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INVESTIGATION IN FEMALE RATS OF THE EFFECTS OF ANDROGEN TREATMENT, DURING THE PRE- AND POSTNATAL PERIODS, ON GROWTH AND REPRODUCTIVE FUNCTION

A thesis presented in partial fulfilment
of the requirements for the
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ELIZABETH MARY SOMMERVILLE
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Abstract of a thesis presented in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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by

Elizabeth Mary Sommerville

In experiments investigating the perinatal period of sensitivity to androgen of hypothalamic functions, testosterone propionate (TP) was administered to female rats at different ages before and after birth and for varying time intervals.

Administration of TP was by subcutaneous injection in oil, or by subcutaneous polydimethylsiloxane (PDS) capsules. Plasma testosterone concentrations were measured by radioimmunoassay in adult female rats after insertion and removal 72h later, of 3 sizes of TP-filled capsule (5, 10 and 20mm crystal length); in 3 day old female neonates after insertion and removal 4h later of a TP-filled capsule (2.5mm crystal length), or after injection of TP (90μg in oil); and in growing female rats after neonatal implantation with a TP-filled capsule (2.5mm crystal length) which was not removed (chronic implantation). The plasma half-life of testosterone estimated after implantation with TP capsules was much shorter in adults (1h) than in neonates (8.6h). After TP injection to 3 day old rats the half-life was 48h and after chronic implantation of TP capsules was 69h.

Exposure of foetal rats to exogenous androgen, achieved by subcutaneous implantation of pregnant rats with TP capsules (3 sizes) for 24h or 72h at varying stages during gestation, did not alter ovarian function, feminine sexual behaviour or growth, despite abnormal development of the external vaginal opening in rats exposed to TP during the last 6 days of gestation.

Female rats aged 2, 3 or 5 days given brief periods of TP treatment by subcutaneous 2.5mm PDS TP-filled capsules, removed after 4, 8 or 24h, were compared with rats given 90μg TP by injection at the same ages. Control of gonadotrophin secretion, as indicated by ovarian morphology and cyclic changes in the vaginal epithelium, was sensitive to alteration by brief periods of exposure to TP. Treatment on days 2 or 3 with 4h implants produced anovulatory sterility at 90 days in at least 50% of animals. Feminine sexual behaviour, assessed by lordosis quotient, was depressed only by treatment longer than 24h at any of the 3 ages. Injection with TP prevented ovulation and depressed the lordosis quotient regardless of day of treatment. Although analysis of variance demonstrated an increased body weight from 14 weeks in rats injected with TP on days 2 or 5, regression analysis did not confirm an increased growth rate. The external vaginal orifice was altered by 4h TP implants given on days 2 or 3, but was unaffected by TP treatment of any duration given on day 5.

Testosterone propionate-filled capsules (2.5mm), implanted in female rats on days 2, 3 or 5 and not removed, prevented ovulation and abolished feminine sexual behaviour. Adult body weight was severely retarded by treatment commencing on day 2, but not altered by treatment beginning on days 3 or 5.

It can be concluded from this investigation that the hypothalamic control of gonadotrophin secretion in the female rat is insensitive to androgen prenatally, and is very sensitive 2 or 3 days after birth, when even brief (estimate of 28h exposure) periods of treatment are able to prevent ovulation. The control of feminine sexual behaviour is insensitive to prenatal TP. Depression of behaviour appears to require a longer neonatal period of exposure to TP (estimate of 48h minimum exposure) than that required to modify gonadotrophin secretion. Stimulation of growth appears to be a variable feature of the androgenized rat.

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CHAPTER 1

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CHAPTER 1

A REVIEW OF SEXUAL DIFFERENTIATION IN THE RAT

1. INTRODUCTION

Most animal species reproduce sexually, a process which allows genetic material from one individual to be recombined with genetic material of another. This occurs during the alternating processes of meiosis and fertilization. The resulting degree of genetic variation is not possible with asexual reproduction in which new cells formed by mitosis are genetically identical to the parent cell. Thus, in evolutionary terms, sexual reproduction is advantageous to the survival of the species since it increases genetic variation, the raw material upon which natural selection acts.

Sexual reproduction in higher animals involves the fusion of gametes from two individuals of opposite sexes. The anatomical, physiological and behavioural differences which distinguish the sexes arise during foetal and postnatal development by a process of sexual differentiation.

2. SEXUAL DIFFERENTIATION OF THE REPRODUCTIVE SYSTEM

The mechanisms determining sex and effecting sexual differentiation have been widely studied and the findings reviewed by many including Jost, Vigier, Prepin and Perchellet (1973), Wilson (1978), Short (1979), Wilson, Griffin and George (1980) and Wilson, Griffin, George and Leshin (1981b).

Chromosomal (genetic) sex, established at the time of fertilization, determines gonadal sex but the presence or absence of hormonal secretion from the foetal gonad governs development of phenotypic sex. Credit for elucidation of this sequential pattern of sexual differentiation is largely due to Alfred Jost (see Jost *et al.*, 1973). Jost's experiments

point out the asymmetry in sexual development of the male and female. His hypothesis that "at every step of sex differentiation maleness has to be forced against a basic feminine program" is supported by studies of sexual dimorphism in the gonad, the genital tract, the external genitalia and the central nervous system.

2.1 Genetic Determination of Sex

In mammals the male is the heterogametic sex (XY) and the female is homogametic (XX), the presence of a Y chromosome conferring maleness which is manifest early in foetal life by the appearance in the primordial gonad of seminiferous tubules. Until this occurs male and female embryos are histologically indistinguishable.

2.2 Differentiation of the Gonads

A gene on the Y chromosome codes for a male-specific antigen, the H-Y antigen, recently identified as a component of cell membranes of males of all mammalian species and thought to induce testicular differentiation in the indifferent gonad (see reviews by Ohno, Nagai, Ciccarese and Iwata, 1979; Haseltine and Ohno, 1981). Genes on the X chromosome are also involved in testicular differentiation and development, and autosomes play a role in gonadal development in both sexes (Wilson, 1978). So, although the sex chromosomes define the sex of the individual, autosomal genes are also involved in expression of the male or female features of the phenotype.

The germ cells originate in the endoderm of the yolk sac and migrate to the genital ridge, which is the anlage of the gonad (see review by Wilson, 1978). Histological evidence of organization appears much earlier in the testis than the ovary. According to Jost and co-workers (1973) the Sertoli cells differentiate first and by swelling and making contact with each other they come to surround the germ cells, thereby forming the future seminiferous tubules. Differentiated Leydig (interstitial) cells appear shortly after. The ovary, by contrast, remains undifferentiated until later in gestation.

2.3 Formation of the Genital Tract

Much of our understanding of the processes involved in the development of the genital tract must be attributed to the research carried out by Wilson and co-workers. Their reviews (Wilson, 1978; Wilson *et al.*, 1980; Wilson, George and Griffin, 1981a; Wilson *et al.*, 1981b) have furnished the material on which this summary of the differentiation and development of the genital tract is based.

Formation of the anlage of the internal genital tracts proceeds in an identical fashion in both male and female foetuses. Two duct systems, Wolffian and Mullerian, are formed and these share with the urinary tract a common opening to the exterior.

During early development the Wolffian ducts appear first and persist in the male as the masculine genital tract while the later formed Mullerian ducts regress. Virilization of the Wolffian ducts produces a rete testis (connecting with the seminiferous tubules of the testis), epididymis and vas deferens.

In the female the opposite process occurs with the Mullerian ducts forming the internal structures of the female (oviducts, uterus and part of the vagina) and the Wolffian ducts degenerating.

The external genitalia in both sexes develop from the urogenital sinus and urogenital tubercle. Thus there is an indifferent period of phenotypic development when both duct systems are present in both male and female foetuses.

2.3.1. Differentiation of the male genital tract

Removal of the gonads from foetal rabbits of either sex before the onset of phenotypic differentiation was shown by Jost and his colleagues (see review, 1973) to result in development of the female phenotype. He regarded masculine differentiation as being imposed on an inherently female pattern, at an early stage, by testicular hormones. He further suggested that two different secretions from the testis were responsible for regression of the Mullerian ducts and for virilization of the Wolffian ducts.

(a) 'anti-müllerian hormone'

Müllerian regression begins soon after the first appearance of seminiferous tubules in the testis. Therefore, cells of the tubules seemed the likely source of a factor causing degeneration of the Müllerian ducts. It now seems certain that 'anti-Müllerian hormone' is a glycoprotein synthesized by the Sertoli cells (Josso, Picard and Tran, 1977; Picard, Tran and Josso, 1978). It is common to all mammalian species and is secreted only during foetal life. The Müllerian ducts undergo antero-posterior degeneration, and Josso and her co-workers (1977) have found that sensitivity to 'anti-Müllerian hormone' is exhibited for a short 'critical period'. This occurs first in cranial segments and progressively in more caudal segments. 'Anti-Müllerian hormone' production is thus the first endocrine function of the testis.

(b) androgen

The onset of virilization of the male Wolffian ducts coincides with the histological differentiation of Leydig cells whose androgen production is believed to be its cause. Studies of androgen metabolism in rat, rabbit, guinea pig and human embryos (see review by Wilson *et al.*, 1981b) have established that although testosterone is responsible for virilization of the Wolffian ducts, its metabolite 5-dihydrotestosterone virilizes the external genitalia.

Administration of exogenous androgen at appropriate times during gestation will mimic the masculinization of the indifferent phenotype by the foetal testis, and female rat embryos thus treated show signs of male genital tract development (Schultz and Wilson, 1974). Anti-androgen treatment at similar times will prevent Wolffian virilization (see Wilson *et al.*, 1981b).

Recent *in vitro* experiments have confirmed that the testes of foetal rabbits (George, Simpson, Milewich and Wilson, 1979) and rats (Warren, Haltmeyer and Eik-Nes, 1973; Picon, 1976) are capable of the *de novo* synthesis of testosterone at the time of sexual differentiation of the Wolffian ducts.

2.3.2. Differentiation of the female genital tract

Maintenance of the Mullerian ducts and regression of the Wolffian ducts occur as the result of absence of a testis and do not require an ovary nor the XX genotype.

Despite the apparent absence of a role for oestrogen in genital tract differentiation, the foetal ovary acquires the capacity for steroid hormone synthesis at the same age as the foetal testis in the rabbit and human (see review by Wilson *et al.*, 1980). At this time the ovary is histologically undifferentiated compared with the testis.

Wilson and co-authors (1981a) suggest that because oestrogen is necessary for implantation and survival of the embryo, oestrogen may be essential to life itself. Any factor which interferes with oestrogen synthesis or action might block the implantation of the embryo and therefore be lethal. Androgen on the other hand is not necessary for survival of the embryo. Several genetic defects which result in inadequate synthesis of testosterone have been identified in humans, and studies of affected individuals have contributed to understanding of the role played by androgens in foetal sexual development (see review by Wilson *et al.*, 1981b).

Oestrogen, formed in the ovary before histological differentiation is apparent, may participate locally in differentiation of the gonad in a manner similar to that of testosterone in the testis (Wilson *et al.*, 1981a). Wilson's group suggests that oestrogen and progesterone may also be involved in growth and development of the internal genitalia of the female during later phases of embryogenesis even if not required for their differentiation.

3. SEXUAL DIFFERENTIATION OF THE BRAIN

3.1 Role of the Hypothalamus in Reproductive Function

Textbooks of physiology (see for example Mountcastle, 1974; Ganong, 1979) emphasize the central role of the hypothalamus in enabling the animal to maintain homeostasis, to respond to change in its environment and to carry out specific functions.

Through its afferent connections from other parts of the brain, the hypothalamus receives information from the sense organs about alterations in the internal and external environment. Its control over body functions is exerted through the endocrine, autonomic, hypothalamo-hypophyseal and somatic neural efferent systems. While it is difficult to adequately describe its multitude of roles - some diffuse and integrative, others specific - it has been said that the hypothalamus is "influenced directly or indirectly by almost all parts of the brain, while it in turn can modify virtually all body functions" (Brooks and Koizumi, 1974).

Among its many functions are those of control and co-ordination of sexual reproduction and, in this respect, hypothalamic function in the female is different from that in the male animal. Two aspects of reproduction in which the hypothalamus is involved are the release of gonadotrophins from the anterior pituitary and sexual behaviour. These two parameters therefore become sexually differentiated during development.

3.1.1. Control of anterior pituitary secretion

Control of secretion from the anterior pituitary is well understood (Ganong, 1979). Specialized hypothalamic cells, with multiple neural inputs, secrete hormones which either stimulate or inhibit (releasing or inhibiting hormones) the secretion by the anterior pituitary of specific pituitary hormones. The releasing (or inhibiting) hormones reach the anterior pituitary by passing along the hypothalamo-hypophyseal portal system. Since it has not been possible to separate different hormones controlling secretion of the two gonadotrophins,

follicle stimulating hormone (FSH) and luteinizing hormone (LH), it is believed that the one gonadotrophin-releasing hormone is responsible for eliciting release of both.

The adult male animal secretes gonadotrophins at a fairly constant rate, with consequent continuous sexual activity, during the mating season. In contrast, all adult mammalian females show cyclic sexual activity, seen as regular cycles in ovarian function, uterine lining, vaginal epithelium and sexual behaviour. The hypothalamus is responsible for this periodicity which represents a basic difference between males and females.

3.1.2. Control of sexual behaviour

The involvement of the hypothalamus in reproduction includes an important role in initiating and co-ordinating sexual behaviour. In adult female rats, 'oestrous' or mating behaviour is displayed only during the ovulatory stage of the oestrous cycle, as a consequence of the sequential exposure of the brain to oestrogen and progesterone from the ovary (Gorski, 1979). In the adult male rat, characteristic male mating behaviour patterns can be elicited continuously following the stimulus of a receptive female.

3.2 Role of Androgen in Sexual Differentiation of the Hypothalamus

Just as the undifferentiated genital tract follows an inherently female pattern of development unless testicular secretions are present to impose differentiation of the male phenotype, so the hypothalamic function is able to be diverted by androgen from a basically female pattern to that of a male type.

Reviews of the research which has led to current concepts of sexual differentiation of the central nervous system include those by Harris, 1964; Barraclough, 1966; Flerko, 1971; Gorski, 1971, 1979; Lieberburg, MacLusky, Roy and McEwen, 1978; McEwen, 1980, 1981; Arnold, 1980; MacLusky and Naftolin, 1981. The rat has been the principal experimental animal used in this area of research and consequently the processes of

sexual differentiation are understood best in this species but may apply to other species.

The reviews cited above acknowledge the original contributions by Pfeiffer (1936) whose series of experiments on neonatal rats provided the basic hypotheses which later investigations have clarified and expanded.

The results of Pfeiffer's experiments have been summarized by Harris (1964):

"1. Male rats castrated at birth, and transplanted with an ovary when adult, showed the capacity to form corpora lutea in this ovarian tissue.

2. Female rats ovariectomized at birth, and implanted with an ovary when adult, showed normal estrous cycles and corpora lutea formation.

3. Male rats in which the testes were transplanted into the neck region at birth, and which were implanted with an ovary when adult, showed no capacity to form corpora lutea in the ovarian tissue.

4. Many female rats into which testes were transplanted at birth failed to show any sign of estrous cycles when they became adult, but entered a state of constant vaginal estrus and failed to show the formation of corpora lutea in the ovaries."

Pfeiffer concluded that at birth the anterior pituitary in both male and female rats was undifferentiated, with an inherent capacity to secrete gonadotrophins in a cyclic fashion. If a testis were present at birth, this pattern could be altered so that constant gonadotrophin secretion occurred. Subsequent research (Harris, 1937; Haterius and Derbyshire, 1937) revealed the role of the hypothalamus in controlling secretion from the anterior pituitary and led to the hypothesis accepted today, that this part of the central nervous system differentiates functionally, under the influence of androgen from the foetal and neonatal testes, to cause the tonic gonadotrophin release pattern typical of the male. Pfeiffer's results were reproduced in rats

using a single injection of testosterone propionate (TP), confirming that androgen was the active testicular component (Barraclough, 1961).

Research which followed (Harris and Jacobsohn, 1952; Segal and Johnson, 1959, as cited by Gorski, 1966) proved that it was not the anterior pituitary which was permanently altered by exposure to androgen, since transplantation of the anterior pituitary from an androgen-treated female rat to a normal ovariectomized female rat resulted in normal pituitary function. The ovaries of androgenized female rats would also function normally and produce corpora lutea when transplanted into ovariectomized, but otherwise normal, female rats (Harris and Levine, 1965; Gorski, 1966). Furthermore, androgen-sterilized rats (primed with progesterone) could be induced to ovulate by electrical stimulation of the hypothalamus (Barraclough and Gorski, 1961), confirming that the anterior pituitary was able to release sufficient luteinizing hormone for ovulation and that the ovaries were able to respond. The weight of the above evidence therefore emphasizes the hypothalamus as the site of sexual differentiation of the control of gonadotrophin secretion.

These early studies demonstrate that the pattern of sexual differentiation of the hypothalamus can be experimentally manipulated during the neonatal period by altering the rat's androgen status. Masculine hypothalamic function may be conferred on a female animal by administration of testosterone during the first few days of life and castration of the male animal immediately after birth induces a feminine pattern in reproductive functions controlled by the hypothalamus. The treatment of the neonatal female rat with testosterone is believed to mimic the masculinizing processes which occur naturally in the male rat, and so can be viewed as a model for studying the mechanism by which testicular hormones impose on the undifferentiated hypothalamus a pattern of development characteristic of the male (Gorski, 1966).

The terms 'organizational' and 'activational' are useful for differentiating two different effects of testosterone. The role of testosterone in imposing permanent changes during these developmental stages may be considered as an 'organizational' effect. In adult life testosterone induces specific changes which disappear on withdrawal of the hormone and in this manner exerts 'activational' effects. The organizational

effects of testosterone may include some alteration to the way in which the animal responds to the activational effects of the hormone.

3.2.1. The androgenized female rat

The terms 'androgenized' or 'androgen-sterilized' are commonly used to describe the female rat which has been treated with an androgen, usually testosterone propionate, during the first few days of life. The following description of an animal so treated is based on the reviews listed at the beginning of Section 3.2 (Chapter 1).

The classical androgenized rat is a healthy genetic female, larger than its non-androgenized sisters, which fails to ovulate and does not show typical oestrous behaviour. The ovaries, when examined after puberty, contain many follicles but no corpora lutea and are smaller than those of control animals. Daily smears of the vaginal epithelium show a pattern of persistent cornification rather than the four or five day cyclic pattern of the normal female rat. When tested with a sexually mature male rat, the adult androgenized rat does not display the typical ear-wiggling and darting of an oestrous female rat. It usually fails to exhibit lordosis when mounted by the male and often aggressively resists his attempts to mount. This failure to show normal feminine sexual behaviour occurs whether she is intact or whether she has been treated with oestrogen and progesterone following ovariectomy. When primed with testosterone as an adult, the ovariectomized androgenized rat shows more evidence of male type behaviour than the normal female.

3.2.2. Steroid hormone production by foetal and neonatal gonads

(a) testis

Differentiation of the male and female brain, as a result of different hormonal environments during a critical perinatal period of development, requires that the testis of the male rat produces androgen around the time of birth and that female rats lack androgen.

The rat testis produces two distinct generations of Leydig cells (Roosen-Runge and Anderson, 1959); the first is seen during foetal and

neonatal life and the second begins at puberty. Recent published research (see below) has established that steroid production in the testis coincides with the presence of these two generations of Leydig cells, and that the male rat has high concentrations of testosterone in the bloodstream during its late prenatal and early postnatal life.

Results of *in vitro* experiments suggest that testosterone production in the rat testis begins as early as 14-15 days of gestation (Picon, 1976; Feldman and Bloch, 1978). It continues to increase, reaching a peak around 17-20 days (Warren *et al.*, 1973; Picon, 1976; Feldman and Bloch, 1978), which corresponds with the pattern of plasma testosterone concentrations (Slob, Ooms and Vreeburg, 1980; Weisz and Ward, 1980). Measurements in plasma before 16 days of gestation are not currently feasible because of the small blood volume in early foetuses (Weisz and Ward, 1980).

Correspondingly, Roosen-Runge and Anderson (1959) discovered that the Leydig cells differentiate morphologically at 15.5-16.5 days, reach a maximum number at 19 days of gestation and the growth rate then declines until birth when a regression results in the nadir of their collective volume (in absolute and relative terms) at day 4 of life. Similarly, Lording and de Kretser (1972) reported that the proportion of the testis occupied by Leydig cells is maximal at 18 days of gestation and declines thereafter to a minimum during the second postnatal week.

Testosterone concentrations are higher in male rat foetuses than in females (Turkelson, Dunlap, MacPhee and Gerall, 1977; Slob, Ooms and Vreeburg, 1978, 1980; Weisz and Ward, 1980). However, appreciable levels of testosterone have been measured in the female foetus (Turkelson *et al.*, 1977; Weisz and Ward, 1980) with some overlap of individual male and female values at all ages (Weisz and Ward, 1980).

In the first few days of life, blood testosterone concentrations in male rats have been found to be consistently higher than in females (Dohler and Wuttke, 1975; Turkelson *et al.*, 1977; Corbier, Kerdelhue, Picon and Roffi, 1978; Butte, Moore and Kakihana, 1979; Forest, 1979; Lieberburg, Krey and McEwen, 1979; Pang, Caggiula, Gay, Goodman and

Pang, 1979; Slob *et al.*, 1980; Weisz and Ward, 1980; Anderson, Fatinikun and Swift, 1982) although again measurable levels were found in females. A surge of testosterone has been measured in male rats at variable times within the first 48 hours of life (Corbier *et al.*, 1978; Slob *et al.*, 1980; Gogan, Slama, Bizzini-Koutznetzova, Dray and Kordon, 1981; Anderson *et al.*, 1982), but not in female rats (Slob *et al.*, 1980; Anderson *et al.*, 1982). Blood testosterone concentrations in the male rat fall during the first days of life and remain low until puberty (Forest, 1979; Pang *et al.*, 1979; Slob *et al.*, 1980; Weisz and Ward, 1980; Corpechot, Baulieu and Robel, 1981; Gogan *et al.*, 1981).

Lieberburg and co-workers (1979) castrated neonatal male rats and found that plasma testosterone concentrations were reduced to within the female range, hence it seems that the testosterone measured in the blood of neonatal male rats is of testicular origin.

(b) ovary

Milewich, George and Wilson (1977) have demonstrated the ability of the foetal rabbit ovary to synthesize oestrogen, the onset of which develops about the same time as development in the foetal rabbit testis of the enzymatic ability to synthesize testosterone. This occurs before histological differentiation of the gonads and follows the appearance of secretory granules in Rathke's pouch, the anlage of the adenohypophysis. There is no evidence that oestrogen produced by the ovary has a role in embryogenesis, although the authors suggest that it may act within the ovary itself. No report of comparable work in the rat is known to this writer.

3.2.3. The aromatization hypothesis

Since exogenous testosterone administered to neonatal female rats causes androgenization, it was naturally assumed that testosterone is the hormone active in normal masculinization of the hypothalamus. However, oestradiol injected into neonatal female rats also causes androgenization, and at a lower dose rate than testosterone (Gorski, 1963). It is now considered that oestradiol, formed by the aromatization of testosterone, is the hormone which induces the development in the hypothalamus of 'masculine' patterns of control of gonadotrophin secretion, sexual behaviour and growth. This apparently paradoxical premise is supported by several lines of evidence.

Androgens such as dihydrotestosterone, which cannot be aromatized to oestrogen, are ineffective in causing androgenization (Luttge and Whalen, 1970; McDonald and Doughty, 1974; Whalen and Rezek, 1974; Booth, 1977). Oestrogen antagonists and inhibitors of aromatization inhibit the testosterone-induced masculinization (McDonald and Doughty, 1973/74; McEwen, Lieberburg, Chaptal and Krey, 1977; Booth, 1979). Aromatase activity has been demonstrated in neonatal rats in central neuroendocrine tissues known to be involved in reproductive function (Naftolin, Ryan, Davies, Reddy, Flores, Petro, Kuhn, White, Takaoka and Wolin, 1975) and ^{3}H-labelled oestradiol has been recovered from limbic and hypothalamic brain tissue of neonatal rats after subcutaneous injection of ^{3}H-labelled testosterone (Lieberburg and McEwen, 1975). Intranuclear oestrogen binding sites are present in the brains of 3-day old rats, and their cell nuclei in the hypothalamus retain more oestrogen than other brain regions (McEwen, Plapinger, Chaptal, Gerlach and Wallach, 1975).

Rat foetuses of both sexes are exposed to oestrogens from the placenta and maternal circulation, and high concentrations of oestrogen have been measured in infantile female rats (Weisz and Gunsalus, 1973; Ojeda, Kalra and McCann, 1975). However, it appears that foetuses and neonates are protected from endogenous oestrogens by an oestrogen binding protein known as α-foetoprotein.

This protein is found in the blood and brain of both male and female foetuses and neonates (Raynaud, Mercier-Bodard and Baulieu, 1971; Plapinger, McEwen and Clemens, 1973; Ali, Kaul and Sahib, 1981). Since α-foetoprotein does not bind testosterone, and its oestrogen-binding capacity can be overwhelmed by large doses of oestrogen, administration of testosterone or pharmacological amounts of oestrogen to the female neonate can cause masculinization of the hypothalamus. Benno and Williams (1978) and Toran-Allerand (1978) report that α-foetoprotein also appears intracellularly in neonatal rat brain, but not in the cells known from autoradiographic studies to contain oestrogen receptors.

Neonatal mice have been rendered anovulatory by the intracranial injection of antibodies to α-foetoprotein (Mizejewski, Vonnegut and Simon, 1980) presumably because the antibody-bound α-foetoprotein is

unable to protect the hypothalamus from the 'androgenizing' effects of circulating endogenous oestrogen.

It therefore seems likely that the perinatal female rat is protected from the androgenizing effects of circulating oestrogens by α-foetoprotein. Exogenous testosterone or large doses of oestrogen may still reach the hypothalamus where the testosterone can be aromatized intracellularly to oestrogen. Oestrogen receptors in the cytoplasm of target cells combine with oestrogen to form a complex which enters the nucleus and influences cell activity. Testosterone from the neonatal and foetal testes acts the same way in the male rat after its aromatization to oestrogen in the relevant cells of the hypothalamus.

3.2.4. Hypothalamic regions involved in sexual differentiation

Once it had been established that the hypothalamus was the tissue which was permanently altered by exposure of the neonatal rat to androgens, Barraclough and Gorski (1961) undertook to define the hypothalamic region involved. In the course of their investigation they accumulated evidence for a dual hypothalamic control system for ovulation. A region around the arcuate-ventromedial nuclei of the median eminence is responsible for the maintenance of a tonic discharge of gonadotrophin, sufficient to maintain oestrogen production in the ovary. The cyclic discharge of gonadotrophin which results in ovulation is caused by an anterior hypothalamic-preoptic area region acting on the arcuate-ventromedial area. This anterior region is influenced by exteroceptive and interoceptive stimuli so that in the normal female the preoptic area responds to appropriate environmental and hormonal stimuli and activates the arcuate-ventromedial region to cause an ovulatory discharge of gonadotrophins from the anterior pituitary. The anterior regions of the hypothalamus are also thought to be responsible for sexual behaviour (Gorski, 1966). Barraclough and Gorski (1961) presumed that the cyclic control centre in the preoptic area differentiates in the presence of androgen, becoming refractory to intrinsic and extrinsic activation so that a tonic pattern of gonadotrophin secretion results.

3.2.5. Morphological evidence of sex differences in the central nervous system

Several scientists have looked for and found evidence of sex differences in the central nervous system.

Raisman and Field (1973) showed, by electron microscopic studies, a sexual dimorphism in the synapses on dendritic spines in the preoptic area. This was susceptible to alteration by neonatal testosterone in the female, or castration of the neonatal male rat.

Other neuro-anatomical and biochemical differences arising as a result of androgenization of the female or as a consequence of normal sexual differentiation have been reviewed by Toran-Allerand (1978).

The most exciting discovery in this field is that of Gorski, Gordon, Shryne and Southam (1978) who described a gross morphological sex difference in the medial preoptic area of the rat brain. This parallels the finding of sexual dimorphism in vocal control areas of the songbird brain (Nottebohm and Arnold, 1976). The sexually dimorphic nucleus (SDN) reported by Gorski and his colleagues is sufficiently obvious to allow sexing, by examination with the naked eye, of a histological section of rat brain. The normal male SDN has a significantly larger volume than the female SDN due to the male nucleus having many more neurons (Gorski, Harlan, Jacobson, Shryne and Southam, 1980).

Manipulation of the neonatal hormone environment (by castration of the 1-day old male rat or administration of TP to the 4-day old female rat) alters the volume of the nucleus.* Testosterone propionate-treated females have a larger SDN than control females and in males castrated at 1 day of age, the SDN does not achieve the dimensions of the control male. Whether or not this nucleus has a specific role in the reproductive process remains to be discovered.

3.2.6. The concept of the 'critical period'

One of the striking points to emerge from this summary of the

research into many aspects of the sexual differentiation of the hypothalamus of the white rat is the existence of a short critical period. Only during this period may the pattern of development of future hypothalamic function be altered by androgen. The duration of the critical period however defies precise definition. The different hypothalamic functions which differentiate according to sex may be sensitive to androgen at different hormone concentrations and over different time spans. Within these time spans there may be a time of maximum sensitivity during which a lower concentration of androgen will masculinize the function, while at an earlier or later period the hypothalamus may still be influenced by supraphysiological doses of androgen. The phenomenon of the critical period must be viewed from different angles - the age of the animal at its onset, its duration, the dose of hormone administered and its persistence within the body, and the endpoint being measured. Gorski (1968) has pointed out the possibility of partial androgenization in his description of the delayed anovulation syndrome (described in Section 4.2.1, Chapter 1), cautioning that the age at observation of the function being investigated must be considered.

Maximal sensitivity to gonadal hormones may be associated with a particular stage of neuronal maturation. In the rat many of the hypothalamic structures thought to be involved in sexual differentiation are poorly differentiated at birth (Toran-Allerand, 1978).

3.2.7. Independence of differentiation of hypothalamic functions

Sexual differentiation of the neural tissues controlling sexual behaviour has been demonstrated to be independent of the differentiation of the control mechanism for gonadotrophin secretion (Barraclough and Gorski, 1962; Mullins and Levine, 1968; Clemens, Hiroi and Gorski, 1969; Luttge and Whalen, 1970; Vreeburg, van der Vaart and van der Schoot, 1977; Christensen and Gorski, 1978; Booth, 1979; Gorski, Christensen and Nance, 1979). Female rats treated neonatally with low doses of androgen, although lacking corpora lutea in their ovaries, may still be sexually receptive when adult.

Because the doses of androgen required to induce depression of female behaviour are higher than those required to produce anovulatory sterility, female sexual behaviour appears to be more resistant to neonatal hormone exposure than the control of ovulation (Christensen and Gorski, 1978; Gorski *et al.*, 1979). Modification of behaviour may require a longer period of exposure to androgen, since anovulation-inducing doses of free testosterone, which has a shorter duration of action than the propionate ester, fails to suppress feminine behaviour (Luttge and Whalen, 1970; McDonald and Doughty, 1974).

Feminine and masculine sexual behaviour can also be independently differentiated, so that it is possible for the same rat to show well developed patterns of both masculine and feminine behaviour (Harris, 1964; Nadler, 1969; Vreeburg *et al.*, 1977; Booth, 1979; Davis, Chaptal and McEwen, 1979).

3.3 Summary

The hypothalamic control of gonadal function, sexual behaviour and some aspects of growth differs between male and female rats. Exposure to aromatizable androgen or oestrogen during a critical perinatal period will result in masculine patterns of gonadotrophin release, sexual behaviour and growth in adulthood. The testes of foetal and neonatal rats synthesize appreciable amounts of testosterone. Protection of the foetal brain from the androgenizing effects of maternal and placental oestrogen may be due to the oestrogen-binding α-foetoprotein identified in the blood and brain of rat foetuses of both sexes. The neonatal female rat treated experimentally with adequate doses of testosterone will, when adult, be anovulatory, fail to display normal female behaviour and grow larger than its untreated counterparts. It provides a model for masculine hypothalamic differentiation. These functional changes are accompanied by neuro-anatomical sex differences in the central nervous system. The organizational actions of androgen are confined to a brief critical period which will be discussed in the following sections.

4. DIFFERENTIATION OF THE CONTROL OF GONADOTROPHIN SECRETION

4.1 Patterns of Gonadotrophin Secretion

The continuous secretion of gonadotrophin by the male rat after puberty is maintained at fairly constant levels and is described as a tonic pattern of release. Production of spermatozoa and synthesis of androgens is therefore stimulated continuously and the male remains fertile at all times.

A cyclic pattern of discharge characterizes female gonadotrophin release and results in cycles of ovarian activity. This release pattern is regulated by feedback from the ovarian hormones acting on the hypothalamus whose releasing hormone evokes the gonadotrophin secretion. In each oestrous cycle the hormonal sequence culminates in a surge of LH which causes ovulation. Two commonly used indices of this phasic pattern of gonadotrophin release are the presence of corpora lutea in the ovary and a regular cycle of cellular change in the vaginal epithelium.

4.2 The 'Critical Period' for Differentiation

Investigation of the critical period in the life of a female rat, during which administration of androgen may cause failure of ovulation after puberty, was initiated by Barraclough (1961). Following up his work in mice (Barraclough and Leathem, 1954), he found that female rats treated at 2 or 5, but not 20, days of age with a single injection of 1.25mg TP were anovulatory in adulthood. Treatment at 10 days produced 50% sterility. He concluded that there is a period between birth and 10 days in the young rat during which testosterone administration can result in subsequent infertility.

4.2.1. Postnatal

Injection of TP on any one of the first 5 days of life of the female rat causes anovulatory sterility (Barraclough, 1961; Gorski and Barraclough, 1963; Swanson and van der Werff ten Bosch, 1964; Gorski, 1968; McDonald and Doughty, 1972). Administration of TP directly into the hypothalamus as late as 11 days prevented ovulation (Lobl and Gorski, 1974). Attempts to identify the most sensitive day for TP treatment have varied in their conclusions, although generally, TP given on days 2 or 3 has been more effective than when given on day 5 or later (Swanson and van der Werff ten Bosch, 1964, 1965; Gorski, 1968; Tarttelin, Shryne and Gorski, 1975; Christensen and Gorski, 1978).

It has been noted that the full anovulatory syndrome may develop after an initial period of normal ovarian activity (Swanson and van der Werff ten Bosch, 1964; Gorski, 1968). Gorski called this phenomenon the 'delayed anovulation syndrome'. When given on or before day 4 of life, 10μg TP induced a high incidence of anovulatory ovaries at 6 weeks of age. However, many of the rats which were treated at day 6 had corpora lutea present at 7 weeks, but absent when examined again at 18 weeks. Treatment with the same dose of androgen in animals 2 days older had delayed the onset of the anovulatory syndrome. Treatment with a higher dose of TP (30μg) at day 5 had been shown in previous experiments (Gorski, 1966) to be effective in causing anovulation with no prior ovulatory period. Gorski concluded that the delayed anovulatory syndrome results from exposure of the brain to subphysiological levels of androgen during the critical period.

4.2.2. Prenatal

Indirect treatment of the female foetal rat with testosterone by injection of the mother during pregnancy has resulted in modification of the external genitalia without alteration to ovarian function (Greene, Burrill and Ivy, 1939; Swanson and van der Werff ten Bosch, 1964). In a later report, Swanson and van der Werff ten Bosch (1965) described the results of injection of much larger doses of TP (10 and 25mg) to pregnant rats. Despite the toxic effects of high doses of androgen

(intrauterine death, delayed parturition and failure to lactate), they were able to successfully rear enough females to demonstrate a high percentage of anovulatory ovaries at 21 weeks, although all had corpora lutea at 8 weeks. This incidence of the delayed anovulation syndrome was attributed to poor transmission of TP from the maternal into the foetal body, since subcutaneous injection of 100μg TP directly into the foetuses while *in utero* late in pregnancy resulted in 9 out of 10 animals with anovulatory ovaries at 14 weeks. A recent paper (Slob, den Hamer, Woutersen and van der Werff ten Bosch, 1983) appears to support the idea that testosterone does not pass from the maternal to the foetal circulation.

Fels and Bosch (1971) failed to alter ovarian function of female rats by prenatal administration of TP (by subcutaneous injection of dams, doses ranging from 5.0-32.5mg TP), but injection of 0.5-1.0mg TP into the amniotic cavity of female foetuses resulted in 64% with anovulatory ovaries. Unfortunately the authors failed to mention the stage of gestation at which treatment was given.

4.3 Minimum Exposure During the 'Critical Period'

Since Barraclough's presumption (1966) "that 24-48 hours of exposure to androgen is all that is necessary to produce permanent sterility", the minimum time required for androgen's action has been investigated by the use of various substances which interfere with steroid action on the central nervous system.

The barbiturates, pentobarbital and phenobarbital, and the anti-androgen, cyproterone acetate, can all prevent androgen-induced sterilization. Arai and Gorski (1968a, b) administered these agents at varying intervals after TP injection to neonatal female rats and concluded that androgen exerts its effects on the hypothalamus within 6-12 hours.

Hayashi and Gorski (1974) placed TP coated tubes in the hypothalamus of 3-day old rats and, by removing them at intervals up to 72 hours, found an increasing incidence of sterility, although only 43% of the

animals were sterile at 50 days even after 72 hours of exposure.

Experiments in which the TP injection site (the tail of the neonatal rat) was removed at a predetermined time after injection, suggest a longer period of TP treatment (24-48 hours) is required for androgenization (Alklint and Norgren, 1970).

4.4 Evidence from the Male Rat

Transplantation of ovarian and vaginal tissues into male rats castrated when adult results in polyfollicular ovaries and a pattern of persistent cornification of the vaginal epithelium (Yazaki, 1960) as a consequence of the tonic gonadotrophin secretion of the normal masculine hypothalamus. However, male rats castrated within the first 2 (Harris, 1964) or 3 (Yazaki, 1960; Gorski and Wagner, 1965) days of life are capable of forming corpora lutea in transplanted ovarian tissue.

Gogan, Beattie, Hery, Laplante and Kordon (1980) observed a cyclic pattern of LH release in male rats, castrated 2 hours after birth, when treated in adulthood with oestradiol. The LH peak was much smaller than that seen in ovariectomized females similarly treated with oestradiol but was absent in neonatally intact males and androgenized females. To account for the partial masculinization of early castrated males, Gogan and co-workers (1980) suggest prenatal action of testosterone on the gonadotrophin control mechanism. Further investigation (Gogan *et al.*, 1981) involved treatment of pregnant females with an antibody to testosterone. The male offspring, castrated when adult, showed an LH peak in response to oestradiol, but again this peak did not match the amplitude of that seen in females after similar treatment. Slow neutralization of the antigen or a prolonged period of sensitivity of the brain to testosterone were suggested by the authors as explanations for this degree of masculinization.

Although the central nervous system of the female rat is susceptible to masculinization by testosterone up until 10 days after birth, the male must be castrated within the first 2 or 3 days of life in order to 'feminize' the programming of brain circuitry involved in control

of gonadotrophin release. This is not a discrepancy between the sexes but reflects the irreversible organizational effect of androgen on the hypothalamus. The hypothalamus of both males and females may be considered to be equally sensitive to androgen until about 10 days, but in the case of the normal male, testosterone has already achieved its effect by the second or third day of life due to secretion of the hormone by the neonatal testes.

