney (nmol/g) and urine (nmol/24h). All animals are multiparous.

Figure 52

Porphyrin content in control female hamsters (C), hamsters at days 8 and 14/15 of pregnancy, and days 1-2 and 6-12 of lactation in Harderian gland (nmol/g), blood (nmol/l) and faeces (nmol/g). All animals are multiparous.

Porphyrin



Porphyrin

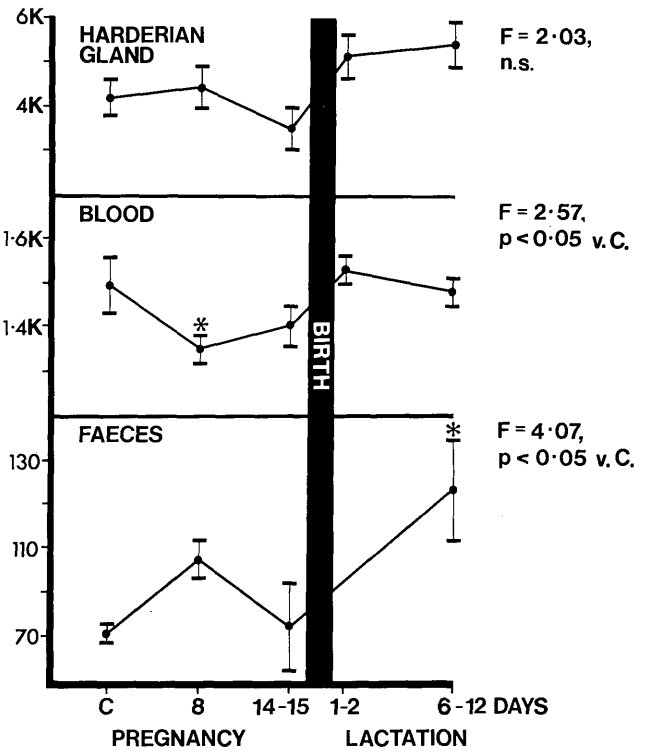
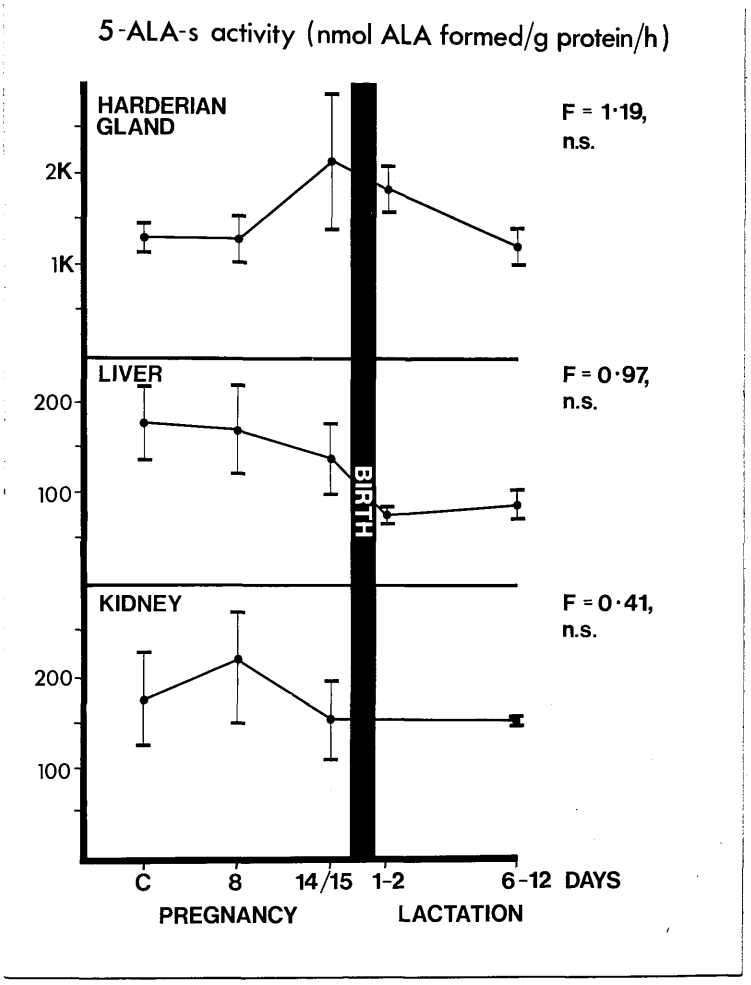


Figure 53

5-ALA-S activity in control female hamsters (C), hamsters at days 8 and 14/15 of pregnancy and days 1-2 and 6-12 of lactation in Harderian gland, liver and kidney (nmol ALA formed/g protein/h). All animals are multiparous.


5-ALA-s activity (nmol ALA formed/g protein/h)
HARDERIAN GLAND
F = 1·19, n.s.
2K
1K
LIVER
F = 0·97, n.s.
200
100
BIRTH
KIDNEY
F = 0·41, n.s.
200
100
C
8
14/15
1-2
6-12
DAYS
PREGNANCY
LACTATION

Figures 54 - 57

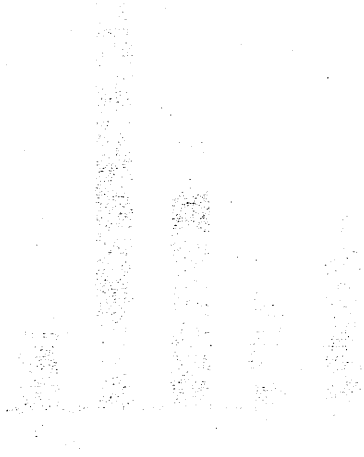
These figures represent changes in Harderian gland porphyrin and 5-ALA-S activity, liver 5-ALA-S activity and blood porphyrin levels in female hamsters in an age series ranging from 2 months to 24 months (Experiment 5). See text for statistical significance levels.

Figure 54 - Harderian gland porphyrin content (nmol/g)

Figure 55 - Harderian gland 5-ALA-S activity (nmol ALA formed/g protein/h)

Figure 56 - Blood porphyrin levels (nmol/l)

Figure 57 - Liver 5-ALA-S activity (nmol ALA formed/g protein /h)