4.5 Summary

Experiments investigating the duration of the androgen-sensitive phase of the gonadotrophin controlling function of the hypothalamus have been performed in both female and male rats. It may be concluded that the hypothalamus can be organized by androgen over a period spanning the last few days of intrauterine life up to about 10 days of age. At the outer extreme of this critical period, the hypothalamus is less sensitive, since greater amounts of androgen are needed for androgenization. It appears to be maximally sensitive around 2 days of age. Estimations of the minimum duration of exposure to TP required for androgenization vary between 6-48 hours.

5. DIFFERENTIATION OF FEMININE SEXUAL BEHAVIOUR

A number of patterns of behaviour show different forms in male and female animals of the same species. These include aggression, active avoidance behaviour, open field behaviour and taste preference (see MacLusky and Naftolin, 1981). The most obvious sexually dimorphic behaviour pattern is that of sexual or mating behaviour.

5.1 Normal Feminine Sexual Behaviour

Beach (1976) has arbitrarily classified the sexual behaviour of female rats into three closely interrelated components - attractivity, proceptivity and receptivity. Elements of proceptivity and receptivity are easily identified and may be used as measures of feminine sexual

behaviour. Proceptive behaviours include the so-called 'soliciting' behaviours - darting, crouching and ear-wiggling. The facility to display lordosis is a quantifiable measure of receptivity which is dependent on ovarian hormones and is seen normally in the female rat at oestrus. Lordosis occurs in response to the male mounting the female, grasping her flanks and thrusting with his pelvis. The female responds by deep ventral arching of the back with dorsal elevation of the head and lateral tail deflection, and this posture is known as the lordosis response. It can be assessed by calculation of the percentage of lordoses resulting from a defined number of mounts, called the lordosis quotient (LQ). The lordosis response is abolished by ovariectomy and restored by treatment with oestradiol and progesterone. These hormones cannot induce the same level of receptivity in the castrated adult male rat (see Young, 1961), hence there appears to exist a difference between the male and female neural systems controlling this behaviour.

Phoenix, Goy, Gerall and Young (1959) were the first to propose that differentiation of the tissues mediating sexual behaviour patterns paralleled the relationship between the differentiation of the genital tract and androgens. As a result of their experiments with female guinea pigs given androgen prenatally, they concluded that; "... modification of behavior follows an alteration in the structure or function of the neural correlates of the behavior. We are assuming that testosterone or some metabolite acts on those central nervous tissues in which patterns of sexual behavior are organized."

Early androgen exposure appears to be responsible for the sex difference in mating behaviour in the rat as well as the guinea pig, since the capacity for lordosis can be suppressed in the female by neonatal androgen treatment (Harris and Levine, 1965) and castration of the neonatal male allows the display of lordosis when the adult animal is treated with ovarian hormones (Harris, 1964).

Masculine sexual behaviour appears to be a natural product of the action of androgen perinatally in the male rat and absence of androgen results in the development of normal feminine mating behaviour. Further consideration of the development of masculine behaviour is beyond the scope of this thesis.

Adult sexual behaviour can be viewed as another sexually dimorphic function which is dependent on modification of the hypothalamus by androgen during the perinatal life of the rat.

5.2 Modification of Sexual Behaviour by Androgen in Female Rats

The potential for feminine mating behaviour in female rats has been successfully modified by both pre- and postnatal treatment with aromatizable androgen or oestrogen and by prenatal anti-androgens and inhibitors of aromatization.

Suppression of feminine behaviour by androgen was illustrated in female guinea pigs by Phoenix and others (1959) and by Goy, Bridson and Young (1964), and involved treatment of the pregnant mother with TP. A similar phenomenon was observed in rats after postnatal treatment with TP (Barraclough and Gorski, 1962) and has been confirmed many times since.

This depressed ability of adult ovariectomized rats to display lordosis after priming with oestrogen and progesterone has been demonstrated in various experiments in which the day or days of treatment and the dosage of androgen administered have varied.

5.2.1. Postnatal

Female rats to which testosterone was administered for several days in the early postnatal period showed a deficit in their adult lordotic behaviour (Goy, Phoenix and Young, 1962; Ward and Renz, 1972; McEwen *et al.*, 1977).

A single injection of a relatively high dose of TP (>100μg) given on one of the first 6 days of life inhibited the adult lordotic response (Harris and Levine, 1965; Whalen and Edwards, 1967; Mullins and Levine, 1968; Hendricks, 1969; Clemens, Shryne and Gorski, 1970; Pfaff and Zigmond, 1971). An enhanced potential for feminine mating behaviour has been demonstrated after a single low dose of TP (50μg or less) on day 5 (Barraclough and Gorski, 1962; Mullins and Levine, 1968),

days 1, 2 or 4 (Clemens *et al.*, 1969) or after placement of testosterone implants in the preoptic area on day 2 (Christensen and Gorski, 1978).

Thus it seems that the neural substrate controlling feminine mating behaviour in the female rat is susceptible to inhibition by large doses of androgen over at least the first six days of life. From the work of Clemens and co-workers (1969), the hypothalamus appeared to be most sensitive to the suppressive effects of androgen on feminine behaviour on days 4 and 6 of life.

5.2.2. Prenatal

Daily injection of the pregnant rat with high doses of TP has been the usual method for exposure of the offspring to prenatal androgen. A reduced potential for the display of feminine mating behaviour, relative to controls, has been achieved by prenatal treatment of female rats during days 16-20 of gestation (Dunlap, Gerall and Carlton, 1978), days 16-21 (Ward and Renz, 1972), days 14-22, 16-22 and 16-20 (Gerall and Ward, 1966). Huffman and Hendricks (1981) found that a single dose of TP on day 19, but not day 16, suppressed feminine behaviour, as did Nadler's (1969) single treatment on day 18. As the other reports listed here involved treatment spans of several days, but all included days 18 and 19, it may be assumed that the hypothalamic control of feminine mating behaviour is responsive to androgen at least as early as day 18 of gestation.

However, Whalen, Peck and LoPiccolo (1966) found the feminine mating behaviour of female rats exposed on days 15-19 of gestation to a dose of progestin (17α-ethinyl-19-nortestosterone), whose androgenic effects were sufficient to severely masculinize the genitalia of the female offspring, to be normal. Revesz, Kernaghan and Bindra (1963) also found no suppression of the capacity for feminine sexual behaviour in the female offspring of rats injected with TP on days 15-20 of pregnancy.

So, despite similarities in treatment, there is disagreement on whether or not the brain control system for feminine sexual behaviour is altered permanently by prenatal androgen. It is possible that inconsistencies in behavioural testing procedures may mask differences.

An observation relevant to discussion of the differentiation of behaviour is that normal female rats are able to display masculine sexual behaviour such as mounting and intromission responses (Clemens, Gladue and Coniglio, 1978). It has been suggested that this behaviour is the result of a degree of masculinization of female foetuses which takes place normally during gestation (Clemens and Coniglio, 1971; Clemens *et al.*, 1978; Gorski *et al.*, 1979) and which may also cause a reduction in the capacity for feminine behaviour. Consequently, normal female rats show both masculine and feminine sexual behaviour patterns to varying extents.

This behavioural masculinization has been suggested by Gorski and colleagues (1979) to be due to small amounts of free oestradiol which may exist in plasma in equilibrium with that bound to α-foetoprotein. Clemens and co-workers (1978) consider the male foetuses in the uterus to be a source of masculinizing agent. In support of their hypothesis, they have demonstrated that proximity to male foetuses during gestation enhances the probability of mounting behaviour in the female rat. Their finding, that the anogenital distance in the female also varied as a function of proximity to a male in the uterus, strongly suggested the involvement of androgen. This was further supported by their observations that an anti-androgen, flutamide, given to the mother during pregnancy, eliminated the androgen-dependent morphological alteration and lowered the probability of masculine sexual behaviour in female offspring. Another anti-androgen, cyproterone acetate, when given prenatally also reduces the capacity of female rats to display masculine behaviour (Ward and Renz, 1972).

Female rats exposed prenatally (*via* the maternal circulation) to an anti-androgen (flutamide) or an inhibitor of aromatization (androst-1,4,6-triene-3,17-dione, ATD), showed higher scores for female behavioural responses (Clemens and Gladue, 1978; Gladue and Clemens, 1978). Both treatments would be expected to reduce the amount

of androgen available to affect the central nervous system.

Most of the evidence presented lends support to the argument that differentiation of the neural tissues mediating feminine sexual behaviour begins before birth in the rat. Exposure of the female foetus to androgen results in a reduced capacity to display oestrous behaviour in adulthood. The critical period for sexual differentiation of feminine mating behaviour appears to include the days immediately before and after birth. Although the process seems to be complete by 6 days of postnatal life, there has been no attempt to define its prenatal limits.

5.3 Modification of Sexual Behaviour in Male Rats

5.3.1. Postnatal

Researchers who have castrated male rats within 24 hours of birth have found that, when treated in adulthood with ovarian hormones, these rats show more feminine sexual behaviour than neonatally intact rats (Harris, 1964; Feder and Whalen, 1965; Grady, Phoenix and Young, 1965; Gerall, Hendricks, Johnson and Bounds, 1967; Whalen and Edwards, 1967; Hendricks, 1969). The LQ exhibited by these early castrated males either did not differ from (Grady *et al.*, 1965; Whalen and Edwards, 1967; Mullins and Levine, 1968; Hendricks, 1969), or was lower than that for females (Gerall *et al.*, 1967). When other criteria of feminine behaviour, such as soliciting or the intensity of the lordosis response, were measured, the genetic males scored less than the females (Grady *et al.*, 1965; Gerall *et al.*, 1967; Hendricks, 1969; Dunlap *et al.*, 1978).

Over the first 10 days of life, castration results in less feminine behaviour as the age at surgery increases (Grady *et al.*, 1965; Gerall *et al.*, 1967). In a comparison, which they acknowledged was not strictly legitimate since the rats were in different experiments, Feder and Whalen (1965) found that those males castrated 16-32 hours after birth showed more feminine behaviour than those castrated at 96 hours.

Administration of an inhibitor of aromatization (ATD) (McEwen *et al.*, 1977; Vreeburg *et al.*, 1977; Davis *et al.*, 1979) or an anti-oestrogen (MER-25 or CI 628) (Booth, 1977; McEwen *et al.*, 1977) to male rats in the first few days of life have also resulted in significantly higher levels of feminine mating behaviour than in control males. However treatment of infant male rats with the anti-androgen cyproterone acetate did not increase the display of feminine behaviour (Ward, 1972).

As feminine behaviour can be elicited from male rats by deprivation of androgen by castration neonatally or by inhibition of its conversion to oestrogen neonatally, it seems that endogenous androgen or its aromatization product is responsible for suppressing feminine behaviour in normal male rats. But, as males castrated at birth are not identical to females in their exhibition of this behaviour, it seems that some degree of suppression has occurred prenatally and/or in the period between birth and surgery or treatment. It is known that the testes of perinatal male rats secrete significant amounts of testosterone which would be available to masculinize androgen sensitive tissues (see Section 3.2.2., Chapter 1).

5.3.2. Prenatal

Information about the contribution of prenatal androgen to suppression of female behavioural potential has come from experiments using aromatase inhibitors and anti-androgens.

Clemens and Gladue (1978) treated pregnant rats with ATD between days 10 and 22 of gestation and found that the male rats from these litters displayed a higher LQ (after castration and oestrogen and progesterone treatment) when adult, than those from control litters, although still lower than that for control females. They observed no proceptive behaviour in the treated males.

Male rats exposed to an anti-androgen prenatally (flutamide on days 10-22, Gladue and Clemens, 1978; or cyproterone acetate on days 13-22, Nadler, 1969) showed higher LQ values than control males. Nadler (1969) found that this LQ was not different from that for control females. Gladue and Clemens (1978) noted that no proceptive behaviour

was exhibited. In contrast, Ward (1972) could show no significant difference in the LQ of males from cyproterone acetate treated mothers (days 10-22 of pregnancy) and those from control litters, although their overall degree of receptivity (which includes measures other than LQ) was greater than control males and less than control females. Combining prenatal and postnatal (birth to 120 days) anti-androgen treatment did not enhance feminine behaviour over and above that produced by prenatal treatment alone. Clemens and co-workers (1978) cite evidence that cyproterone acetate has androgenic and progestagenic side effects which make its influence upon behaviour difficult to interpret. This may explain the slight discrepancy in the results of similar experiments conducted by Ward (1972) and Gladue and Clemens (1978).

Thus there is evidence that, if protected from the masculinizing influence of androgen, the neural substrate mediating sexual behaviour can allow feminine mating behaviour in the male rat. It also seems that the control mechanism is normally suppressed by testicular androgen acting both pre- and postnatally.

5.4 Summary

If the neural centres controlling sexual behaviour are regarded as bipotential in the developing rat, evidence from both females and males supports the hypothesis that the control of feminine sexual behaviour undergoes an androgen-sensitive phase during the late prenatal and early neonatal period at which time the presence of androgen or its metabolic product of aromatization, oestrogen, can suppress the capacity for display of feminine behaviour in adult life.

Results from experiments in the female rat suggest that the period of androgen sensitivity extends from day 18 of gestation, possibly earlier, until at least the sixth day after birth. The period has been even less precisely defined in the male but again appears to span the periods immediately preceding and following birth.

6. EFFECTS OF EARLY ANDROGEN ON GROWTH

6.1 Sexual Dimorphism in Body Weight

Growth in animals is a complex process, difficult to define, and subject to environmental, nutritional, genetic and hormonal influences. Its assessment by measurement of body weight and dimensions is simple to perform and provides a mathematical description of body size but suffers from the limitations that the body is a composite of many parts whose growth may vary independently. Control of growth is similarly complex and one of the factors affecting body size is the presence or absence of testosterone during the perinatal period.

A difference in adult body size is one of the most obvious expressions of sexual dimorphism, the male being heavier and larger than the female in many species, including the rat (Swanson and van der Werff ten Bosch, 1963; Bell and Zucker, 1971; Slob and van der Werff ten Bosch, 1975). Barraclough's observation (1961) that the female rat treated neonatally with TP grew larger than control animals led to the hypothesis that this sexual dimorphism in size may be due to early androgen exposure in the male.

Gonadal hormones are involved in body weight control at later ages, as illustrated by experiments involving gonadectomy and hormone replacement. In the female rat ovariectomy increases body weight and food intake, and oestrogen treatment reverses this effect (Tarttelin and Gorski, 1973; Wade, 1975). Adult male rats however eat less and lose weight after castration (Kakolewski, Cox and Valenstein, 1968; Gentry and Wade, 1976a) and testosterone reverses these changes (Gentry and Wade, 1976a; Gray, Nunez, Siegel and Wade, 1979). The dual nature of testosterone in its organizational and activational roles is thus again illustrated - testosterone organizes a body weight control mechanism in the perinatal animal and activates other weight controlling measures after puberty.

6.2 Growth of the Androgenized Female Rat

Neonatal female rats treated with androgen grow into larger adults than do those treated with the hormone vehicle alone (Barraclough, 1961; Swanson and van der Werff ten Bosch, 1963; Harris and Levine, 1965; Ward, 1969; Beatty, Powley and Keesey, 1970; Bell and Zucker, 1971; Pollak and Sachs, 1975; Tarttelin, Shryne and Gorski, 1975, 1976; Dubuc, 1976; McEwen *et al.*, 1977; Somana, Visessuwan, Samridtong and Holland, 1978).

Results of work by McEwen and colleagues (1977) suggest that the growth promoting actions of perinatal androgen depend on its conversion to oestrogen. Treatment of neonatal rats with the aromatization inhibitor ATD (administered in polydimethylsiloxane capsules) on the day prior to an androgenizing dose of testosterone abolished any growth differences between treated and control females. ATD alone had no effect.

6.3 Growth of the Neonatally Castrated Male Rat

The role of androgen in organizing growth in early life is also borne out by the results of neonatal castration of male rats. Rats castrated on day 1 or day 2 of life do not grow as fast or as large as intact or sham-operated males (Harris, 1964; Slob and van der Werff ten Bosch, 1975; Tarttelin *et al.*, 1975; Somana *et al.*, 1978).

When the body weight of early castrated males was compared with that of androgenized female rats, Tarttelin and co-workers (1975) found that the two groups were not significantly different after 11 weeks of age. Slob and van der Werff ten Bosch (1975) raised female rats whose hormonal status resembled that of the normal male rat by spaying at birth and giving TP before and after birth. Such animals showed a growth pattern indistinguishable from that of male rats castrated 20 days after birth. Ward (1969), using a similar treatment regimen but without spaying, noted that body weights of the experimental females were not different from those of normal males at 100 days of age. This evidence points to both pre- and postnatal actions of androgen in its effects on growth.

6.4 The 'Critical Period' for Sexual Differentiation of Growth

The postnatal limit of the critical period for the organizing effects of TP on the growth of female rats has been defined as being 3 days after birth (Tarttelin *et al.*, 1975). Androgen given after this age was ineffective in promoting body weights. Barraclough (1961) used a higher dose of TP (1.25mg) than the highest dose (270μg) used by Tarttelin's group and found that treatment on days 2 or 5, but not days 10 or 20, resulted in female rats whose body weights at 100 days of age were significantly greater than those of their litter-mate controls. Swanson and van der Werff ten Bosch (1963) also treated on days 2 or 5 (with 0.3, 0.5 or 1.0mg TP) and were able to demonstrate a greater body weight than controls from 80 days onwards. Therefore, the body weight of a female rat can be increased by TP treatment during the first 5 days of life, although higher doses are required as the animal becomes older.

Results from experiments involving male rats support this definition of the critical period since castration on days 1 or 2 of life but not on days 3-7 resulted in reduced body weight compared with intact controls (Harris, 1964).

Timing of the prenatal action of androgen is more difficult. Ward (1969) and Ward and Renz (1972) showed that female rats exposed to TP prenatally (by injections given to the pregnant mother) were significantly lighter than control animals at 100 and 90 days of age respectively. Slob and van der Werff ten Bosch (1975) have suggested that prolonged androgen treatment of the mother rat may 'damage' the placenta and therefore interfere with the nutrition of the foetus. Certainly a higher than normal incidence of abortions or resorbed foetuses has resulted from androgen treatment during pregnancy (Greene *et al.*, 1939; Swanson and van der Werff ten Bosch, 1965; Gerall and Ward, 1966; Fels and Bosch, 1971; Schultz and Wilson, 1974).

The observation that sex differences in body weight are already present at birth (Bruce and Norman, 1975; Slob and van der Werff ten Bosch, 1975) argues for some prenatal influences on body weight control.

The increased body size of female rats after neonatal androgen treatment is due to a general increase in size of all body components, rather than to obesity (Swanson and van der Werff ten Bosch, 1963; Beatty *et al.*, 1970; Dubuc, 1976), although Beatty's team (1970) did find that the inguinal fat depot was larger than in controls.

6.5 Mechanism of Action of Early Androgen on Growth

There have been various theories put forward to explain how perinatal androgen alters growth.

The well-known anabolic action of androgen on protein metabolism does not seem to be responsible since the period of influence is limited to a 'critical' time around birth, later androgen treatment of the same duration (i.e. single injection) being ineffective. Instead, androgen is exerting an 'organizing' action, whose effect is permanent and evident only later in life.

Barraclough (1961), the first to describe body weight differences between androgenized and normal female rats, attributed the discrepancy in size to a difference in metabolism. Changes in liver enzymes and metabolism have been identified after early androgen action (Denef and DeMoor, 1968) and have been suggested as contributing to differences in body weight (Bell and Zucker, 1971).

Other workers (Harris, 1964; Dubuc, 1976) have favoured the hypothesis that early androgen alters hypothalamic control of growth hormone secretion, just as it alters gonadotrophin control. In support of this theory, Somana and co-workers (1978) found that pituitaries of androgenized female rats had a higher mean content of growth hormone than those from normal females when measured during the first 5 months of age. Male rats castrated within 3 days of birth had a lower pituitary growth hormone content than their intact counterparts. There was no difference between the mean growth hormone content from androgenized females and normal intact males. Dubuc (1976) cites a report of similar findings (Kurcz, Nagy, Gerhardt and Baranyai, 1968). Clark

and Tarttelin (1975) reported elevated growth hormone levels in TP treated rats (90μg TP on day 3) when killed at 15 weeks of age.

Swanson and van der Werff ten Bosch (1963) consider that the effect of perinatal androgen on growth in female rats is secondary to reduced ovarian production of oestrogen.

Several authors have suggested that perinatal androgen exerts its effects on growth by decreasing the responsiveness of the animal to oestrogen (Bell and Zucker, 1971; Gentry and Wade, 1976b; Tarttelin *et al.*, 1975, 1976). This conclusion was drawn from experiments involving ovariectomy and oestradiol benzoate (OB) treatment of both androgenized and control female rats. A less pronounced body weight increase after ovariectomy, and a smaller decrease in body weight after oestrogen treatment, were noted in the androgenized than in control groups.

Beatty and co-workers (1970) and Dubuc (1976) disagree with this hypothesis since they found no difference in the body weight response to either ovariectomy or OB treatment between TP-treated and control female rats. Beatty's group ascribes growth changes following perinatal androgen to a reorganization of the basic mechanisms responsible for body weight regulation, which remain normally sensitive to the effects of oestrogen.

Tarttelin and his colleagues (1976) provided further evidence for the theory of altered responsiveness to oestrogen by showing that the ovaries appear to exert a weight-suppressing effect on growth from an early age. Their neonatally spayed rats (treated with either TP or oil) were heavier than intact females from 9 weeks of age. No difference was found between the body weights of neonatally ovariectomized rats treated with either TP or oil, suggesting that androgen had not altered the basic growth regulating processes.

6.6 Summary

From the foregoing evidence it seems that the presence or absence of androgen during the perinatal period in the rat is at least partly responsible for the normal body weight differences between males and females. During this period androgen exerts organizational actions, probably on hypothalamic centres involved in body weight control, and possibly involving sensitivity of these tissues to gonadal hormones. 'Androgenization' of growth occurs only if sufficient aromatizable androgen is present in the body during a limited critical period which appears to end sometime in the first 5 days of life, but whose prenatal limit is uncertain.

CHAPTER 2

PLASMA TESTOSTERONE CONCENTRATIONS AFTER IMPLANTATION OF TESTOSTERONE PROPIONATE-FILLED POLYDIMETHYLSILOXANE CAPSULES FOR VARYING PERIODS IN ADULT AND NEONATAL FEMALE RATS, AND AFTER INJECTION OF TESTOSTERONE PROPIONATE IN NEONATAL FEMALE RATS

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CHAPTER 2

PLASMA TESTOSTERONE CONCENTRATIONS AFTER IMPLANTATION OF TESTOSTERONE PROPIONATE-FILLED POLYDIMETHYLSILOXANE CAPSULES FOR VARYING PERIODS IN ADULT AND NEONATAL FEMALE RATS, AND AFTER INJECTION OF TESTOSTERONE PROPIONATE IN NEONATAL FEMALE RATS

1. INTRODUCTION

The role of androgen in causing masculine differentiation of the rat hypothalamus during the perinatal period has been widely investigated by administering testosterone propionate to the neonatal female rat (see Chapter 1, Section 3.2). This process is thought to mimic that which occurs naturally in the male rat, and recent work has confirmed the presence of testosterone in plasma during the perinatal period in the male rat (see Chapter 1, Section 3.2.2).

The hypothalamus is able to be irreversibly altered by androgen during a short 'critical period' in the early life of the rat (see Chapter 1, Section 3.2.6), and experiments described in this thesis aim to define more clearly this interval of androgen sensitivity.

Classical androgenization is typically induced by a single subcutaneous injection of TP in oil. This treatment might be expected to produce a high initial plasma testosterone concentration which would gradually decline as the pool of hormone was absorbed. However, the length of time for which the testosterone level is raised after such an injection has not, to the knowledge of the author, been reported, and is pertinent to any study of the critical period. The present experiment aimed to provide a profile of plasma testosterone concentration in the neonatal rat after injection of an androgenizing dose of TP.

Polydimethylsiloxane (PDS) ('Silastic', Dow Corning Corp.) capsules containing TP were used in experiments described in this thesis, to provide a removable source of hormone. They allowed precise timing of

TP treatment, which is not possible with injections of TP in oil. Such capsules were used to deliver TP to neonatal female rats and, indirectly, to rat foetuses by placement in the pregnant female.

Polydimethylsiloxane is well tolerated by tissues (Kincl and Rudel, 1971; Stratton, Ewing and Desjardins, 1973; Berndtson, Desjardins and Ewing, 1974) and is permeable to a wide range of steroids, including testosterone (Dziuk and Cook, 1966; Kincl, Benagiano and Angee, 1968). It has been shown that the rate of passage of steroids through PDS is unrelated to the weight of steroid contained in the capsule (Dziuk and Cook, 1966) and that the *in vivo* rate of absorption in rats is proportional to the surface area of the capsule (Kincl and Rudel, 1971; Damassa, Kobashigawa, Smith and Davidson, 1976; Moger, 1976; Gay and Kerlan, 1978).

The present experiment investigated:

1. the plasma testosterone profile following insertion and later removal of TP-filled PDS implants of different sizes in adult female rats,
2. the plasma testosterone decay profile in neonatal female rats after removal of a TP-filled PDS capsule which had been in place for 4 hours,
3. the plasma testosterone profile in neonatal female rats after injection of an androgenizing dose (90μg) of TP,
4. the plasma testosterone concentrations in growing rats bearing TP-filled PDS capsules inserted subcutaneously at 3 days of age.

2. MATERIALS AND METHODS

2.1 Animals

The experimental subjects were:

(a) Adult female rats of the Simonsen strain of Sprague-Dawley rats obtained from the Small Animal Production Unit, Massey University, housed in a temperature- (23°C) and light- (14 hours) controlled room in polycarbamate cages with pelleted rat food and tap water available *ad libitum*.

(b) Neonatal rats were obtained from timed matings (based on daily examinations for presence of spermatozoa in vaginal smears) of female rats of the above strain.

2.2 Polydimethylsiloxane Capsules

Capsules were prepared from PDS tubing (i.d. 1.57mm, o.d. 3.18mm), cut to predetermined lengths, filled with crystalline TP (Sigma Chemical Co.) and sealed with 'Silastic adhesive Type A' (Dow Corning Corp.). The capsule size specified refers to the length of the contained crystals, and not the cut length of tubing which allowed for sealant at both ends. Crystalline TP from 10 capsules was weighed and averaged 10mg/mm crystal length. Capsules were pre-incubated in phosphate buffered saline at 37°C for 48 hours (h) prior to insertion to prevent an initial high peak in plasma hormone level (Karsch, Dierschke, Weick, Yamaji, Hotchkiss and Knobil, 1973).

2.3 Experimental Procedures (Summarized in Table 2.I)

2.3.1. Adult rats

Adult female rats of mixed ages (250-400g body weight) were anaesthetized with ether for subcutaneous insertion of PDS capsules

TABLE 2.I

SUMMARY OF EXPERIMENTAL DESIGN

CLASS OF RAT	TREATMENT (at t=0)	SAMPLING TIMES (t)
Adults	Implant for 72h TP 5,10,20mm° or cholesterol 10mm	0 4 8 12 24 48 72 76 80 96 120h
Neonates, day 3	Implant for 4h TP 2.5mm or empty	0 0.5 1 2 4 5 6 9 12 16 22 28 34h
Neonates, day 3	Injection TP 90μg	4 8 24 48 72 96h 6 7 8days
Neonates, day 3	Implant TP 2.5mm or empty	24 48h 2 3 6 9 12 15weeks

° Length of crystals in the PDS capsule

containing TP (5mm, 10mm or 20mm), or cholesterol (10mm) for controls. Blood samples of 1.5-2.0ml were collected into heparinized syringes, up to 3 samples being collected from each rat, by cardiac puncture under ether anaesthesia. Plasma was stored at -20°C until analysis.

2.3.2. Neonatal rats

Plasma testosterone concentrations were measured in neonatal rats implanted with TP-filled PDS capsules for brief (4h) or prolonged (up to 15 weeks) periods. Three day old female rats were anaesthetized by hypothermia, and implanted subcutaneously in the left flank with 2.5mm TP-filled or empty (controls) PDS capsules. Another group of neonatal rats was injected subcutaneously in the nape of the neck with either 90μg TP in 0.05ml arachis oil, or oil only (controls), on day 3.

Blood was collected by exsanguination of the anaesthetized hypothermic rats into a heparinized tube. To obtain an adequate plasma volume for assay, blood from between 3 and 10 individual rats was pooled. The separated plasma was stored at -20°C until analysis.

Subcutaneous implants (2.5mm TP-filled or empty PDS capsules inserted on day 3) were left in position in a group of 'chronically implanted' rats. At intervals after implantation (see Table 2.I) blood was collected by cardiac puncture from rats anaesthetized with chloroform. Blood from several neonatal animals was pooled, but rats over two weeks of age each contributed one sample. Blood was processed and stored as described above.

2.4 Assay Procedures

Testosterone was measured by radioimmunoassay (Wilson and Lapwood, 1978) of plasma samples extracted with toluene:hexane (1:2) and incubated with rabbit antiserum to testosterone-3-(O-carboxymethyl)-oxime-BSA (S250, courtesy of Dr G.D. Niswender, Colorado State University, U.S.A.). The free and bound steroids were separated with dextran coated charcoal. Extracted samples were analysed in triplicate. Interassay coefficient of variation varied from 23.3% for a plasma pool of low concentration

of testosterone to 11.7% for a pool of high concentration of testosterone: sensitivity ranged from 0.08-0.11ng ml^{-1}.

2.5 Statistical Analyses

Analysis of the regression of the logarithm of the concentration of plasma testosterone at the various sampling times was employed to summarize the data and to estimate the half-life of plasma testosterone. A series of 't' tests was used to determine the period for which plasma testosterone in neonatal rats remained elevated above control levels after removal of capsules, or after injection of TP. The duration of elevation of plasma testosterone in young rats whose TP implants were not removed was estimated by plotting the 95% confidence limits about the regression line. The time at which the lower confidence limit intercepted the upper 95% confidence limit for control values was taken to be the time at which testosterone concentration had returned to normal.

3. RESULTS

3.1 Adult Rats

The plasma concentrations of testosterone following implantation of PDS capsules (5mm, 10mm and 20mm) filled with TP are illustrated in Figures 2.1, 2.2 and 2.3. Figure 2.1 shows the concentrations during the first 24h; Figure 2.2 the concentrations from 24-72h and Figure 2.3 the decay in plasma concentrations following removal of capsules.

The peak concentrations of plasma testosterone attained by the 5, 10 and 20mm TP capsules were 7.1 ± 0.4, 9.8 ± 1.1 and 12.8 ± 0.9ng ml^{-1} (24.6 ± 1.4, 34.0 ± 3.8 and 44.4 ± 3.1nmol $litre^{-1}$) respectively. For the two smaller capsules these peak levels appear to be reached at or soon after 4h and then declined for the remainder of their 72h residence (Figs 2.1 and 2.2). The 20mm capsules produced at 8h a

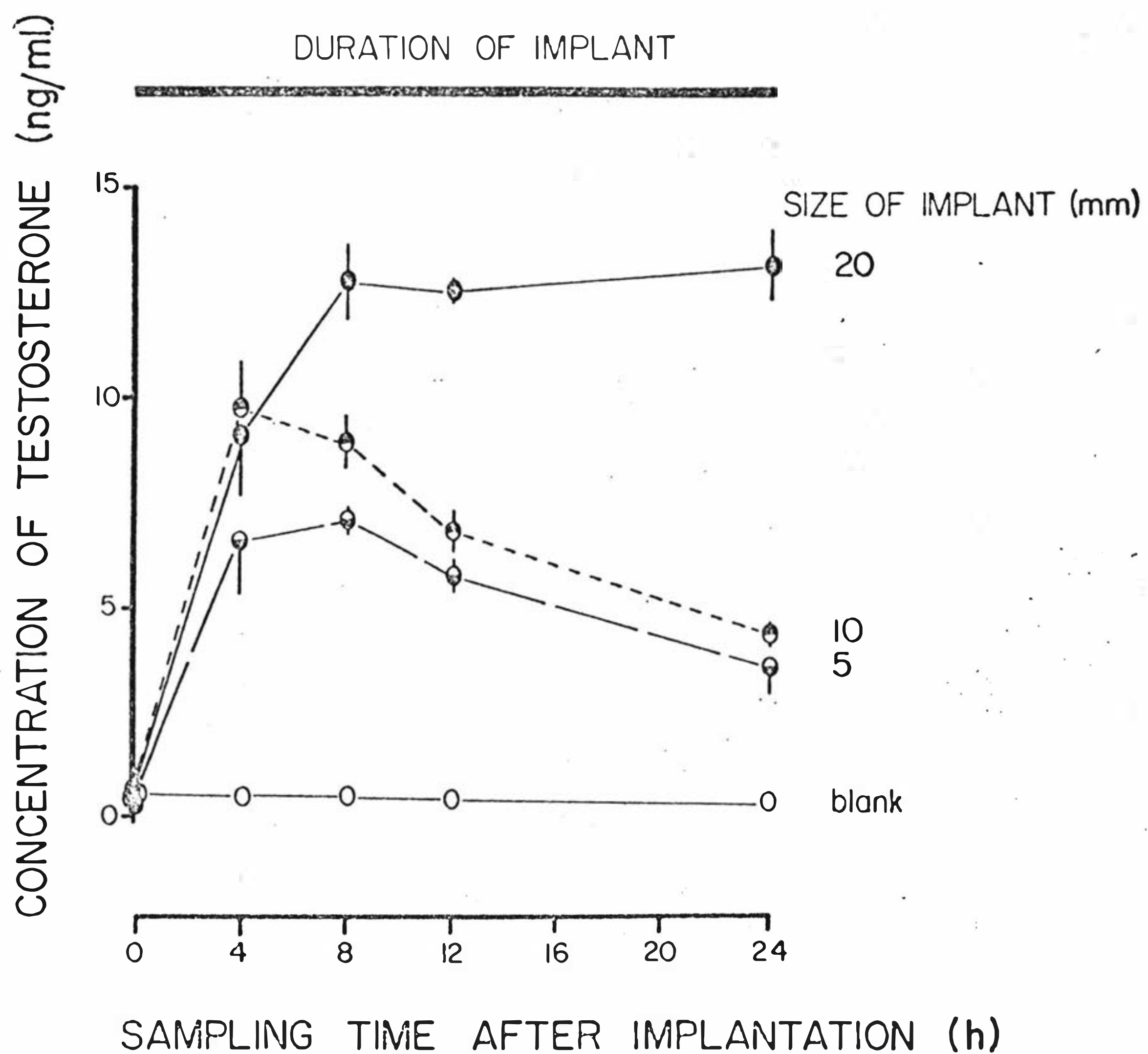


Figure 2.1 PLASMA CONCENTRATIONS OF TESTOSTERONE IN ADULT FEMALE RATS OVER A 24h PERIOD FOLLOWING THE IMPLANTATION OF THREE DIFFERENT SIZES OF PDS CAPSULES CONTAINING TP

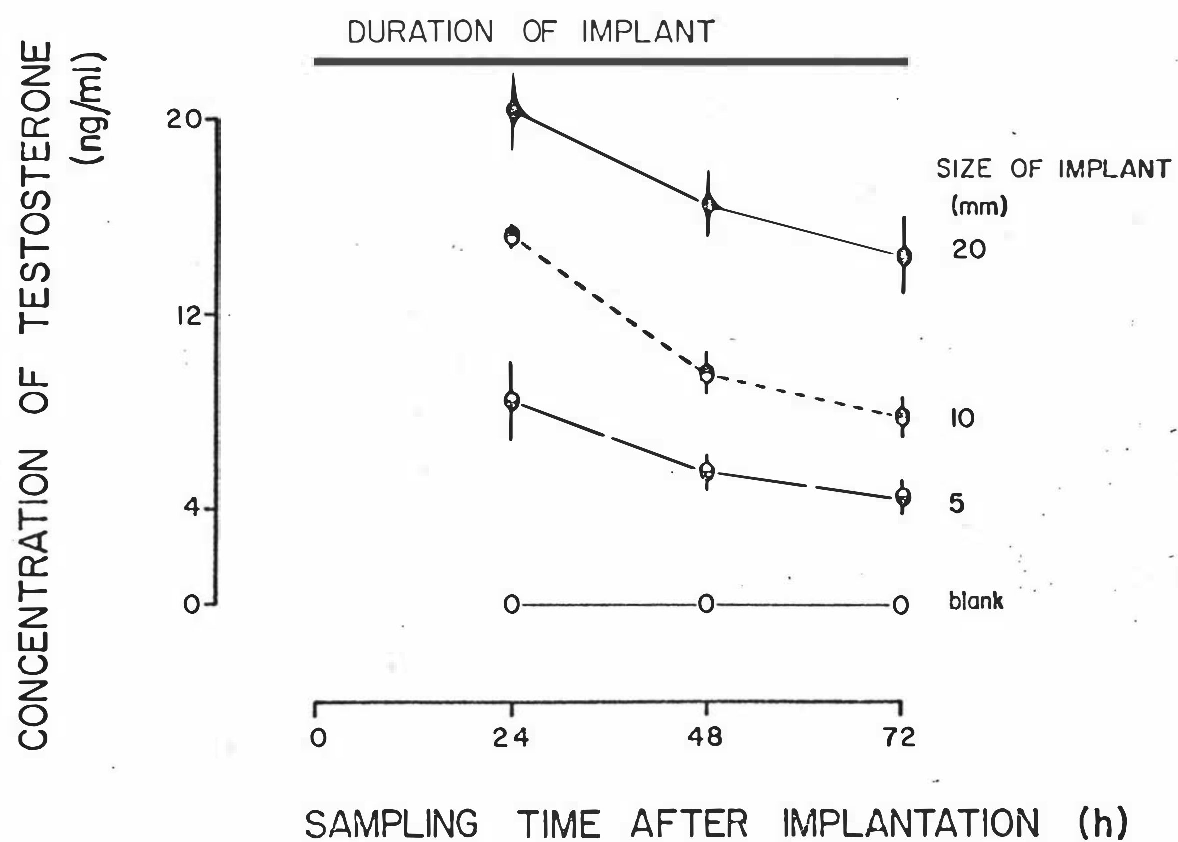


Figure 2.2 PLASMA CONCENTRATIONS OF TESTOSTERONE IN ADULT FEMALE RATS OVER A 72h PERIOD FOLLOWING THE IMPLANTATION OF THREE DIFFERENT SIZES OF PDS CAPSULES CONTAINING TP

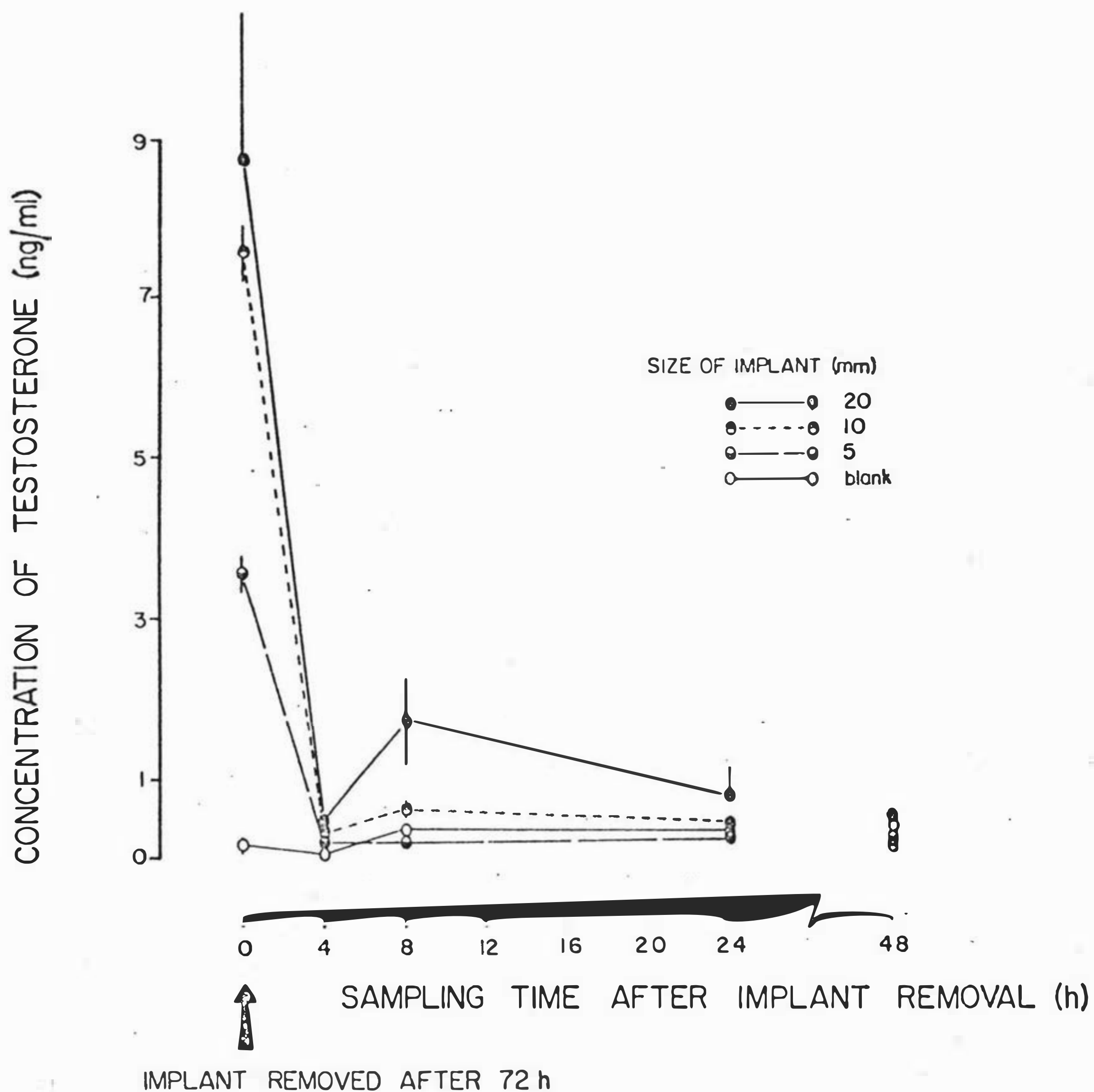


Figure 2.3 PLASMA CONCENTRATIONS OF TESTOSTERONE IN ADULT FEMALE RATS OVER A 48h PERIOD FOLLOWING THE REMOVAL OF TP-FILLED PDS CAPSULES IMPLANTED FOR 72h

peak concentration which was maintained to 24h and thereafter declined (Figs 2.1 and 2.2).

After removal of the capsules (Fig. 2.3) the plasma testosterone concentration rapidly declined and had reached control levels at the 4h sampling time. The high mean concentration of plasma testosterone in the 20mm capsule group at 8h was accounted for by one individual rat with high values at 8 and 24h. (The remaining 5 rats had levels of 1.2 ± 0.3ng ml^{-1} at 8h and 0.39 ± 0.03ng ml^{-1} at 24h).

There were no significant differences between the estimates of the half-life of plasma testosterone for the three sizes of capsule (5mm, 1.2h; 10mm, 0.9h; 20mm, 0.9h). The mean half-life was 1.0h.

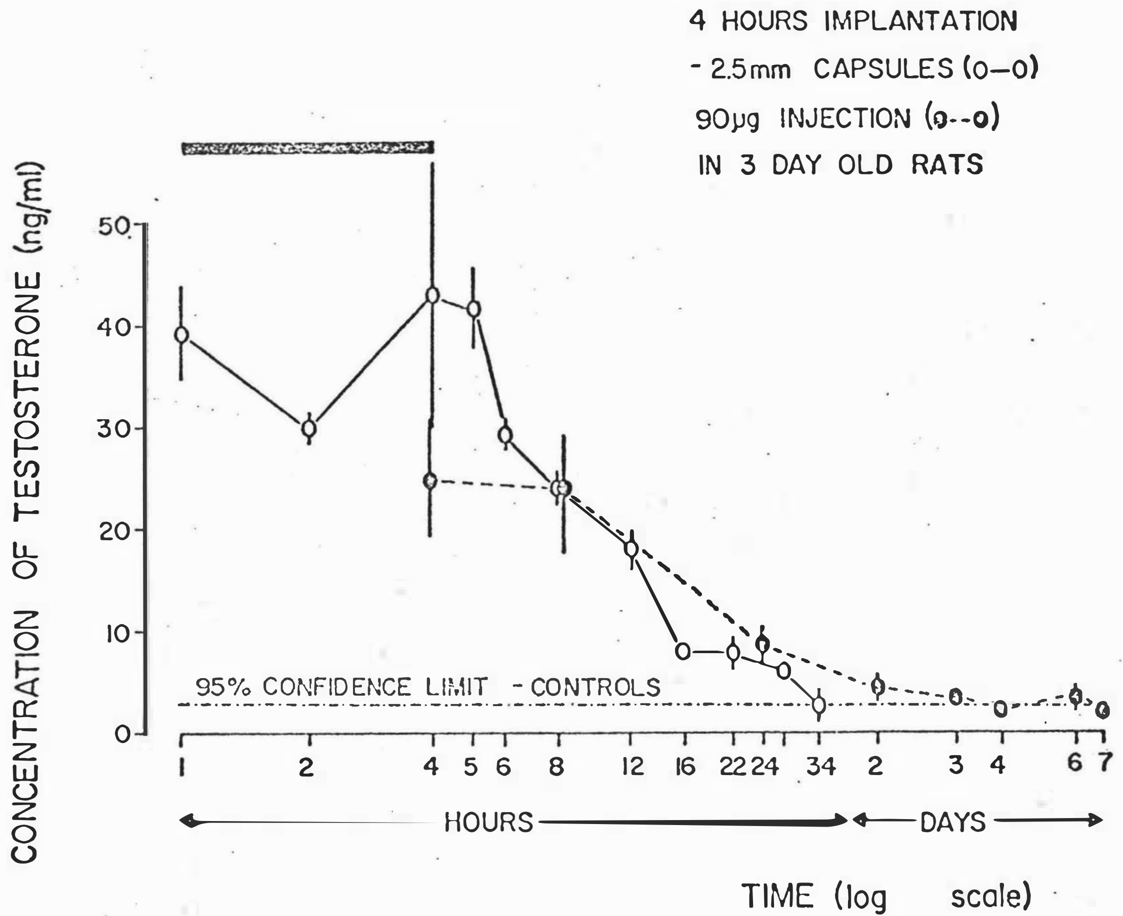
3.2 Neonatal Rats

3.2.1. Four hour TP implants and TP injection

Plasma testosterone concentrations in neonatal female rats bearing 2.5mm TP capsules for 4h, or injected with 90μg TP, are illustrated in Figure 2.4. Four hours after the beginning of treatment plasma testosterone levels were higher in TP-injected than in TP-implanted rats.

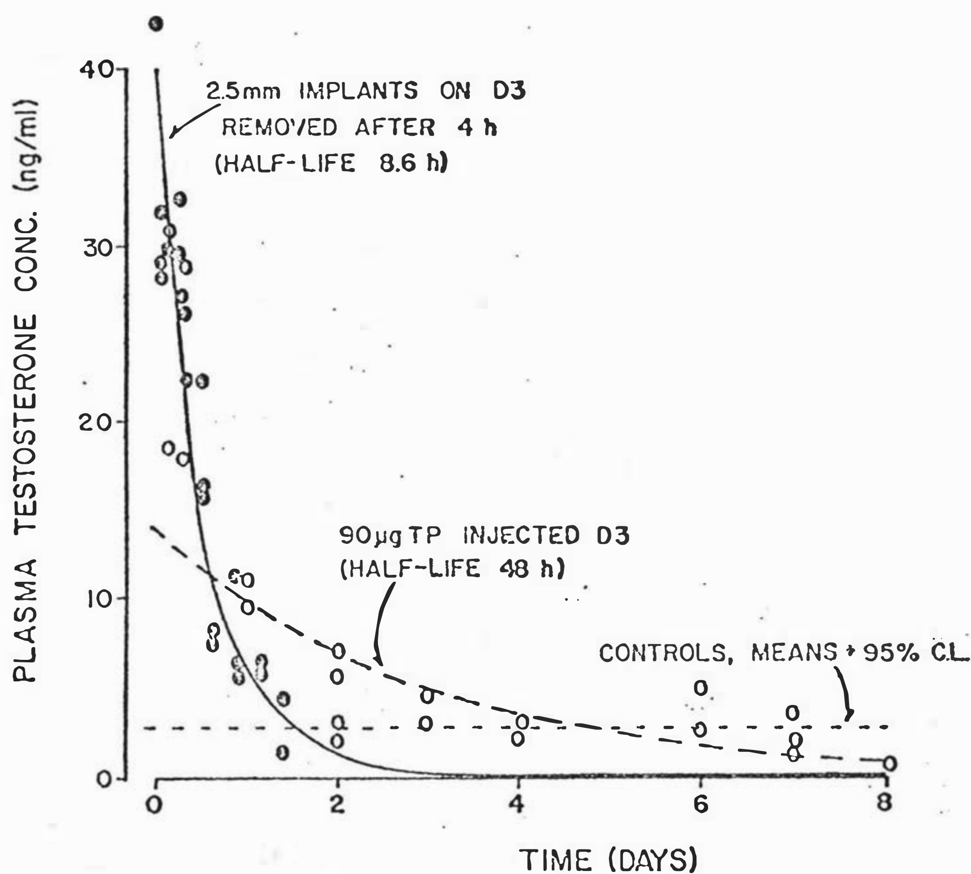
The calculated regression lines for TP-injected and TP-implanted rats are shown in Figure 2.5. In both cases the regression of log concentration on time was highly significant ($P<0.001$). The calculated plasma half-life of testosterone after removal of the PDS capsule was 8.6h and the half-life of testosterone after subcutaneous injection was 48h.

After removal of the capsules, testosterone concentration declined to control levels in 24h. In the TP-injected group testosterone concentration remained significantly higher than in controls until 192h (8 days) after injection.



The horizontal dashed line shows the upper 95% confidence limit of the level in control animals.. The 2.5mm capsule was removed after 4h.

Figure 2.4 PLASMA LEVELS OF TESTOSTERONE IN NEONATAL FEMALE RATS AFTER INJECTION OF 90ug TP AND AFTER INSERTION OF A TP-FILLED PDS CAPSULE



The horizontal dashed line shows the upper 95% confidence limit of the level in control animals. The curves are the linear regressions with a logarithmic scale for testosterone concentration. The 2.5mm capsule was removed after 4h.

Figure 2.5 PLASMA LEVELS OF TESTOSTERONE IN NEONATAL FEMALE RATS AFTER INJECTION OF 90µg TP (open symbols) AND AFTER INSERTION OF A TP-FILLED PDS CAPSULE (closed symbols)

3.2.2. Chronic TP implants

Plasma testosterone concentration in rats bearing 2.5mm PDS capsules of TP for up to 40 days (measurement was made up to 15 weeks) is illustrated in Figure 2.6 with the measured data plotted about the calculated curve as in Figure 2.5. (The regression coefficient was highly significant, $P<0.001$). The calculated half-life of plasma testosterone for this treatment was 69h. Plasma testosterone concentration declined exponentially and reached control levels in 10 days.

4. DISCUSSION

The present experiment has demonstrated a dose-dependent (related to size of capsule) elevation of plasma testosterone with TP-filled PDS capsules in adult rats, thus confirming previously described work using a radioimmunoassay (Damassa *et al.*, 1976; Moger, 1976) or a relatively insensitive bioassay (Chang and Kincl, 1970). Damassa and co-workers (1976) used, in male rats, implants of a comparable size to those used in the present experiment, but reported substantially lower plasma testosterone concentrations (for example, for a 10mm implant after 3 days *in situ*, 0.75ng ml^{-1} compared with 7.80 ± 0.5ng ml^{-1} in the present experiment). However their capsules were filled with free testosterone, and higher concentrations of plasma testosterone result from TP-filled than from testosterone-filled capsules of the same length (Gay and Kerlan, 1978). The present experiment used female rats which may differ in their plasma clearance rate for testosterone.

Plasma testosterone concentration reached a peak 4-8 hours after implantation and fell to control levels within 4 hours after removal, which agrees with the reports of Damassa *et al.* (1976).

It has been reported that after subcutaneous implantation of testosterone-filled PDS capsules, plasma testosterone concentration remains constant for 13 weeks in rabbits (Stratton *et al.*, 1973) and 3 weeks in castrated male rats (Damassa *et al.*, 1976) but in the present experiments the plasma testosterone concentration declined

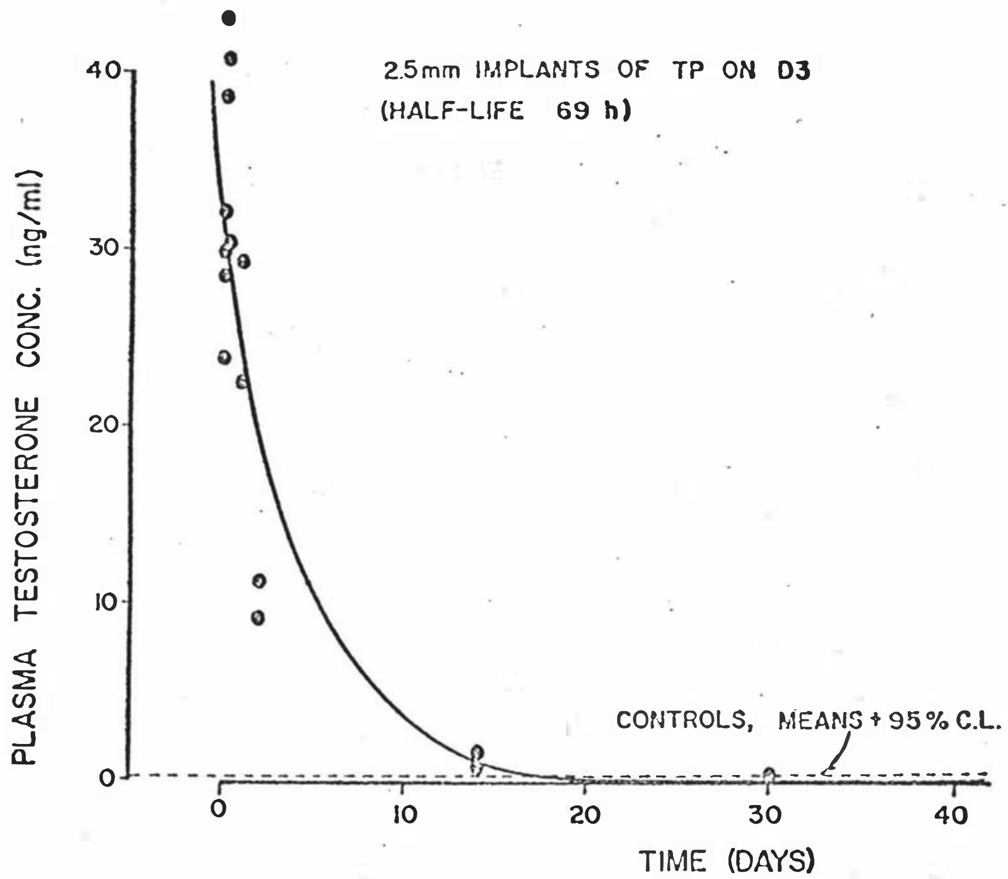


Figure 2.6 PLASMA LEVELS OF TESTOSTERONE IN FEMALE RATS AFTER IMPLANTATION OF A TP-FILLED PDS CAPSULE ON DAY 3

over the 3 days of sampling in adult female rats. Moger (1976) reported peak elevation of serum testosterone concentration in young castrated male rats at the earliest sampling period, 24 hours, and a progressive decline at subsequent sampling times (3, 5 and 10 days).

Clearly, in adult rats testosterone was rapidly absorbed from PDS capsules and its rapid clearance from the plasma was reflected in a half-life of 1 hour calculated from the present data. Results obtained with the radioimmunoassay system used here indicated that the duration of exposure of adult female rats to elevated plasma testosterone concentrations from the subcutaneously placed TP-filled PDS capsules of the dimensions described was not more than 4 hours longer than the period for which the capsule was in place.

The half-life of plasma testosterone after removal of 2.5mm TP-filled PDS capsules, in neonates, was 8.6 hours, which is considerably longer than that in the adult. Biotransformation pathways involving microsomal enzymes are known to be deficient in neonates and excretion processes are not well developed (Baggot, 1977) so that delayed elimination of large quantities of testosterone from the neonatal body would be expected. The half-life of plasma testosterone following subcutaneous injection of 90μg TP in neonates was 48 hours. The whole body half-life for a single intramuscular injection of ^{14}C-labelled TP in adult rats, reported by James, Nicholls and Roberts (1969), was 3.75 days. However these authors measured ^{14}C excretion which would include many metabolites of testosterone. They also used a larger dose of TP in a larger volume of oil (1mg in 0.1ml ethyl oleate) than used in the present experiment and this could contribute to the longer half-life measured.

Plasma testosterone concentration in rats injected with 90μg TP remained elevated for at least 7 days. When TP capsules were removed after 4 hours, plasma testosterone levels declined and reached control levels in 24 hours. In defining the period of exposure to TP therefore, 24 hours must be added to the time for which the implants were left *in situ*.

The half-life of plasma testosterone in chronically implanted neonates was 69 hours. Some of the capsules examined after being in position for 12-15 weeks were found to be empty. An *in vitro* study by Kincl and others (1968) enabled theoretical calculation of the rate of passage of TP through the walls of the PDS capsules used in the present experiment. This calculation predicted that the capsule would be empty at 18.5 weeks. Clearly, *in vivo* the rate of release is much greater and the rapid absorption of TP from the small capsule within the rapidly growing rat, which provides an exponentially increasing pool for the steroid, limits the effectiveness of PDS capsules as a long-term method of hormone treatment. When compared with TP injection in the neonate however, they provided a longer effective concentration of hormone and they had the added advantage that termination of treatment by removal of the capsule could be carried out after the desired period of treatment.

Information regarding the plasma testosterone concentration after administration of TP to rats, either by injection or PDS implants has enabled the usual techniques of androgenization to be assessed in terms of the duration of exposure of the animal to androgen. This has been an important contribution to the studies, described in Chapters 3, 4 and 5, investigating the critical period for sexual differentiation of the rat hypothalamus.

CHAPTER 3

THE EFFECTS ON FEMALE OFFSPRING OF TESTOSTERONE PROPIONATE, ADMINISTERED TO PREGNANT RATS BY POLYDIMETHYLSILOXANE CAPSULES AT VARYING TIMES DURING PREGNANCY

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5. CONCLUSIONS

CHAPTER 3

THE EFFECTS ON FEMALE OFFSPRING OF TESTOSTERONE PROPIONATE, ADMINISTERED TO PREGNANT RATS BY POLYDIMETHYLSILOXANE CAPSULES AT VARYING TIMES DURING PREGNANCY

1. INTRODUCTION

The effects of neonatally administered androgen on the ovarian function, sexual behaviour and growth of female rats is well established (see Chapter 1). Prenatal androgen treatment, while long known to masculinize the genital tract of developing females (Greene *et al.*, 1939) has produced conflicting evidence for a prenatal period of hypothalamic sensitivity to androgen (Revesz *et al.*, 1963; Swanson and van der Werff ten Bosch, 1964, 1965; Gerall and Ward, 1966; Fels and Bosch, 1971; Slob *et al.*, 1983).

Both direct and indirect methods of administering androgen to the foetus have been used in these experiments. Treatment of the foetus is assured by its direct injection in the uterus during anaesthesia of the mother, but introduces new variables and is potentially hazardous. Indirect treatment, by injection or oral treatment of the mother, has frequently involved such a high dosage of testosterone that abortion, foetal death and deficient lactation have resulted (Swanson and van der Werff ten Bosch, 1965; Gerall and Ward, 1966; Fels and Bosch, 1971; Schultz and Wilson, 1974). Treatment by injection also has the disadvantage of producing an inconstant elevation of hormone concentration for an unknown period.

In the present experiment androgen was administered to the pregnant rat by way of PDS capsules which offer the advantages of providing a continuous and fairly constant source of hormone for an interval determined by the time of removal of the capsule. Different doses were supplied by different sizes of capsule.

Chapter 2 described the profiles of plasma testosterone resulting from the use of such capsules. Confirmation, by radioimmunoassay, of elevated testosterone concentration in foetal blood after treatment of the mother with TP was not considered feasible since the small volume of blood obtainable from each foetus would mean the sacrifice of large numbers of animals. Evidence of masculinization of the external genitalia was assumed to indicate that the female foetuses had been exposed to androgen. However, a recently published paper (Slob *et al.*, 1983), which is discussed later in this Chapter, challenges this assumption.

Pregnant rats were treated with TP-filled capsules for 72 or 24 hours at various stages of pregnancy from 10 days, summarized in Figure 3.1, and the development of female offspring observed. Attention was focussed on the rats born after treatment during the 3 days preceding birth (days 19-21 of gestation) as this period was considered the most likely to be involved if the hypothalamus is sensitive to androgen prenatally.

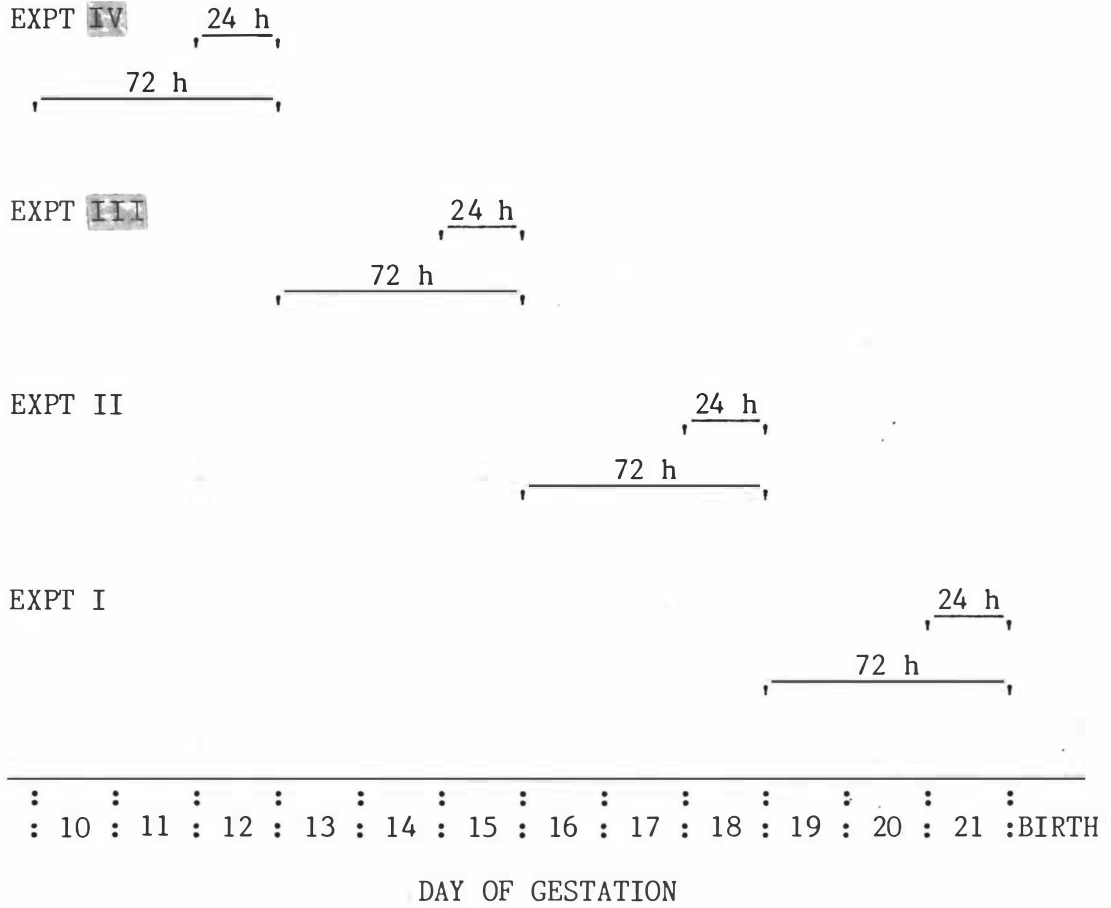
The daughters of rats treated with TP during pregnancy were studied for evidence of 'masculinization' of the external genitalia, and of the control of gonadotrophin secretion, feminine sexual behaviour and growth.

2. MATERIALS AND METHODS

2.1 Animals and Animal Management

The experimental subjects were Sprague-Dawley rats of the Simonsen strain, obtained from the Small Animal Production Unit, Massey University. They were kept in a temperature- (23°C) and light- (14h) controlled room with pelleted rat food and tap water available *ad libitum*.

Adult female rats were housed three to a cage with one male, and vaginal smears were examined daily for spermatozoa. The morning on which spermatozoa were found was designated day 1 of pregnancy and the rat was removed from the male. Pregnancy was confirmed by abdominal



Pregnant rats were implanted for 24 or 72h with a PDS capsule containing TP (5, 10 or 20mm) or cholesterol (10mm)

Figure 3.1 EXPERIMENTAL DESIGN: PERIODS OF EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

palpation at day 10 and the rat assigned to a treatment group and housed in a polycarbamate cage until its litter was weaned. In most cases litters were born on day 22.

On the expected day of parturition the mother rat was checked regularly and the time of arrival of the litter recorded. Each pup was sexed, weighed and each female identified by a simple code requiring amputation of one or two toes. During these procedures pups were kept under a 60 watt lamp to maintain their body temperature. Litter size was adjusted to 12 at this time by removal of excess males or addition of males from other litters of the same age. The female rats were weaned at 21 days of age, weighed, ear-marked and allocated randomly to stainless steel colony cages, 7 rats to each cage.

2.2 Administration of Testosterone Propionate

Treatment of the pregnant rat involved subcutaneous insertion, under ether anaesthesia, of a PDS capsule (dimensions and preparation described in Chapter 2) containing either crystalline TP or cholesterol (controls). The capsule was removed under anaesthesia after either 24h or 72h.

2.3 Experimental Design

This study consisted of four sections, each involving treatment with TP during a different period of gestation: days 19-21, 16-18, 13-15 and 10-12. Within each section each pregnant female was assigned to one of eight subgroups determined by duration of treatment (72h or 24h) and the size of the implant (5, 10 or 20mm or cholesterol 10mm). Table 3.I shows details of the numbers of female offspring whose reproductive function and growth were subsequently studied.

The four sections of this experiment were conducted sequentially starting with the latest stage of pregnancy and progressing to successive earlier periods. The findings from each section to some extent determined the experimental procedures followed in the subsequent sections, therefore each section differed. Experiment I (treatment on days 19-21) is

TABLE 3.I

SUMMARY OF EXPERIMENTAL DESIGN
NUMBER OF RATS INVESTIGATED

	EXPERIMENT							
	I		II		III		IV	
	STAGE OF GESTATION AT TREATMENT (days)							
	21-19		18-16		15-13		12-10	
TREATMENT	DURATION (h)							
	72	24	72	24	72	24	72	24
IMPLANT								
5mm TP	10	10	0	6	6	11	10	7
10mm TP	10	10	5	4	5	9	3	4
°10mm TP			10					
20mm TP	10	14	9	6	8	2	12	2
10mm Cholesterol	10	10	8	0	13	10	0	2
°10mm Cholesterol			10					

° Supplementary study group, see Section 2.4

described here in detail and the experimental procedures and the age at which they occurred are summarized in Table 3.II. Table 3.III shows the measurements and observations recorded for all sections of the experiment and also serves to indicate where the other sections differ from the Experiment I protocol.

Some results obtained from Experiment II (treatment during days 16-18) were considered questionable (details in Results section), therefore this section of the experiment was repeated with pregnant females implanted for 72h with 10mm TP- or cholesterol-filled implants.

2.4 Assessment of Responses

The experimental procedures and the ages at which they were conducted are summarized in Tables 3.II and 3.III.

The body weight and anogenital distance (AGD) were recorded on the day of birth. Rats were weighed weekly from weaning (3 weeks) until the end of the experiment (18 weeks). Daily examination for vaginal opening commenced at 4 weeks. Beginning at 6 weeks, vaginal smears were taken daily for 2 weeks from rats with normal vaginae, and examined microscopically. At 6 weeks the right ovary was examined for corpora lutea by laparotomy under ether anaesthesia. Bilateral ovariectomy was carried out at 13 weeks and the ovaries weighed. Histological sections prepared from representative ovaries were used to confirm the presence or absence of corpora lutea.

Administration of OB (Sigma Chemical Co.) in subcutaneous 10mm PDS capsules to half of the rats at 16 weeks permitted assessment of growth response to oestrogen and also provided hormonal preparation for mating behaviour testing.

Mating behaviour was tested 4 days after the beginning of oestrogen administration (16 weeks). The rats under test received a subcutaneous injection of 0.5mg progesterone (Sigma Chemical Co.) in arachis oil and were tested 5h later during the early portion of their normal dark phase. Testing was carried out under 'Safelight' illumination (Wratten orange filter)

TABLE 3.II

SCHEDULE OF EXPERIMENTAL PROCEDURES
EXPERIMENT I

MEASUREMENT OR OBSERVATION		AGE
External Genitalia	anogenital distance	birth
	appearance	18weeks
Ovarian Function	age at vaginal opening	---
	vaginal smear	6weeks
	inspection of R ovary for CL°	6weeks
	weight of ovaries	13weeks
	histology of ovaries	13weeks
Sexual Behaviour	lordosis quotient	16weeks
Growth	birth weight	birth
	weekly body weight	3-18weeks
	organ weights	18weeks

° Corpora lutea

TABLE 3.III

SUMMARY OF EXPERIMENTAL PROCEDURES

MEASUREMENT OR OBSERVATION	EXPERIMENT I		II			III		IV	
	STAGE OF GESTATION AT TREATMENT (days) 21-19		18-16			15-13		12-10	
	DURATION (h) 72	24	72		24	72	24	72	24
External Genitalia									
AGD at birth	+	+	+	+°	+	+	+	+	+
examination at slaugter	+	+	+	+°	+	+	+	+	+
Ovarian Function									
'age at vaginal opening	+	+	+	+°	+	+	+		
vaginal smears	+	+				+	+		
inspection of R ovary	+	+		+°		+			
weight of ovaries	+	+		+°	+	+	+	+	+
histology of ovaries	+	+		+°		+			
Sexual Behaviour									
LQ	+	+		+°		+			
Growth									
birth weight	+	+	+	+°	+	+	+	+	+
weekly body weight	+	+		+°		+			
organ weights	+	+				+			

°Supplementary group
'In rats with abnormal development of external vagina it was often impossible to establish a date for vaginal opening.

in mating arenas constructed of clear perspex (35cm x 50cm x 50cm). Two male rats in each arena were allowed a 1h period of adaptation before the introduction of a female rat. Each female was observed for 10 mounts, the number of lordoses in response to these mounts was recorded and the LQ calculated. (LQ = percentage of mounts in which lordosis was elicited.)

At 18 weeks all rats were killed with chloroform. The weights of liver, kidneys, adrenal glands, brain, pituitary, uterus and preputial glands were recorded. The external genitalia were examined and classified from their gross appearance as normal, abnormal or lacking a vaginal orifice.

2.5 Statistical Analyses

2.5.1. General

Results were subjected to one-way analysis of variance. When a significant ($P<0.05$) 'F' ratio was obtained 't' tests were carried out, according to an *a priori* scheme, to identify those groups which contributed to the significant 'F' ratio. The computation of 't' used the difference between the means (numerator) and the pooled error estimate from analysis of variance (denominator) (Sokal and Rohlf, 1969a).

2.5.2. Body weight data analysis

Body weight data (g) were first transformed into logarithms. The variance of the body weight measurement increases with age and use of transformation to $\log_{10}$ of the body weight removes this heteroscedasticity (inequality of variance) of the data allowing legitimate statistical analysis. For a more detailed discussion of the advantages of the $\log_{10}$ transformation of body weight, see Clark and Tarttelin (1978).

Initially a one-way analysis of variance was applied to log_{10} body weight measurements at each week. When a significant ($P<0.05$) 'F' ratio was obtained, 't' tests, as described above, were used to identify the group(s) which contributed to it.

2.5.3. Regression analysis of body weight

When consistently significant 'F' ratios were obtained for a number of consecutive weeks in the analysis of variance, further analyses using regression techniques were carried out according to the methods of Sokal and Rohlf (1969b).

Regression analyses were carried out on individual log_{10} body weight data. The sum of squares of groups at each week of measurement was calculated. The regression of rat body weight ($log_{10}Y$) on the reciprocal of age (1/X) is linear (Clark and Tartte1in, 1978) and was calculated for each group. The proportion of the variance among weeks which was explained by linear regression was very much greater when the regression used the reciprocal of age, rather than untransformed age, as the independent variable. For a typical analysis (Chapter 5, day 2 TP-injected group, data shown in Appendix Table I), the proportion of the among week variance due to regression of age was 84.9%, and due to regression on the reciprocal of age was 99.6%. For the periods following ovariectomy, and during OB treatment, the transformation of age to its reciprocal gave no improvement in the fit of the regression line, and conclusions were based on analyses using untransformed age as the independent variable.

From the regression data the statistics Σx^2 and Σxy were obtained enabling computation of $\Sigma\hat{y}^2$ (the sum of squares of deviations of estimated values of Y from the mean, also called the 'explained sum of squares'). The difference between the sum of squares of groups and $\Sigma\hat{y}^2$ gives an estimate of $\Sigma d^2y.x$ (the sum of squares of observed values of Y from the values estimated by the regression, also called the 'unexplained sum of squares'). The 'explained mean square' (mean square due to linear regression, based on one degree of freedom) represents the variation in Y which can be accounted for by variation of X. The 'unexplained mean square' (n-2 degrees of freedom) represents

the residual variation, in other words the deviation from the regression line. The 'F' value obtained from the ratio of these two mean squares tests the significance of the regression, which was very highly significant ($P<0.001$) in all cases.

The regression coefficients (b) were compared by the calculation shown below.

Among b's SS $\quad \Sigma\Sigma\hat{y}^2 - \frac{(\Sigma\Sigma xy)^2}{\Sigma\Sigma x^2} \quad ; \quad$ d.f. $g-1$

Within b's SS $\quad \Sigma\Sigma d^2y.x \quad ; \quad$ d.f. $\Sigma^g a-2g$

g = number of treatment groups

a = number of weeks

When significant ($P<0.05$) 'F' ratios were obtained, the groups whose regression coefficients contributed to the significance were identified by further 'F' tests as shown below:

$$F = \frac{(b_1 - b_2)^2}{\frac{\Sigma x_1^2 + \Sigma x_2^2}{\Sigma x_1^2 \cdot \Sigma x_2^2} \cdot \bar{S}^2y.x}$$

$$\bar{S}^2y.x = \frac{\Sigma\Sigma d^2y.x}{\Sigma^g a - 2g}$$

3. RESULTS

The results of all four sections of the experiment are described together under headings corresponding to the aspects of sexual differentiation being investigated. For most observations or measurements a table summarizes the findings.

The treatment of pregnant rats with TP implants for 72h or 24h during the second half of gestation did not appear to interfere with the maintenance of pregnancy nor with parturition, and the offspring were healthy at birth.

Since the first experiment involved the last 72h or 24h of gestation and since the actual time of parturition could not be predicted, the duration of exposure of litters to TP *in utero* varied between 5-24h for the '24h' treatment, and between 50-72h for the '72h' treatment. This variability in the duration of treatment should be borne in mind when considering the results of this section of the experiment.

3.1 Ovarian Function

3.1.1. Age at vaginal opening

The ages at vaginal opening are presented in Table 3.IV.

Several groups of rats (72h, 10mm and 20mm) receiving TP during the 3 days preceding birth (19-21) showed abnormal vaginal development which prevented accurate determination of the timing of vaginal opening. Statistical analysis of the ages at which the vaginae canalized was therefore limited to the remaining groups. Of these, only the 5mm TP (24h) group differed from the control, being significantly older ($P<0.01$) when the vaginae opened (41.3 ± 0.9 days compared with 35.7 ± 0.9 days). The age at vaginal opening was not obtained for one rat in this group (5mm TP, 24h) whose vaginal opening was abnormal.

TABLE 3.IV

AGE (days) AT VAGINAL OPENING

TREATMENT	STAGE OF GESTATION AT TREATMENT (days)								
	21-19			18-16			15-13		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT									
5mm TP	10	41.3 ***°	0.9				6	37.5 *°	1.0
10mm TP	10	37.2	0.4	5	38.0	1.3	5	37.6 *°	2.1
20mm TP	10	37.0	1.1	9	38.4	0.7	8	44.1	1.0
10mm CL[3]	10	35.7	0.9	8	40.1	0.4	13	41.8	1.2
72 HOUR IMPLANT									
5mm TP	9	39.9	1.0	6	–[2]		11	39.6	1.3
10mm TP	10	–[2]		4	–[2]		9	37.3 **°	0.9
				[1]10	–[2]				
20mm TP	14	–[2]		6	–[2]		2	44.5	0.5
10mm CL	10	37.5	0.9				10	42.0	1.1
				[1]10	43.0	0.5			
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS "F"		5.32***			1.45ns			3.95**	

°Significance of difference between TP mean and corresponding CL mean (t-test)

[1]Supplementary group

[2]Abnormality of vaginal structure prevented identification of day of opening.

[3] Cholesterol

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

All rats exposed to TP for 72h during days 16-18 of gestation showed abnormal vaginal development, and it was not possible to determine accurately the day of vaginal opening. Analysis of variance of the age of vaginal opening of rats from mothers receiving TP treatment for 24h found no significant differences.

Of the rats treated for 24h during days 13-15, those in the 5mm or 10mm TP groups had significantly ($P<0.05$) earlier vaginal opening times than the 24h control (37.5 ± 1.0 and 37.6 ± 2.1 days compared with 41.8 ± 1.2 days). Within the 72h groups, the 10mm group had a significantly ($P<0.01$) earlier vaginal opening date than the control group (37.3 ± 0.9 days compared with 42.0 ± 1.1 days).

3.1.2. Vaginal smears

Vaginal smears were taken from rats with normal vaginal orifices and all showed normal cyclic patterns of cell types in the vaginal epithelium.

3.1.3. Corpora lutea

Ovaries from all control animals and those animals exposed to TP on days 19-21, 13-15 and 10-12 of gestation contained corpora lutea. They were evident at day 45 in those rats subjected to laparotomy, and in ovaries removed at ovariectomy or slaughter from all rats. Histological examination of a representative sample of ovaries confirmed the presence of corpora lutea.