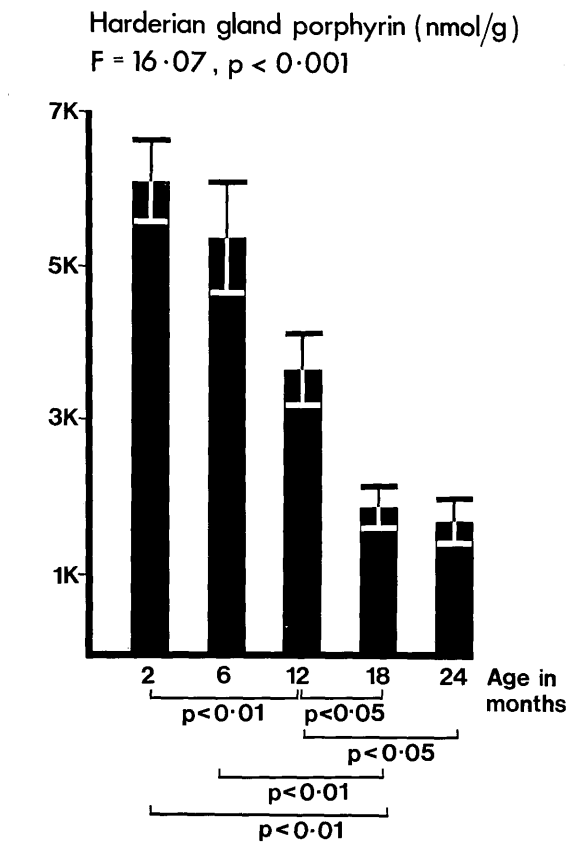


Figure 54

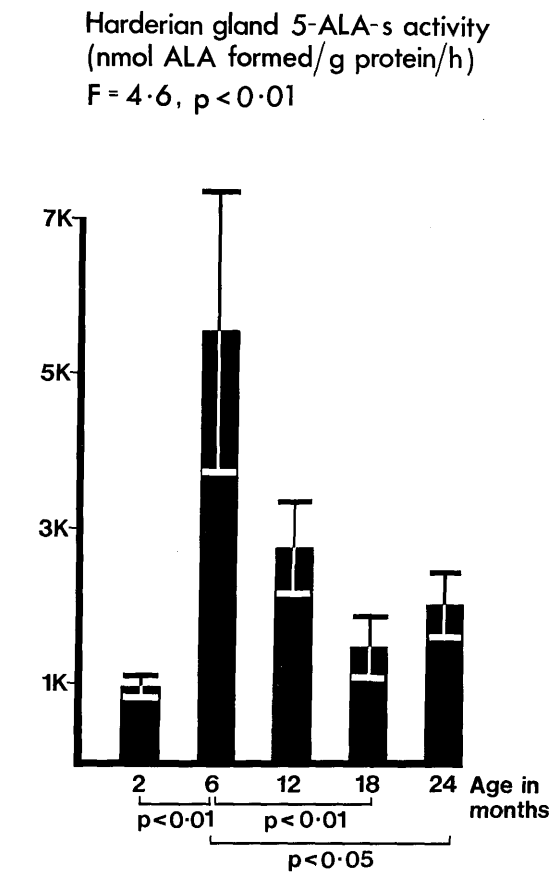


Figure 55

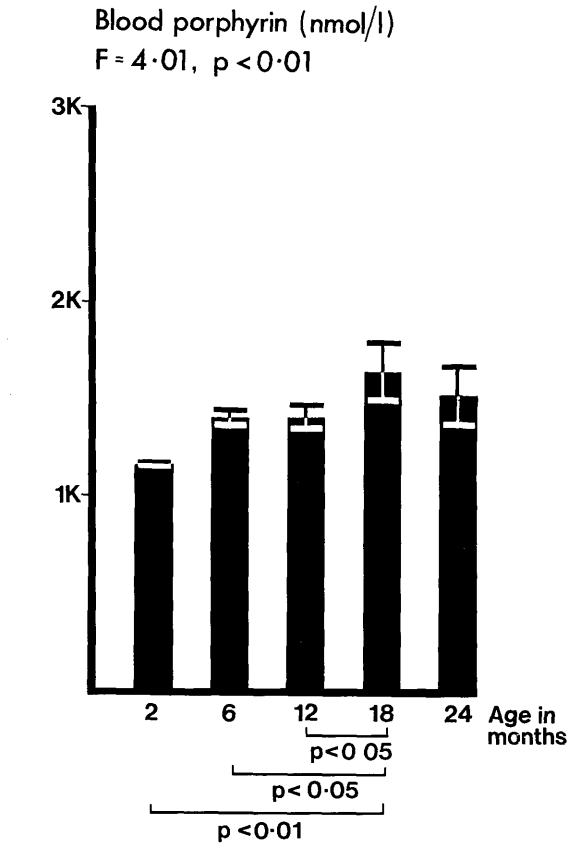


Figure 56

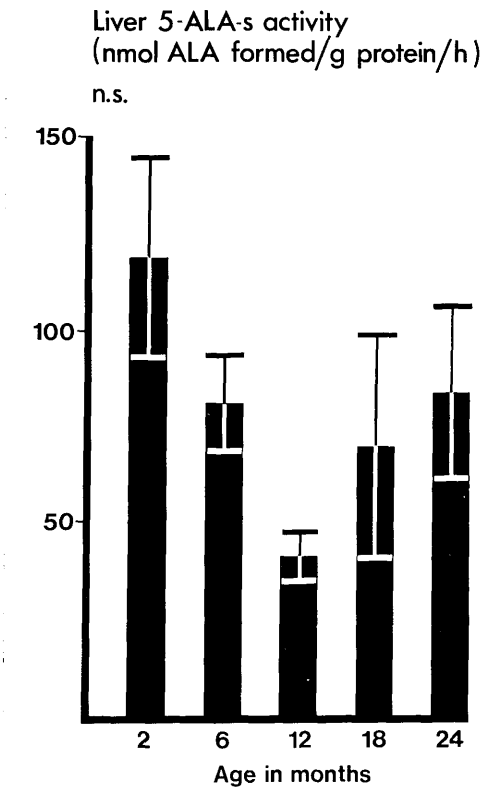


Figure 57

Figures 58 - 61

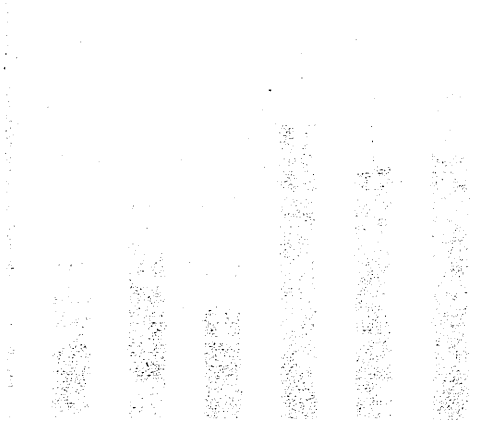
These figures represent changes occurring in Harderian gland porphyrin and 5-ALA-S activity, liver 5-ALA-S activity and blood porphyrin in male hamsters in an age series ranging from 2 months to 30 months (Experiment 5). See text for statistical significance levels.

Figure 58 - Harderian gland porphyrin (nmol/g)

Figure 59 - Harderian gland 5-ALA-S activity (nmol ALA formed/g protein/h)

Figure 60 - Blood porphyrin levels (nmol/l)

Figure 61 - Liver 5-ALA-S activity (nmol ALA formed/g protein/h)