Treatment during days 16-18 of gestation produced equivocal results. While all control animals and rats from mothers treated with 5mm TP or 20mm TP for 24h had normal ovaries containing corpora lutea, 2 rats from each of the 10mm TP groups and 3 from the 20mm 72h group had small ovaries lacking corpora lutea. In view of the failure to alter ovarian function by treatment during the periods of gestation flanking this period, it was decided to repeat this section of the experiment in more detail. A supplementary group of pregnant female rats was treated during days 16-18 of pregnancy with 10mm implants containing TP or cholesterol. Twenty female offspring (10 controls,

10 TP-treated) were subjected to the experimental procedures described previously. All of these rats had normal ovaries with corpora lutea present. No explanation can be offered for this discrepancy, and in view of the failure to replicate it, the finding of anovulatory sterility after TP treatment during days 16-18 must be regarded as spurious.

3.1.4. Ovarian weights

The weights of ovaries are shown in Appendix Table II.

The ovarian weights of 10 rats from the groups treated on days 16-18 (8 from 24h cholesterol, 2 from 24h 10mm TP groups), and 2 rats from the group treated on days 10-12 with cholesterol for 72h, were inadvertently lost. As the rats were of widely differing ages, analysis of variance was not applied to data from the experiments involving treatment on days 16-18 or days 10-12.

Four rats treated for 72h on days 19-21 (3 from 10mm TP, 1 from 20mm TP group) died before ovariectomy.

For those groups treated on days 19-21, or 15-13, there were no significant differences between the TP-treated and control rats in the weights of ovaries.

The ovaries of those rats receiving TP during days 16-18 and which lacked corpora lutea were correspondingly small. As these rats were not scheduled for routine ovariectomy, the discovery of anovulatory ovaries was made at slaughter, and records of ovarian weights are incomplete. When this section of the experiment was repeated (10mm TP or cholesterol for 72h), the ovaries were normal and there was no significant difference between the weights of ovaries from control and TP-treated animals.

On the basis of the results of inspection of the ovaries, all rats appeared to be ovulating normally, with the exception mentioned above (5 rats receiving TP on days 16-18). Vaginal smears, when obtained, gave confirmatory evidence of ovarian cyclicity.

3.2 External Genitalia

The measurements of the anogenital distance (AGD) at birth are summarized in Appendix Table III. Where the numbers of animals shown is higher than the numbers given in Table 3.I (summary of experimental design; number of rats investigated), not all animals were retained for subsequent study, although all were measured for AGD at birth.

No difficulty was encountered in distinguishing males from females at birth. The anogenital distance was difficult to measure accurately at birth in the active neonates, and no clear pattern of differences emerges from the results. Although 't' tests of weighted means revealed some significant differences in the *a priori* tests applied, they were inconsistent.

Table 3.V summarizes the appearance of the external genital structures at the time of slaughter.

All rats from mothers treated during days 10-15 of pregnancy had normal external genital structures as did all rats from cholesterol-treated litters from all treatment ages. However there was clear evidence of external genital masculinization in some rats from mothers treated with TP during the period 16-21 days. In many cases abnormalities were noted when the animals were first checked for vaginal opening at 30 days.

Of the rats receiving TP during days 19-21 of gestation, all those exposed for 72h to 10mm and 20mm implants and 2 rats of the 10 in each of the 5mm groups showed some abnormality of vaginal structure. Twenty-four hour treatment with 10mm and 20mm implants did not alter the vaginal structure.

The vaginal structure of the affected rats of the 5mm groups constituted the mildest form of abnormality recognized. A strip of mucosa extended anteriorly from the vaginal opening toward the genital papilla, although still separated from the base of the papilla by normal hairy skin.

TABLE 3.V

APPEARANCE OF EXTERNAL GENITALIA AT SLAUGHTER

TREATMENT	STAGE OF GESTATION AT TREATMENT (days) 21-19				18-16				15-13				12-10			
	n	N	A	NV	n	N	A	NV	n	N	A	NV	n	N	A	NV
24 HOUR IMPLANT																
5mm TP	10	8	2	-					6	6	-	-	10	10	-	-
10mm TP	10	10	-	-	5	3	2	-	5	5	-	-	3	3	-	-
20mm TP.	10	10	-	-	9	-	9	-	8	8	-	-	12	12	-	-
10mm CL°	10	10	-	-	8	-	8	-	13	13	-	-				
72 HOUR IMPLANT																
5mm TP	10	8	2	-	6	3	3	-	11	11	-	-	7	7	-	-
10mm TP	10	-	10	-	4	-	3	1	9	9	-	-	4	4	-	-
					'10	-	-	10								
20mm TP	14	-	14	-	6	-	6	-	2	2	-	-	2	2	-	-
10mm CL	10	10	-	-					10	10	-	-	2	2	-	-
					'10	10	-	-								

°Cholesterol

'Supplementary group

N = normal vaginal orifice
A = abnormal
NV = no vaginal orifice

Rats exposed to 10mm capsules for 72h all exhibited a similar but more extensive alteration, the mucosal tissue extending all the way back to the genital papilla.

Rats from mothers treated for 72h with 20mm capsules of TP typically exhibited a 'vaginal opening' at the posterior aspect of the base of the genital papilla. The papilla itself appeared to be cleft into bilateral lobes. Between the anomalous opening and the anus, the perineum was covered in normal hairy skin.

Four rats experienced urine scalding and excoriation of the ventral skin, associated with their unusual genital and urethral development and were destroyed at 8 weeks of age. Three had been exposed to 10mm TP capsules for 72h, the fourth to 20mm for 72h.

The abnormalities of the external genitalia of rats treated with TP on days 16-18 varied in degree between treatments. In general the more severe abnormalities were seen in those rats exposed to larger doses of TP for longer periods.

Many of the female rats from mothers treated during days 16-18 with 5mm TP capsules for 72h or 10mm TP capsules for 24h, showed the mildest form of abnormality described above. A fine frenulum dividing the vaginal opening in an antero-posterior direction frequently accompanied this form of abnormal development.

Those females which had experienced greater TP exposure (10mm and 20mm implants for 72h) had the 'vaginal opening' situated at the base of the posterior aspect of the genital papilla. The opening lacked the distensible features of a normal rat vagina. One rat from the original 10mm 72h TP treatment group, and all 10 rats from the TP-treated supplementary group (also 10mm, 72h) had no external vaginal orifice.

3.3 Sexual Behaviour

Rats which had received prenatal treatment during days 19-21 or 13-15 (72h only) were tested with adult males and their lordosis response recorded. Not every rat in these groups was tested since, as noted in Section 2.4 (Chapter 3), not all were given OB implants. All rats of the supplementary group treated on days 16-18 of gestation were tested for feminine sexual behaviour. Results are shown in Appendix Table IV. Analyses of variance applied to data from groups treated on days 19-21, and days 13-15, did not give significant 'F' ratios. Rats in the two groups treated on days 16-18 all showed 100% LQ.

3.4 Growth

3.4.1. Birth weight

The weights ($\log_{10}$) of rats at birth are summarized in Appendix Table V. Again the numbers of rats exceed the numbers detailed in Table 3.I, as all rats were weighed at birth but not all were retained for further study.

Of the rats exposed to TP during days 19-21 of gestation, the females from mothers receiving 10mm and 20mm implants for 24h were significantly ($P<0.05$) lighter than control rats.

At birth, female rats from mothers treated during days 16-18 for 24h with TP (regardless of size of capsule) were significantly ($P<0.001$) heavier than the rats from similarly cholesterol-treated mothers. Seventy-two hour treatment with 10mm and 20mm TP implants produced rats significantly ($P<0.01$ and $P<0.001$ respectively) lighter than controls. Overall rats treated with TP for 72h were significantly ($P<0.001$) lighter than those treated for 24h.

Rats from mothers exposed to 10mm TP implants for 72h on days 13-15 of gestation were significantly ($P<0.05$) heavier at birth than control animals, while those from 20mm TP-implanted mothers were significantly ($P<0.001$) lighter.

Rats from mothers treated for 24h with 10mm TP implants during days 10-12 of pregnancy were significantly ($P<0.001$) heavier than control animals but no other significant differences were found.

No consistent pattern is evident in these differences, and reference to the birth records (not presented here) suggests that litter size might account for weight differences. Rats from large litters tended to weigh less at birth than those from smaller litters and in this experiment all rats in the same litter inevitably fell into the same treatment group.

3.4.2. Weekly body weight

The mean ($\log_{10}$) body weight for each group for each weekly weighing appears in Appendix Tables VI and VII. Summaries of the regression data are contained in Appendix Tables VIII and IX.

Analysis of variance of the body weights of rats from mothers treated during days 19-21 showed there to be a significant overall difference in body weight during the 3-6 week period. Although the 24h 5mm group was smaller than its control group, treatment with both 5 and 10mm capsules (but not 20mm) for 72h resulted in rats which were significantly ($P<0.01$) heavier than their controls over these ages. When the effect of the duration of treatment was examined, 72h treatment gave heavier rats in those exposed to 5mm capsules, but smaller rats in the 20mm groups. Other significant differences were inconsistent.

Comparison of the slopes of the regression lines of $\log_{10}$ body weight *versus* reciprocal of age for the period of 3-12 weeks showed there to be no significant differences between the groups receiving treatment during the last 3 days of gestation. This is illustrated in Figure 3.2 which shows the regression coefficients and their 95% confidence limits. One-way analysis of variance of body weight during the periods following spaying (13-15 weeks) and OB treatment (16-18 weeks) yielded 'F' ratios which were not consistently significant hence regression analysis was not applied to these periods of body weight measurement.

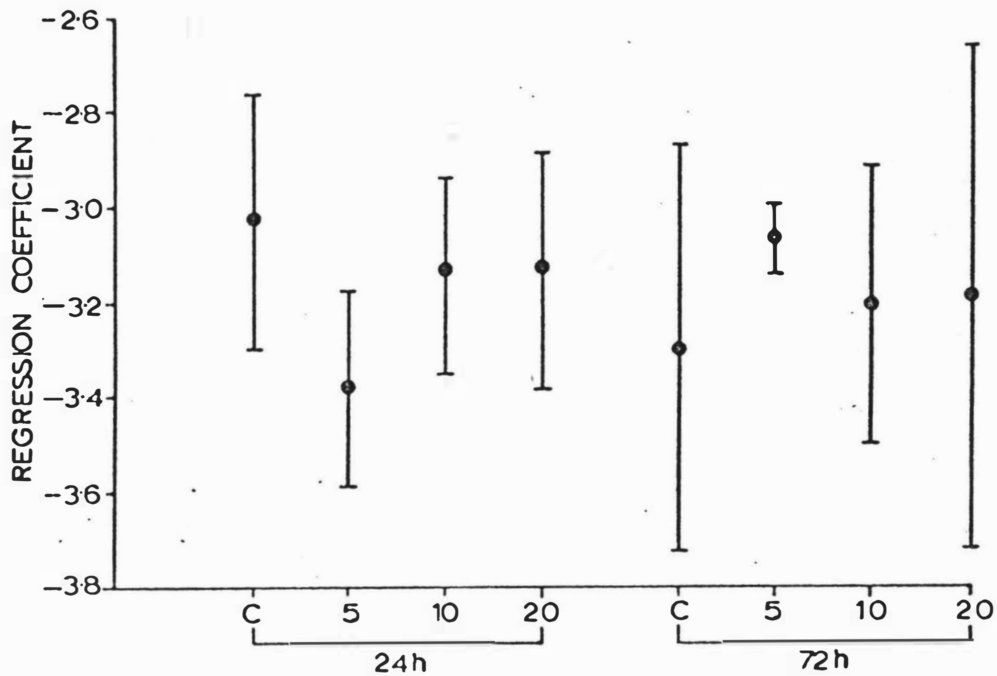


Figure 3.2 BODY WEIGHT GROWTH WEEK 3 TO 12 WITH PRENATAL TREATMENT AT GESTATIONAL AGE 19-21 DAYS
95% CONFIDENCE LIMITS FOR LINEAR REGRESSION COEFFICIENTS
(X = 1/AGE, Y = log(base 10) BW)

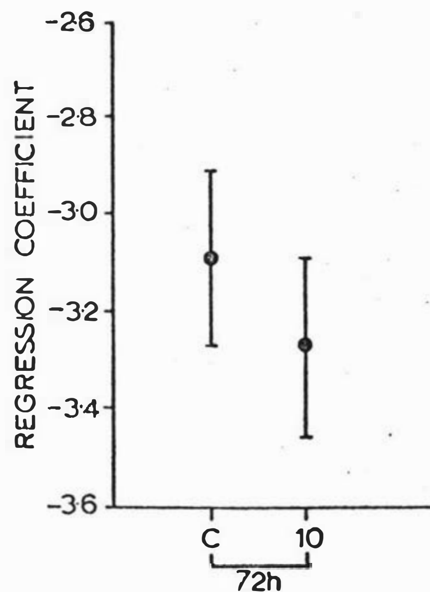


Figure 3.3 BODY WEIGHT GROWTH WEEK 3 TO 12 WITH PRENATAL TREATMENT AT GESTATIONAL AGE 16-18 DAYS
95% CONFIDENCE LIMITS FOR LINEAR REGRESSION COEFFICIENTS
(X = 1/AGE, Y = log(base 10) BW)

Treatment code: C = cholesterol capsule implanted
5,10 or 20mm = length of TP capsule implanted
24 or 72h = duration of implant

Student's 't' tests were used to compare the weekly body weight measurements for weeks 3-12 of rats born of mothers receiving 10mm TP or cholesterol capsules during days 16-18 of pregnancy. Rats from TP-treated mothers were significantly ($P<0.05$) heavier than control animals for all of this period except at week 4. One-way analysis of variance applied to the body weight following ovariectomy (13-16 weeks) and during OB treatment (17-20 weeks) showed that the two groups were not significantly different. Regression analysis for the period 3-12 weeks showed there to be no significant difference between regression coefficients (see Fig. 3.3).

One-way analysis of variance of the body weights of rats from mothers receiving 72h treatment during days 13-15 of pregnancy produced significant 'F' ratios for weeks 3 and 5 only. At 3 weeks, each TP-treated group was significantly ($P<0.01$) heavier than its control. At 5 weeks only the 5mm and 10mm groups were still significantly heavier than controls and by 6 weeks of age there were no significant differences in body weight between groups. Regression analysis was therefore not performed.

3.4.3. Organ weights

(a) treatment during days 19-21 of gestation

Separate analysis of variance was applied to several organ weights (adrenal, preputial gland, uterus, pituitary, brain, liver, kidney) from the oestrogen-treated and the non-oestrogen-treated groups.

There were no significant differences between groups in the weights of adrenal glands, preputial glands, uterus or pituitary. A summary of these measurements is given in Appendix Tables X, XI, XII and XIII.

Analysis of variance of liver weights (summarized in Table 3.VI) of oestrogen-treated rats showed there to be no significant differences but a significant ($P<0.05$) 'F' value for the untreated animals indicated that there were differences within this group. The livers of TP-treated groups were lighter than those of controls, but for only the 5mm 24h and 10mm 72h groups was this significant ($P<0.05$). Livers of oestrogen-

TABLE 3.VI

WEIGHT OF LIVER (g) AT 130 DAYS

TREATMENT	STAGE OF GESTATION AT TREATMENT (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB[1]			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	12.63	0.66	5	10.79	0.50 *°						
10mm TP	3	11.94	0.62	7	11.56	0.26						
20mm TP	6	12.99	0.76	4	11.42	0.52						
10mm CL[2]	5	13.40	0.43	5	12.58	0.55						
72 HOUR IMPLANT												
5mm TP	5	14.30	1.04	3	11.62	1.08	6	12.07	0.40	5	12.16	0.16
10mm TP	4	12.43	0.26	3	10.66	0.24 *°	5	13.78	0.48	4	12.09	0.67
20mm TP	8	12.65	0.46	5	11.45	0.40	2	12.07	0.07			
10mm CL	4	13.96	0.63	6	12.49	0.53	5	13.10	0.74	5	11.93	0.40
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS												
"F"		1.24ns			2.48*					2.03ns		

°Significance of difference between TP mean and corresponding CL mean (t-test)
[1]Oestradiol benzoate
[2]Cholesterol

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

treated groups were all larger than those of non-oestrogen-treated.

The weights of brains are summarized in Table 3.VII. The brains of oestrogen-treated groups were lighter than untreated groups. There were no significant differences within the oestrogen-treated groups, but differences did exist in untreated groups. Rats receiving 24h treatment with 5mm and 72h treatment with 5mm and 10mm implants had significantly ($P<0.05$) lighter brains than controls, and 72h TP treatment overall resulted in brains which were significantly ($P<0.01$) lighter than those receiving TP treatment for 24h.

Analysis of variance of the weights of kidneys (see Table 3.VIII) showed there to be significant differences within both oestrogen-treated and untreated groups. However 't' tests of weighted means of oestrogen-treated groups showed no significant differences in the *a priori* comparisons. Amongst the untreated groups, 10mm and 20mm 24h groups had significantly ($P<0.05$) heavier kidneys than their controls, while the 10mm 72h group had significantly ($P<0.05$) lighter kidneys. Overall, 72h treatment resulted in significantly ($P<0.05$) lighter kidneys than did TP treatment for 24h.

(b) treatment during days 13-15 of gestation

The weights of liver, brain, kidneys, adrenals, preputial glands, uterus and pituitary are shown in Tables 3.VI, 3.VII, 3.VIII and Appendix Tables X, XI, XII and XIII respectively. There were no significant differences between groups in the weights of preputial glands, adrenals, liver or kidneys. Oestrogen treatment resulted in significantly heavier pituitaries and uteri, and lighter brains.

TABLE 3.VII

WEIGHT OF BRAIN (g) AT 130 DAYS

TREATMENT	STAGE OF GESTATION AT TREATMENT (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB[1]			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	1.922	.029	5	1.900	.064 *°						
10mm TP	3	1.935	.040	7	2.045	.024						
20mm TP	6	1.870	.017	4	1.960	.051						
10mm CL[2]	5	1.983	.023	5	2.032	.044						
72 HOUR IMPLANT												
5mm TP	5	1.890	.022	3	1.856	.050 *°	6	1.998	.025 *°	5	2.038	.009
10mm TP	4	1.896	.062	3	1.886	.049 *°	5	1.946	.008	4	2.040	.047
20mm TP	8	1.853	.024	5	1.922	.030	2	1.775	.045 **°			
10mm CL	4	1.912	.024	6	2.007	.025	5	1.918	.026	5	1.962	.040
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS												
"F"		1.18ns			3.34**				5.86**			

°Significance of difference between TP mean and corresponding CL mean (t-test)
[1]Oestradiol benzoate
[2]Cholesterol

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE 3.VIII

WEIGHT OF KIDNEYS (g) AT 130 DAYS

TREATMENT	STAGE OF GESTATION AT TREATMENT (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB[1]			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	2.078	.169	5	1.934	.126						
						**°						
10mm TP	3	2.060	.175	7	2.084	.021						
						*°						
20mm TP	6	2.146	.079	4	2.045	.023						
10mm CL[2]	5	1.896	.040	5	1.802	.049						
72 HOUR IMPLANT												
5mm TP	5	2.310	.104	3	2.135	.005	6	2.053	.099	5	2.180	.045
						*°						
10mm TP	4	1.797	.054	3	1.660	.079	4	2.277	.148	4	2.220	.087
20mm TP	8	1.941	.051	5	1.902	.077						
10mm CL	4	2.112	.154	6	1.946	.068	5	2.024	.036	5	1.975	.048
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS												
"F"		2.40*			3.62**				1.84ns			

°Significance of difference between TP mean and corresponding CL mean (t-test)
[1] Oestradiol benzoate
[2] Cholesterol

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

4. DISCUSSION

Treatment of the pregnant rat with TP for 24 or 72 hour periods during the second half of pregnancy caused 'masculinization' of the external genitalia of female offspring but did not alter their reproductive function, their display of feminine sexual behaviour nor their growth pattern.

It was shown in Chapter 2 (Section 3.3.1.) that high concentrations of plasma testosterone were achieved soon after the subcutaneous implantation of TP-filled capsules in adult rats and that this testosterone was rapidly cleared from the plasma so that the periods of exposure to the hormone exceeded the duration of implantation by no more than 4 hours. Although androgen levels in the foetuses were not measured, it was assumed that testosterone had crossed the placenta, since marked alteration of the external genital features occurred. Since androgen appeared to have reached the foetus and yet did not alter sexual differentiation of hypothalamic function, it may be concluded that the hypothalamus of the rat is not susceptible to androgenization prenatally with the doses of TP used. However, a recent paper by Slob and others (1983) claiming that testosterone does not cross the placenta raises some interesting questions which are discussed later in this section.

4.1 External Genitalia

Modification of the external genitalia occurred in the present experiment in female rats treated indirectly with TP during days 16-21 of gestation, but not as a result of earlier treatment. With two exceptions (5mm 24h on day 21), rats receiving TP within 24h before birth showed no sign of genital 'masculinization'. Since the time of birth could not be accurately predicted, most of these litters experienced less than 24 hours treatment, and some only a few hours. However, Swanson and van der Werff ten Bosch (1964, 1965) also found that genital masculinization was rare in rats from mothers injected with TP on days 21 or 22 or pregnancy. As injection into the foetus itself caused profound virilization when given 1 or 2 days before birth, they suggested then that there may be interference with the passage of androgen from

the maternal circulation to the foetus at this particular time.

Swanson and van der Werff ten Bosch (1965) describe, with the aid of photographs, a range of hypospadic conditions similar to the forms seen in the present experiment.

Many other instances of masculinization of the genital structures of the female rat as a result of prenatal androgen, given indirectly by way of the mother, are recorded in the literature. In 1939, Greene and colleagues gave a detailed account of the masculinization of the reproductive tract which resulted from treatment with TP and other androgens before birth. Other authors have confirmed the modification of the external genitalia of female offspring, by multiple daily injections of androgen to the mother rat during the latter part of gestation (Revesz *et al.*, 1963; Swanson and van der Werff ten Bosch, 1964, 1965; Gerall and Ward, 1966; Ward, 1969; Ward and Renz, 1972), and of a single injection on day 18 (Nadler, 1969), day 19, but not day 16 (Huffman and Hendricks, 1981), and, days 19 or 20, but not days 21 or 22 (Swanson and van der Werff ten Bosch, 1964).

Thus the tissues which form the genital tubercle and those which normally form the vaginal opening at puberty are susceptible to alteration by androgen during the last 5 days of prenatal life in the female rat, and exposure of the mother to TP for 24-72 hours is sufficient to disturb normal female development. Slob and co-workers (1983) suggest that the metabolite of testosterone, androsterone, is responsible, since they found this to be the principal androgen in foetal rat tissues after injection of TP into the mother (described in more detail later in this Discussion).

4.2 Gonadotrophin Secretion

In contrast to the masculinization of external genitalia, no disturbance of ovarian function was found in the daughters of rats which were treated with TP for 72 or 24 hour periods during the second half of pregnancy. Corpora lutea were present in the ovaries, the weights of which were not significantly different from those of control animals.

Vaginal cell types followed a normal cyclic pattern. Thus the hypothalamic control of gonadotrophin secretion appears to be insensitive to androgen prenatally. However, there has been evidence to the contrary, and the recent report, that testosterone apparently does not cross the placenta (Slob *et al.*, 1983), must be taken into consideration.

Attempts to androgenize ovarian function by prenatal administration of androgen *via* the maternal circulation have met with both success and failure, occasionally using identical treatment regimens. Anovulatory sterility has been achieved by repeated daily injections of the mother rat during the last trimester of pregnancy with 2mg TP (Ward and Renz, 1972) or 2mg (but not 1mg) androstenedione (Popolow and Ward, 1978). A single injection of 10 or 25mg TP (but not 2.5mg) given to the mother on either day 19, 20, 21 or 22 was also successful (Swanson and van der Werff ten Bosch, 1965). Failure to induce anovulatory sterility by androgen treatment of the pregnant rat has been reported after repeated daily injections on days 16-20 (2mg TP, Ward, 1969; Slob *et al.*, 1983); and after a single injection on day 18 (2.5mg TP, Nadler, 1969); day 16 or day 19 (1mg TP, Huffman and Hendricks, 1981). Corpora lutea were present in ovaries of all female rats born of mothers treated with a range of doses of TP given as single or multiple injections at varying times during gestation (Greene *et al.*, 1939).

Experiments involving oestrogen treatment of the pregnant rat have also produced conflicting evidence for the notion of prenatal androgenization. When given to the mother rat daily during days 16-20 of pregnancy, large doses of the synthetic oestrogen RU 2858 (50μg) or oestradiol (1250μg) caused anovulatory sterility in 100% of female offspring (Vannier and Raynaud, 1980). However, when diethylstilboestrol (DES) was administered for 72 or 24 hours by PDS capsule to pregnant rats during the last 3 days of gestation, there was no evidence of androgenization (Tarttelin, M.F., personal communication). Female rats treated with DES on days 1 or 5 after birth in the same study were clearly anovulatory. That DES given to the pregnant mouse or hamster is absorbed by the foetus is known from several reports (Bengtsson and Ullberg, 1963; Shah and McLachlan, 1976; Rustia and Shubik, 1979), and severe modification of the external genital structures in the female offspring of treated mothers in

Tarttelin's study would appear to confirm this in the rat.

Androgen injected directly into the foetus can cause anovulatory sterility. This has been demonstrated by Swanson and van der Werff ten Bosch (1965) who injected TP into foetuses 1, 2 or 4 days before birth, and Fels and Bosch (1971) who injected TP into the amniotic cavities. Gorski (1968) mentions an "unpublished preliminary study" in which TP injected into rat foetuses 1-4 days before birth induced anovulatory sterility.

According to Slob and colleagues (1983), the reason for failure of prenatal androgen, administered indirectly through the mother rat, to cause androgenization is its inability to cross the placenta unless an extremely large dose is used. Their experiments involved injection of the pregnant rat daily with 2mg TP on days 16-20 of pregnancy, and measurement of plasma testosterone concentrations in the mother and the foetuses on day 22. While the maternal concentration was elevated 20-fold, the foetal plasma testosterone concentration did not rise significantly above oil injected control levels. Parenthetically, in earlier experiments (Swanson and van der Werff ten Bosch, 1964, 1965), maternal injection with TP on days 21 or 22 rarely produced masculinization of the external genitalia of female offspring whereas earlier treatment did. The authors postulated that on these days of gestation the placenta may not permit the passage of androgen. In view of this observation measurements of foetal testosterone concentrations at earlier ages would be of interest.

When a large dose of TP (10mg) was injected into a pregnant rat on day 20, she had a higher plasma testosterone concentration on day 22, and her 4 foetuses also showed substantially higher plasma testosterone concentrations, than after the same dose of TP given by 5 daily injections (Slob *et al.*, 1983). Hence Slob and co-workers suggested that only when a large dose of TP is given to the mother rat will the foetuses experience elevated testosterone concentrations. The placenta is active in steroid hormone metabolism and it has been shown in the human to have enzymes whose activities prevent the passage of significant amounts of androgen or excessive oestrogen to the foetus (Gower and Fotherby, 1975).

Slob's group (1983) also measured the ^{3}H-labelled testosterone and its metabolites in the plasma and tissues of day 18 foetuses after infusion of ^{3}H-labelled testosterone into the mother's bloodstream. As was found also in guinea pigs (Vreeburg, Woutersen, Ooms and van der Werff ten Bosch, 1981), the principal labelled androgen recovered from the foetuses was androsterone; testosterone and its other derivatives were present in only small amounts and labelled oestrogens were undetectable. The androsterone, they suggest, is responsible for the masculinization of the reproductive tract, since it is a non-aromatizable steroid (therefore unable to androgenize the hypothalamus), known to be capable of masculinizing the genital organs of female rat foetuses (Greene *et al.*, 1939). Results of this experiment appear to indicate that at day 18, as well as at day 22, the placenta prevents the passage of testosterone.

While these experiments suggest that only very large doses of testosterone administered to the pregnant rat will elevate foetal testosterone concentrations, prenatal androgenization has been reported after a dose considered low (2mg TP daily for 6 days, Ward and Renz, 1972) by Slob and colleagues (1983).

Despite considerations of placental testosterone transfer, prenatal treatment with either testosterone or oestrogen has resulted in anovulatory sterility (Swanson and van der Werff ten Bosch, 1965; Fels and Bosch, 1971; Ward and Renz, 1972; Vannier and Raynaud, 1980). Although this appears to confirm the concept of prenatal androgenization, it is possible, as suggested by Gorski (1968), that testosterone (or oestrogen) reaching the foetus before birth, persists in the tissues until after birth when it exerts its effects on the androgen-sensitive hypothalamus. In Chapter 2 it was reported that neonatal rats were very much slower than adults to clear testosterone from the bloodstream and after injection of TP it took 8 days for plasma testosterone concentration to reach control levels. The steroid metabolic machinery in the foetus would possibly be slower than in the neonate. Thus although some reports suggest that the foetal hypothalamus may be organized by testosterone (or oestrogen), the possibility of a carry-over of the hormone into the postnatal period has not been eliminated.

4.3 Sexual Behaviour

Suppression of feminine sexual behaviour was not found in female rats whose mothers were given TP for 72 or 24 hour periods during days 13-21 of pregnancy. Similar negative findings have been reported after daily injections of pregnant rats with 0.5-1.0mg TP on days 15-20 (Revesz *et al.*, 1963); with daily injections (days 15-19) of a progestin whose androgenic properties were sufficient to cause genital virilization (Whalen *et al.*, 1966); or after a genitally virilizing dose of DES given on days 19-21 (Tarttelin, M.F., personal communication).

However, suppression of feminine behaviour following prenatal androgen has been demonstrated following daily injection of the mother rat during the last trimester of pregnancy (Gerall and Ward, 1966; Ward and Renz, 1972; Dunlap *et al.*, 1978; Popolow and Ward, 1978) and by a single injection of TP on day 19, but not day 16 (Huffman and Hendricks, 1981) nor day 18 (Nadler, 1969). Studies designed to investigate the effects on feminine behaviour of androgens from male litter-mates also support the concept of prenatal organization of mating behaviour by androgen (Clemens and Gladue, 1978; Clemens *et al.*, 1978; Gladue and Clemens, 1978). Once again, the possibility of persistence of androgen in the tissues until a sensitive neonatal period must be considered.

That the present experiment failed to demonstrate a prenatal influence on behaviour may be due to a difference in testing and assessment procedures.

Many of the experiments described above were concerned solely with effects on mating behaviour so that testing was more extensive and more stringent, often including qualitative assessment of proceptive and receptive behaviour as well as a quantitative measure of lordosis response. Testing was frequently repeated over several weeks. The simple lordosis testing used in the present experiment may not have detected fine differences in the quality of sexual behavioural responses between treated and control rats.

De-feminization of the hypothalamic control of mating behaviour has been reported in the female offspring of rats treated during pregnancy with doses of TP which Slob and others (1983) consider too low to raise foetal plasma testosterone concentrations (2mg TP daily for 5-7 days, Gerall and Ward, 1966; Ward and Renz, 1972; Dunlap *et al.*, 1978).

When given neonatally, much less androgen is required to disrupt ovarian function than to suppress feminine sexual behaviour (Barraclough and Gorski, 1962; Swanson and van der Werff ten Bosch, 1964; Nadler, 1968; McDonald and Doughty, 1974; Christensen and Gorski, 1978). While some studies have shown suppression of both feminine behaviour and ovulation as a result of prenatal androgen treatment (Ward and Renz, 1972; Popolow and Ward, 1978), there are three reported instances of prenatal androgen suppressing feminine sexual behaviour without suppressing ovulation (Nadler, 1969; Popolow and Ward, 1978; Huffman and Hendricks, 1981).

Several explanations may account for the discrepancies noted in the last two paragraphs. As mentioned earlier, differences in methods of assessing behaviour may confound the comparison of different experiments. Different strains of rat may vary in the placental conversion of androgens so that after maternal injection with TP, foetal concentrations of testosterone and other androgenic metabolites may differ. The period of sensitivity to androgen may differ for the hypothalamic control of ovarian function and feminine sexual behaviour, the control of behaviour being more androgen-sensitive than control of the ovary before birth, and less androgen-sensitive after birth. Finally, different androgens (to which perhaps the placenta is differentially permeable) may be involved in androgenization of these two hypothalamic functions.

Interestingly with regard to the last point, androsterone, the non-aromatizable androgen found by Slob and others (1983) in the foetus after administration of testosterone to the mother, was reported to cause significantly reduced feminine sexual behaviour but normal ovarian function, when given to neonatal rats on the first 5 days of life (McDonald and Doughty, 1974). If the de-feminization of behaviour which occurs after prenatal TP treatment of the mother were due to androsterone, this would explain not only those cases of suppressed feminine behaviour in rats whose

ovaries were functioning normally, but also the reason for suppression of behaviour by administration to the pregnant rat of doses of TP which Slob and his team found to be too low to raise foetal testosterone levels. It suggests however that aromatization is not necessary for alteration of the tissues controlling behaviour, and numerous studies have implicated aromatization in the neonatal masculine differentiation of neural tissues mediating adult sexual behaviour (McEwen *et al.*, 1977; Booth, 1977, 1979; Clemens and Gladue, 1978; Gladue and Clemens, 1980).

4.4 Growth

The longitudinal studies of body weight conducted here have failed to detect any change in growth patterns in female rats whose mothers received TP during pregnancy.

Several significant but inconsistent differences in birth weight between prenatally TP-treated and cholesterol-treated rats were found. Low birth weights were associated with large litter sizes and since each treatment group contained rats from only two or three litters, differences were attributed to differences in litter size. Although there are several reports (Slob and van der Werff ten Bosch, 1975; Popolow and Ward, 1978; Slob *et al.*, 1983) of prenatal androgen treatment causing a reduced birth weight, presumed to be a consequence of the reduced weight of the placenta (Slob *et al.*, 1983), this was not confirmed by the present experiment. Perhaps the treatments used in this experiment were insufficient to affect the placenta as no instances of foetal death, which might indicate foetal insufficiency and which have been associated with TP treatment of the pregnant rat (Swanson and van der Werff ten Bosch, 1965; Gerall and Ward, 1966; Fels and Bosch, 1971; Schultz and Wilson, 1974), were recognized.

Lower body weights at 90-100 days of age (Ward, 1969; Ward and Renz, 1972) and between 30-40 days (Slob *et al.*, 1983) have been reported and this reduced postnatal growth has been attributed to the lower birth weight resulting from prenatal TP treatment (Slob *et al.*, 1983).

The organ weights of rats from mothers given TP during the last 3 days of gestation (days 19-21) showed no consistent pattern of alteration in the present experiment. Although heavier adrenal glands, anterior pituitaries and livers have been reported after neonatal TP treatment (Barraclough, 1961; Gorski and Barraclough, 1962; Swanson and van der Werff ten Bosch, 1963; Wagner, Erwin and Critchlow, 1966), there are also reports of no difference in these organ weights (Brown-Grant, 1964; Bradshaw and Critchlow, 1966).

Thus it seems that prenatal androgen alone is unable to 'masculinize' growth. Slob and van der Werff ten Bosch (1975) found that, in order to mimic the growth patterns of male rats (castrated at 20 days) in female rats, it was necessary to give TP postnatally (for 20 days) to females which had received prenatal TP (*via* the mother) and were spayed at birth. It therefore appears that the immediate postnatal period is more important than the period preceding birth for androgenic effects on growth.

5. CONCLUSIONS

Despite masculinization of the external genital tissues caused by androgen given prenatally *via* the maternal circulation during the last 6 days of gestation, the mechanisms regulating gonadotrophin secretion, feminine sexual behaviour and growth were unaffected in this experiment. The evidence for a prenatal period of hypothalamic sensitivity to androgen, suggested by reported instances of prenatal androgenic alteration of ovarian function and mating behaviour, was considered to be inconclusive, but could be accounted for by a persistence of testosterone in the foetal rat tissues until the sensitive neonatal period of differentiation.

CHAPTER 4

THE EFFECTS ON FEMALE RATS OF VARYING DURATIONS OF TREATMENT WITH TESTOSTERONE PROPIONATE AT DIFFERENT NEONATAL AGES

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4.2.2 Sexual behaviour
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5. CONCLUSIONS

CHAPTER 4

THE EFFECTS ON FEMALE RATS OF VARYING DURATIONS OF TREATMENT WITH TESTOSTERONE PROPIONATE AT DIFFERENT NEONATAL AGES

1. INTRODUCTION

Early experiments investigating 'androgenization' of the neonatal female rat and mouse showed that the sensitivity to androgen was a temporary phenomenon. When given at later ages androgen was not able to exert the same organizational effects, and the idea of a 'critical period' for hypothalamic androgen sensitivity was proposed (Barraclough and Leathem, 1954; Barraclough, 1961).

The critical period refers to the neonatal period when exogenous androgen given to the female rat is able to masculinize various hypothalamic functions - the control of gonadotrophin secretion and mating behaviour are the endpoints usually considered. Different sexually differentiated central nervous system functions will not necessarily be sensitive to androgen during the same period, and the fact that control of gonadotrophin secretion, sexual behaviour and growth can be independently masculinized (Barraclough and Gorski, 1962; Swanson and van der Werff ten Bosch, 1964; Mullins and Levine, 1968; Clemens *et al.*, 1969; Luttge and Whalen, 1970; McDonald and Doughty, 1973/74; Tarttelin *et al.*, 1975; Christensen and Gorski, 1978; Gorski *et al.*, 1979) suggests differing susceptibilities. Within the critical period there may be a time of maximum sensitivity to androgen, when a lower dose of androgen is more effective than during the remainder of the critical period. The minimum time for which androgen must be present in order to be effective may vary for each parameter and may vary at different times within the critical period.

The critical period is a concept rather than an identifiable stage of development. The characteristics of the endpoint of androgenization under study, as well as the experimental procedures used to induce it, need careful definition. The 'delayed anovulation syndrome' illustrates

the importance of this. Threshold doses of TP produce a partial androgenization; the rat has apparently normal oestrous cycles after puberty, but becomes anovulatory at some later age (Swanson and van der Werff ten Bosch, 1964; Gorski, 1968) so that the age at observation may affect the conclusion drawn. Because of the variable factors involved in both the means of androgen administration and the assessment of hypothalamic androgenization, the absolute length of the postnatal critical period cannot be categorically stated. Anovulatory sterility has been induced by TP given as late as day 10 by subcutaneous injection of a high dose (Barraclough, 1961) or day 11 by intracranial implantation (Lobl and Gorski, 1974).

Plasma testosterone measurements described in Chapter 2 (Section 3.2.1.) demonstrate that a typical androgenizing dose of TP (90μg), given to a neonatal rat by injection in oil, results in elevated, but gradually diminishing, testosterone concentrations for many days (192h). Clearly this route of administration does not allow ready manipulation of the period of exposure to testosterone. In order to examine some aspects of the critical period in more detail, the present experiment made use of subcutaneously placed PDS capsules filled with TP. Because these were easily removed, timing of exposure to testosterone could be accurately manipulated. As described in Chapter 2 (Section 3.2.1.) plasma testosterone concentrations reached control levels in the neonate 24 hours after removal of such a capsule.

A study of Figures 2.3 and 2.4 shows that the peak concentration of plasma testosterone achieved by a TP-filled PDS capsule was not dissimilar to that for a subcutaneous injection of 90μg TP in oil, at the times selected for the initial measurements. Different effects resulting from the two methods of hormone administration are therefore likely to be a function of the different durations of elevated androgen levels in the body.

Chapter 2 (Section 3.2.1.) established the decay profiles of plasma testosterone in 3 day old rats whose TP implants were inserted for 4 hours and then removed. Study of the plasma testosterone concentration following the three different periods of implantation at the three ages investigated in this experiment would require the sacrifice of huge numbers of baby

rats to furnish sufficient blood samples for a complete investigation. Experiments using adult rats (Chapter 2, Section 3.1) showed that the rate of decay of plasma testosterone was the same regardless of the duration of implantation. There is an increase in the metabolic clearance of testosterone with age (Chapter 2, Section 4) but the age difference in the present experiment is only three days. It is therefore assumed that the decay profiles after 4 hour implants in 3 day old neonates will be representative of the 4, 8 and 24 hour periods of implantation given at 2, 3 or 5 days.

Use of these TP-filled capsules permitted investigation of two facets of the critical period concept - the period of maximum sensitivity to androgen, and the minimum exposure time. Each was examined with respect to ovarian function, feminine sexual behaviour, growth and development of the external vagina.

Subcutaneous PDS capsules filled with androgen have been used previously in neonatal rats to provide a constant source of hormone over a period of time (Whalen and Rezek, 1974; McEwen *et al.*, 1977). Oestrogen-filled PDS capsules have provided short-term hormone treatment to adult female rats (Johnston and Davidson, 1979) and guinea pigs (Bullock, 1970) in experiments to determine the minimum exposure required for specific oestrogenic effects.

2. MATERIALS AND METHODS

2.1 Animals and Animal Management

The Sprague-Dawley rats used in these experiments were from the same source and housed under the same conditions as those described in Chapter 2 (Section 2.1). Adult females were time-mated (as described in Chapter 3, Section 2.1). Litter numbers were adjusted to 12 by removal of excess males or cross-fostering of males from a litter of the same age. The litters were examined daily for the first week after surgery and any dead or sickly animals removed. At weaning (21 days) the experimental subjects were weighed, identified by ear code and

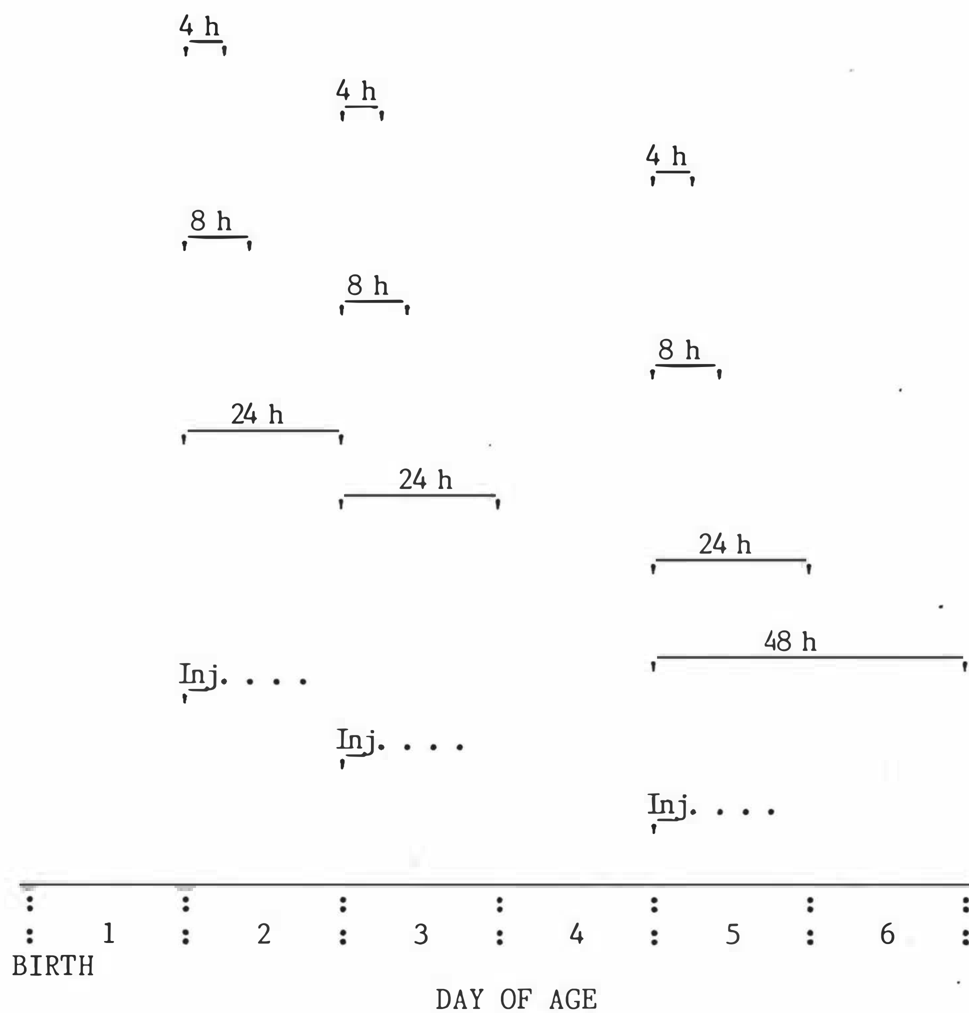
randomly allocated to stainless steel colony cages.

2.2 Experimental Design

As illustrated in Figure 4.1 the experiment was conducted in three parts, according to whether the experimental treatment was given at the age of 2, 3 or 5 days. Within each part the duration of treatment was varied by removing the PDS capsule at one of three predetermined times after insertion (4, 8 or 24h). Each part of the experiment therefore consisted of six groups of animals with implanted capsules (TP-filled or empty). An additional group, in which the capsules remained in place for 48h, was included for rats treated on day 5. In addition, there were two groups of animals injected (90μg TP or oil) at each age. Table 4.I gives details of the experimental design and the numbers of animals used for the assessment of responses. Twelve extra rats treated on day 5 were maintained until vaginal opening, and contribute to measurements made before 6 weeks.

2.3 Administration of Testosterone Propionate

Near the expected day of delivery the pregnant rat was observed frequently and the date of birth of the litter recorded. At the assigned age the neonates were temporarily removed from their dam, sexed, and the females weighed, identified by toe removal and anaesthetized by hypothermia. A 2.5mm PDS capsule (described in Chapter 2, Section 2.2), which had been pre-incubated in sterile buffered saline, was placed subcutaneously in each female rat through a small flank skin incision, the wound was sutured with 5/0 silk, and the rat was warmed under a 60 watt lamp until mobility was restored. The neonate was returned to its mother along with its similarly treated female and untreated male litter-mates. When the elected time period (4, 8, 24 or 48h) had elapsed, the babies were again taken from their dam, re-anaesthetized by hypothermia and the suture and capsule removed and the wound resutured. Care was taken to restore normal body temperature as it was found that the dam would sometimes cannibalize young which were returned to her cold.



Rats were implanted with 2.5mm PDS capsules (TP or empty) or injected (with 90μg TP or with oil)

Figure 4.1 EXPERIMENTAL DESIGN: PERIOD OF TREATMENT OF NEONATAL FEMALE RATS WITH TESTOSTERONE PROPIONATE

TABLE 4.I

SUMMARY OF EXPERIMENTAL DESIGN
NUMBER OF RATS INVESTIGATED

TREATMENT	DAY OF TREATMENT D2	D3	D5
4 HOUR IMPLANT°			
TP	11	11	10
BLANK	9	9	10
8 HOUR IMPLANT			
TP	7	10	10
BLANK	5	7	10
24 HOUR IMPLANT			
TP	7	10	9
BLANK	6	10	9
48 HOUR IMPLANT			
TP			9
BLANK			10
INJECTION			
TP'	11	8	7
OIL	12	7	8

° 2.5mm PDS capsule
' 90 µg TP in oil

Neonates in the injected groups received, at the appropriate ages, a subcutaneous injection of 0.05ml of 90µg TP in arachis oil or oil alone (controls) in the nape of the neck, the site being sealed with acrylic resin skin dressing ('Nobecutaine', BDH Pharmaceuticals Ltd) to prevent leakage.

2.4 Assessment of Responses

Body weight was recorded at weaning (3 weeks) and weekly thereafter.

From the age of 30 days each rat was checked daily for vaginal opening and its date recorded. Daily vaginal smears were taken and examined microscopically, for a period of 10 days beginning at 9 weeks, from each rat whose external vagina was open.

Bilateral ovariectomy under ether anaesthesia was performed when the rats reached 90 days of age, and the ovaries dissected, weighed and examined macroscopically for corpora lutea. Ovaries from all TP-treated rats and a sample from control animals were histologically processed and examined to confirm the presence or absence of corpora lutea.

Three weeks after ovariectomy the rats were anaesthetized with ether and implanted subcutaneously with a 10mm PDS capsule filled with crystalline OB. Three days later a subcutaneous injection of 0.5mg progesterone was administered to each rat and 4-5 hours later mating behaviour tests, as described in Chapter 3 (Section 2.4), were conducted.

During assessment of mating behaviour in the previous experiment (Chapter 3) subtle behavioural differences in mating behaviour were not recorded. Therefore in addition to measurement of LQ, the proceptivity and receptivity of each rat was scored on a simple scale of 1 to 5:

1. normal oestrous behaviour including darting, lordosis posture readily elicited and well maintained,
2. absence of soliciting behaviour, yet ready to adopt lordosis posture when mounted,

3. passive, no proceptive behaviour, lordosis response only when firmly mounted,
4. no proceptive behaviour, avoided being mounted and sometimes fended off the male,
5. strong resistance to male's advances, often jumping, rolling over and fighting.

Problems were encountered with the male rats used for testing. They were easily deterred by unenthusiastic females and would then refuse to attempt to mount anything other than a strongly oestrous female. For the experiment in which TP treatment was given on day 5, the males became unco-operative and it was found impossible to test every female. Instead, a random sample from each group was tested.

The animals were killed with chloroform at the age of 18 weeks. The external genitalia were examined and any abnormalities recorded.

2.5 Statistical Analyses

The statistical analyses performed were the same as those used and described in Chapter 3, Section 2.5.

3. RESULTS

Neonatal female rats given TP implants were exposed to high plasma testosterone concentrations during the period of implantation and to exponentially declining levels thereafter. Plasma testosterone was estimated (Chapter 2, Section 3.2.1.) to have returned to control levels 24h after removal of the implant. Where reference is made to the 'treatment' period (duration of implantation) it should be remembered that the rats were exposed to elevated testosterone concentrations for a maximum of 24h after implant removal. The 'exposure' time (= duration of implantation + 24h) is probably a generous estimate of the actual period of TP treatment, but as the minimum effective concentration of plasma testosterone is unknown, the effective exposure time cannot be more precisely defined.

3.1 Sequelae to Implantation

The subcutaneous implantation of PDS implants was not without complication. Despite careful skin preparation, some rats suffered wound infection with small abscess formation or skin necrosis and subsequent sloughing. These reactions were not limited to any particular treatment group and controls were also affected. Any rat which did not appear to be strong and feeding well, or was losing weight, was destroyed. Chronic respiratory disease was a problem in the experiment involving treatment on day 3 and six rats either died or were destroyed because of the illness. One other rat (day 2, 24h TP) died during the course of the experiment.

3.2 External Genitalia

Table 4.II summarizes the appearances of the external genitalia at slaughter (18 weeks). Data from 7 rats which died during the course of the experiment are not included.

While control animals in all groups showed normal vaginal canalization, all rats treated with TP on days 2 or 3 (but not day 5), regardless of means or duration of treatment, exhibited some vaginal abnormality, ranging from a small contracted opening to a complete absence of the external vaginal orifice.

Although all rats treated with TP on day 2 for 4h had open vaginae, they had small contracted openings. With 8h and 24h treatment periods, some vaginae failed to open at all and the remainder had small orifices. Of the rats injected with TP, half lacked vaginal openings and half had abnormally small openings.

All groups of rats treated with TP on day 3 contained both animals lacking vaginal openings and animals with abnormally small vaginal orifices.

Rats treated on day 5 with TP, either by implant or injection, had normal vaginal morphology.

TABLE 4.II

APPEARANCE OF EXTERNAL GENITALIA AT SLAUGHTER (126 days)

TREATMENT	DAY OF TREATMENT D2				D3				D5			
	n	N	A	NV	n	N	A	NV	n	N	A	NV
4 HOUR IMPLANT												
TP	11	-	11	-	9	1	7	1	10	10	-	-
BLANK	9	9	-	-	9	9	-	-	10	10	-	-
8 HOUR IMPLANT												
TP	7	-	5	2	9	-	6	3	10	10	-	-
BLANK	5	5	-	-	6	6	-	-	10	10	-	-
24 HOUR IMPLANT												
TP	6	-	2	4	10	-	8	2	9	9	-	-
BLANK	6	6	-	-	10	10	-	-	9	9	-	-
48 HOUR IMPLANT												
TP									9	9	-	-
BLANK									10	10	-	-
INJECTION												
TP	11	-	6	5	7	-	6	1	7	7	-	-
OIL	12	12	-	-	6	6	-	-	8	8	-	-

N = normal vaginal orifice
A = abnormal
NV = no vaginal orifice

3.3 Ovarian Function

3.3.1. Age at vaginal opening

The ages at which vaginal opening occurred appear in Table 4.III.

The vaginae of rats treated with TP at 2 or 3 days of age did not form normally and this caused difficulty in accurately identifying the day on which the opening occurred. In many rats vaginal opening did not occur at all, so that few animals contributed to the mean in some groups. Only one group was significantly different from its control ($P<0.05$; day 3 4h TP) and this finding probably has no physiological significance.

Normal vaginal structures were present in rats treated on day 5. Testosterone propionate treatment (all groups combined) caused vaginal opening to occur significantly ($P<0.01$) earlier. When the individual treatment groups were compared with their respective controls, the 4h, 8h and 24h implanted groups showed significantly earlier vaginal opening ages, while the 48h implanted and TP-injected groups did not differ significantly from controls.

3.3.2. Vaginal smears

Records of vaginal cytology are incomplete for groups treated with TP on day 2 and day 3 of life where some vaginae failed to open.

The results of vaginal smearing are presented in Table 4.IV. Three patterns were evident:

- (i) normal cyclic changes;
- (ii) predominantly cornified smears classified as 'persistent-oestrous';
- (iii) a pattern of vaginal cell change which was neither clearly cyclic, nor consistently cornified, classified as indeterminate.

TABLE 4.III

AGE (days) AT VAGINAL OPENING

TREATMENT		DAY OF TREATMENT D2			D3			D5		
		n	Mean	SE	n	Mean	SE	n	Mean	SE
4 HOUR IMPLANT	TP	'9	37.6	1.0	'6	40.6 **°	0.9	17	35.0 *°	0.8
	BLANK	9	38.1	0.8	9	37.4	0.6	10	38.4	1.3
8 HOUR IMPLANT	TP	'5	37.0	1.0	'3	41.3	1.7	10	36.2 **°	0.9
	BLANK	5	40.0	0.8	6	40.7	0.7	12	40.0	1.2
24 HOUR IMPLANT	TP	'2	36.0	0.0	'4	39.3	0.5	9	33.7 *°	0.8
	BLANK	6	39.5	1.1	10	38.0	0.6	9	37.3	1.3
48 HOUR IMPLANT	TP							9	36.7	1.0
	BLANK							15	37.4	0.8
INJECTION	TP	'6	38.5	0.9	'6	38.5	1.1	7	34.9	0.6
	OIL	12	37.8	0.6	6	37.2	1.0	8	37.1	1.0
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS "F"			1.236ns			3.581**			3.364**	

°Significance of difference between TP mean and control mean (t-test)

'Excludes rats where the vagina did not have a normal external orifice.

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE 4.IV

VAGINAL CYCLICITY AS DETERMINED BY CYTOLOGICAL STUDY OF VAGINAL SMEARS FOR A TEN DAY PERIOD (D63-73).

TREATMENT	DAY OF TREATMENT D2				D3				D5			
	n	N	C	I	n	N	C	I	n	N	C	I
4 HOUR IMPLANT												
TP	11	1	8	2	10	6	3	1	10	6	3	1
BLANK	9	7	-	2	9	9	-	-	10	10	-	-
8 HOUR IMPLANT												
TP	5	1	2	2	7	-	6	1	10	7	2	1
BLANK	5	5	-	-	6	6	-	-	10	10	-	-
24 HOUR IMPLANT												
TP	2	-	1	1	8	-	8	-	9	3	4	2
BLANK	6	6	-	-	10	10	-	-	9	9	-	-
48 HOUR IMPLANT												
TP									9	7	2	-
BLANK									10	10	-	-
INJECTION												
TP	6	1	5	-	6	-	6	-	7	-	7	-
OIL	12	12	-	-	6	6	-	-	8	8	-	-

N = normal, showing 4 day cyclicity
C = cornified, a persistent oestrous smear
I = indeterminate

With two exceptions (both day 2 rats with an indeterminate pattern) control animals were all cycling normally. All rats injected with TP had persistently cornified vaginal smears except for one day 2 animal. Animals implanted with TP on days 2 or 3 generally had persistent-oestrous or indeterminate patterns. A high proportion of rats implanted with TP on day 5 showed normal cycles with few animals classified persistent-oestrous.

3.3.3. Corpora lutea

The incidence of corpora lutea in the ovaries of 90 day old rats is shown in Table 4.V.

Corpora lutea were present in all control rats from all three age groups. All TP treated groups contained some animals whose ovaries lacked corpora lutea. The incidence of anovulatory ovaries was affected by duration and age of exposure to TP. Apart from one exceptional animal treated on day 2, TP injected rats all lacked corpora lutea. In general, TP implants left in place for a longer period were more effective in preventing corpora lutea formation than were short term implants. Treatment with TP implants on day 5 caused fewer anovulatory ovaries than did treatment on days 2 or 3.

3.3.4. Ovarian weights

The weights of the ovaries removed at 90 days are presented in Figure 4.2 and Appendix Table XIV. The measurements for two rats, one given an 8h TP implant on day 3, and the other a 24h TP implant on day 2, are missing.

The ovarian weights reflected the presence or absence of corpora lutea.

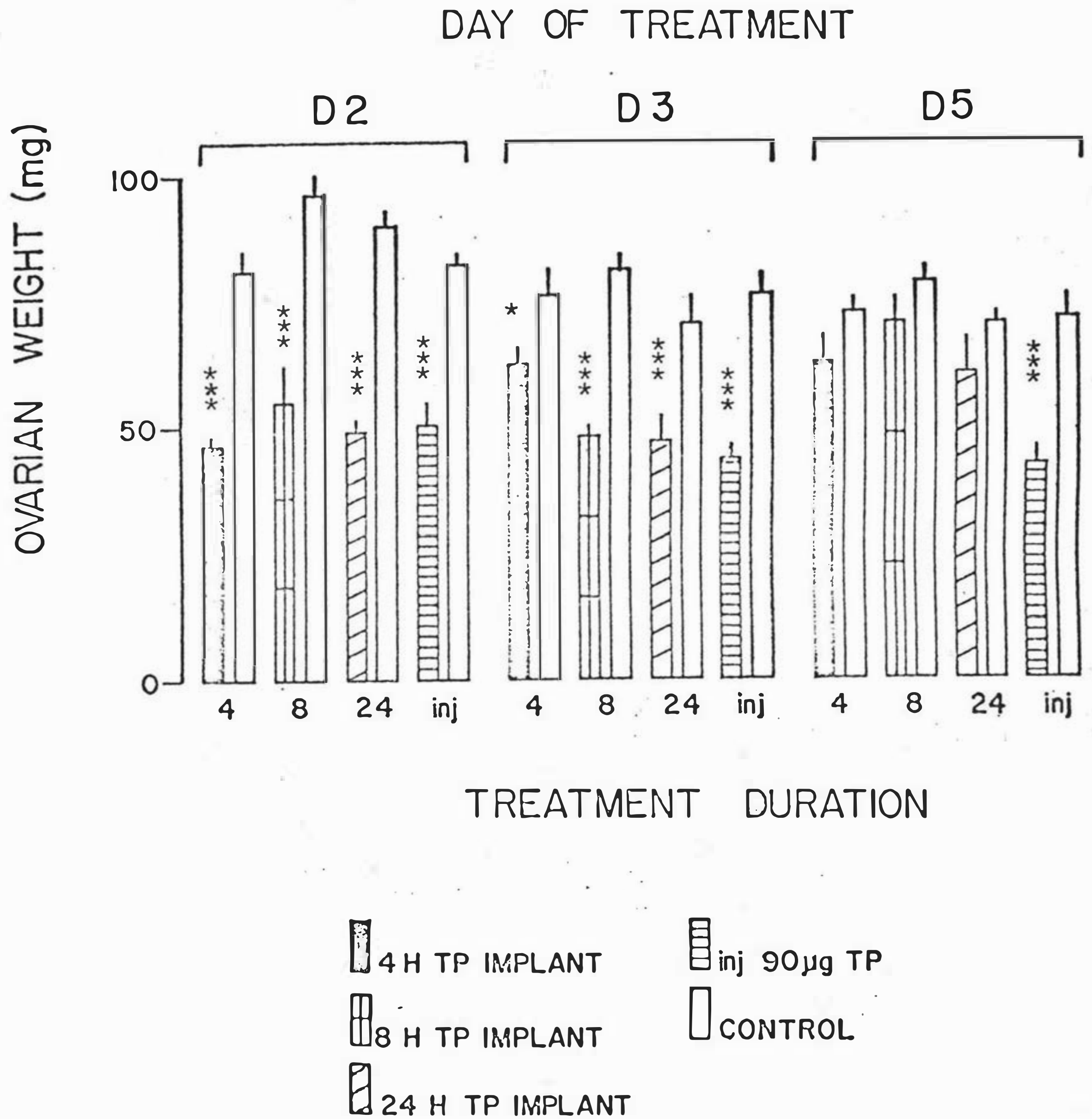
All groups of rats treated with TP on day 2, whether by injection or implant, had ovaries which were significantly ($P<0.001$) lighter than those of their respective control groups. There was no significant effect of mode of administration of TP.

TABLE 4.V

PRESENCE OF CORPORA LUTEA AT 90 DAYS

TREATMENT	DAY OF TREATMENT D2			D3			D5		
	n	P	A	n	P	A	n	P	A
4 HOUR IMPLANT									
TP	11	3	8	11	6	5	10	8	2
BLANK	9	9	–	9	9	–	10	10	–
8 HOUR IMPLANT									
TP	7	2	5	10	–	10	10	8	2
BLANK	5	5	–	7	7	–	10	10	–
24 HOUR IMPLANT									
TP	7	–	7	10	–	10	9	5	4
BLANK	6	6	–	10	10	–	9	9	–
48 HOUR IMPLANT									
TP							9	5	4
BLANK							10	10	–
INJECTION									
TP	11	1	10	8	–	8	7	–	7
OIL	12	12	–	7	7	–	8	8	–

P = corpora present
A = corpora absent



*** P< 0.001; ** 0.001< P <0.01; * 0.01< P< 0.05

Figure 4.2 OVARIAN WEIGHT AT 90 DAYS: MEAN AND SE FOR EACH GROUP AND SIGNIFICANT DIFFERENCES BETWEEN TREATED AND CONTROL GROUPS

Rats treated with TP on day 3 also had ovaries which were significantly lighter than their controls'. This effect was most marked ($P<0.001$) in the case of groups treated for 8h, 24h or by injection. The ovaries of injected rats weighed significantly ($P<0.05$) less than did those of rats receiving TP implants.

When the ovarian weights of rats implanted with TP on day 5 were compared with those of control animals, only the injected group was significantly ($P<0.001$) different. The ovarian weights of the injected group were significantly less ($P<0.001$) than those of implanted rats.

3.3.5. Incidence of sterility

The classification of a rat as anovulatory was based on macroscopic and histological examinations of the ovaries at 90 days of age. Rats whose ovaries lacked corpora lutea were classified as sterile. The occurrence of persistent-oestrous vaginal smears from anovulatory rats provided further confirmation of non-cyclic ovarian activity. Rats with indeterminate vaginal smears were variable in their ovarian luteinization. A summary of the incidence of sterile rats is given in Table 4.VI. All control rats showed normal cyclic ovarian activity and ovulation.

(a) Day 2 treatment

In all TP-treated groups more than 50% of the rats were sterile. For those rats allowing vaginal smears, the vaginal epithelium confirmed the state of the ovaries.

(b) Day 3 treatment

Roughly half of the rats receiving TP implants for 4h, and all rats in the remaining TP groups (8h, 24h and injected) were judged to be anovulatory. These results confirmed by the persistent-oestrous pattern of vaginal smears from those rats with a patent vaginal orifice. Rats with corpora lutea showed cyclic vaginal epithelial changes.

TABLE 4.VI

INCIDENCE OF STERILITY AT 90 DAYS

TREATMENT		DAY OF TREATMENT D2		D3		D5	
		n	S%	n	S%	n	S%
4 HOUR IMPLANT							
	TP	11	73	11	45	10	20
	BLANK	9	0	9	0	10	0
8 HOUR IMPLANT							
	TP	7	71	10	100	10	20
	BLANK	5	0	7	0	10	0
24 HOUR IMPLANT							
	TP	7	86	10	100	9	44
	BLANK	6	0	10	0	9	0
48 HOUR IMPLANT							
	TP					9	44
	BLANK					10	0
INJECTION							
	TP	11	91	8	100	7	100
	OIL	12	0	7	0	8	0

S% = percentage of n animals classified as sterile

(c) Day 5 treatment

Less than 50% of the rats receiving TP treatment by implantation were sterile. All rats injected with TP lacked corpora lutea.

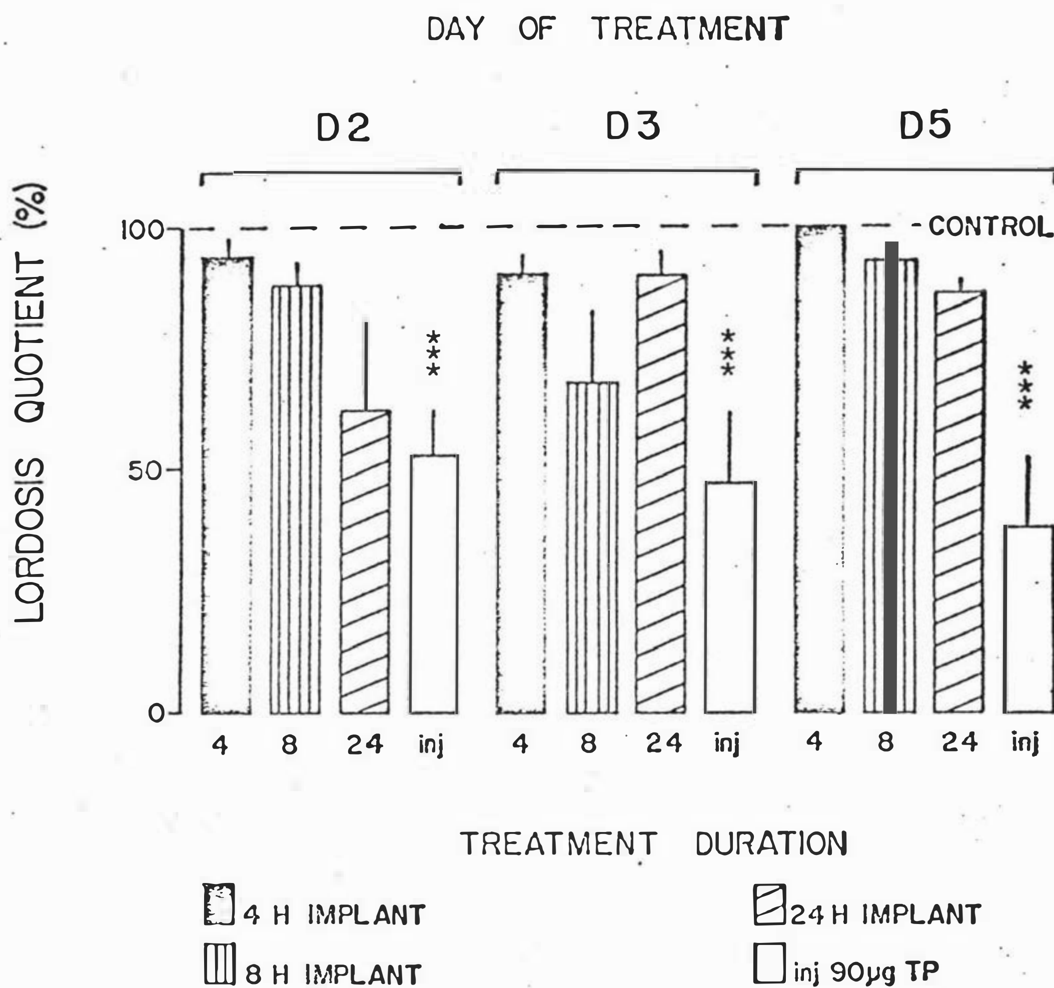
Two discrepancies between ovarian and vaginal observations were seen in this group. One rat (4h TP implant) judged ovulatory on the basis of its ovarian histology had a persistent-oestrous vaginal smear. No satisfactory explanation can be offered for this discrepancy and the rat was presumed to be cycling. Within the 48h TP implant group two rats showed normal vaginal cycles (at 63-73 days) but lacked corpora lutea (at 90 days). These rats are assumed to have experienced a delayed anovulation syndrome.

3.4 Sexual Behaviour

Analyses of these results were complicated by the lack of variance seen in the control groups (in most cases all control animals responded to each mount with lordosis) and the large variance in TP-treated groups, in many of which some animals showed 100% LQ while others showed somewhat less. For this reason, 't' tests comparing each TP-treated group with its control group were applied, to provide a strict test of differences. Results are presented in Figure 4.3 and Appendix Table XV. Data are missing for three rats treated for 4h on day 2, and for the seven which died during the course of the experiment. As already noted (Section 2.4, Chapter 4), results for rats treated on day 5 are incomplete.

Although all TP-treated groups, except the day 5 24h TP implant group, contained some rats which showed a LQ less than 100%, only the TP-injected rats (all 3 ages) were significantly ($P<0.05$) different from their corresponding controls.

Examination of the receptivity scores summarized in Table 4.VII shows that control animals, as expected by definition of the scoring procedure, were normal in their display of oestrous behaviour patterns. All 4h TP treatment groups were also essentially normal, while treatment for 8 or 24h on any of the days, resulted in slightly reduced female behaviour. Rats injected with TP on any one of the three days showed the most marked behavioural deficits.



*** P < 0.001; ** 0.001 < P < 0.01; * 0.01 < P < 0.05

Figure 4.3 SEXUAL BEHAVIOUR (LQ): MEAN AND SE FOR EACH GROUP AND SIGNIFICANT DIFFERENCES BETWEEN TREATED AND CONTROL GROUPS

TABLE 4.VII

RECEPTIVITY SCORES (1-5) FOR SEXUAL BEHAVIOUR

TREATMENT	DAY OF TREATMENT D2 n	Median	Range	D3 n	Median	Range	D5 n	Median	Range
4 HOUR IMPLANT									
TP	11	2	2-3	9	1	1-4	6	1	---
BLANK	7	1	---	9	1	---	4	1	---
8 HOUR IMPLANT									
TP	7	2	1-3	9	3	2-4	8	2	1-3
BLANK	5	1	---	6	1	1-2	6	1	---
24 HOUR IMPLANT									
TP	7	2	1-5	10	2	1-4	7	2	1-3
BLANK	6	1	---	10	1	---	4	1	---
48 HOUR IMPLANT									
TP							4	1	1-3
BLANK							4	1	---
INJECTION									
TP	11	4	2-5	7	4	1-5	4	4	---
OIL	12	1	---	6	1	---	3	1	---

Scoring of receptivity is defined in Section 2.4

3.5 Growth

A summary of the mean body weights ($\log_{10}$) for each group is given in Appendix Tables XVI, XVII and XVIII. The regression data are summarized in Appendix Tables XIX, XX, XXI and XXII.

3.5.1. Treatment on day 2

One-way analysis of variance of the group means of weekly weighings revealed some significant 'F' ratios. However, *a priori* comparisons carried out by 't' tests applied to the weighted means showed that the TP-treated groups were not different from their respective control groups until 12 weeks of age, from which time only the TP-injected group was significantly ($P<0.05$) heavier than its control at all subsequent weighings.

Analysis of variance of the regression coefficients obtained from regression analysis of the body weights over weeks 3-12 showed there to be no significant differences for the different groups (Table 4.VIII). Figure 4.4, showing the regression coefficients and 95% confidence limits for each group, illustrates this. Similarly, for the periods after ovariectomy (weeks 13-15) and during OB treatment (weeks 16-18), the regression coefficients were not significantly different.

3.5.2. Treatment on day 3

While there were significant differences between groups at the first 6 weekly weighings on a one-way analysis of variance, the *a priori* comparisons lacked a consistent pattern. Testosterone propionate-treated groups did not differ significantly from their individual control groups during this time period.

Regression coefficients obtained from regression analysis of the body weights recorded during weeks 3-12 were subjected to analysis of variance and no significant differences disclosed (see Table 4.VIII and Fig. 4.5). One-way analysis of variance for the period following ovariectomy and the period of OB treatment yielded 'F' values which were not significant, so that regression analysis was not carried out for these periods.

TABLE 4.VIII

GROWTH OF BODY WEIGHT FROM 3 TO 12 WEEKS

THE SLOPE OF THE REGRESSION OF Log(base 10) BW ON 1/AGE

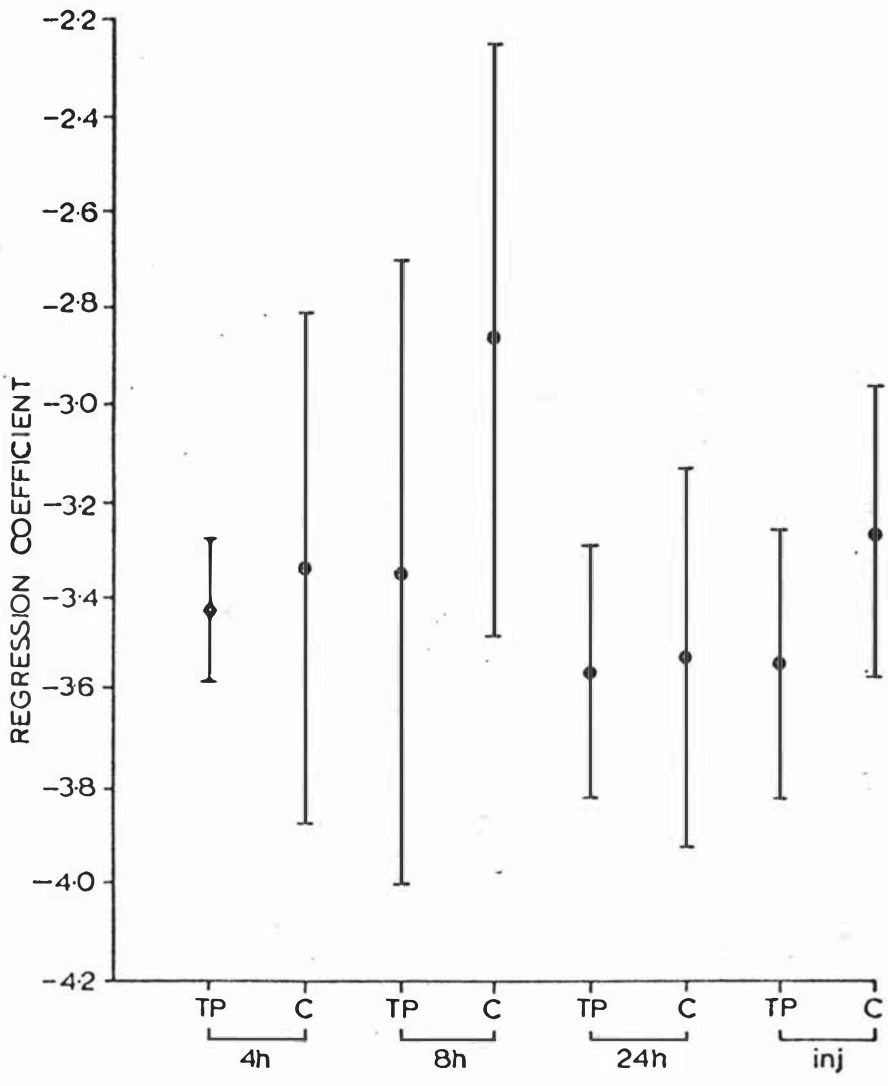
ANALYSIS OF VARIANCE OF THE DIFFERENCES BETWEEN REGRESSIONS FOLLOWING TREATMENT ON DAY 2

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO
Between subclasses	7	0.018310	1.21ns
Within subclasses	64	0.015181	

ANALYSIS OF VARIANCE OF THE DIFFERENCES BETWEEN REGRESSIONS FOLLOWING TREATMENT ON DAY 3

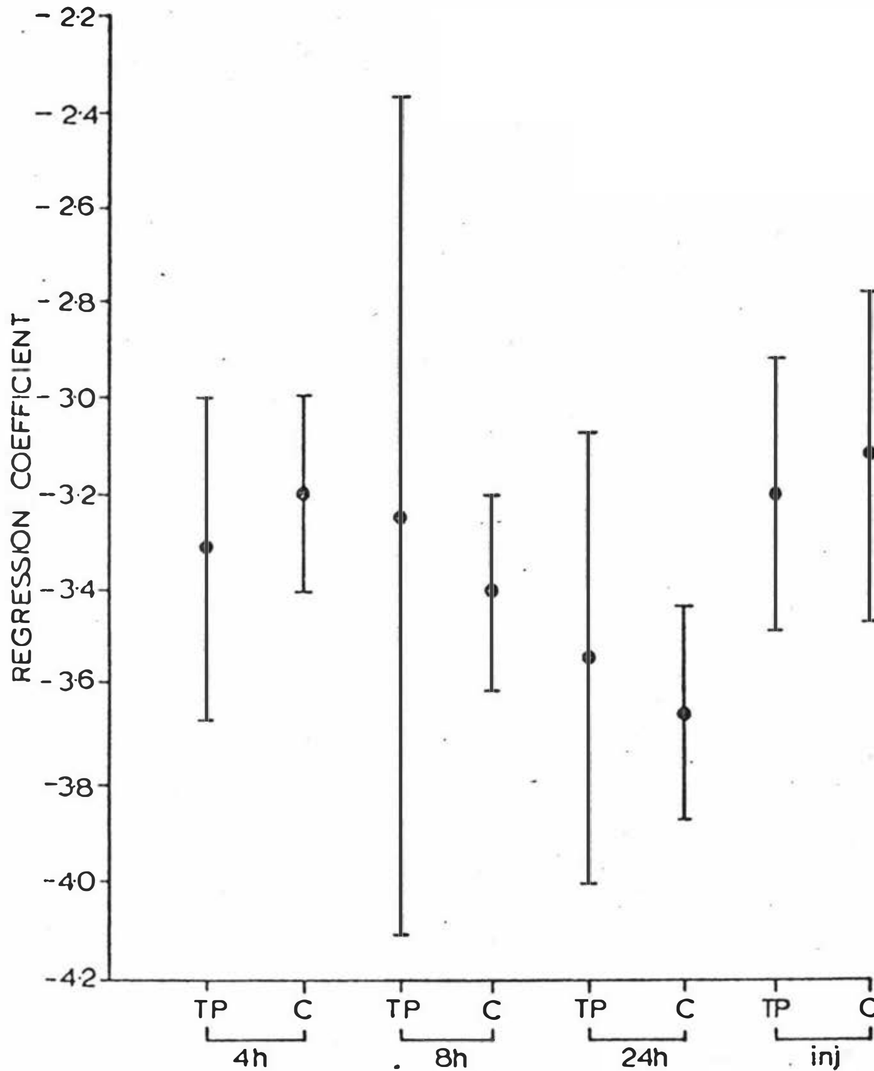
SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO
Between subclasses	7	0.017421	0.95ns
Within subclasses	64	0.018276	

ns $P > 0.05$



Treatment by 2.5mm capsule filled with TP or empty (C), or by injection of 90µg TP or of oil (C)

Figure 4.4 BODY WEIGHT GROWTH WEEK 3 TO 12 FOR RATS TREATED ON DAY 2
95% CONFIDENCE LIMITS FOR LINEAR REGRESSION COEFFICIENTS
(X = 1/AGE, Y = log(base 10) BW)



Treatment by 2.5mm capsule filled with TP or empty (C), or by injection of 90μg TP or of oil (C)

Figure 4.5 BODY WEIGHT GROWTH WEEK 3 TO 12 FOR RATS TREATED ON DAY 3
95% CONFIDENCE LIMITS FOR LINEAR REGRESSION COEFFICIENTS
(X = 1/AGE, Y = log(base 10) BW)

3.5.3. Treatment on day 5

A one-way analysis of variance of weekly weighings of the rats treated on day 5 showed there to be significant differences at all weighings except those at 3, 7 and 8 weeks. By 7 weeks, all TP-treated group means were heavier than their corresponding control groups but further investigation by 't' testing of weighted means established that TP-treated groups did not differ significantly from their respective control groups until 14 weeks. At 14 weeks both the 24h TP-treated group and the TP injected group were significantly ($P<0.05$) heavier than their controls and continued so to 17 weeks, after which the injected group alone was significantly ($P<0.05$) heavier than its control for the two subsequent weighings.