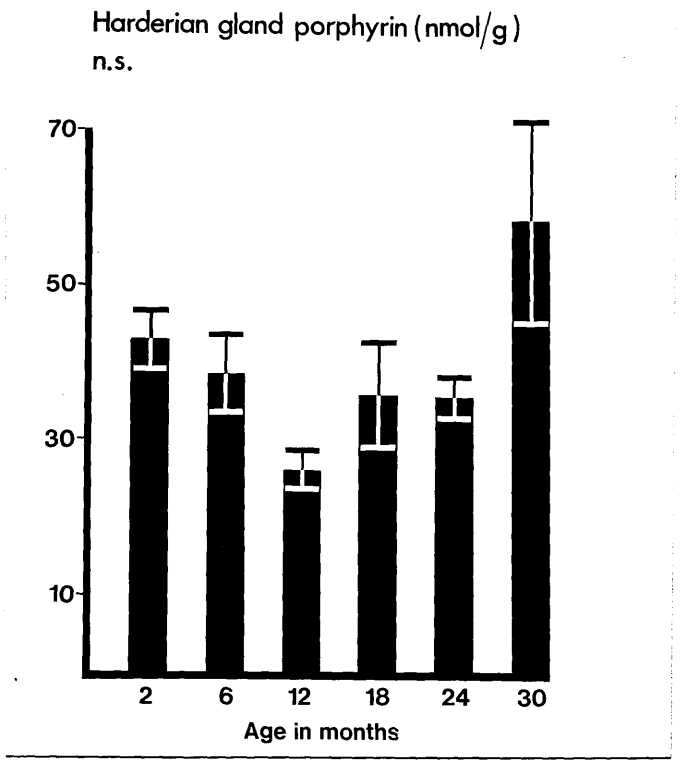


Figure 58

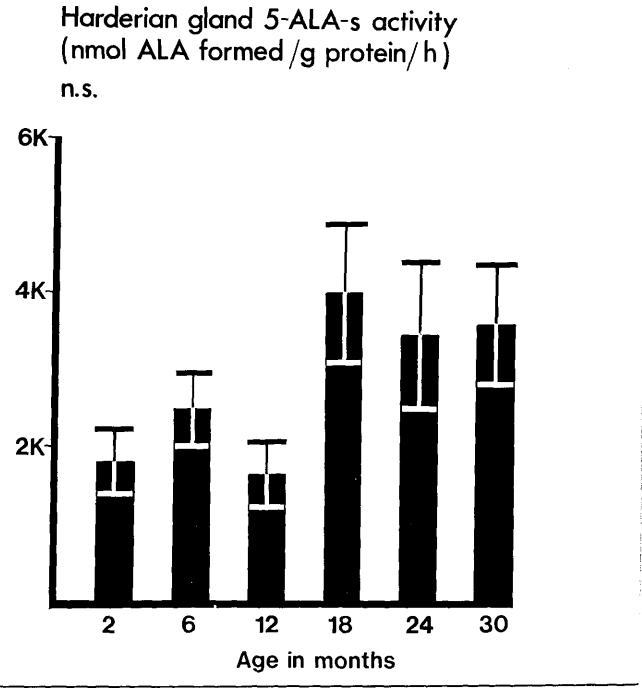


Figure 59

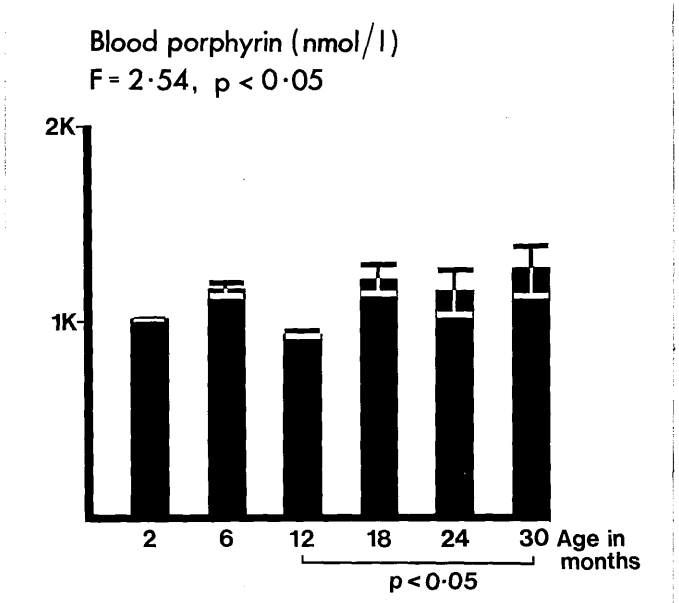


Figure 60

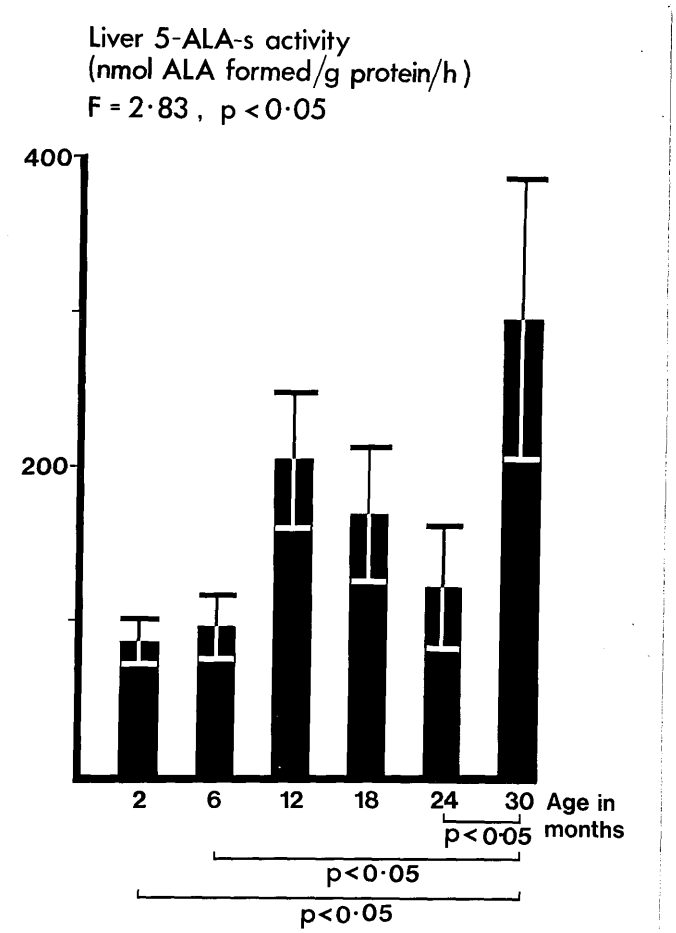


Figure 61

Figure 62

Graph showing the correlation (r = 0.43) between the number of intraluminal deposits/mm^2 of a 5 µm thick tissue section and the number of interstitial mast cells/mm^2 in female hamsters at different ages (m = months). Assuming a linear relationship $y = 0.28 + 0.63x$. (Experiment 5).

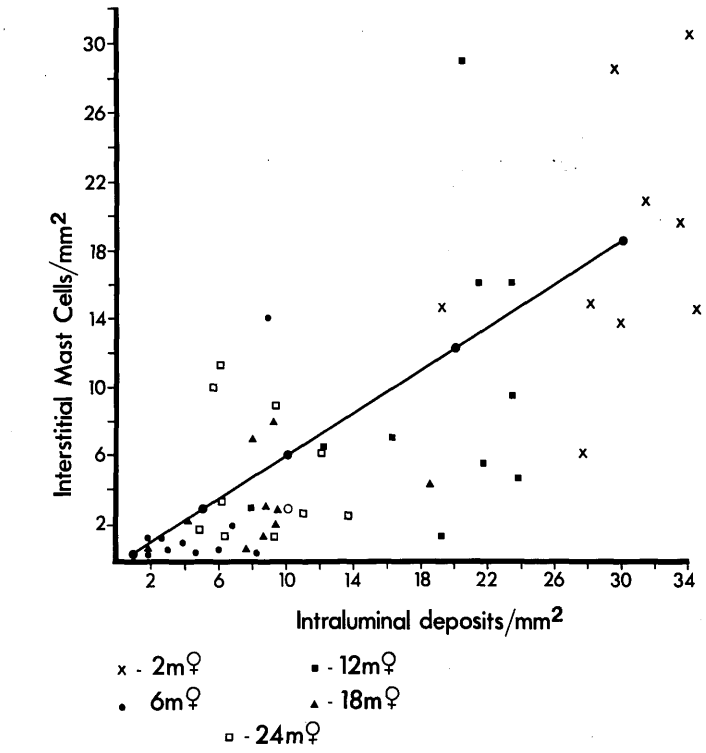
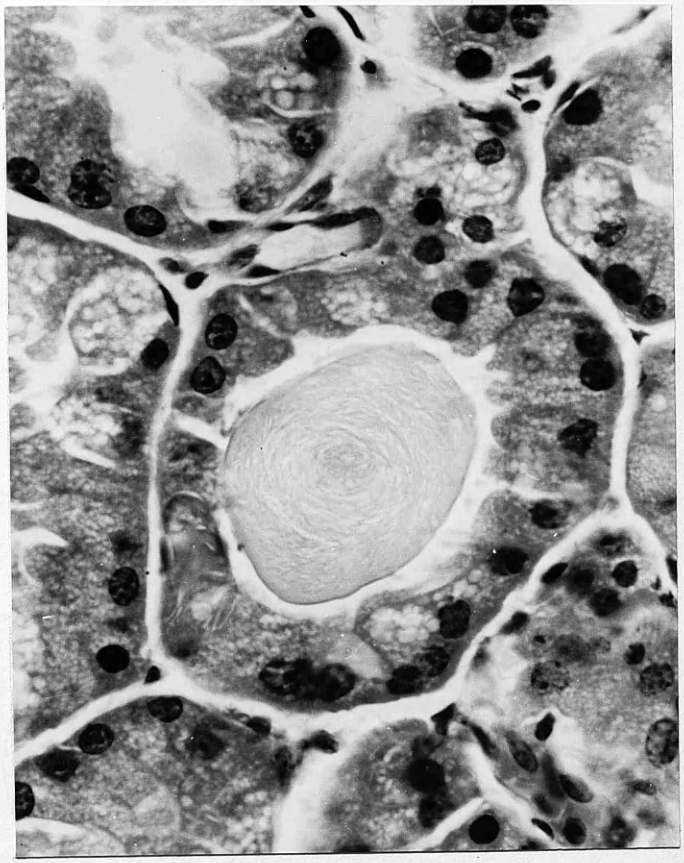

Interstitial Mast Cells/mm2
30
26
22
18
14
10
6
2
2
6
10
14
18
22
26
30
34
Intraluminal deposits/mm2
x - 2m♀
▪ - 12m♀
• 6m♀
▲ - 18m♀
▫ - 24m♀

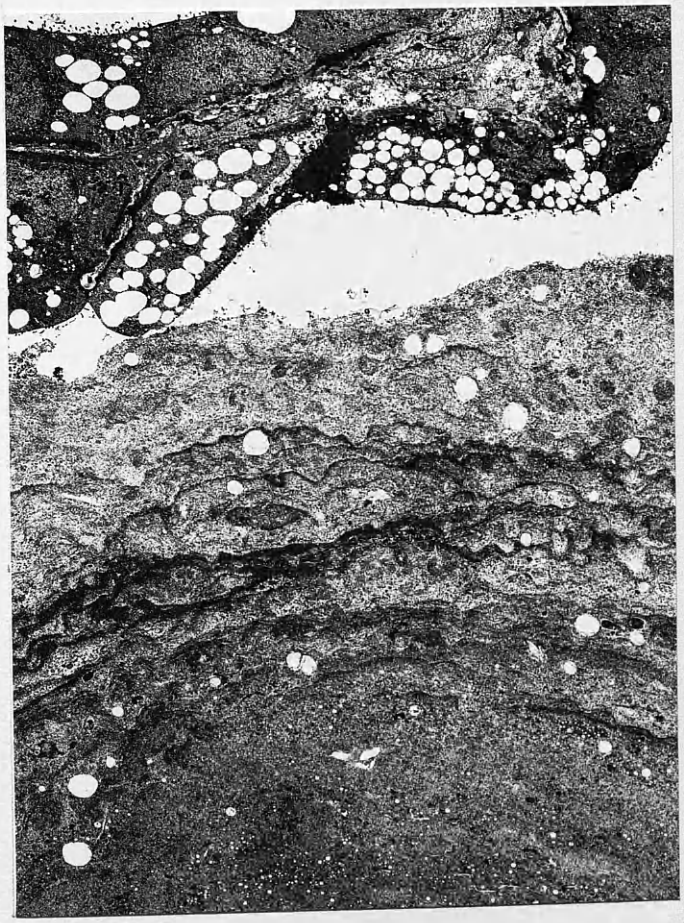
Figure 63

Light micrograph of an intraluminal deposit in the Harderian gland of a 36 month old male hamster. Type I and Type II cells are visible in the tubule (5 μm thick tissue section, stained with H & E). x 500

Figure 64

Electron micrograph of an intraluminal deposit in a 36 month male hamster, showing its laminar nature and lipid vacuoles, which are similar in size to those in the surrounding epithelial cells. x 1,900





Figures 65 - 68

These histograms show the effect of castration ± steroid administration on Harderian gland porphyrin content and 5-ALA-S activity, liver 5-ALA-S activity and blood porphyrin levels at 2, 4 and 6 weeks post-operatively (Experiment 6). At each time interval the 4 experimental groups are represented in the following order (from L to R):

1. castrates (C)
2. castrates + oestrogen (O)
3. castrates + progesterone (P)
4. castrates + testosterone propionate (T)

Reference figures for intact males and intact females are included.