Regression analysis was not conducted for this experiment since weekly one-way analyses of variance were not consistently significant.

4. DISCUSSION

This experiment examined two aspects of the 'critical period' concept of neonatal hypothalamic androgen sensitivity - the time of maximum sensitivity to androgen and the minimum exposure to androgen required. The effects of brief exposure to TP neonatally on the external genitalia are also discussed.

4.1 Age of Maximum Sensitivity to Androgen

The age at which the hypothalamic control of reproductive function is most sensitive to alteration by androgen has been studied by administration of various dosages of TP at varying neonatal ages. Experiments which involve a single injection of esterified testosterone in oil do not allow fine definition of the sensitive period since, as the results presented in Chapter 2 (Section 3.2.1.) show, plasma testosterone remains elevated for several days following injection in the neonatal rat. The present experiment avoided this problem by removing the hormone source, and earlier radioimmunoassay of plasma had defined the duration of

testosterone elevation (Chapter 2, Section 3.2.1.). Low (threshold) doses of androgen given at different ages may demonstrate that one age is more susceptible to alteration than others.

Since numerous studies have suggested that ovulatory and behavioural control mechanisms and growth can be differentiated independently (Barraclough and Gorski, 1962; Swanson and van der Werff ten Bosch, 1964; Mullins and Levine, 1968; Clemens *et al.*, 1969; Luttge and Whalen, 1970; McDonald and Doughty, 1973/74; Tarttelin *et al.*, 1975; Christensen and Gorski, 1978; Gorski *et al.*, 1979), each of these hypothalamic functions is discussed separately.

4.1.1. Control of gonadotrophin secretion

All durations of TP treatment given by PDS capsules caused higher incidences of sterility when given on days 2 or 3 than when given on day 5. Injection of TP was equally effective regardless of the day of treatment. The hypothalamic control of gonadotrophin secretion therefore appeared to be most sensitive to androgen on the second and third day of life.

A single subcutaneous injection of TP can cause anovulatory sterility when injected on any one of the first 5 days of the life of the female rat (Barraclough, 1961; Gorski and Barraclough, 1963; Swanson and van der Werff ten Bosch, 1964; Gorski, 1968; McDonald and Doughty, 1972). This critical period for induction of anovulatory sterility is related to the biological age of the hypothalamus rather than to the time of birth as was shown in an experiment in which female rats, delivered after a shortened or lengthened period of gestation, were treated with TP neonatally (Butterstein and Freis, 1978).

Studies which compared the effectiveness of TP injection at 2 or 5 days of age (Barraclough, 1961), and 1, 2 or 4 days of age (Swanson and van der Werff ten Bosch, 1964), found no differences in the incidence of sterility. Testosterone propionate implanted into the hypothalamus at 2 or 5 days was equally effective in preventing ovulation (Christensen and Gorski, 1978). However, injection of a small dose of TP on days 1, 2, 3 or 4 (Gorski, 1968), or day 2 (Swanson and van der

Werff ten Bosch, 1964), was more effective than on day 5, even when the dose rate was adjusted to allow for the body weight increase between the ages (Swanson and van der Werff ten Bosch, 1964). Tarttelin and others (1975) found low doses of TP to be more effective on days 2 and 3 than on days 4 and 5.

Taken together with the results of the present experiment, these studies suggest that the control of gonadotrophin secretion by the female rat hypothalamus is maximally sensitive to alteration by androgen during the first 3 or 4 days of life. After this age, larger doses of androgen or longer periods of treatment are required to affect this function.

4.1.2. Sexual behaviour

Testosterone propionate injection on any one of the three neonatal ages caused a significant depression of LQ and clearly deficient displays of oestrous type behaviour. All the TP treatments of shorter duration, while clearly causing some depression of feminine mating behaviour, did not result in LQs which were significantly different from those of their respective control groups. These results suggest that the hypothalamic control of feminine sexual behaviour is equally sensitive to androgen on days 2, 3 and 5 of life in the female rat.

Reduced feminine sexual behaviour in adults after priming with oestrogen and progesterone has been recorded in female rats given a single injection of TP on day 1, 2, 3, 4, 5 or 6 of life (Barraclough and Gorski, 1962; Harris and Levine, 1965; Gerall, 1967; Whalen and Edwards, 1967; Mullins and Levine, 1968; Clemens *et al.*, 1969; Hendricks, 1969; Clemens *et al.*, 1970; Edwards and Thompson, 1970; Pfaff and Zigmond, 1971; McDonald and Doughty, 1972; Tarttelin *et al.*, 1975). The results obtained by Tarttelin and his colleagues (1975) suggest that TP treatment on days 2 or 3 is more effective than on day 5 in inhibiting the adult display of feminine sexual behaviour. Implantation of TP directly into the ventromedial hypothalamus on day 2, but not day 5, caused a slight reduction in lordosis response (Christensen and Gorski, 1978). However, the findings of McDonald and Doughty (1972) and Clemens and co-workers (1969) place the most sensitive period later (days 4-6)

in the neonatal period.

An alteration of the hypothalamic control of sexual behaviour cannot be as precisely identified as alteration of control of gonadotrophin secretion. A tonic rather than a cyclic pattern of gonadotrophin discharge is readily determined by the absence of corpora lutea in the ovary, and threshold treatment is manifest in the later onset of the effect - i.e. by the delayed anovulation syndrome (seen in the present experiment as an absence of corpora lutea at ovariectomy after cyclic vaginal change at an earlier age). Threshold effects on mating behaviour are more difficult to quantify and assessment may vary according to the testing procedures used, environmental conditions and the hormonal priming of the test subject (Clemens *et al.*, 1969). Consequently different studies may not be directly comparable.

4.1.3. Growth

Analysis of variance of the weekly body weight measurements showed that TP injection when given on day 2 or day 5, but (inexplicably) not day 3, resulted in rats which were significantly heavier than oil-injected controls from 12 or 14 weeks onwards. Analysis of the regression of $\log_{10}$ body weight on age (or its reciprocal) was not able to demonstrate any significant differences in growth rates for rats subjected to neonatal TP treatments of varying duration.

Treatment with a single subcutaneous injection of TP on any one of the first 5 days of life has resulted in adult female rats which were significantly heavier than controls (Barraclough, 1961; Swanson and van der Werff ten Bosch, 1963; Harris and Levine, 1965; Beatty *et al.*, 1970; Bell and Zucker, 1971; Slob and van der Werff ten Bosch, 1975; Tarttelin *et al.*, 1975, 1976; Dubuc, 1976). Swanson and van der Werff ten Bosch (1963) remarked that injection on day 2 or day 5 had similar effects on subsequent growth. A study which systematically compared the adult body weights after treatment at different neonatal ages determined that treatment after day 3 at the dosages used was unable to effect changes in body weight (Tarttelin *et al.*, 1975). This does not necessarily contradict those studies which found increased body weight after TP treatment on days 4 or 5, since these used larger doses of TP than the maximum used by Tarttelin and colleagues (Barraclough, 1961;

Swanson and van der Werff ten Bosch, 1963; Harris and Levine, 1965; Bell and Zucker, 1971). Tarttelin and co-workers used a range of doses (10-270μg TP) and concluded that mechanisms controlling growth were sensitive to TP during a critical period in the first 3 days of life.

The results of the present experiment are not directly comparable with those of the previously published reports described above, since the statistical treatment of the body weight data is different. Conclusive statements on the growth of neonatally TP-treated rats compared with controls were in some cases not supported by statistical analysis and in some cases were based on body weight measurements at a single age. Statistical analysis, where used, was carried out on untransformed data. Although Tarttelin and colleagues (1975) scrutinized the frequency distribution of their data and deemed it suitable for analysis of variance, it has been claimed that the distribution of body weight is a lognormal distribution (Bliss, 1967; Clark and Tarttelin, 1978). Plotting the standard deviation against the mean of the untransformed body weight demonstrates a distinct correlation which is removed after the transformation of body weight to logarithms. A basic tenet of statistical inference is that the samples of observations are drawn from a population with the same variance, and for this reason all statistical analyses of body weight in the present experiment were computed using the logarithms of body weight.

Regression analysis of growth data was not used in any of the publications cited above, but was applied to the present experiment, since, with suitable transformations, it provided an accurate description of the increase in body weight with age, and made use of all the body weight measurements. Comparison of slopes of regression lines allowed comparison of the growth rates of different groups of rats.

While not suggesting that significant differences in body weight did not exist in the references cited above, analysis of growth data should be examined critically. The methods used in the present experiment are considered to be statistically sound (Clark and Tarttelin, 1978) and were unable to detect any alteration to growth rates as a result of TP treatment of neonatal female rats. Other studies have also failed to demonstrate stimulation of growth after neonatal TP (Brown-

Grant, 1964; Bradshaw and Critchlow, 1966; Wagner *et al.*, 1966).

The findings of the present experiment have not identified a neonatal age which is more sensitive than others to the organizational effects of TP on growth regulation.

The failure of the present experiment to demonstrate increased growth in rats given TP implants on any one of three days during the neonatal period indicates that control of growth is more resistant to modification by neonatal TP than is the control of gonadotrophin secretion. This was observed also by Tarttelin and others (1975) for their lowest dose of TP (10μg) and for all doses given on days 4 or 5. Although sufficient to induce sterility in more than 50% of the animals, these treatments did not result in consistently significantly heavier body weights. The increased body weight usually resulting from neonatal TP is therefore probably independent of the persistent oestrous condition.

No differences in growth were seen between TP-treated groups and control groups after ovariectomy or during OB treatment.

The hypothesis that decreased ovarian secretion from the small ovaries is responsible for the increased growth of neonatally TP-treated rats seems to have been largely superseded by the view that neonatal androgen has a direct organizational effect on the regulation of growth. Oestrogen has a restraining effect on growth (see, for example, Tarttelin and Gorski, 1973; Wade, 1975), and it has been suggested that the effects of early androgen include a decreased sensitivity to oestrogen (Tarttelin *et al.*, 1975, 1976; Gentry and Wade, 1976b) or to oestrogen and progesterone (Bell and Zucker, 1971). Other authors consider the effect to be independent of oestrogen (Beatty *et al.*, 1970; Dubuc, 1976). The present results confirm neither of these views.

4.2 Length of Exposure to Androgen

Other studies which have examined the minimum exposure necessary for androgenization have looked at only one endpoint - the control of ovarian function. For this reason, most of this section is devoted to discussion of this aspect of neonatal androgen action. The effects of short exposure to TP on sexual behaviour and growth are briefly discussed.

4.2.1. Control of gonadotrophin secretion

Treatment with TP for 4 hours (maximum exposure time 28 hours) during a maximally sensitive period (days 2 and 3 of life) in the present experiment was sufficient to induce anovulatory sterility at 90 days in at least 50% of rats. On the other hand at 5 days of age exposure for 72 hours did not achieve the same level of sterility.

Barraclough (1966), in reviewing the evidence for a critical period of hypothalamic androgen sensitivity, speculated (correctly it now would seem) that "... although the length of time that androgen remains in circulation is unknown, it is reasonable to presume that 24-48 hours of exposure to androgen is all that is necessary to produce sterility."

Since then, several studies have investigated the minimum duration of exposure to testosterone necessary for androgenization of ovarian function, using various methods to manipulate the treatment period. Arai and Gorski (1968a, b) relied on the administration of agents which interfered with the action of TP. In different studies they injected a barbiturate (either phenobarbital or pentobarbital) and an anti-androgen (cyproterone acetate) at varying intervals after injection of an androgenizing dose of TP to 5 day female rats. On the basis of their results they concluded that androgen can exert its effects on the brain in 6-12 hours. A similar interval was suggested by a study in which the antibiotics actinomycin-D and puromycin, injected after TP, interfered with androgenization (Kobayashi and Gorski, 1970).

Alklint and Norgren (1970) reported an experiment in which the presence of androgen in 5 day old female rats, injected with TP in the tail, was terminated by removal of the tail at varying intervals after injection. An exposure time of 72 hours, in 5 day old rats injected with 250μg TP, was needed to alter reproductive function in 50% of animals.

Another study by the same authors (Alklint and Norgren, 1971) compared non-esterified testosterone, known to have a shorter duration of action than TP, and TP in their effectiveness in inducing anovulatory sterility when given to neonatal female rats. A single injection of testosterone on day 5 caused androgenization in 50% of rats (compared with 100% when TP given on day 5), but 100% became sterile when a second dose of testosterone was administered on day 10 as well. On this basis the authors argue that a minimal exposure time greater than a few hours is required to produce androgenization in 5 day old female rats. McDonald and Doughty (1974) reach a similar conclusion from their work in which small doses of androgen injected over several days was more effective in preventing cyclic ovarian function than a single injection.

Hayashi and Gorski (1974), who placed TP coated tubes into the hypothalamus of 3 day old female rats and later removed them, found that exposure for 48 hours was needed to cause sterility in at least 50% of the animals at 100 days. In comparing this with the minimum exposure time estimate by Arai and Gorski (1968a, b) they point out several possible differences in the extent and duration of hypothalamic TP exposure between the different experimental approaches.

These estimates of the minimum time of exposure to TP necessary to cause androgenization of gonadotrophin secretion vary from 6-72 hours. Since the methods used are so different, it is difficult to compare them with the present experiment which has certain advantages over the other techniques. Termination of the hormonal action is specific - simply by removal of the source - so it does not rely on the action of an interfering drug, whose onset, duration and mechanism of action may not be known. The interfering substance may exert hormonal effects of its own. Secondly, the time period during which hormone concentrations are elevated by this technique has been defined by measurement of plasma testosterone concentrations. Thirdly, the hormone is placed in a site

from which uptake and subsequent systemic distribution is comparable to that of the injection used in traditional androgenization studies.

Most closely allied to the present experiment is the one in which rats' tails, containing the injection site, were amputated at different times after injection of TP (Alklint and Norgren, 1970). This was reported in abstract form. The authors made no allowance for persistence of the hormone in the body tissues after removal of the tail, and results reported in Chapter 2 suggest that this may lengthen their exposure time considerably. Moreover, peculiarities of the vasculature of the tail make it hard to relate 'subcutaneous uptake' from the tail to uptake from any other subcutaneous site. The rats used were of an age (5 days) considered to be late in the sensitive period when higher doses of TP are required to induce sterility than at an earlier age (Gorski, 1968).

Experiments which involve the use of agents which interfere with the action of androgen, such as those of Arai and Gorski (1968a, b) make no allowance in their estimations of a minimal exposure time for the time taken for the onset of action of the interfering agent. The authors (1968a) qualify their findings, pointing out that they apply only to androgen-barbiturate interactions in 5 day Sprague-Dawley female rats treated with 30μg TP. Again, as they explain, day 5 is near the end of the androgen-sensitive period so that the minimum duration of exposure may be different from that at an earlier age.

The present experiment, which demonstrated that exposure to testosterone for 28 hours could cause anovulatory sterility in at least 50% of rats, cannot exclude the possibility of a shorter minimum exposure period. However the use of any method involving systemic treatment with TP must anticipate prolonged elevation of blood testosterone concentrations, even after removal of the source of the hormone, and exposure times less than 24 hours would be difficult to achieve. Since the proportion of rats classified as sterile at 90 days was around 50% with 28 hour exposure at 2 or 3 days of age, a shorter exposure might be expected to be even less effective. While this experiment did not specifically look for evidence of the delayed anovulation syndrome, it was recognized in two rats which received 72 hour exposure to TP on day 5, and evidence of it would further support the idea that the

durations of TP exposure given by implant provided threshold treatment.

The exposure times quoted in this experiment define the maximum period during which plasma testosterone concentrations are significantly higher than control levels, according to measurements using a radioimmunoassay with sensitivity of 0.08-0.11ng ml^{-1}. A more sensitive assay system would indicate longer durations. The exposure times include an interval during which high concentrations are maintained (while the implant is in place), followed by a period of rapidly declining levels. In the absence of information regarding the nature of the hormone stimulus to which the sexually differentiating nervous tissues respond, it is not possible to define the duration of 'effective testosterone concentration'.

4.2.2. Sexual behaviour

The brief periods of TP treatment by PDS capsule given at any of the three neonatal ages were insufficient to significantly depress the LQ in adult rats. The scores obtained for most of the groups approached significance, so there appeared to have been some depression of the normal oestrous behavioural response. Since no significant reduction in behavioural response was obtained with even the longer periods of TP implantation (exposure to elevated plasma testosterone for up to 48 hours) on any of the three days, and yet the exposure resulting from TP injection (192 hours) was sufficient to cause marked depression, it must be assumed that the control of mating behaviour requires exposure to elevated plasma testosterone concentration for some period of between 2 and 8 days to be significantly de-feminized.

Results obtained from the present experiment support previous suggestions that feminine sexual behaviour is more resistant than gonadotrophin secretion to modification by neonatal androgen (Barraclough and Gorski, 1962; Nadler, 1968; Luttge and Whalen, 1970; McDonald and Doughty, 1974; Christensen and Gorski, 1978; Gorski *et al.*, 1979). Some treatments which induced anovulatory sterility in the majority of rats (for example, 4 and 8 hour TP implants on day 2) failed to significantly depress the display of feminine sexual behaviour as assessed by LQ in adults after hormonal priming.

4.2.3. Growth

Rats exposed *via* PDS implants to TP for 32 hours and 48 hours on days 3 or 5, or 48 hours on day 2, were heavier than their counterparts which received empty implants, but apart from a few isolated weighings, these differences were not significant. These periods of exposure were apparently insufficient to alter growth patterns in these rats. The longer period of treatment afforded by TP injection produced heavier rats when it was given on days 2 or 5, but unaccountably failed to cause significant weight increases when given on day 3.

The minimum period of exposure to androgen necessary to stimulate growth would appear to be longer than that required to alter control of feminine sexual behaviour or gonadotrophin secretion. An experiment by Sheridan, Zarrow and Denenberg (1973) found that to increase adult body weight, a period of exposure to androgen longer than that necessary to induce anovulatory sterility, was required. A dose of 10μg TP, injected in daily or twice-daily doses over days 1-10, significantly increased adult body weight, whereas a single injection of 10μg TP on day 1 had no effect (but caused a high incidence of sterility).

4.3 External Genitalia

The timing of vaginal opening and the structural development of the vagina are discussed here together. In many animals in this experiment an abnormal vaginal structure obscured identification of the age at vaginal opening so that it is difficult to discuss the time of vaginal opening alone.

The age at vaginal opening in the female rat has traditionally been considered an index of ovarian function, specifically indicating the onset of puberty. In normal female rats the first oestrus and ovulation usually occur 24 hours later (Justo, Colillas and Tramezzani, 1970). However its significance in this regard is doubtful since vaginal opening can occur without being followed by ovulation, in rats with deafferentation of the mediobasal hypothalamus (Ramaley and Gorski, 1967), or in fully androgenized rats (Wagner *et al.*, 1966), and can occur

even in the absence of ovaries (Colombo, 1968; Lobl and Gorski, 1974).

It has been found that the age at vaginal opening is advanced in rats given TP neonatally (Harris, 1964; Harris and Levine, 1965; Wagner *et al.*, 1966; Nadler, 1968; Lobl and Gorski, 1974). This was confirmed in the present experiment for TP given on day 5 for even a brief period. However, TP given on days 2 or 3 altered the vaginal morphology obscuring the time of opening. Tramezzani, Voloschin and Nallar (1963), in their detailed study of the effects of a single injection of TP given at different neonatal ages on subsequent vaginal development, made similar observations.

In their experiment, treatment on day 1 prevented vaginal opening, and on days 2 or 3 caused precocious opening of a very small orifice, visible only with the help of a dissecting microscope. Day 4 treatment resulted in half of the rats having the 'pinpoint' opening typical of day 2 or 3 treatment, and the other half having normal but precocious opening. Testosterone propionate given on days 5 or 10 also caused precocious but structurally normal vaginal opening.

Other workers have also reported an abnormally small vaginal orifice or a failure of the distal vagina to canalize when TP was given on one of the first three days of life (Swanson and van der Werff ten Bosch, 1963, 1964; Alklint and Norgren, 1971; McDonald and Doughty, 1972; Young, Nance and Gorski, 1979). When given on days 4 or 5 vaginal opening was advanced (Tramezzani *et al.*, 1963; Harris and Levine, 1965; Colombo, 1968; Mullins and Levine, 1968; McEwen *et al.*, 1977). No alteration of vaginal structure resulted from TP given on days 4 or 5 (Swanson and van der Werff ten Bosch, 1963, 1964; Alklint and Norgren, 1971).

The effects of TP on the timing of vaginal opening have been attributed to central nervous system or ovarian mediation, or local tissue effects (see below).

Claims for central nervous system involvement in the early vaginal opening seen in androgenized rats have been made on the basis of

experiments in which TP was placed in the hypothalamus (Wagner *et al.*, 1966; Nadler, 1968; Lobl and Gorski, 1974). Wagner and his colleagues (1966) suggested that the mechanisms involved were independent of those controlling pituitary-ovarian cyclicity, while Nadler (1968) considered that the controls of vaginal opening and ovarian function merely have different sensitivities to androgen.

Other workers argue that TP induces early vaginal opening by a local action on the genital tissues (Tramezzani *et al.*, 1963; Zarrow, Naqvi and Denenberg, 1969). This theory is supported by experiments in which dihydrotestosterone appeared to mimic the effects of TP on vaginal opening (McDonald and Doughty, 1972). Since dihydrotestosterone cannot be aromatized it is unable to cause androgenization so its actions are assumed to be peripheral. McDonald and Doughty (1972) suggest that the effects of early TP on the vagina are due to its conversion to dihydrotestosterone.

It has been suggested that opening of the vaginal membrane is an indication of the onset of higher levels of ovarian secretion (Ramaley and Gorski, 1967) so it is possible that TP induces early vaginal opening through the elevation of plasma oestrogen seen early in life in androgenized rats (Cheng and Johnson, 1973/74). Certainly administration of OB to immature female rats has been shown to advance vaginal opening (Ramirez and Sawyer, 1965; Porterfield and Stern, 1974), although in one study the time of opening was the same whether OB treatment began on day 5 or day 26 (Ramirez and Sawyer, 1965). However precocious vaginal opening has also been recorded in TP-treated rats lacking ovaries, although in this case the author (Colombo, 1968) suggested that the dose of TP furnished oestrogenic metabolites which were responsible for the early canalization.

No single explanation seems to account satisfactorily for the early vaginal opening which results from neonatal TP administration. However, regardless of the mechanisms involved, it is clear that the time of vaginal introitus is not a useful index in the study of androgenization.

From the experimental results obtained, it may be concluded that the normal vaginal structure can be altered by TP given on days 2 or 3, but not day 5, of life in the female rat. Even brief elevation of plasma testosterone (28 hours) during this sensitive period was able to interfere with normal development.

5. CONCLUSIONS

The results of the experiments described in this Chapter allow some general comments regarding the temporal aspects of the action of TP in modifying the sexual differentiation of the hypothalamus and the final development of the external vagina in neonatal female rats.

The neuroendocrine control of ovarian function appeared to be most sensitive to TP during days 2 and 3 of life in the neonatal female rat and exposure for as little as 28 hours during this time caused anovulatory sterility at 90 days in more than 50% of the rats. De-feminization of mating behaviour appeared to require a longer duration of treatment (between 48 and 192 hours), and no difference in sensitivity to TP between the three ages was detected.

A relatively long period of exposure to testosterone appeared to be required to stimulate growth, since only when given by injection (on days 2 or 5) were increased body weights recorded. However regression analysis did not demonstrate significantly increased growth rates even in the TP injected rats.

The development of the vaginal orifice was disturbed by even brief exposure to TP on days 2 or 3, but at day 5 was apparently insensitive, as at this age no TP treatment, regardless of duration, altered its structure.

The results of this experiment in general agree with other reported experiments using comparable methods of investigation, although there is no general agreement on the precise period of maximum sensitivity for modification of feminine sexual behaviour, and the androgen-sensitive period for growth has received little attention.

Evidence from this experiment confirms the independence of differentiation of several sexually dimorphic characters. While the female rat androgenized by a conventional dose of TP on day 2 or 3 of life displays a characteristic syndrome, different features of this can be independently manipulated by altering the timing and dosage of androgen treatment.

CHAPTER 5

THE EFFECTS ON FEMALE RATS OF PROLONGED TESTOSTERONE PROPIONATE TREATMENT COMMENCING AT DIFFERENT NEONATAL AGES

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CHAPTER 5

THE EFFECTS ON FEMALE RATS OF PROLONGED TESTOSTERONE PROPIONATE TREATMENT COMMENCING AT DIFFERENT NEONATAL AGES

1. INTRODUCTION

Hypothalamic function in the female rat was modified by brief exposure to androgen during the neonatal period, as described in Chapter 4. Gonadotrophin secretion was clearly abnormal and the display of feminine sexual behaviour was reduced. However, injection of TP was still more effective than the short-term TP implants and produced the classical androgenization syndrome. It was therefore of interest to investigate the effects of prolonged treatment with TP during the neonatal and prepubertal periods by leaving the subcutaneous TP-filled PDS capsules in place. It was expected that the capsules would provide, at least initially, a more constant elevation of testosterone concentration than the single injection of TP, and would avoid the stress and fluctuating hormone levels resulting from repeated injections of TP given, daily or every second day, in previously reported experiments in which prolonged treatment regimens were used (Ward, 1969; Ward and Renz, 1972; McDonald and Doughty, 1974; Pollak and Sachs, 1975; Slob and van der Werff ten Bosch, 1975). From experiments reported in Chapter 2 (Section 3.2.2.), it was estimated that the 2.5mm TP-filled capsules would produce elevation of plasma testosterone concentrations for about 10 days.

The results of experiments in which the duration of androgen action was prolonged by injecting small amounts over a number of days led Sheridan and others (1973) to comment that androgenization is a slow process. They considered that their injection procedure in the female rat provided a better model for the study of sexual differentiation of the brain than single injection of a large amount of androgen, since it more closely paralleled the supposed continuous endogenous androgen secretion of the male. Of interest is their finding that the maintenance

of androgen in the body for a long period, achieved by twice daily injection of TP over 10 days, resulted in increased rat body weight whereas the same dose given in one injection did not.

Measurements of plasma testosterone in the male rat between birth and puberty (about 40 days) show a moderately high concentration at birth, declining levels with possibly a small peak at about 20 days, and rising again from about 35 days to adult levels at 45 days (Resko, Feder and Goy, 1968; Miyachi, Nieschlag and Lipsett, 1973; Podesta and Rivarola, 1974; Dohler and Wuttke, 1975; Forest, 1979). Studies of the rat testis at different ages suggest that testosterone is the major secretory product of the Leydig cells from birth to 10 days, but from then until puberty metabolites of 5α-reduction are produced in greater quantity (Purvis, Clausen and Hansson, 1980 ; Corpechot *et al.*, 1981).

In the present experiment TP-filled capsules were inserted subcutaneously at three different ages (2, 3 or 5 days) and the subsequent growth of the rats was followed by regular weekly weighing. The evidence for androgenization of gonadotrophin secretion and feminine sexual behaviour was examined.

2. MATERIALS AND METHODS

2.1 Animals and Animal Management

Female Sprague-Dawley rats were time-mated and housed, as described in Chapter 3, Section 2.2.1., until the litter was weaned. The litter size was adjusted to 12 where possible. At three weeks the female rats were weaned, identified by ear marking and housed in stainless steel colony cages.

2.2 Experimental Design

Treatment was carried out on day 2, 3 or 5 of life (the day of birth being designated day 1) as shown in Figure 5.1. Table 5.I gives details of the final numbers of animals in each group. Rats whose implants were missing when examined at slaughter were excluded.

2.3 Administration of Testosterone Propionate

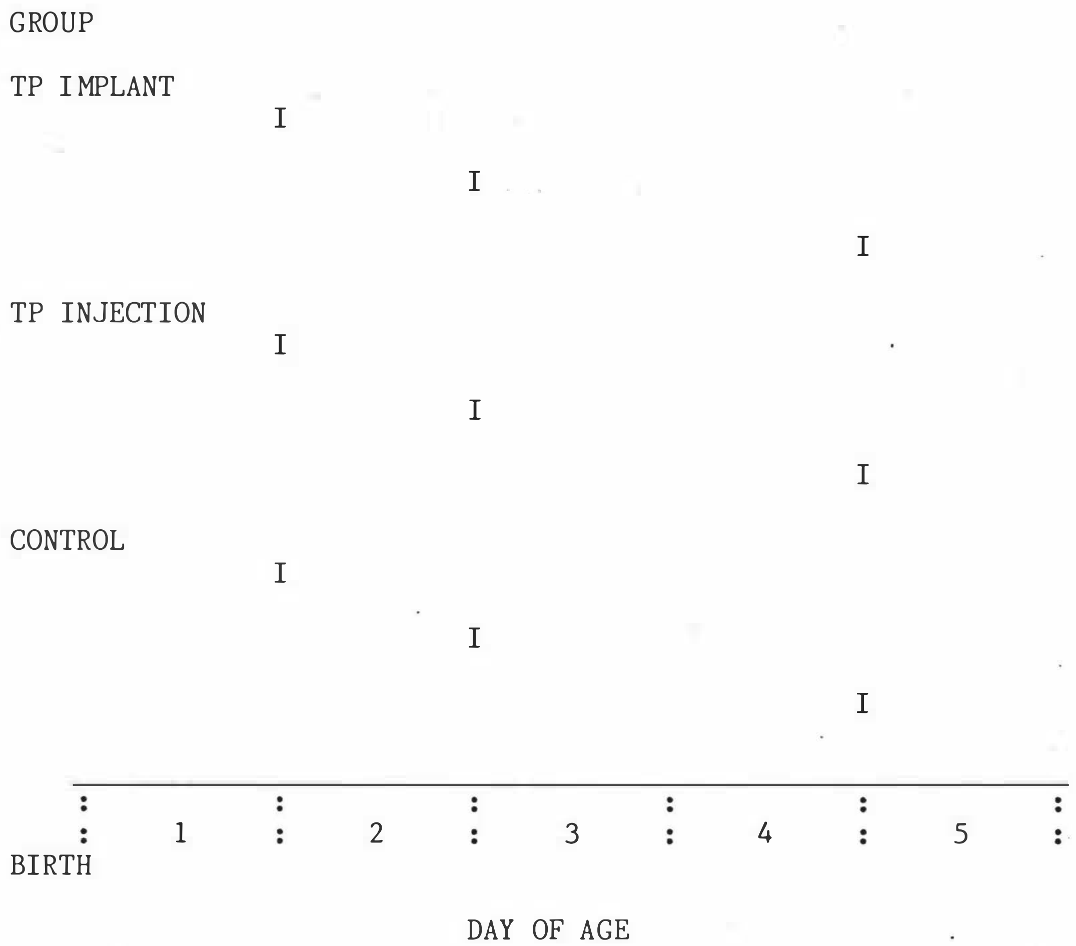
At the assigned age (2, 3 or 5 days) the litter was sexed, female pups weighed and anaesthetized by hypothermia and identified by toe code. Testosterone propionate treatment was either implantation with a 2.5mm TP-filled PDS capsule (described in Chapter 2, Section 2.2.2.) or subcutaneous injection with 90μg TP in oil. Testosterone propionate-implanted rats received an injection of oil, and TP-injected rats received an empty PDS capsule. Control animals were implanted with empty capsules and injected with oil. Thus each rat received both an implant and an injection, which allowed TP-injected rats to constitute a directly comparable group since they were also subjected to the stress of surgery and implantation.

Particular attention was paid to sterility during surgery. Implants were autoclaved and incubated in sterile saline for 48 hours prior to insertion. The skin was cleaned with alcohol before surgery and aseptic conditions were maintained as far as possible. Surgery and recovery from anaesthesia were as described in Chapter 4 (Section 2.2.3.).

2.4 Assessment of Responses

Body weight was recorded weekly from three weeks of age. At weaning (21 days) the anogenital distance was measured.

Daily observation for vaginal opening was begun at weaning and the date recorded. Vaginal smearing was not carried out since many of the TP-treated rats failed to form an external vaginal orifice.



All rats were implanted with 2.5mm PDS capsules (TP filled or empty) and all were injected (TP or oil)

Figure 5.1 EXPERIMENTAL DESIGN: TIME AT WHICH TREATMENT WITH TESTOSTERONE PROPIONATE WAS COMMENCED IN NEONATAL RATS

TABLE 5.I

SUMMARY OF EXPERIMENTAL DESIGN
NUMBERS OF RATS INVESTIGATED

TREATMENT	DAY OF TREATMENT D2	D3	D5
TP IMPLANT[1]	4	6	11
TP INJECTION[2]	7	4	7
CONTROL[3]	11	8	12

[1] 2.5mm TP-filled PDS capsule plus 0.05ml oil injection
[2] 90µg TP in 0.05ml oil plus an empty capsule
[3] 0.05ml oil injection plus an empty capsule

When 14 weeks old, the rats were ovariectomized under ether anaesthesia through bilateral flank incisions. The ovaries were weighed and examined macroscopically (and, following histological processing, microscopically) for corpora lutea. Nose-anal length was recorded while the rats were anaesthetized. Three weeks after ovariectomy (17 weeks) each rat was again anaesthetized with ether to allow subcutaneous placement of a 10mm OB implant. At 19 weeks, mating behaviour tests were conducted (4 hours after subcutaneous injection of 0.5mg progesterone in oil) as described in Chapter 3 (Section 2.4), and receptivity was assessed as described in Chapter 4 (Section 2.4).

At the age of 23 weeks, all rats were killed using chloroform. The degree of development of the vagina was assessed and recorded. The presence of both PDS capsules (TP and OB) was confirmed by palpation or if necessary by dissection.

2.5 Statistical Analyses

The statistical analyses used are described in Chapter 3.

3. RESULTS

3.1 Sequelae to Implantation

Occasional cases of skin necrosis or abscessation about the wound area were encountered. If extensive, or if deemed unhealthy for other reasons, the affected pup was destroyed. At slaughter some implants could not be found and results from these animals were not included. This particularly affected groups given TP implants on day 2 or day 3.

3.2 Ovarian Function

3.2.1. Age at vaginal opening

The mean ages at which the vaginae opened are presented in Table 5.II. Vaginal opening dates are missing for two rats given TP injection on day 5. Since many of the TP-treated animals failed to show vaginal opening, the results were not statistically analysed.

The vaginae of control animals opened normally some time between 31 and 44 days. Implantation with TP-filled capsules on any day resulted in early vaginal opening (before 29 days) or a failure to open at all. Rats injected with TP on days 2 or 3 had no visible vaginal opening, and those injected on day 5 opened at the lower range of control values.

Vaginal smears were not made since many rats lacked a patent vagina.

Measurements of the anogenital distance at weaning (shown in Appendix Table XXIII) gave a significant 'F' ratio for an overall analysis of variance, but no group was significantly different from its control in the *a priori* comparisons.

3.2.2. Corpora lutea

The presence of corpora lutea at 100 days is shown in Table 5.III. Ovaries of all control animals contained corpora lutea. Corpora lutea were absent from the ovaries of all of the TP-injected, and all but one of the TP-implanted rats (day 3), irrespective of the day of treatment.

3.2.3. Ovarian weights

The ovarian weights (presented in Fig. 5.2 and Appendix Table XXIV) reflect the presence or absence of corpora lutea. Ovarian weights are missing for one rat from each of three groups (day 3 TP implant, day 3 control, day 5 control).

TABLE 5.II

AGE (days) AT VAGINAL OPENING

TREATMENT	DAY OF TREATMENT D2 n	Mean	SE	D3 n	Mean	SE	D5 n	Mean	SE
TP IMPLANT	4	25.0	1.3	6	--(4)'		11	22.1	0.8
TP INJECTION	7	--(7)'		4	--(2)'		5	34.8	2.5
CONTROL	11	38.8	0.8	8	35.4	1.6	12	39.3	1.2

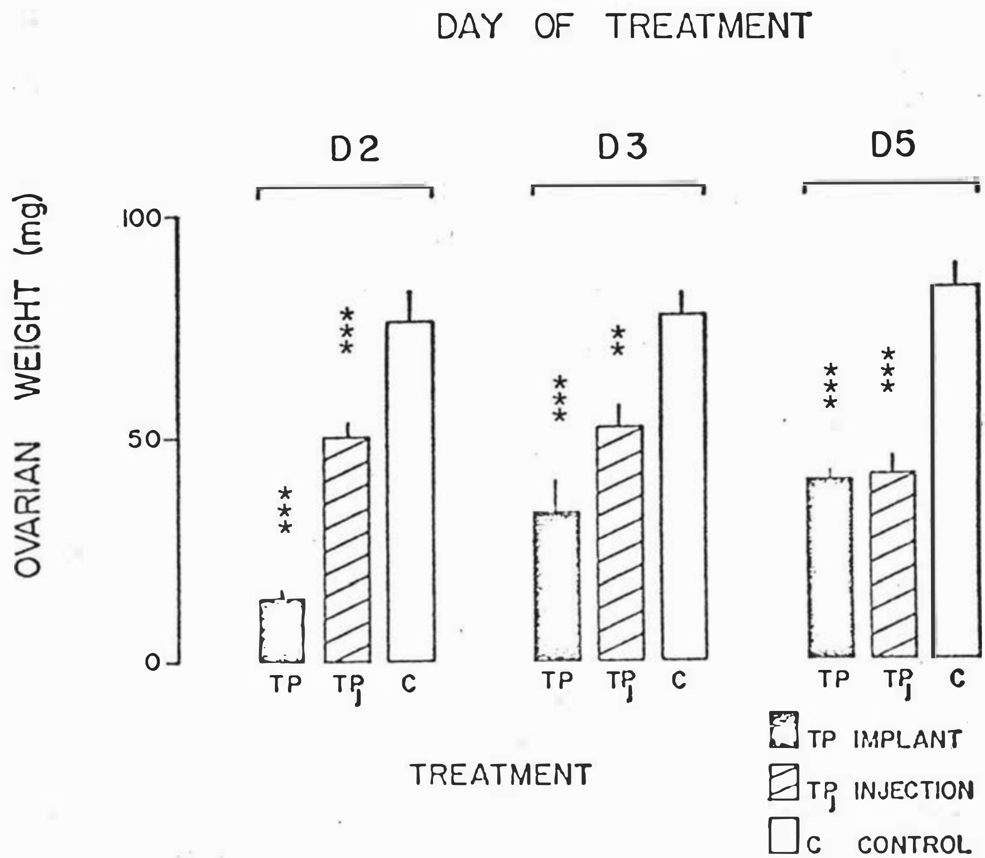
' Number of rats, in parentheses, lacking a normal external vaginal orifice.