F values are given in the appropriate text.

* $p < 0.05$ vs intact males

** $p < 0.01$ vs intact males

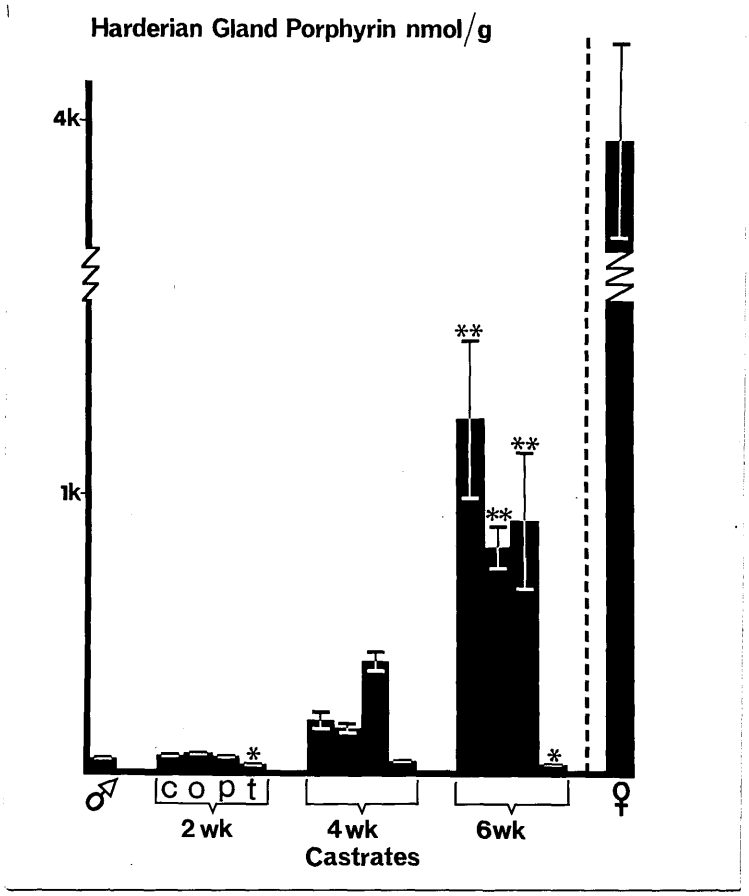


Figure 65

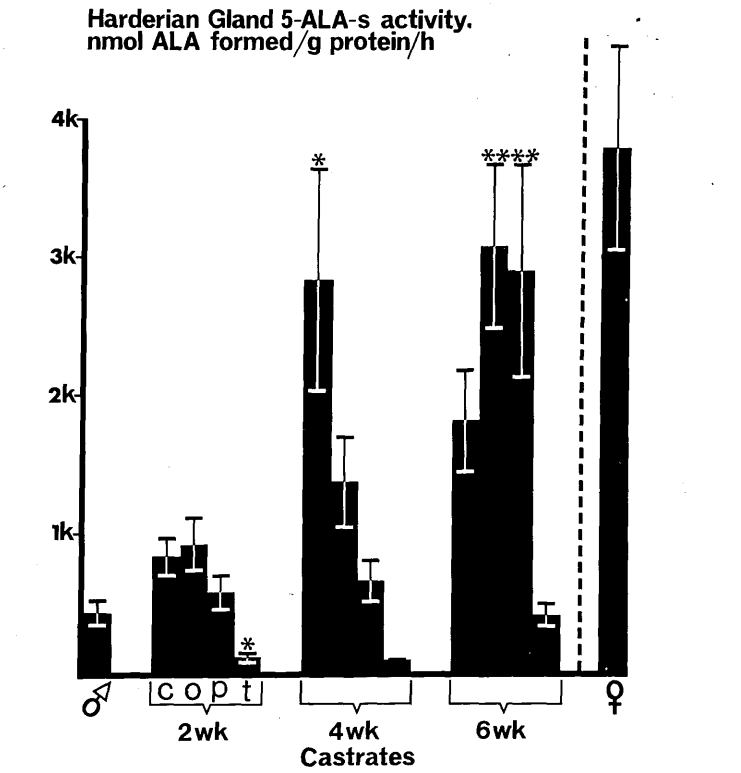


Figure 66

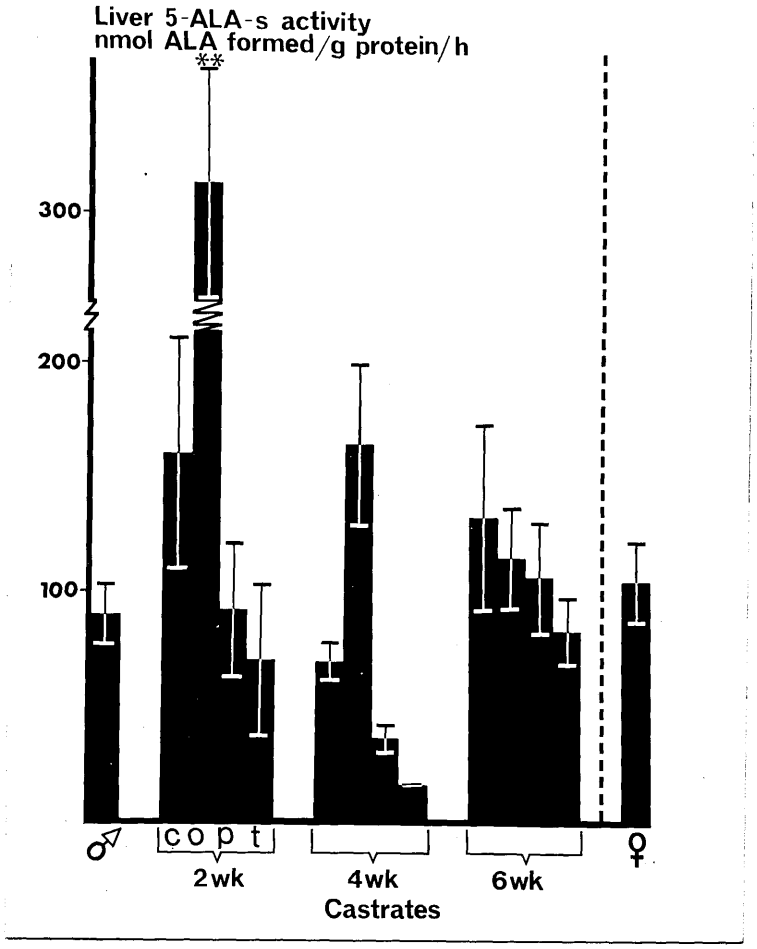


Figure 67

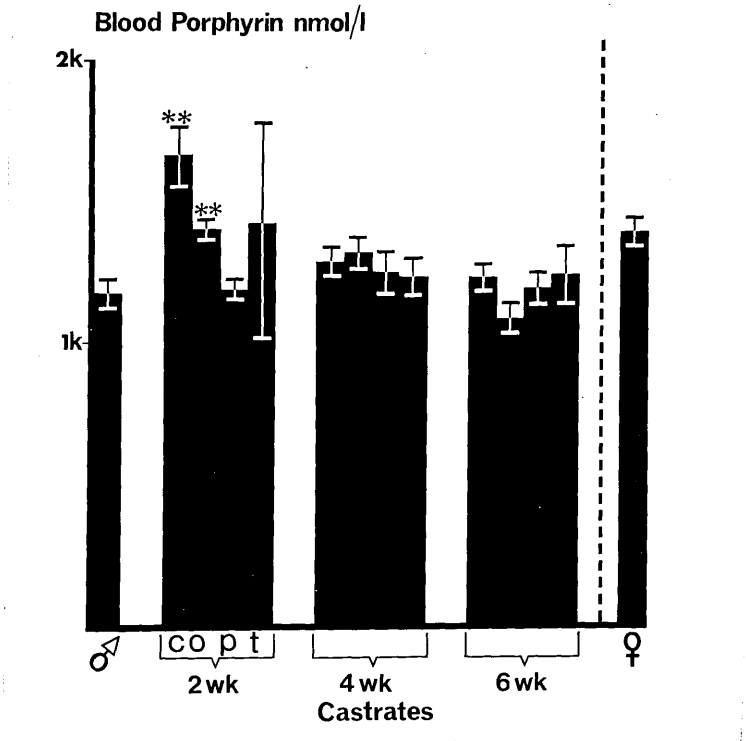


Figure 68

Figures 69 - 73

These histograms show the changes occurring in liver porphyrin content and 5-ALA-S activity, kidney porphyrin content and 5-ALA-S activity and blood porphyrin levels in female and male hamsters Harderianectomised for 5 months (5mHx ♀ & 5mHx ♂, respectively) compared to intact females (♀) and males (♂) (Experiment 7a).

Figure 69 - Liver porphyrin (nmol/g)

Figure 70 - Liver 5-ALA-S activity (nmol ALA formed/g protein/h).

Figure 71 - Kidney porphyrin (nmol/g)

Figure 72 - Kidney 5-ALA-S activity (nmol ALA formed/g protein/h)

Figure 73 - Blood porphyrin (nmol/l)

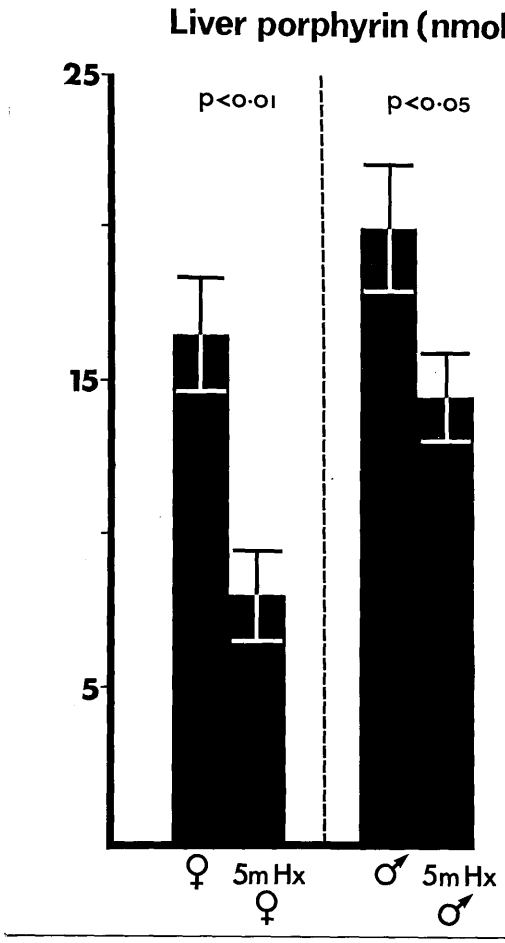


Figure 69

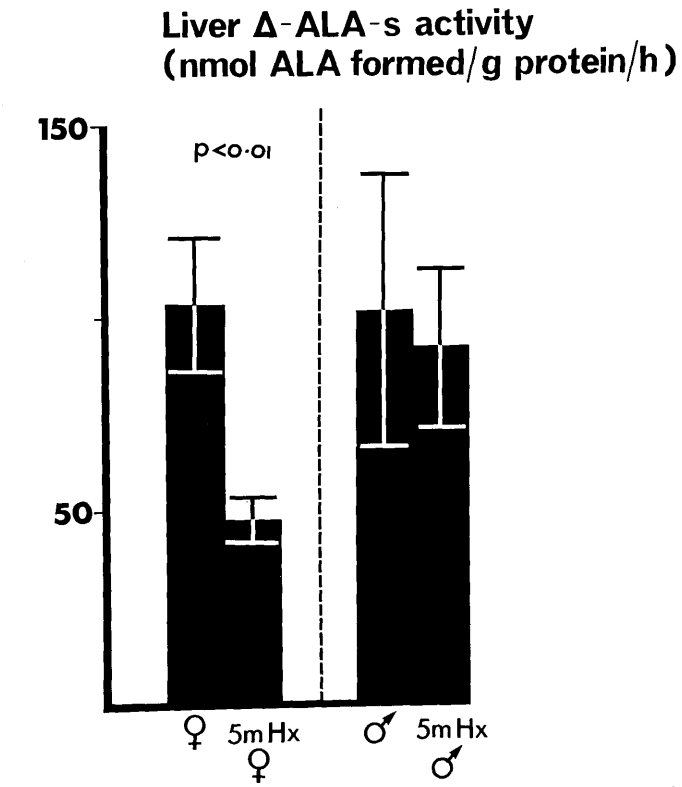


Figure 70

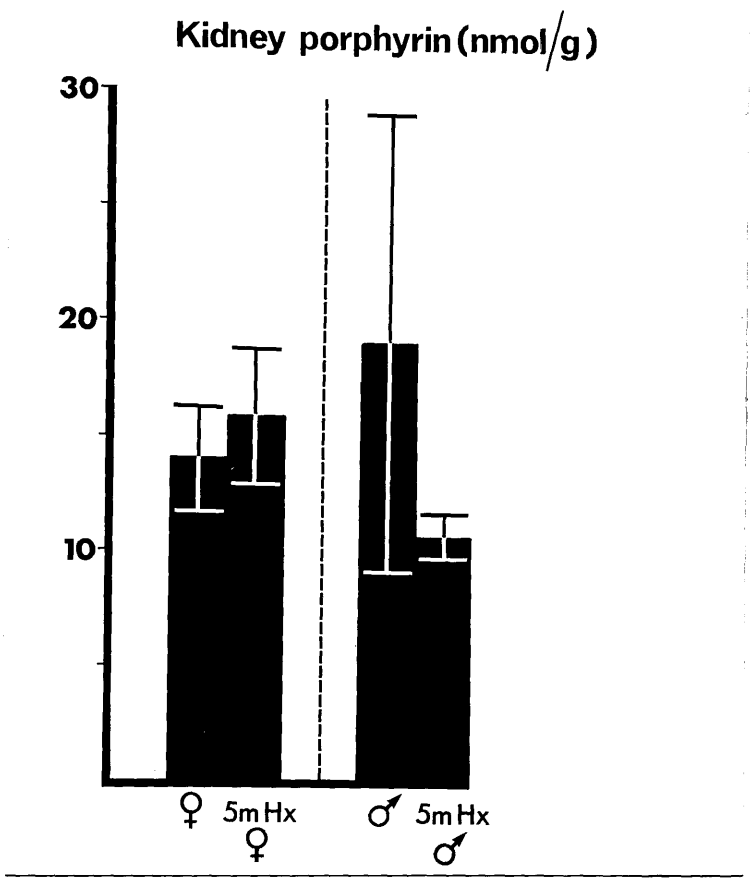


Figure 71

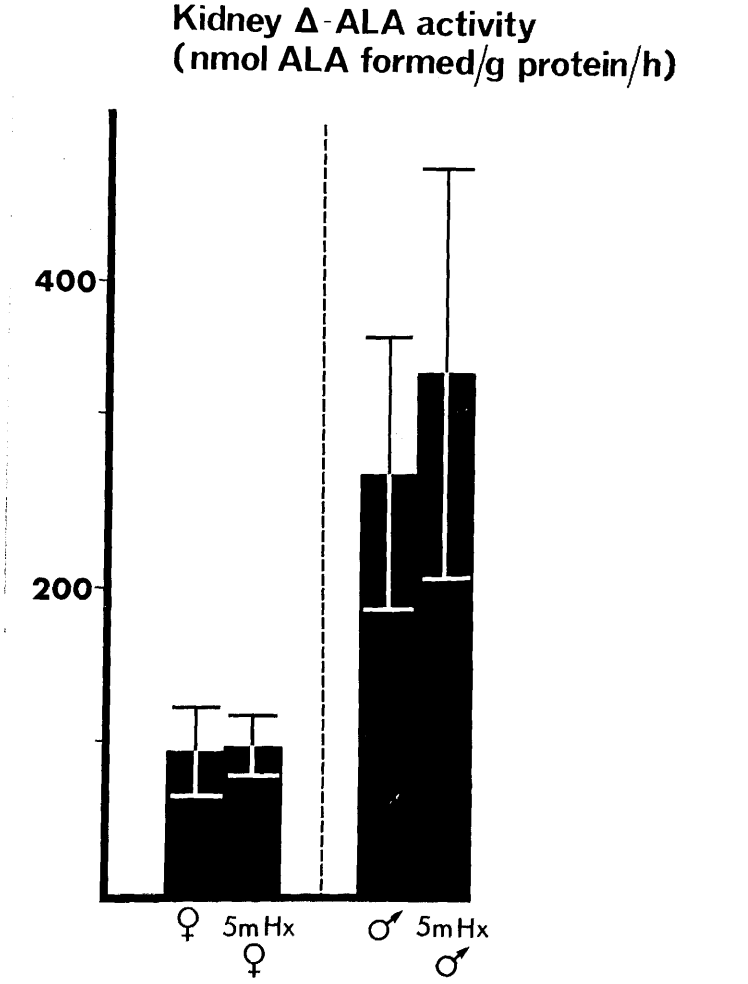


Figure 72

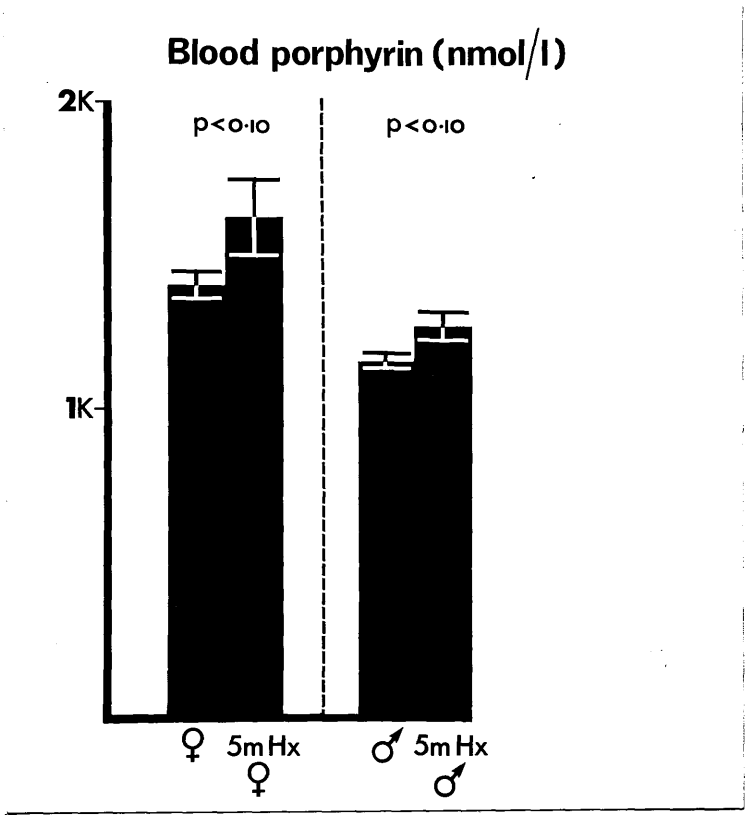


Figure 73

Figures 74 - 78

These histograms show the time course of changes occurring in blood porphyrin levels, liver porphyrin content and 5-ALA-S activity, and kidney porphyrin content and 5-ALA-S activity in female hamsters Harderianectomised for 1, 2 and 5 months (1mHx♀, 2mHx♀, and 5mHx♀ respectively) compared to control intact females (C) (Experiment 7b).

Figure 74 - Liver porphyrin (nmol/g)