TABLE 5.III

PRESENCE OF CORPORA LUTEA AT 100 DAYS

TREATMENT	DAY OF TREATMENT D2 n	P	A	D3 n	P	A	D5 n	P	A
TP IMPLANT	4	0	4	6	1	5	11	0	11
TP INJECTION	7	0	7	4	0	4	7	0	7
CONTROL	11	11	0	8	8	0	12	12	0

P = corpora present
A = corpora absent



*** $P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$

Figure 5.2 OVARIAN WEIGHT AT 100 DAYS: MEAN AND SE FOR EACH GROUP AND SIGNIFICANT DIFFERENCES BETWEEN TREATED AND CONTROL GROUPS

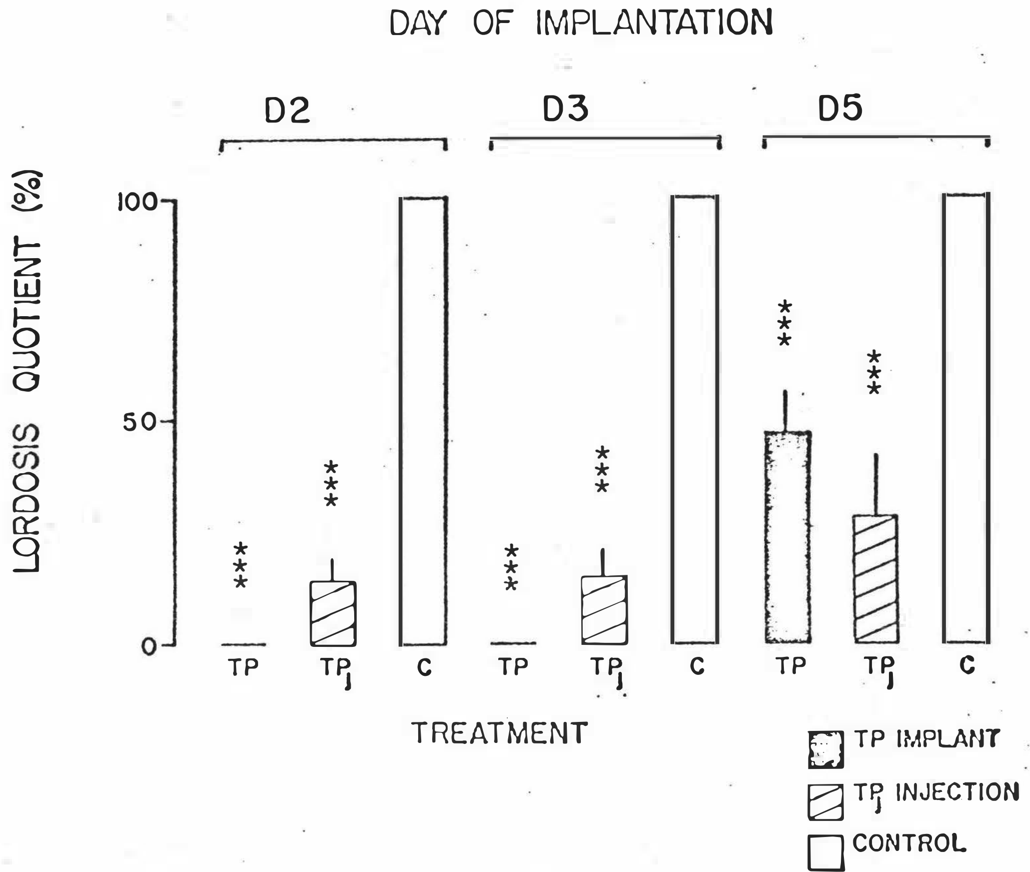
Overall analysis of variance gave a highly significant 'F' ratio ($P<0.001$) and all TP-treated groups had ovaries which weighed significantly ($P<0.001$) less than those of control animals. Insertion of a TP-filled implant on day 2 caused the most profound depression of the ovarian weights, which were significantly ($P<0.01$) lighter than those of rats given TP implants on days 3 or 5.

The incidence of sterility (based on the ovarian findings) for all TP-injected groups was 100% and, with the exception of one rat treated on day 3, was 100% for all rats given TP implants.

3.3 Sexual Behaviour

Results of mating behaviour tests are shown in Figure 5.3 and Appendix Table XXV. Data are missing for four rats, two of which died before mating behaviour testing (one each from day 2 and day 3 TP implant groups) and two from the day 5 TP-injected group. Control animals all responded with 100% LQ. Lordosis quotients were low for injected groups which were all highly significantly ($P<0.001$) lower than their respective control groups. The suppression of mating behaviour was the same no matter at which age the injection was given. Rats given TP implants on days 2 or 3 of life all scored 0 for lordosis testing, in other words they did not respond with a single lordosis when mounted. Obviously these scored very highly significantly lower than their control groups, as did the group given implants on day 5, although the mean score for this group was significantly ($P<0.001$) higher than that for the day 2 and day 3 TP-implanted groups.

The deficits in normal 'oestrous' behaviour are also illustrated in the 'Receptivity Scores' (Table 5.IV). While control animals were all fully receptive (scoring 1) all animals treated with TP by implant or injection scored higher than 1, and many scored 5 (the maximum score reserved for rats which actively resisted mounting by fighting and fending off the male).



*** P < 0.001; ** 0.001 < P < 0.01; * 0.01 < P < 0.05

Figure 5.3 SEXUAL BEHAVIOUR (LQ): MEAN AND SE FOR EACH GROUP AND SIGNIFICANT DIFFERENCES BETWEEN TREATED AND CONTROL GROUPS

TABLE 5.IV

RECEPTIVITY SCORES (1-5) FOR SEXUAL BEHAVIOUR

TREATMENT	DAY OF TREATMENT D2			D3			D5		
	n	Median	Range	n	Median	Range	n	Median	Range
TP IMPLANT	3	4	3-5	5	5	4-5	11	3	2-4
TP INJECTION	7	4	4-5	4	4	---	5	3	3-4
CONTROL	10	1	---	5	1	---	12	1	---

Scoring of receptivity defined in Chapter 4, Section 2.4

3.4 Growth

A summary of the mean body weights ($\log_{10}$) for each group appears in Appendix Table XXVI. The regression data are summarized in Appendix Tables XXVII, XXVIII and XXIX. The regression lines ($\log_{10}$ body weight vs age) for the period 3-14 weeks are illustrated, together with bar charts representing the mean body weights at three selected ages, in Figures 5.4, 5.5 and 5.6. Note that regression analyses were carried out using the reciprocal transformation of age, but illustrations show regression lines based on untransformed age data as the positive slopes make for clearer interpretation.

3.4.1. Weekly body weight measurements

A significant ($P<0.05$) overall 'F' ratio was obtained at all weekly weighings except weeks 4 and 5. No differences were found among the three control groups. When TP-treated groups were compared with their control groups, the rats implanted on day 2 were significantly ($P<0.01$) lighter from 6 weeks onwards. The rats implanted on day 3 were also lighter than their controls but this difference did not reach significance ($P<0.01$) until 15 weeks and then persisted for the remainder of the experiment. In contrast, the group given TP implants on day 5 was consistently heavier than controls, and significantly so ($P<0.05$) for weeks 12-15. By 10 weeks, all TP-injected groups were heavier than corresponding control groups and remained so throughout the experiment, but generally without the difference becoming significant.

Differences in body weight attributable to the age at treatment were also found. Insertion of a TP-filled capsule on day 2 caused a marked depression of body weight, significantly different ($P<0.001$ from 7 weeks onwards) from the groups given the same treatment on days 3 or 5. From 12 weeks, rats given TP implants on day 3 were significantly (P varying <0.05-<0.001) lighter than those implanted on day 5.

There were no significant differences in body weight among groups injected with TP at different ages.

3.4.2. Regression analysis

Regression lines were fitted to the $\log_{10}$ body weight *vs* the reciprocal of age for the period 3-14 weeks. For the periods following ovariectomy (15-17 weeks), and during OB treatment (18-20 weeks), regression lines were fitted to the $\log_{10}$ body weight *vs* age. The regression data are summarized in Appendix Tables XXVII, XXVIII and XXIX and the regression lines for the period 3-14 weeks ($\log_{10}$ body weight *vs* age) are illustrated in Figures 5.4, 5.5 and 5.6. The regression coefficients and their 95% confidence limits for the period 3-14 weeks are illustrated in Figure 5.7.

Analysis of variance of the slopes of the regression lines for the period 3-14 weeks showed there to be significant ($P<0.001$) differences (Table 5.V).

Further investigation showed that for rats treated on day 2 the group given TP implants had a slope significantly ($P<0.001$) less than the control group (Figures 5.4, 5.7). The slope for the injected group did not differ from that for the control group.

For rats treated on day 3, neither TP treated group differed significantly from the control (Figures 5.5, 5.7).

When treatment began at day 5, the TP injected group showed a significantly ($P<0.001$) steeper slope than that of the control group (Figures 5.6, 5.7). Examination of Figure 5.6 shows that the TP injected group was lighter than the control group at 3 weeks and heavier at 14 weeks, so the difference in slope can be explained, at least partly, by 'catch-up' growth (discussed in Clark and Tarttelin, 1978). The slope for the TP implanted group did not differ from that for the control group.

TABLE 5.V

GROWTH OF BODY WEIGHT FROM 3 TO 14 WEEKS

THE SLOPE OF THE REGRESSION OF Log(base 10) BW ON 1/AGE

ANALYSIS OF VARIANCE OF THE DIFFERENCES BETWEEN REGRESSIONS

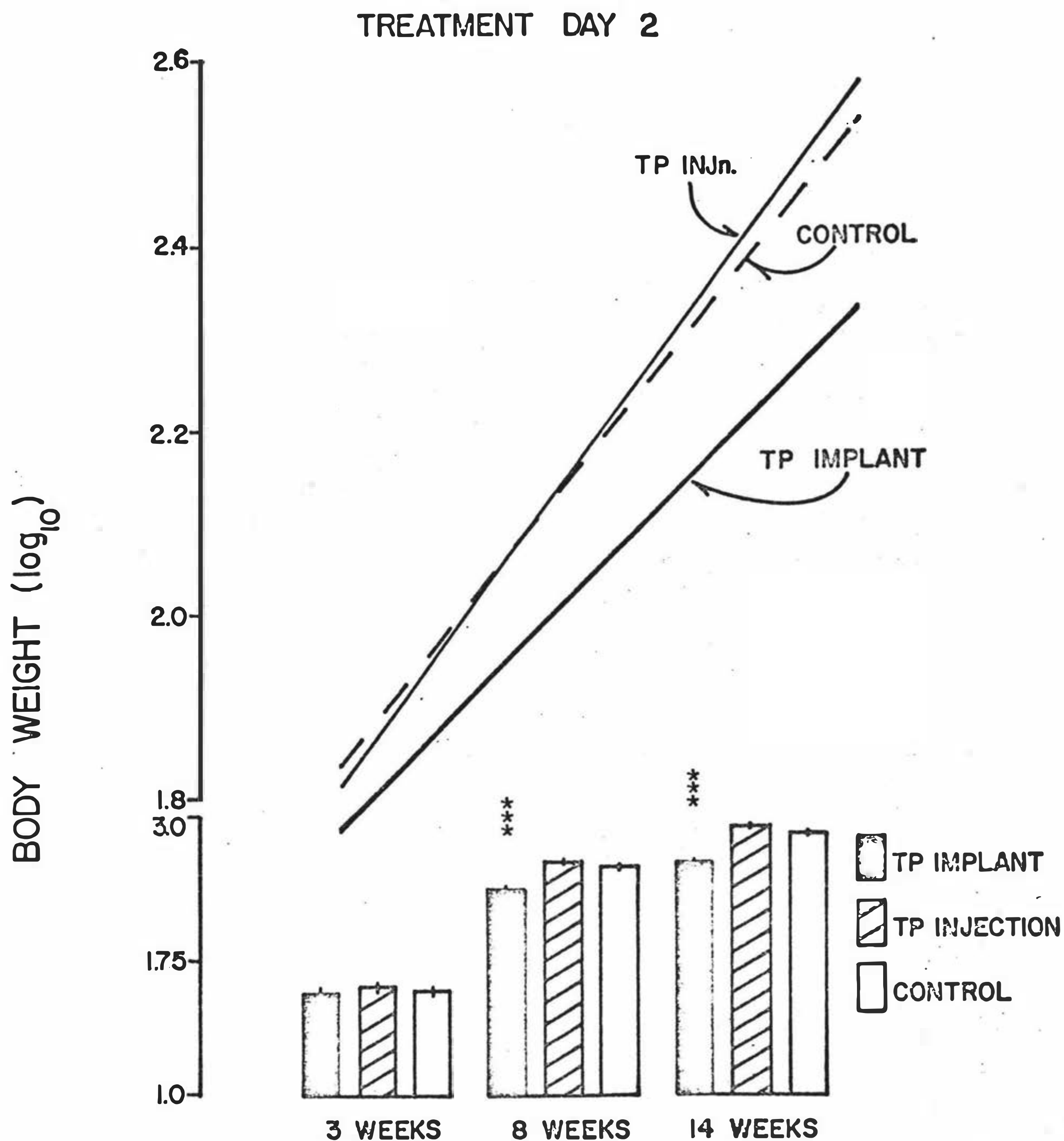
SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO
Between subclasses	8	0.03212	4.78***
Days of treatment°	2	0.00555	0.83ns
Groups within days'	6	0.04098	6.10***
Within subclasses	90	0.00672	

CONTRASTS OF TP GROUPS WITH CONTROL GROUPS WITHIN DAYS (F RATIOS)[2]

	DAY 2	DAY 3	DAY 5
TP IMPLANT vs CONTROL	15.74***	0.07ns	0.99ns
TP INJECTION vs CONTROL	0.11ns	2.19ns	18.95***

° Days 2, 3 and 5 when treatments were applied
' TP implant, TP injection and control groups within each day
[2] *A priori* comparisons, with one degree of freedom, calculated from the squared difference between the regression coefficients and the within subclass mean square weighted by the sum of squares of 1/age for the two regressions (Weight = (SS1 + SS2)/(SS1 x SS2))

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$



*** Significant ($p < 0.001$) difference from the control group

Figure 5.4 BODY WEIGHT GROWTH WEEK 3 TO 14 FOR RATS TREATED ON DAY 2: LINEAR REGRESSIONS, X = AGE, Y = log(base 10) BW AND BAR DIAGRAMS FOR SELECTED AGES

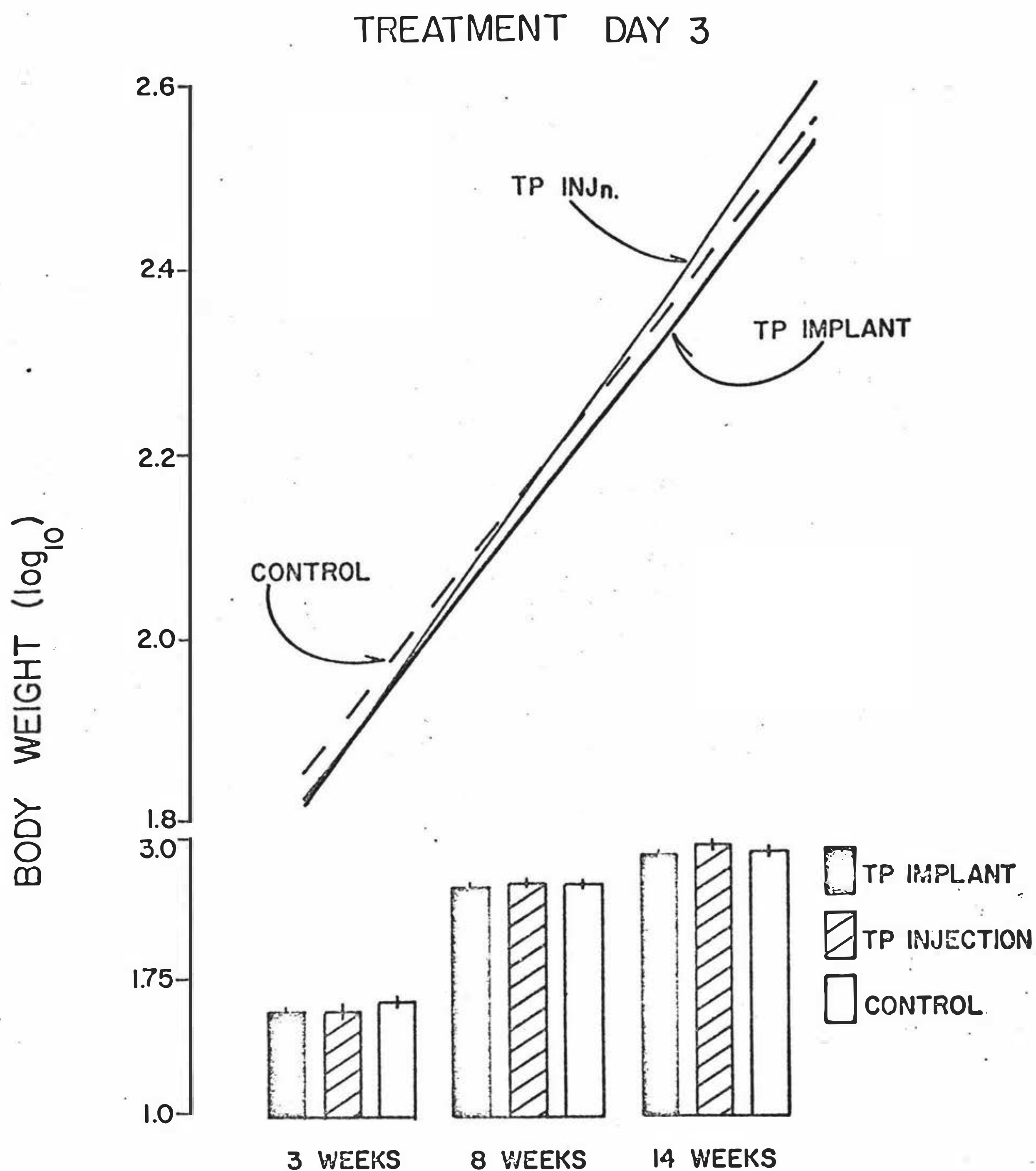
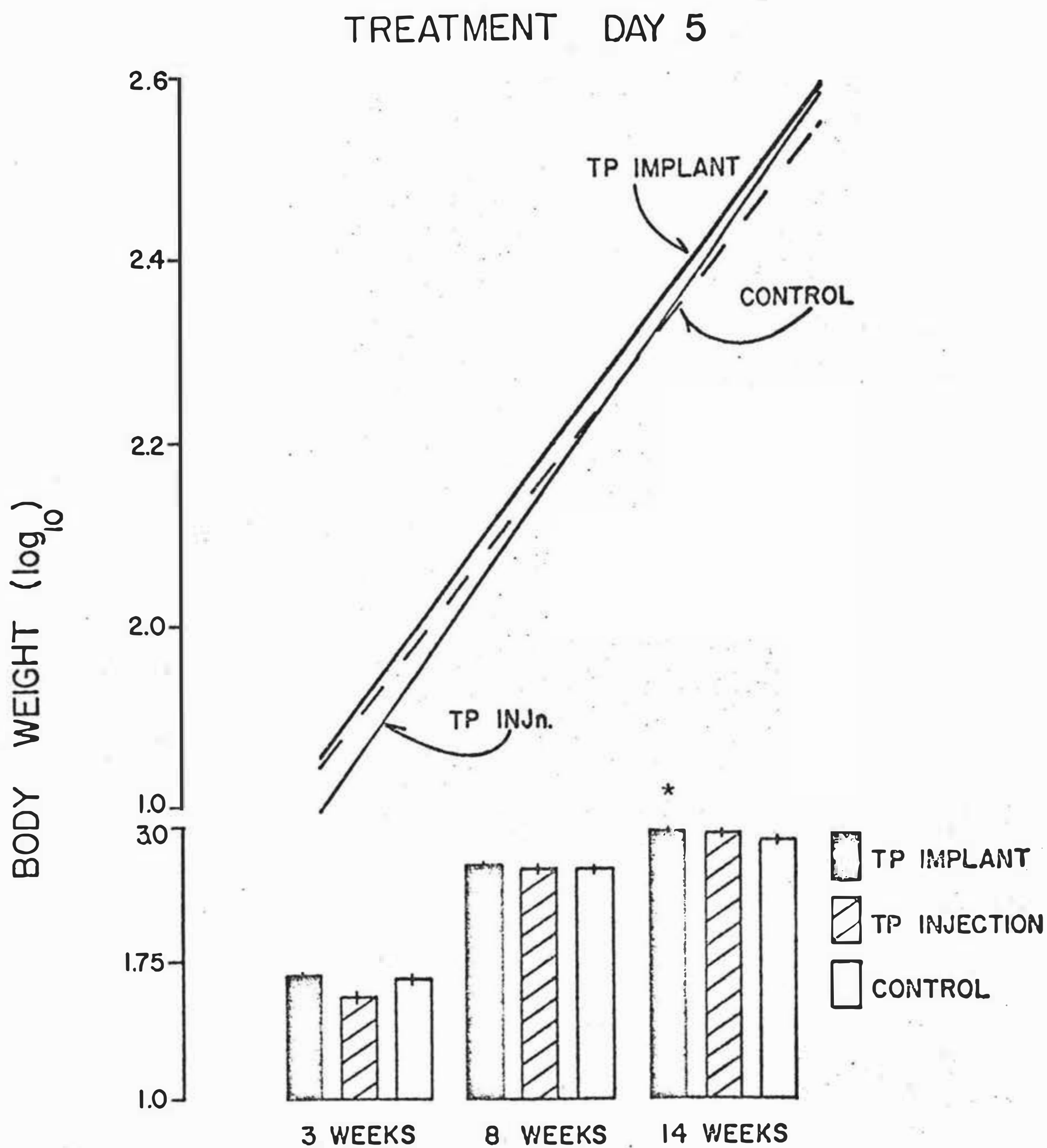
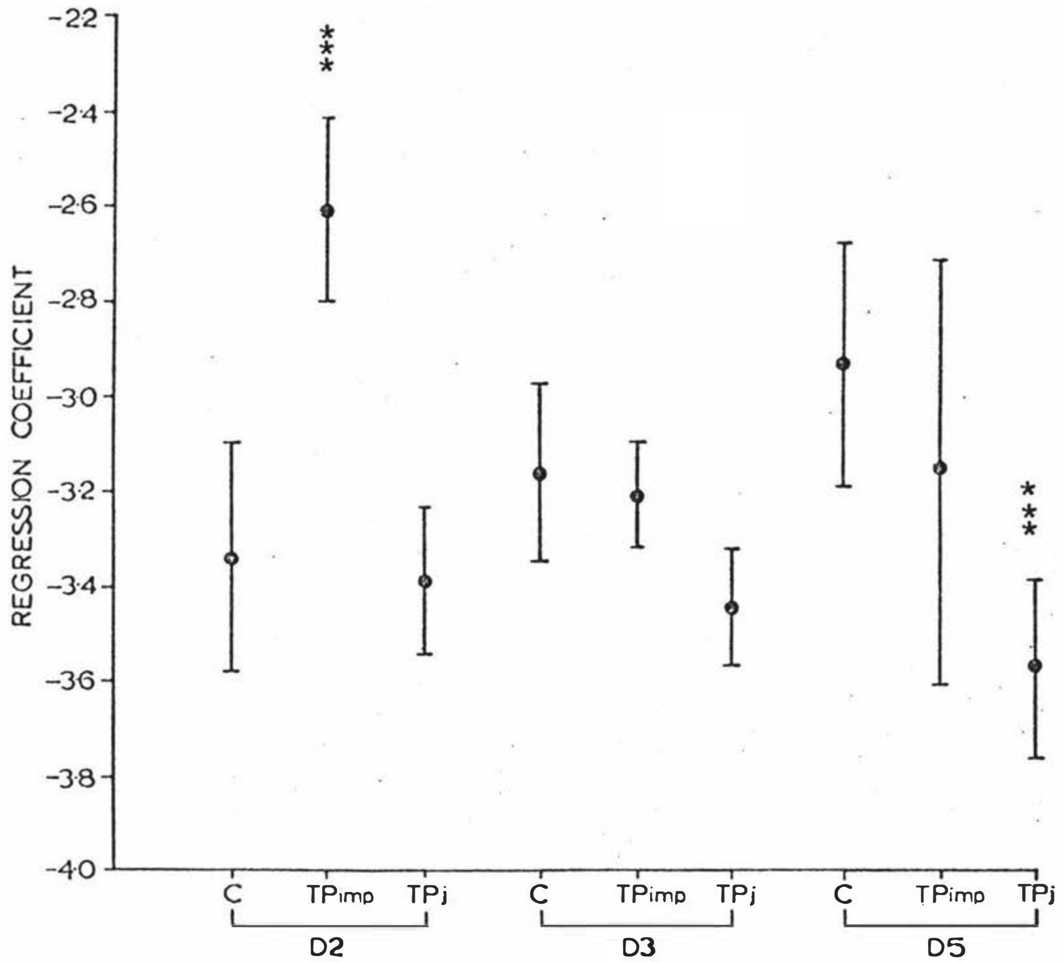


Figure 5.5 BODY WEIGHT GROWTH WEEK 3 TO 14 FOR RATS TREATED ON DAY 3: LINEAR REGRESSIONS, X = AGE, Y = log(base 10) BW AND BAR DIAGRAMS FOR SELECTED AGES



* Significant ($p < 0.05$) difference from the control group

Figure 5.6 BODY WEIGHT GROWTH WEEK 3 TO 14 FOR RATS TREATED ON DAY 5: LINEAR REGRESSIONS, X = AGE, Y = log(base 10) BW AND BAR DIAGRAMS FOR SELECTED AGES



Significant differences between treated and control groups shown thus
*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

Treatment by 2.5mm capsule filled with TP or empty (C),
or by injection of 90µg TP or of oil (C)

Figure 5.7 BODY WEIGHT GROWTH WEEK 3 TO 14 FOR RATS WITH TREATMENT BEGINNING ON DAY 2, 3 OR 5
95% CONFIDENCE LIMITS FOR LINEAR REGRESSION COEFFICIENTS
(X = 1/AGE, Y = log(base 10) BW)

For the two week period following ovariectomy, the slope of the regression line for the group given TP implants on day 2 was significantly ($P<0.05$) less than that of the control group (Table 5.VI, Appendix Table XXVIII). The group given TP implants on day 5 had a significantly ($P<0.01$) smaller slope than its control. All other groups did not differ significantly from their controls.

The regressions of $\log_{10}$ body weight *vs* age for the period of OB treatment were in most cases not significant.

3.4.3. Skeletal growth

The nose-anal length measurements at 100 days are given in Table 5.VII. Data are missing for 15 rats treated at day 5 (9 TP-implanted, 6 controls) and one day 3 control rat.

Insertion of a TP implant on day 2 depressed skeletal growth so that this group was significantly ($P<0.001$) shorter in nose-anal length at 100 days than its control. It was also significantly ($P<0.01$) shorter than the groups given TP implants on days 3 or 5. No other significant differences were recorded in this measurement.

4. DISCUSSION

All three of the parameters of hypothalamic sexual differentiation investigated were seriously disturbed by treatment of neonatal female rats with TP for a prolonged period. That ovulation was prevented and feminine sexual behaviour nearly abolished, was not surprising in view of the evidence presented in Chapter 4, but the effects on growth were unexpected and intriguing.

TABLE 5.VI

GROWTH OF BODY WEIGHT FROM 15 TO 17 WEEKS

THE SLOPE OF THE REGRESSION OF Log(base 10) BW ON AGE

ANALYSIS OF VARIANCE OF THE DIFFERENCES BETWEEN REGRESSIONS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO
Between subclasses	8	0.0008244	3.69*
Days of treatment°	2	0.0000432	0.19ns
Groups within days'	6	0.001048	4.86*
Within subclasses	9	0.0002232	

CONTRASTS OF TP GROUPS WITH CONTROL GROUPS WITHIN DAYS (F RATIOS)[2]

	DAY 2	DAY 3	DAY 5
TP IMPLANT vs CONTROL	7.13*	1.60ns	13.76**
TP INJECTION vs CONTROL	0.05ns	4.47ns	0.0003ns

° Days 2, 3 and 5 when treatments were applied
' TP implant, TP injection and control groups within each day
[2] *A priori* comparisons, with one degree of freedom, calculated from the squared difference between the regression coefficients and the within subclass mean square weighted by the sum of squares of age for the two regressions (Weight = (SS1 + SS2)/(SS1 x SS2))

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE 5.VII

NOSE-ANAL LENGTH (mm) AT 100 DAYS

TREATMENT	DAY OF TREATMENT D2			D3			D5		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
TP IMPLANT	4	194.5 *°	0.6	6	219.5	6.2	2	216.0	19.0
TP INJECTION	7	225.9	1.7	4	229.0	3.9	7	225.1	1.6
CONTROL	11	222.1	1.6	7	225.7	4.6	3	216.3	3.7
SIGNIFICANCE OF DIFERENCES BETWEEN GROUP MEANS "F"		5.40**			0.50ns			0.76ns	

°Significance of difference between TP mean and control mean (t-test)

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

4.1 Effects on Gonadotrophin Secretion and Sexual Behaviour

With one exception, all rats implanted with TP in this experiment were anovulatory. In both this experiment and those described in Chapter 4, TP injected on either day 2, 3 or 5 also caused 100% sterility. Since the implants provided a longer exposure to high concentrations of plasma testosterone than did injection (of 90μg TP), this effect of TP implants on ovarian function was not unexpected. The ovarian weights were consequently depressed in all TP-treated groups. Those rats receiving TP implants on day 2 had ovaries which were significantly smaller than those of rats receiving TP implants on days 3 or 5.

The suppression of adult feminine sexual behaviour, reported after neonatal TP injection in Chapter 4 (Section 3.4), was again seen in this experiment. Long-term TP treatment from subcutaneous capsules inserted on days 2 or 3 appeared to completely abolish feminine sexual behaviour. The same treatment given on day 5, while depressing the LQ, did not have such a severe effect, which may indicate a lesser sensitivity to androgen at this age.

The effects on gonadotrophin secretion and feminine sexual behaviour confirm the findings of other experiments in which the female rat was exposed to androgen for a prolonged period (at least five days) beginning soon after birth (Ward and Renz, 1972; Sheridan *et al.*, 1973; McDonald and Doughty, 1974; Whalen and Rezek, 1974; McEwen *et al.*, 1977). Two reports of no alteration to sexual behavioural response, despite a high incidence of sterility, used daily injections of non-esterified testosterone rather than TP (Luttge and Whalen, 1970; McDonald and Doughty, 173/74). When non-esterified testosterone was given by PDS capsule rather than by injection, feminine sexual behaviour was suppressed (McEwen *et al.*, 1977), suggesting that daily injection of free testosterone does not elevate testosterone concentrations in the rat long enough to modify behavioural control mechanisms.

While the ovaries of rats given TP implants on day 2 were atrophic, and significantly lighter than the anovulatory ovaries of rats given TP implants on days 3 or 5, they all clearly showed development of follicles. In this respect they differed from the ovaries, described

by Arai (1971), of Wistar rats given neonatal treatment with pharmacological doses of oestrogen or androgen. These ovaries resembled those of weanling or hypophysectomized rats, showing no follicular growth nor corpus luteum formation. Vaginal smears from such rats were persistently dioestrous, but lack of an external vaginal orifice in many rats in the present experiment prevented this procedure.

As was the case with the brief TP exposure described in Chapter 4, chronic implantation with TP resulted in either very early vaginal opening or no spontaneous opening. This finding has been reported in other experiments in which androgen administration continued over several days in the neonatal period (Ward, 1969; Ward and Renz, 1972; Sheridan *et al.*, 1973; McDonald and Doughty, 1973/74, 1974; Whalen and Rezek, 1974; McEwen *et al.*, 1977).

4.2 Effects on Growth

Subcutaneous TP implants inserted on day 2 severely depressed growth. This was evident both from the analyses of variance of weekly body weight measurements, and of the slopes of the regression lines. Rats with the same implants placed subcutaneously on day 3, although weighing significantly less than control rats from 15 weeks onwards, were shown by regression analysis to be no different in their growth rate from control animals. Implantation with TP on day 5 did not significantly alter growth according to regression analysis, although for four weeks (weeks 12-15) analysis of variance showed these animals to be significantly heavier than their equivalent controls. Rats injected with TP on day 5 were found by regression analysis to have greater growth rates than their respective controls, although this could be accounted for by catch-up growth. In this case the injected group at 3 weeks was lighter (but not significantly so) than the control group, and at 14 weeks was heavier (but again not significantly so) than the corresponding control group.

The striking feature to emerge from these analyses of rat body weight is the depression of growth resulting when TP implants were given to rats on day 2 of life.

Several questions are raised by this finding. Firstly, why was growth retarded by TP administration? Secondly, why did the same treatment commencing one or three days later not cause a similar depression of growth? And thirdly, why should TP treatment by injection or by implantation, given on the same day and apparently elevating plasma testosterone concentrations for similar periods, have different effects on growth?

Attempts to answer these questions with the present information available are purely speculative. The effect may be due to androgen or a metabolite, and the hormonal action demonstrated may be an organizational or activational one, or perhaps neither. All of these possibilities are considered.

It seems unlikely that testosterone is depressing growth by an organizational action, as it is largely without precedent. Two exceptions have been noted in published literature. Depressed body weight was seen in male rats given 0.5mg TP on either day 2 or day 5 (Swanson and van der Werff ten Bosch, 1963), but this difference was significant only at 40-60 days and was not accompanied by decreased skeletal length. Another report of depressed adult (110 days) body weight after neonatal androgen treatment involved subcutaneous injection of female rats daily for the first five days with 10μg androstenedione-enol-propionate (an aromatizable steroid) or 10μg 5α,3β-androstanediol dipropionate (non-aromatizable) (McDonald and Doughty, 1974). Other experiments in which TP was given for at least five days soon after birth (by injection daily or every second day, or by PDS implant) have invariably reported increased growth (Ward, 1969; Ward and Renz, 1972; Sheridan *et al.*, 1973; McDonald and Doughty, 1974; Pollak and Sachs, 1975; McEwen *et al.*, 1977). In the present experiment, treatment by TP implant given on days 3 or 5 did not cause the same depression as implantation on day 2 which argues against an organizational effect. However, in considering the possibility of a hypothalamic effect, the profound depression of ovarian weights seen in rats given TP implants on day 2 (significantly lighter than those of rats given implants on days 3 or 5), does suggest unusually marked disruption of the hypothalamic-pituitary axis.

In its activational role testosterone's actions are anabolic, manifest in the greater size of intact male animals compared with their castrated counterparts. This is true for male rats and is in contrast to the female rat whose body weight increases after ovariectomy (see for example, Kakolewski *et al.*, 1968). This action is obviously not relevant to the depression of growth being discussed here. However, long-term administration of high doses of TP to adult male rats has been found to inhibit growth (Gentry and Wade, 1976a) and there is evidence that this effect is due to conversion of TP to oestrogen (Gray *et al.*, 1979).

Inhibition of growth is an activational effect of oestrogen (see for example, Wade, 1975), and being a metabolite of testosterone, it may be involved in the depression of growth seen in the present experiment.

Injection of 240μg OB (but not 30-120μg) into 5 day old male rats resulted in lower body weight at 45 days (Kincl *et al.*, 1965). Depression of body weight resulting from an organizational action of oestrogen has not been confirmed in studies involving female rats injected neonatally, but OB has, like TP, stimulated growth (Bell and Zucker, 1971).

If the large amount of androgen used in the present experiment were supplying significant amounts of oestrogen which were causing inhibition of growth by an activational action, then recovery of normal body weight would be expected after the source of oestrogen had gone. At 10 days, plasma testosterone concentrations were no higher than in control animals, so that elevated levels of oestrogen would have ceased at about the same time, yet the depression of body weight was permanent.

However, permanent reduction in adult body weight by underfeeding rats during the suckling period has been reported (Kennedy, 1957; Kennedy and Mitra, 1963). Therefore any influence capable of inhibiting growth at this early age might produce permanent stunting. However, recent work (Tarttelin, M.F., personal communication) has demonstrated that rats exposed to DES during the neonatal period are undersized at three weeks and need to be weaned at five or six weeks to ensure survival yet regression analysis has proven them to grow significantly faster than controls (catch-up compensatory growth). It is presumed in these

studies that the cause of early stunting is reduced milk supply in the dam, who was also exposed to DES in the course of the experiment.

Depression of body weight and decreased body length have been recorded with prepubertal administration of OB (Schwartz and Wade, 1981). Nunez and Grundman (1982) found that a daily low dose of TP (0.2mg) given to prepubertal male rats was relatively more effective than a high dose (1mg) in stimulating body weight gain, food intake, and body length. They suggested that oestrogenic metabolites resulting from the high dose of TP might be responsible for the different responses. However, in both of these experiments hormone treatment began at a later age (27 days, Schwartz and Wade, 1981; 22 days, Nunez and Grundman, 1982) than in the present experiment.

It has been claimed (Wade and Zucker, 1970) that control of body weight is insensitive to the inhibitory effects of oestrogen before about 7 weeks of age. If this is the case, then oestrogenic metabolites of testosterone resulting from the high dose of TP cannot account for the depression of growth seen in the present experiment. However, the results of an experiment mentioned above (Schwartz and Wade, 1981) do not support the hypothesis of Wade and Zucker (1970), and suggest that oestrogen can inhibit body weight before puberty. In the present experiment the body weight difference between day 2 TP-implanted rats and control animals did not reach significance until 7 weeks, although the implanted animals were lighter at weaning and each subsequent weighing.

An argument against the involvement of oestrogen in the depression of growth seen in the present experiment, is an apparent insensitivity to oestrogen indicated by the lack of increased growth response seen in the stunted rats after ovariectomy. It is also possible however, that the atrophic ovaries of these rats were producing amounts of oestrogen too small to have had any restraint on growth prior to ovariectomy.

Thus the evidence for oestrogens, resulting from aromatization of large amounts of TP, causing before weaning an inhibition of growth sufficient to result in permanent stunting, is inconclusive.

A further explanation for the growth phenomenon occurring in this experiment is the possibility of a generalized toxic effect of high doses of TP, perhaps affecting liver metabolism, and resulting in retardation of growth.