Figure 75 - Liver 5-ALA-S activity (nmol ALA formed/ g protein/h)

Figure 76 - Kidney porphyrin (nmol/g)

Figure 77 - Kidney 5-ALA-S activity (nmol ALA formed/ g protein/h)

Figure 78 - Blood porphyrin (nmol/l)

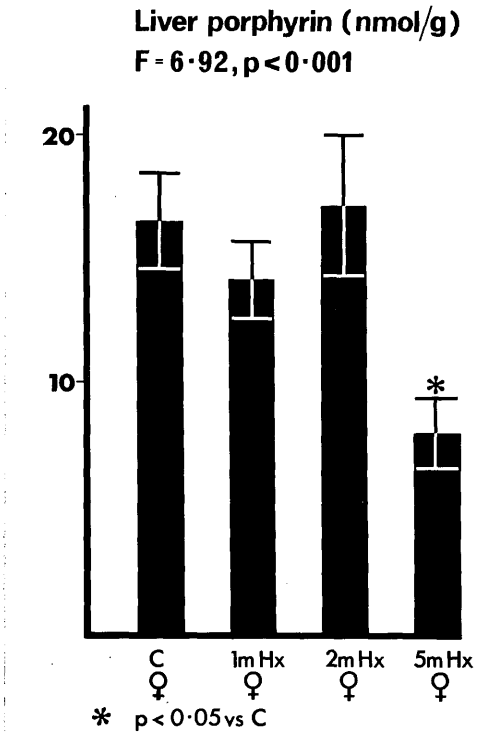


Figure 74

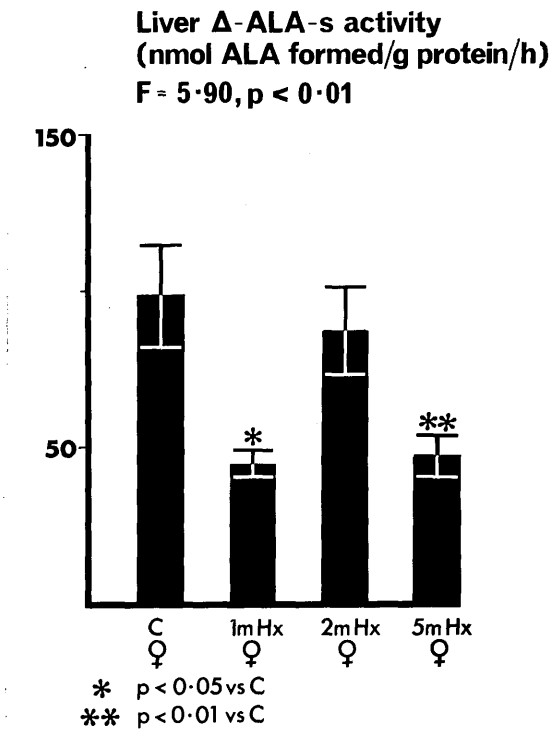


Figure 75

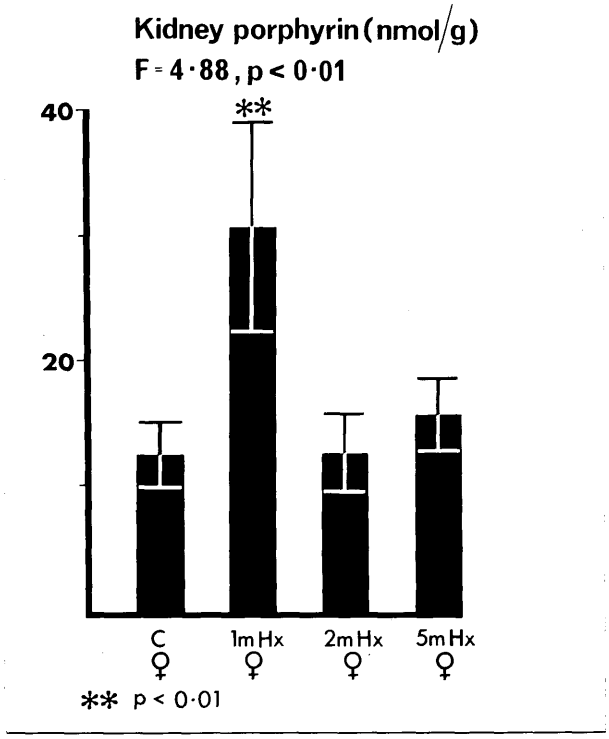


Figure 76

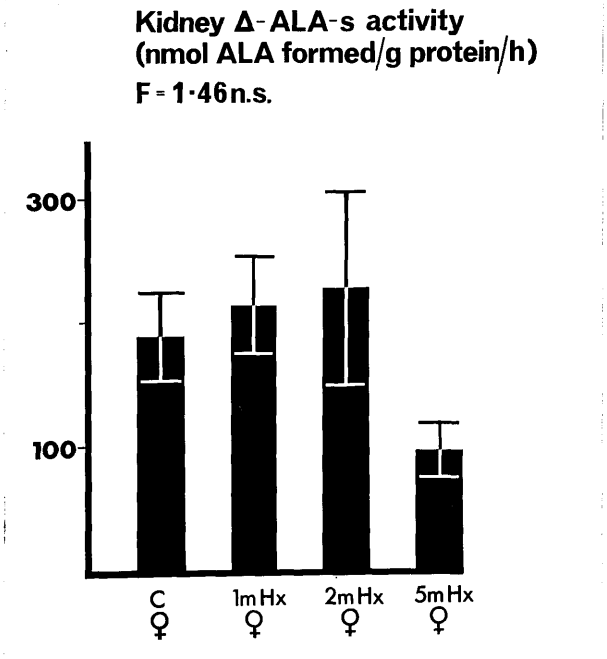


Figure 77

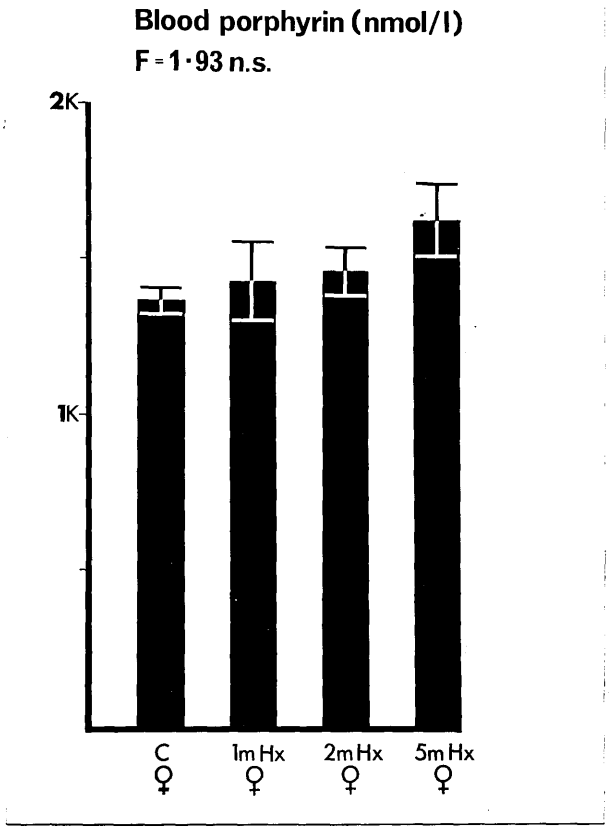


Figure 78

Figures 79 - 83

Histograms showing the changes in liver porphyrin content and 5-ALA-S activity, kidney porphyrin content and 5-ALA-S activity and blood porphyrin levels in sham Harderianectomised and sham ovariectomised females (♀), sham Harderianectomised and ovariectomised females (⚲), Harderianectomised and sham ovariectomised females (Hx ♀), and Harderianectomised and ovariectomised females (Hx ⚲).
Significance levels, where appropriate, are given in the text.

Figure 79 - Liver porphyrin (nmol/g)

Figure 80 - Liver 5-ALA-S activity (nmol ALA formed/ g protein/h)

Figure 81 - Kidney porphyrin (nmol/g)

Figure 82 - Kidney 5-ALA-S activity (nmol ALA formed/ g protein/h)

Figure 83 - Blood porphyrin (nmol/l)

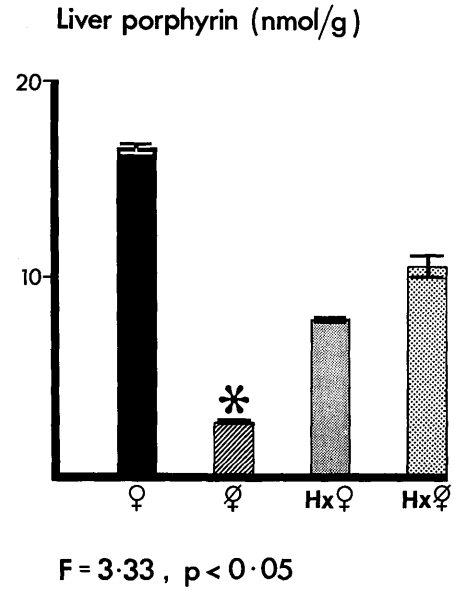


Figure 79

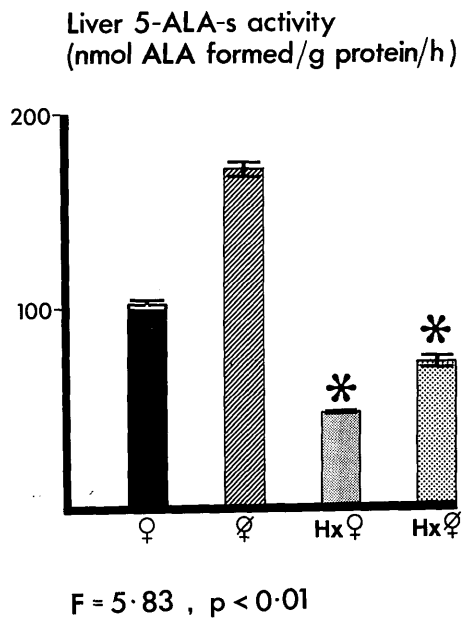


Figure 80

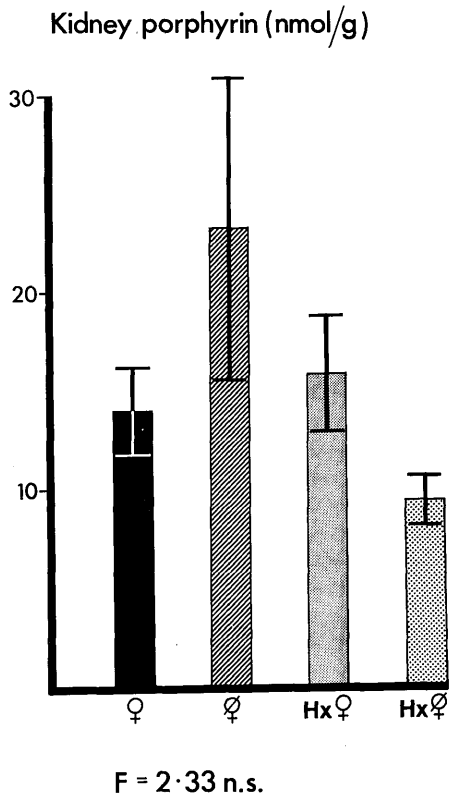


Figure 81

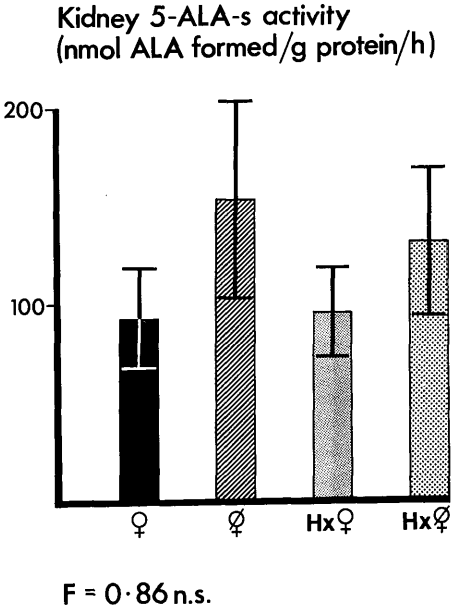


Figure 82

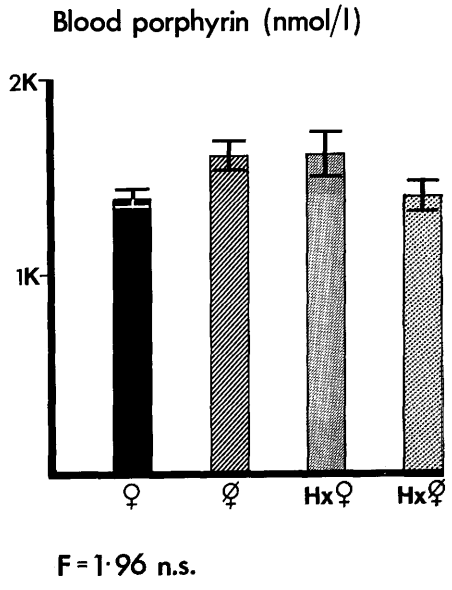


Figure 83

APPENDIX 1

Reagents required for 5-aminolaevulinic acid synthase assay (Thompson et al., 1984).

a) The following reagents were weighed out for the Reaction Mixture, giving a total volume of 250ml:-

0.5 mM glycine (9.5mg/250ml)

0.5 mM sodium maleate (22.25mg/250ml)

2.0 mM $MgCl_2$ (0.108g/250ml)

0.4 mM pyridoxal phosphate (66mg/250ml)

The following solutions were also made up:-

(i) KH_2PO_4 (3.4g/500ml)

(ii) $K_2H\ PO_4$ (4.355g/500ml)

The glycine, sodium maleate, $MgCl_2$ and pyridoxal phosphate were dissolved in approximately 100ml of $K_2H\ PO_4$ solution and the pH adjusted to 6.8 with the KH_2PO_4 solution, and added to a 250ml volumetric flask.
The remaining 400ml of K_2HPO_4 was then adjusted to pH 6.8 by the addition of KH_2PO_4 and this solution was used to make the above solution in the volumetric flask up to the mark. 250 µCi , 2-14C Glycine (Specific Activity 54mCi/mmol, Amersham International plc) was then added to 20ml of this reaction mixture.

b) The following reagents were weighed out for the Incubation Buffer giving a total volume of 1 litre:-

0.5M sucrose (171.15g/l)

0.025M Mg Cl_2 (5.08g/l)

0.2mM EDTA (0.744g/l)

8mM Mercaptoethanol - (volume 558 µl/l)

1mM glycine (0.075g/l)

The following solutions were then made up:-

(iii) K_2HPO_4 (20.902/l)

(iv) KH_2PO_4 (16.320/l)

The sucrose, $MgCl_2$, EDTA and glycine were then dissolved in about 700ml of the K_2HPO_4 and the pH adjusted to 7.0 with the addition of KH_2PO_4, then added to a 1 litre volumetric flask.

The remaining 300ml of K_2HPO_4 was then adjusted to pH 7.0 by the addition of KH_2PO_4 and this solution was then used to make the contents in the flask up to 1 litre.

The chemicals mentioned above were all supplied by BDH Chemicals Ltd., unless stated otherwise.

APPENDIX 2

Reagents required for protein estimation (Lowry et al., 1951)

The stock protein reagent is 2% Na_2CO_3 in N/10 NaOH which is made up by weighing out 20g sodium carbonate (Na_2CO_3) and 4g sodium hydroxide (NaOH) and making this up to 1 litre with distilled water. On the day of the assay, to each 100ml of this stock reagent used, 1ml of 1% copper sulphate ($CuSO_4$) and 1ml 2% sodium tartrate $2H_2O$ are added, as this mixture only lasts for one day.

The stock protein standard is bovine serum albumin (200mg/l), and on the day of the assay the following dilutions are made:-

Standard concentration (mg/l)	Volume of stock protein standard (ml)	Volume of distilled water (ml)
200	1	0
100	0.5	0.5
50	0.25	0.75
25	0.125	0.875

The colouring agent, Folin & Ciocalteau's Phenol Reagent, is diluted 1:1 with distilled water on the day of the assay.

The above mentioned chemicals were all supplied by BDH Chemicals Ltd.

APPENDIX 3: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF HAMSTER HARDERIAN GLANDS

INTRODUCTION

HPLC analyses of the female hamster Harderian gland has shown that protoporphyrin XI forms 95% of the total porphyrin content (Payne et al., 1982a). While ovariectomy resulted in a significant decrease in new porphyrin synthesis, as shown by the decrease in 5-ALA-S activity, the porphyrin content of ovariectomised females was not significantly different from that in intact females, presumably reflecting low clearance rates of porphyrin already formed there (Experiment 2). This decreased porphyrin production may result in an altered pattern of porphyrin synthesis: the glands of intact females were therefore compared with those of females ovariectomised for 5 months. Since castration of males resulted in greatly increased porphyrin synthesis and the appearance of porphyrin pigment, which is never seen in sexually mature males (Experiment 6), the pattern of porphyrin synthesis may also change. The porphyrin profiles of intact males and males castrated for 6 weeks were therefore also examined.

MATERIALS AND METHODS

Following total porphyrin extractions of Harderian glands, the remaining solvent, consisting of 95:5 methanol:conc. HCl, was prepared to form the porphyrin methyl ester, which is more stable than the free porphyrin in the solvent. Samples were prepared from the following animals; intact females

(n = 10), females ovariectomised for 5 months (n = 6), intact males (n = 7) and males castrated for 6 weeks (n = 5). The method of preparation of the porphyrin methyl esters is described in "GENERAL MATERIALS AND METHODS".

A porphyrin methyl ester standard (Porphyrin Products, Logan, Utah) was injected into a silica column (SI 100 Hewlett Packard) and eluted with a solvent which separated the constituent porphyrins and showed their relative proportions. The solvent mixture consisted of n-heptane, 600ml
ethylacetate, 250ml
chloroform, 125ml
methanol, 25ml
The flow rate through the column was 1ml per minute.

The standard contained six porphyrins which were eluted in the following order: protoporphyrin, coproporphyrin, pentacarboxylic porphyrin, hexacarboxylic porphyrin, heptacarboxylic porphyrin and uroporphyrin. The porphyrins were detected with a Fluorimeter (Perkin Elmer 3000) with settings of 396nm excitation and 594nm emission. These component peaks and their elution times were printed out on a Stumadzu C-RIB Chromatopac.

Several standards were injected until reproducible results were obtained. Ten µl of the reconstituted Harderian gland samples were injected into the column, and each sample was repeated three times. Every 6th sample was a standard, to ensure that the elution times had not changed significantly.

RESULTS (Table - see page 242)

In the female gland, protoporphyrin IX occurred in every trace, accounting for an average of 93% of the total porphyrin content. Trace amounts of coproporphyrin, pentacarboxylic porphyrin, hexacarboxylic porphyrin, heptacarboxylic porphyrin and uroporphyrin were also present in some individuals. Pentacarboxylic porphyrin occurred most consistently.

In ovariectomised females, 100% protoporphyrin IX occurred in four of the six animals. In the remaining two animals, pentacarboxylic porphyrin showed small traces in addition to protoporphyrin, and one of these females also had a small trace of heptacarboxylic porphyrin.

In intact males, protoporphyrin IX occurred in six of the seven traces, and the average amount present, 71.95%, was considerably less than in the intact females. Coproporphyrin, penta- and hexa-carboxylic porphyrins were present in greater proportions than in the female gland. No detectable traces of heptacarboxylic porphyrin or uroporphyrin were obtained in these samples.

The major component in castrate males was also protoporphyrin XI, and in 6 week castrates the amount present, 84.28%, was intermediate between male and female values. There was a higher percentage of coproporphyrin and a lower percentage of pentacarboxylic porphyrin in 6 week castrates compared to intact males. No detectable traces of hexa-, hepta-carboxylic porphyrins or uroporphyrin were obtained from these samples.

SUMMARY

The porphyrin profiles of both intact and ovariectomised females shows that protoporphyrin forms more than 90% of the total, while coproporphyrin, penta-, hexa-, and hepta-carboxylic porphyrins and uroporphyrin may be present in small amounts.

The male gland contains considerably less protoporphyrin but more coproporphyrin, penta- and hexacarboxylic porphyrins than the female showing that decarboxylation does not proceed so completely in the male gland. Castration produces changes in the porphyrin profile and shows that decarboxylation proceeds more completely than in the intact male since only protoporphyrin and coproporphyrin peaks are significantly detectable.

	Intact females (n=10)	Ovariect-omised females (n=6)	Intact males (n=7)	Castrated males (n=5)
Protoporphyrin	93.10±4.01	97.62±	71.95±13.05	84.28±4.07
Coproporphyrin	3.29±3.23	0	10.70±5.74	15.35±4.06
Pentacarboxylic porphyrin	2.29±0.67	2.35±	8.80±9.51	0.37±0.33
Hexacarboxylic porphyrin	0.39±0.21	0	8.54±9.22	0
Heptacarboxylic porphyrin	0.26±0.17	0.03±	0	0
Uroporphyrin	0.46±0.24	0	0	0

Table

High Performance Liquid Chromatographic analyses of porphyrin methyl esters from the Harderian glands of intact females, females ovariectomised for 5 months, intact males and males castrated for 6 weeks. All figures are mean percentages ± SEM.

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LIST OF PUBLICATIONS

1. A quantitative assessment of porphyrin loss from Harderian glands during routine histological processing. R.C. Spike, J. McGadey & A.P. Payne. Journal of Anatomy, 1983, 137, 817.

2. The effects of ovariectomy and androgen administration on the histology and porphyrin content of the Harderian gland in the female golden hamster. R.C. Spike, J. McGadey, H.S. Johnston & A.P. Payne. Journal of Anatomy, 1983, 137, 807.

3. The effects of hormone manipulation on the porphyrin content and histology of the Harderian gland in the female golden hamster. R.C. Spike, J. McGadey, H.S. Johnston, A.P. Payne, G.G. Thompson & M.R. Moore. Biochemical Society Transactions, 1985, 13, 206-207.

4. Quantitative studies on the effects of hormones on structure and porphyrin biosynthesis in the Harderian gland of the female golden hamster. I. The effects of ovariectomy and androgen administration. R.C. Spike, H.S. Johnston, J. McGadey, M.R. Moore, G.G. Thompson & A.P. Payne. Journal of Anatomy, 1985, 142, 59-72.

5. Quantitative studies on the effects of hormones on structure and porphyrin biosynthesis in the Harderian gland of the female golden hamster. II. The time course of changes after ovariectomy. R.C. Spike, H.S. Johnston, J. McGadey, M.R. Moore, G.G. Thompson & A.P. Payne. Journal of Anatomy, 1986, 145, 67-77.

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8. The prevention of porphyrin loss from tissues during routine histological processing: quantitative studies on the Harderian gland. R.C. Spike, J. McGadey, H.S. Johnston, A.P. Payne, G.G. Thompson & M.R. Moore. Histochemical Journal, in press.

LIST OF POSTERS AND SPOKEN PRESENTATIONS

July, 1983. - Anatomical Society, Glasgow.

1. A quantitative assessment of porphyrin loss from Harderian glands during routine histological processing. Spike, R.C., McGadey, J. & Payne, A.P. Journal of Anatomy 137, 817.
2. The effects of ovariectomy and androgen administration on the histology and porphyrin content of the Harderian gland in the female golden hamster. Spike, R.C., McGadey, J., Johnstone, H.S. & Payne, A.P. Journal of Anatomy 137, 807.

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The effects of hormone manipulation on the porphyrin content and histology of the Harderian gland in the female golden hamster. R.C. Spike, J. McGadey, H.S. Johnston, A.P. Payne, G.G. Thompson & M.R. Moore. Biochemical Society Transactions 13, 206-207.

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Changes in the Harderian gland of the golden hamster after ovariectomy. R.C. Spike, H.S. Johnston, J. McGadey, M.R. Moore, G.G. Thompson & A.P. Payne. C.U.P.

September, 1985. - Tetrapyrrole Discussion Group, Edinburgh.
The effects of Harderianectomy on the porphyrin content and 5-aminolaevulinic acid synthase activity in the liver and kidney of the golden hamster. R.C. Spike, A.P. Payne & M.R. Moore.