The second question raised by these results concerns the difference in growth responses occurring when TP implants were inserted at different ages within a four day period. Although implantation at day 3 or day 5 did not cause severe inhibition of growth, the body weight data do suggest a progressive change. While long-term treatment beginning on day 2 caused marked depression of body size, treatment beginning on day 3 caused a moderate depression of body weight (significantly lighter from 15 weeks), and treatment beginning on day 5 caused a slightly increased body weight (significant at weeks 12-15). Regression analysis did not confirm this observation.

The explanations offered for the reduced size of rats given TP implants on day 2 would appear to apply just as well to those treated one or three days later. The increased body size of the growing rats over this interval would reduce the hormone dose a little but it would still constitute a large dose. The number of rats in the day 2 TP-implanted group was small (4) but nevertheless these animals were from three different litters and their weights were remarkably consistent as borne out by statistical analysis which showed their weight and length to be highly significantly smaller than those of animals given TP implants at 3 or 5 days of age.

Kennedy and Mitra (1963) noted that the first three weeks of age was a period of "very rapid development of new ossification centres, when one day in age caused a marked change in the appearance of the skeleton", so perhaps the likelihood of dramatic changes occurring as a result of treatment beginning on three close days during this period should not be dismissed lightly.

The third question posed by results of this experiment asks why TP injection and PDS implants containing TP had different effects on growth. Although rats injected with TP on day 5 demonstrated a compensatory growth after low weaning weights, this did not occur in rats given TP implants on day 2. Growth was severely depressed by TP implants inserted on day 2, and yet not by TP injection on the same day, despite similar periods of elevation of plasma testosterone concentrations. The similarity in periods of hormone elevation may in fact be an artifact resulting from the 'sensitivity' of the measurement of plasma hormone. The calculated plasma half-lives after TP injection and TP implants were different (48 hours and 69 hours respectively, Chapter 2, Section 3.2). As discussed earlier (Chapter 4, Section 4.2.1.), greater sensitivity in the radioimmuno-assay would result in an apparently longer period of elevation of plasma concentrations. Therefore the relative insensitivity of the plasma measurements may mask greater differences in the persistence of testosterone in plasma after the two methods of administration. A more prolonged elevation of plasma testosterone after TP implant, or differences in hormone profiles after the two treatments, may explain the differences in effect on growth.

5. CONCLUSIONS

Female rats given TP implants during the first five days of life were exposed to elevated testosterone concentrations for at least ten days. As adults they showed no cyclic ovarian activity and did not display normal feminine sexual behaviour when appropriately stimulated. Those treated on day 2 grew significantly less than either controls or rats given TP implants on days 3 or 5. Retardation of growth at a time when rapid development normally occurs can result in permanent stunting and the possibility of this resulting from a generalized 'toxic' effect of excessive androgen, or from the growth-inhibiting actions of testosterone-derived oestrogen, is discussed. An organizational effect occurring at the hypothalamic level during a very restricted period of time may be considered.

Different growth responses were seen after the same TP implants given at 2 days were inserted at 3 or 5 days of age. Reasons for this may lie in the rapid developmental changes occurring at this time, so that the same treatment which inhibited growth, either may not alter, or may stimulate, growth when begun three days later.

While TP injection of female rats on day 2 has stimulated rat growth in many studies, TP implants introduced at the same age had the opposite effect in this experiment. The explanation for this anomaly may lie in differing plasma testosterone profiles.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

GENERAL DISCUSSION

Temporal aspects of the organizational effects of testosterone on the hypothalamus of the perinatal female rat were investigated in the studies presented in this thesis. Measurements of plasma testosterone concentration after administration of TP by various means (Chapter 2) made it possible to relate the effects on sexually dimorphic characters to the duration of elevated testosterone concentration.

Subcutaneous injection of neonatal female rats with a classically 'androgenizing' dose of TP (90μg) resulted in elevated plasma testosterone concentrations for 192 hours (8 days). Much briefer exposure to TP was achieved by TP-filled PDS capsules which were removed after 4, 8 or 24 hours. However, the long half-life (8.6 hours) of plasma testosterone in neonatal rats following removal of the capsule indicated a much slower clearance rate than in adult rats (half-life of 1 hour after removal of TP-filled capsule), and elevated testosterone levels, although declining, continued above control levels for up to 24 hours. Rats chronically implanted with TP-filled PDS implants as neonates had elevated plasma testosterone concentrations for 10 days, which allowed observation of the effects of more prolonged neonatal TP treatment.

Emphasis was placed on identifying the period of elevated plasma hormone concentrations and, in view of some later findings, more detailed comparisons of the profiles of hormone concentrations in neonates after different treatments would be of interest. While the testosterone concentrations in the plasma of foetal and neonatal rats is well documented (see Chapter 1, Section 3.2.2.), there has been a paucity of information on the levels resulting from treatments used to effect androgenization. Experiments described here provide information for the neonate, and the recent paper by Slob and colleagues (1983) provides information for pregnant rats and their foetuses. There is scope for further definition of androgen treatments in terms of resulting hormone levels.

That the hypothalamus is insensitive to androgen before birth was the conclusion drawn from experiments described in Chapter 3. Treatment of the pregnant rat with TP for intervals of 72 or 24 hours during the last trimester of pregnancy caused marked alteration to the external genitalia of the daughters, but was without apparent effect on the ovarian function, feminine sexual behaviour or growth. However, the results of experiments by Slob and others (1983) suggest that testosterone injected into the mother does not cross the placenta unless very large doses are given, and that virilization of the genitalia is due to androsterone, a non-aromatizable androgen which they measured in the foetus after TP injection to the mother. Difficult to reconcile with this idea is the finding of androgenization of gonadotrophin secretion and sexual behaviour after treatment of the pregnant rat with a dose of TP considered low by Slob's group (Ward and Renz, 1972). Since different strains of rat were used in the two studies, replication in Sprague-Dawley rats of foetal testosterone measurements after maternal TP injection would be useful.

Direct injection of TP into foetuses *in utero* ensures their treatment with androgen and has resulted in anovulatory sterility (Swanson and van der Werff ten Bosch, 1965; Fels and Bosch, 1971). This is not necessarily proof of prenatal hypothalamic androgen sensitivity, since it is likely that TP injected before birth would persist in the tissues for some days, and so could effect hypothalamic androgenization during the neonatal period. Neonatal clearance of plasma testosterone was found to be slow (Chapter 2, Section 3.2.1.) and foetal clearance may be even slower.

Further experiments which do not support the theory of a prenatal period of hypothalamic sensitivity have recently been carried out. Pregnant rats were treated for brief periods with diethylstilboestrol (DES), a potent androgenizing agent when given neonatally to female rats (Tarttelin, M.F., personal communication). Severe modification of the external genitalia indicated that DES had reached the foetus, and other reports confirm its ability to cross the placenta in mice and hamsters (Bengtsson and Ullberg, 1963; Shah and McLachlan, 1976; Rustia and Shubik, 1979). Preliminary results show no effects on ovarian function, sexual behaviour or growth. Thus there appears to be no conclusive evidence that the critical period for hypothalamic androgenization

begins before birth.

The brief periods of exposure to androgen, made possible by the removable TP-filled PDS capsules used in experiments described in Chapter 4, confirmed the independence of differentiation of sexually dimorphic hypothalamic functions. Testosterone was able to exert its organizational effects on the control of gonadotrophin secretion within 28 hours in at least 50% of female rats of 2 and 3 days of age. Elevation of plasma testosterone for intervals from 28 to 48 hours caused a perceptible but not significant depression of the display of feminine sexual behaviour. However, depression of both of these characters occurred when plasma testosterone elevation persisted for 192 hours as it did after injection of 90μg TP at any one of the three ages. Vaginal development could be altered by very brief TP treatments (maximum 28 hours exposure) on days 2 or 3, but was refractory to any TP treatment on day 5.

After implantation of TP-filled capsules in the neonatal rat for a brief period (4 hours) plasma testosterone concentration did not return to control levels for 24 hours (Chapter 2, Section 3.2.1.). Investigation of the effects of briefer periods of exposure would be difficult to achieve by this method because of the slow clearance of plasma testosterone in neonates. Inhibiting or interfering substances have been used to terminate androgenic action but interpretation of results is complicated by unknown factors such as the duration of action, the delay before the onset of action and the mechanism of action of the interfering substance. However, since the brief exposure periods investigated in Chapter 4 caused approximately 50% sterility compared with the 100% induced by TP injection, it seems likely that shorter treatment periods would be ineffective. The threshold nature of the effects caused by the brief periods of exposure could perhaps be demonstrated by identification of the delayed anovulation syndrome (by examining ovaries for corpora lutea at intervals starting from puberty). This was not attempted in the study described in Chapter 4 because of problems previously encountered with adhesions developing between ovaries and surrounding tissues after exploratory laparotomy.

Previous experiments which investigated the minimum period of exposure to testosterone required for androgenization have produced varying estimates of its duration, which is not surprising considering the different approaches used (see Chapter 4, Section 4.2). Results of experiments described in Chapter 4 support the view that complete androgenization of all sexually dimorphic hypothalamic functions is a gradual process. While control of gonadotrophin secretion can be altered by testosterone present for 28 hours or less, control of feminine mating behaviour and growth probably require testosterone to be present for some days rather than hours. Evidence from studies (Barnea and Lindner, 1972) in which macromolecular synthesis in the hypothalamus was blocked for up to 24 hours after injection of TP, without preventing androgenization of 5 day old female rats, also suggests a relatively slow process. Barnea and Lindner (1972) also reported that uptake of [^{3}H]-TP by the brain after subcutaneous injection to neonatal female rats is slow and reaches a peak at least 8 hours after injection.

Studies in the male rat provide further evidence that androgen acts slowly, since castration within 48 hours after birth permits cyclic release of gonadotrophin (evident as corpora lutea formation in transplanted ovaries, Yazaki, 1960; Harris, 1964), despite appreciable plasma testosterone concentrations present immediately before and after birth (see Chapter 1, Section 3.2.2.).

Single injections of small doses of esterified testosterone in neonatal female rats clearly provide the necessary prolonged exposure to androgen whereas the metabolism of injected free testosterone is so rapid that much larger doses, or repeated injections, are required for androgenization (Kincl, Pi, Maqueo, Lasso, Oriol and Dorfman, 1965; Alklint and Norgren, 1971). By measuring the levels of radioactivity in the brain after injection of [^{3}H]-TP and [^{3}H]-testosterone (free testosterone), Barnea, Weinstein and Lindner (1972) demonstrated that free testosterone is taken up rapidly and declines again rapidly, whereas the uptake of esterified testosterone is more gradual and prolonged. A large dose of free testosterone, or repeated small doses, presumably cause anovulatory sterility because levels in the brain persist sufficiently long. The possibility that changes in the uptake of testosterone by the hypothalamus could account for the critical period was considered

by Barnea and co-workers (1972), but discounted, since they found that the decline in effectiveness of TP in producing androgenization was not closely paralleled by changes in the uptake.

The molecular and cellular events which are the basis for the critical period are being investigated in studies of steroid hormone receptors in the brain, of sexually dimorphic structures in the brain, and of the effect of steroid hormones on the growth in tissue culture of neuronal processes (reviewed by Toran-Allerand, 1978), and may eventually provide explanations for the brief existence of a period of androgen sensitivity.

Although able to induce 'masculinization' of the hypothalamus, a single injection of TP has been considered to be an inadequate model for sexual differentiation, as it does not parallel the continuous androgen secretion of the male rat (Sheridan *et al.*, 1973). Neither can the release of testosterone from PDS capsules used in Chapter 5 be claimed to mimic closely the endogenous secretion from the testis. Measurements in the male rat (see Chapter 1, Section 3.2.2.) show moderately high plasma testosterone concentrations at birth declining to low levels in the first few days and persisting until just before puberty, although androgens other than testosterone may be present in larger amounts during this period (Dohler and Wuttke, 1975; Purvis *et al.*, 1980; Corpechot *et al.*, 1981).

The body weights of female rats which were treated prenatally or in infancy with TP were subjected to regression analysis in the present experiments. This is a statistical tool which has not been used in comparable studies referred to in this thesis, and which has certain advantages already outlined in Chapter 4 (Section 4.1.3.). When used in combination with weekly analyses of variance of body weight, an overall picture of growth and body weight is obtained. The requirement for samples of observations to be drawn from a population with the same variance to permit legitimate statistical analysis, was met in these statistical analyses by transformation of body weight to logarithms. Significant increases in growth rate in androgen-treated female rats were not demonstrated by regression analysis in experiments described in Chapter 4, but neither did one-way analysis of variance, carried

out at each weekly weighing, demonstrate consistently significant weight increases. Clearly, in these rats early TP treatment did not stimulate growth, and the reasons for this are unknown, but not without precedent. Other studies have also shown no change in body weight after neonatal TP (Brown-Grant, 1964; Bradshaw and Critchlow, 1966; Wagner *et al.*, 1966) or an increase which was not apparent until a relatively late age (150 days, Brown-Grant, Munck, Naftolin and Sherwood, 1971). The extent to which the variation in growth response reflects the use of different strains of rat and different diets can only be a matter for speculation.

Regression analysis did demonstrate a profound depression in the growth rate of female rats given prolonged TP treatment beginning on day 2 (Chapter 5). These animals were severely and permanently stunted and yet the same treatment administered at days 3 or 5 did not have the same dramatic results. A more detailed study of the plasma testosterone concentration after treatment with TP-filled capsules inserted at the three different ages might indicate if this effect were due to widely different hormone levels during the neonatal period. These could come about by changes in size of the animal, or by changes in steroid metabolism occurring with increasing age. An alternative explanation would imply a difference in the sensitivity of the hypothalamus itself during this period.

Injection of TP on day 2, while elevating plasma testosterone concentrations for nearly as long as the PDS implants, did not cause similar depression of growth rates, and has been widely reported to stimulate growth (see for example, Tarttelin *et al.*, 1975). Perhaps TP implants saturated the animal with hormone so that the fat depots provided a continuing source of low levels of androgen, not detected by the plasma radioimmunoassay, but sufficient to have physiological effects. Neither did injection of TP on day 2 result in such marked depression of ovarian weights as seen in rats with TP implants inserted on day 2, which indicates that the implants on day 2 caused a profound disruption of hypothalamic-pituitary relationships. Further plasma hormone studies comparing more closely the plasma profiles of rats treated with TP injection with rats treated with long-term TP-filled capsules might explain the differences in growth responses seen when the two different treatments were given at the same age.

Important developmental changes occur in the life of the rat during a short period around the time of birth. Remarkable co-ordination of hormone production with the stage of development of the hypothalamus ensures that the male animal differentiates physiologically and, in some respects, behaviourally. Studies presented in this thesis have examined this 'critical period' of hypothalamic androgen sensitivity and attempted to define, in terms of the duration of plasma testosterone concentration, the stimulus which induces androgenization in the female model for masculine sexual differentiation.

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TABLE I

REGRESSION ANALYSIS OF BODY WEIGHT AND AGE:
COMPARISON OF AGE AND THE RECIPROCAL OF AGE AS THE X-VARIABLE

Illustrative data from Chapter 5: group injected with TP on day 2

ANALYSIS OF VARIANCE OF log(base 10) BW FOR AGES 3 TO 12 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO
Between weeks	11	0.51476	233.98***
Linear regression (Age)	1	4.80475	2183.98***
Deviations from regression	10	0.08576	38.98***
Linear regression (1/Age)	1	5.64285	2564.93***
Deviations from regression	10	0.00195	0.89ns
Within weeks	72	0.00220	

*** $p < 0.001$, ns $p > 0.05$

TABLE II

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

OVARIAN WEIGHT (mg)

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days)											
	21-19			18-16			15-13			12-10		
	AGE AT OVARIECTOMY (days)											
	90			55-90			75			40-60		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	10	72.9	2.2				6	86.5	4.9	10	69.1	4.4
10mm TP	10	77.0	3.3	3	86.9	4.0	5	83.2	5.5	3	83.8	5.8
20mm TP	10	84.7	2.3	9	66.8	2.0	8	77.6	2.4	12	51.1	2.1
10mm CL°	10	76.2	2.2				13	76.7	2.5			
	AGE AT OVARIECTOMY (days)											
	90			40-90			65			40-65		
72 HOUR IMPLANT												
5mm TP	10	81.2	2.7	6	66.9	4.9	11	81.3	3.7	7	85.7	4.8
10mm TP	7	74.4	3.6	4	32.8	5.0	9	71.8	4.3	4	82.4	12.5
				'10	90.1	5.1						
20mm TP	13	84.9	2.3	6	36.3	3.2	2	70.0	7.0	2	81.5	10.5
10mm CL	10	77.3	2.7				10	78.1	2.5			
				'10	83.8	2.9						
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS												
"F"	2.16 ns			-			1.53 ns			-		

°Cholesterol
'Supplementary group

ns $p > 0.05$

TABLE III

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

ANOGENITAL DISTANCE (mm) AT BIRTH

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days)											
	21-19			18-16			15-13			12-10		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	12	2.84 **°	0.06	16	2.64 ***°	0.09	10	2.92	0.09	12	2.69	0.06
10mm TP	14	2.58	0.07	10	2.88	0.06	14	2.72	0.05	3	2.40 **°	0.06
20mm TP	13	2.45	0.05	14	2.78 *°	0.07	24	2.97	0.06	12	2.68	0.08
10mm CL'	14	2.57	0.06	17	2.98	0.06	17	2.82	0.06	19	2.83	0.04
72 HOUR IMPLANT												
5mm TP	16	2.61	0.06	13	3.02 **°	0.04	12	2.65	0.07	9	2.61	0.03
10mm TP	15	2.72 **°	0.07	5	2.92	0.12	14	2.96 **°	0.05	7	2.43 *°	0.12
20mm TP	15	2.59	0.06	18	3.14 ***°	0.08	6	2.73	0.11	7	2.64	0.08
10mm CL	23	2.46	0.06	4	2.58	0.05	9	2.69	0.08	10	2.66	0.07
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS												
"F"		4.23 ***			5.66 ***			3.30 **			3.41 **	

°Significance of difference between TP mean and corresponding CL mean, determined by t-test.

'Cholesterol

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE IV

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

SEXUAL BEHAVIOUR (lordosis quotient)

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days)								
	21–19			18–16			15–13		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT									
5mm TP	5	96.0	2.4						
10mm TP	3	96.7	3.3						
20mm TP	6	100.0	0.0						
10mm CL°	5	96.0	4.0						
72 HOUR IMPLANT									
5mm TP	4	100.0	0.0				6	93.0	4.2
10mm TP	4	100.0	0.0	'10	100.0	0.0	5	90.0	6.3
20mm TP	8	98.8	1.3				2	100.0	0.0
10mm CL	4	100.0	0.0	'10	100.0	0.0		100.0	0.0
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS									
"F"		1.01 ns						1.11 ns	

°Cholesterol

'Supplementary group

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE V

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

BIRTH WEIGHT (g, log base10)

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days)											
	21-19			18-16			15-13			12-10		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	12	0.791	.010	16	***° 0.767	.007	10	0.740	.012	12	0.757	.010
10mm TP	14	*° 0.763	.009	10	***° 0.838	.017	14	0.723	.051	3	***° 0.827	.011
20mm TP	13	*° 0.776	.008	14	***° 0.794	.006	24	0.727	.004	12	0.713	.010
10mm CL'	14	0.797	.007	17	0.729	.005	17	0.745	.006	19	0.732	.006
72 HOUR IMPLANT												
5mm TP	16	0.766	.008	13	0.773	.014	12	0.778	.007	9	0.749	.023
10mm TP	15	0.769	.012	5	**° 0.691	.009	14	*° 0.789	.012	7	0.694	.018
20mm TP	15	0.790	.014	20	***° 0.688	.006	6	**° 0.675	.009	7	0.717	.034
10mm CL	23	0.766	.005	4	0.758	.011	9	0.750	.013	10	0.729	.009
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS												
"F"		2.29 *			28.78 ***			9.80 ***			4.82 ***	

°Significance of difference between TP mean and corresponding CL mean, determined by t-test.

'Cholesterol

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE VI

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE: DAYS 19-21

BODY WEIGHT (log base 10) FOR AGES 3 TO 17 WEEKS: GROUP MEANS AND STANDARD ERRORS

Group	Week	3	4	5	6	7	8	9	10	11	12	13	14	15	16 OB'	16 C[2]	17 OB	17 C
10mm CL°																		
24h	Mean	1.649	1.885	2.062	2.163	2.225	2.282	2.323	2.353	2.375	2.404	2.422	2.462	2.487	2.465	2.515	2.475	2.531
	SE	.006	.013	.009	.008	.009	.009	.007	.006	.007	.006	.007	.008	.008	.011	.011	.010	.011
72h	Mean	1.646	1.746	1.979	2.118	2.201	2.269	2.318	2.349	2.380	2.404	2.419	2.467	2.496	2.469	2.526	2.477	2.545
	SE	.005	.008	.008	.007	.007	.008	.008	.008	.007	.007	.010	.007	.007	.010	.008	.009	.008
5 mm TP																		
24h	Mean	1.594	1.799	2.002	2.133	2.214	2.278	2.328	2.362	2.382	2.406	2.409	2.452	2.482	2.478	2.495	2.486	2.502
	SE	.017	.025	.022	.016	.014	.015	.013	.012	.012	.012	.013	.011	.010	.019	.005	.018	.012
72h	Mean	1.632	1.904	2.063	2.166	2.229	2.290	2.329	2.359	2.384	2.402	2.423	2.456	2.495	2.484	2.498	2.493	2.511
	SE	.013	.014	.014	.013	.010	.011	.011	.009	.010	.010	.006	.009	.008	.012	.004	.010	.016
10 mm TP																		
24h	Mean	1.658	1.843	2.043	2.168	2.231	2.291	2.334	2.364	2.387	2.414	2.436	2.483	2.488	2.476	2.525	2.479	2.536
	SE	.014	.023	.014	.008	.007	.006	.005	.005	.006	.005	.008	.008	.014	.008	.011	.011	.013
72h	Mean	1.608	1.852	2.032	2.153	2.215	2.277	2.316	2.351	2.377	2.396	2.421	2.447	2.463	2.457	2.464	2.459	2.486
	SE	.020	.015	.012	.011	.009	.012	.010	.010	.010	.008	.008	.011	.012	.013	.019	.010	.011
20 mm TP																		
24h	Mean	1.632	1.872	2.069	2.176	2.234	2.298	2.337	2.362	2.386	2.409	2.420	2.464	2.488	2.459	2.508	2.463	2.532
	SE	.011	.019	.011	.010	.011	.012	.014	.015	.012	.012	.012	.012	.012	.020	.019	.020	.009
72h	Mean	1.699	1.766	1.985	2.127	2.218	2.283	2.330	2.366	2.398	2.428	2.430	2.468	2.488	2.472	2.508	2.478	2.525
	SE	.007	.006	.008	.008	.006	.007	.007	.006	.004	.009	.005	.006	.006	.007	.009	.006	.012
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																		
"F"		7.66	12.74	8.41	4.49	1.36	0.84	0.53	0.47	0.72	1.22	0.84	1.61	0.84	3.76		5.27	
		***	***	***	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	**		***	

*** p <0.001; ** 0.001 <p< 0.01; * 0.01 <p< 0.05; ns p > 0.05 ° cholesterol ' oestradiol benzoate [2] control

TABLE VII

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

DAYS 16-18, 72h — BODY WEIGHT (log base 10) FOR AGES 3 TO 20 WEEKS: GROUP MEANS AND STANDARD ERRORS

Group	Week	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CL'	Mean	1.625	1.831	2.032	2.155	2.234	2.295	2.337	2.368	2.391	2.408	2.437	2.469	2.498	2.515	2.486	2.505	2.508	2.515
10mm	SE	.012	.007	.006	.007	.005	.006	.005	.006	.006	.007	.006	.006	.006	.007	.006	.006	.006	.007
TP	Mean	1.693	1.881	2.071	2.187	2.258	2.319	2.358	2.391	2.415	2.433	2.454	2.485	2.516	2.537	2.512	2.519	2.521	2.530
10mm	SE	.013	.025	.005	.006	.007	.007	.006	.006	.006	.007	.006	.007	.007	.007	.007	.007	.007	.007
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																			
"t"		3.71	1.92	5.17	3.36	2.81	2.72	2.52	2.69	2.87	2.41	2.02	1.67	1.92	2.21	2.69	1.63	1.40	1.43
		**	ns	***	**	*	*	*	*	*	*	ns	ns	ns	*	*	ns	ns	ns

DAYS 13-15, 24h — BODY WEIGHT (log base 10) FOR AGES 3 TO 18 WEEKS: GROUP MEANS AND STANDARD ERRORS

Group	Week	3	5	6	7	8	9	10	11	12	13	14	15	16	17 OB°	17 C'	18 OB	18 C
CL	Mean	1.646	1.992	2.119	2.230	2.244	2.293	2.323	2.359	2.368	2.389	2.401	2.437	2.477	2.472	2.495	2.480	2.518
10mm	SE	.012	.009	.010	.010	.011	.008	.009	.008	.011	.009	.007	.008	.008	.006	.015	.005	.014
TP	Mean	1.708	2.047	2.152	2.238	2.253	2.295	2.313	2.339	2.351	2.378	2.386	2.411	2.452	2.425	2.496	2.443	2.519
5mm	SE	.008	.012	.010	.017	.010	.010	.009	.013	.011	.010	.009	.011	.011	.018	.007	.019	.006
TP	Mean	1.700	2.031	2.149	2.218	2.264	2.311	2.329	2.362	2.369	2.392	2.398	2.428	2.468	2.461	2.484	2.470	2.510
10mm	SE	.010	.015	.012	.016	.010	.009	.010	.008	.010	.009	.009	.009	.008	.012	.013	.013	.014
TP	Mean	1.694	2.015	2.130	2.260	2.250	2.290	2.312	2.345	2.321	2.357	2.364	2.400	2.427	2.424		2.432	
20mm	SE	.013	.006	.000	.000	.000	.000	.004	.005	.010	.014	.008	.010	.017	.014		.010	
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																		
"F"		8.11	3.83	2.27	0.64	0.65	0.83	0.65	1.00	1.55	1.06	1.48	1.85	1.81	4.16		5.89	
		***	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**		**	

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$ — ° oestradiol benzoate — ' control — ' cholesterol

TABLE VIII

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE: DAYS 19-21

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 3-12)
X=reciprocal of age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TP IMPLANT 24h 5mm	10mm	20mm
Between weeks	9	6.6900***	5.7519***	5.7758***
Linear regression	1	6.6530***	5.7115***	5.7221***
Deviations	8	0.0370	0.0404**	0.0537**
Within weeks	10(n-1)-m	0.2416	0.1044	0.1525
Number of rats (n)		10	10	10
Missing values (m)		0	0	0
Regression coefficient		-3.3883	-3.1395	-3.1353

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TP IMPLANT 72h 5mm	10mm	20mm
Between weeks	9	5.4932***	5.3633***	8.4207***
Linear regression	1	5.4882***	5.2972***	8.0873***
Deviations	8	0.0050	0.0661**	0.3334***
Within weeks	10(n-1)-m	0.1220	0.1126	0.0800
Number of rats (n)		10	9	14
Missing values (m)		0	5	5
Regression coefficient		-3.0775	-3.2134	-3.1943

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y CHOLESTEROL IMPLANT (10mm) 24 HOUR	72 HOUR
Between weeks	9	5.3066***	6.4836***
Linear regression	1	5.2439***	6.3240***
Deviations	8	0.0627***	0.1596***
Within weeks	10(n-1)-m	0.0567	0.0482
Number of rats (n)		10	10
Missing values (m)		1	0
Regression coefficient		-3.0295	-3.3035

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE IX

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE: DAYS 16-18°

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 3-12)
X=reciprocal of age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y	
		72 HOUR IMPLANT	
		CL' 10mm	TP 10mm
Between weeks	9	6.23800***	5.55456***
Linear regression	1	6.20797***	5.52651***
Deviations	8	.03003***	.02805**
Within weeks	90	.04438	.10057
Number of rats		10	10
Regression coefficient		-3.2731	-3.0882

ANALYSIS OF VARIANCE OF THE DIFFERENCES BETWEEN REGRESSIONS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F RATIO
Between groups²	1	0.009903	2.73ns
Within groups	16	0.003629	

° Supplementary study group
' Cholesterol
² CL 10mm and TP 10mm

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE X

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

WEIGHT OF ADRENAL GLANDS (mg) AT 130 DAYS[2]

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB°			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	79.1	5.7	5	65.4	2.6						
10mm TP	3	74.8	5.4	7	78.8	5.6						
20mm TP	6	82.0	4.8	4	73.0	6.2						
10mm CL'	5	84.7	5.8	5	85.7	4.9						
72 HOUR IMPLANT												
5mm TP	5	85.4	5.0	3	65.8	2.6	6	86.2	6.1	5	92.9	4.5
10mm TP	4	77.2	3.9	3	64.1	2.4	5	80.9	5.9	4	86.7	6.1
20mm TP	8	78.8	3.0	5	76.1	4.8	2	62.5	0.9			
10mm CL	4	79.7	3.1	6	79.6	5.7	5	87.9	2.3	5	82.6	4.1

TABLE XI

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

WEIGHT OF PREPUTIAL GLANDS (mg) AT 130 DAYS[2]

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB°			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	133	16	5	145	13						
10mm TP	3	119	14	7	116	7						
20mm TP	6	120	8	4	123	10						
10mm CL'	5	124	8	5	142	19						
72 HOUR IMPLANT												
5mm TP	5	122	6	3	118	13	6	119	12	5	117	6
10mm TP	4	129	10	3	125	17	5	129	8	4	131	14
20mm TP	8	128	8	5	143	23	2	97	5			
10mm CL	4	124	4	6	127	8	5	109	8	5	110	10

°Oestradiol benzoate 'Cholesterol

[2] All of the *a priori* contrasts were non-significant ($p > .05$)

TABLE XII

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

WEIGHT OF UTERUS (mg) AT 130 DAYS[2]

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB°			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	582	49	5	135	1						
10mm TP	3	648	17	7	136	6						
20mm TP	6	769	23	4	108	19						
10mm CL'	5	714	47	5	134	11						
72 HOUR IMPLANT												
5mm TP	5	635	58	3	126	12	6	663	34	5	138	10
10mm TP	4	574	23	3	113	14	5	622	54	4	125	4
20mm TP	8	651	49	5	115	9	2	564	50			
10mm CL	4	753	74	6	138	1	5	606	31	5	130	13

TABLE XIII

EXPOSURE OF FOETAL RATS TO TESTOSTERONE PROPIONATE

WEIGHT OF PITUITARY (mg) AT 130 DAYS[2]

TREATMENT	STAGE OF GESTATION OF EXPOSURE (days) 21-19						15-13					
	POST-OVARIECTOMY TREATMENT											
	OB°			untreated			OB			untreated		
	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
24 HOUR IMPLANT												
5mm TP	5	37.4	4.8	5	15.5	1.1						
10mm TP	3	34.7	4.3	7	18.4	1.5						
20mm TP	6	43.4	4.1	4	18.0	0.9						
10mm CL'	5	41.5	4.3	5	16.6	1.8						
72 HOUR IMPLANT												
5mm TP	5	46.6	4.8	3	17.0	1.0	6	38.8	3.3	5	13.7	1.1
10mm TP	4	54.2	8.4	3	13.5	0.4	5	47.6	4.2	4	17.6	1.6
20mm TP	8	49.3	3.0	5	16.5	1.6	2	32.7	2.4			
10mm CL	4	45.8	4.1	6	17.4	0.7	5	38.7	3.5	5	16.3	0.9

°Oestradiol benzoate 'Cholesterol

[2] All of the *a priori* contrasts were non-significant ($p > .05$)

TABLE XIV

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

OVARIAN WEIGHT (mg) AT 90 DAYS

TREATMENT		DAY OF TREATMENT								
			D2			D3			D5	
		n	Mean	SE	n	Mean	SE	n	Mean	SE
4 HOUR	TP IMPLANT°	11	45.8	1.8	11	61.9	4.2	10	62.8	5.8
			***			*			ns	
	BLANK IMPLANT'	9	80.6	4.2	9	75.8	5.8	10	72.4	3.0
8 HOUR	TP IMPLANT	7	55.4	7.7	9	48.8	2.3	10	70.7	4.7
			***			***			ns	
	BLANK IMPLANT	5	96.0	3.8	7	80.8	4.1	10	78.5	2.6
24 HOUR	TP IMPLANT	6	49.0	2.0	10	47.3	3.6	9	59.9	7.1
			***			***			ns	
	BLANK IMPLANT	6	90.3	2.4	10	69.8	6.1	9	70.6	2.2
48 HOUR	TP IMPLANT							9	62.0	6.4
									ns	
	BLANK IMPLANT							10	69.3	1.6
INJECTION	TP[2]	11	50.0	4.5	8	43.7	2.5	7	42.5	3.8
			***			***			***	
	OIL[3]	12	82.3	2.0	7	75.8	3.7	8	71.4	4.3

° 2.5mm TP-filled PDS capsule
' 2.5mm empty PDS capsule
[2] 90μg TP in 0.05ml oil
[3] 0.05ml oil

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$
Significance of differences between TP groups and their appropriate control groups (analysis of variance followed by t-test)

TABLE XV

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

SEXUAL BEHAVIOUR (lordosis quotient)

TREATMENT		DAY OF TREATMENT								
		D2			D3			D5		
		n	Mean	SE	n	Mean	SE	n	Mean	SE
4 HOUR	TP IMPLANT°	9	94.4	4.0	9	90.0	4.4	6	100.0	0.0
			ns			ns			ns	
	BLANK IMPLANT'	8	100.0	0.0	9	100.0	0.0	4	100.0	0.0
8 HOUR	TP IMPLANT	7	88.6	4.7	8	68.8	15.3	8	93.1	2.8
			ns			ns			ns	
	BLANK IMPLANT	5	100.0	0.0	6	100.0	0.0	6	100.0	0.0
24 HOUR	TP IMPLANT	6	61.8	18.1	10	90.0	5.4	7	93.5	4.1
			ns			ns			ns	
	BLANK IMPLANT	6	100.0	0.0	10	100.0	0.0	9	70.6	2.2
48 HOUR	TP IMPLANT							4	95.0	0.0
									ns	
	BLANK IMPLANT							4	100.0	0.0
INJECTION	TP²	11	52.8	10.8	7	47.4	14.6	4	36.3	17.2
			*			*			*	
	OIL³	12	100.0	0.0	6	100.0	0.0	3	100.0	0.0

° 2.5mm TP-filled PDS capsule
' 2.5mm empty PDS capsule
² 90µg TP in 0.05ml oil
³ 0.05ml oil

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$
Significance of differences between TP groups and their appropriate control groups (t-test)

TABLE XVI

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 2

BODY WEIGHT (log base 10) FOR AGES 3 TO 18 WEEKS: GROUP MEANS AND STANDARD ERRORS

Group	Week	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
4 HOUR IMPLANT																	
TP	Mean	1.596	1.811	2.018	2.141	2.227	2.296	2.336	2.374	2.400	2.418	2.421	2.445	2.477	2.489	2.485	2.491
	SE	.017	.018	.012	.010	.009	.009	.009	.007	.007	.005	.006	.007	.006	.006	.007	.007
BLANK	Mean	1.603	1.817	2.020	2.141	2.226	2.290	2.338	2.368	2.394	2.409	2.408	2.440	2.475	2.494	2.492	2.502
	SE	.019	.020	.020	.020	.015	.014	.015	.015	.011	.010	.012	.013	.013	.011	.009	.009
8 HOUR IMPLANT																	
TP	Mean	1.666	1.867	2.065	2.203	2.274	2.334	2.373	2.417	2.437	2.463	2.469	2.491	2.522	2.540	2.527	2.535
	SE	.024	.032	.027	.010	.009	.009	.011	.011	.011	.011	.011	.008	.008	.009	.008	.009
BLANK	Mean	1.724	1.923	2.095	2.209	2.269	2.340	2.379	2.417	2.445	2.461	2.465	2.498	2.527	2.552	2.535	2.540
	SE	.018	.022	.016	.010	.017	.008	.006	.010	.009	.011	.010	.008	.010	.011	.011	.011
24 HOUR IMPLANT																	
TP	Mean	1.600	1.784	2.012	2.157	2.225	2.308	2.350	2.392	2.394	2.440	2.447	2.474	2.513	2.527	2.524	2.539
	SE	.029	.023	.015	.013	.015	.015	.015	.017	.034	.016	.018	.015	.015	.015	.015	.017
BLANK	Mean	1.600	1.777	2.011	2.140	2.225	2.307	2.349	2.383	2.410	2.428	2.423	2.463	2.503	2.516	2.509	2.518
	SE	.022	.026	.023	.014	.009	.005	.003	.005	.006	.007	.008	.004	.004	.005	.003	.003
INJECTION																	
TP	Mean	1.606	1.790	2.020	2.155	2.233	2.310	2.357	2.395	2.420	2.445	2.447	2.472	2.506	2.524	2.517	2.520
	SE	.018	.010	.008	.008	.007	.009	.008	.009	.007	.009	.011	.007	.008	.009	.010	.010
OIL	Mean	1.636	1.819	2.025	2.156	2.232	2.292	2.335	2.370	2.393	2.411	2.409	2.447	2.480	2.493	2.485	2.490
	SE	.010	.012	.010	.008	.008	.007	.007	.008	.007	.009	.010	.007	.007	.007	.007	.008
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																	
"F"		4.10	4.35	2.50	4.10	2.73	2.93	2.35	3.10	2.63	4.65	4.61	5.08	4.85	5.65	4.64	4.49
		**	**	*	**	*	*	ns	*	*	**	**	***	***	***	**	**

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE XVII

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 3

BODY WEIGHT (log base 10) FOR AGES 1 TO 18 WEEKS: GROUP MEANS AND STANDARD ERRORS

Week		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
4 HOUR IMPLANT																			
TP	Mean	1.124	1.424	1.618	1.795	2.040	2.157	2.245	2.254	2.320	2.366	2.393	2.420	2.435	2.462	2.495	2.478	2.490	2.492
	SE	.010	.008	.009	.023	.007	.009	.015	.007	.008	.007	.008	.009	.010	.010	.008	.008	.007	.009
BLANK	Mean	1.099	1.441	1.636	1.841	2.050	2.160	2.250	2.264	2.330	2.371	2.389	2.415	2.435	2.464	2.500	2.479	2.492	2.488
	SE	.024	.016	.008	.010	.010	.009	.010	.012	.010	.009	.009	.009	.009	.011	.009	.008	.009	.009
8 HOUR IMPLANT																			
TP	Mean	1.091	1.411	1.597	1.859	2.072	2.184	2.277	2.289	2.342	2.389	2.420	2.445	2.461	2.452	2.513	2.504	2.512	2.513
	SE	.010	.011	.018	.009	.008	.009	.011	.014	.013	.013	.011	.010	.010	.028	.010	.009	.010	.012
BLANK	Mean	1.055	1.390	1.587	1.836	2.039	2.162	2.257	2.251	2.332	2.379	2.401	2.427	2.442	2.462	2.495	2.483	2.494	2.502
	SE	.027	.034	.036	.033	.028	.023	.021	.019	.018	.020	.022	.024	.025	.020	.022	.021	.022	.023
24 HOUR IMPLANT																			
TP	Mean	1.091	1.400	1.570	1.771	2.003	2.131	2.233	2.260	2.314	2.373	2.385	2.430	2.440	2.467	2.495	2.482	2.492	2.495
	SE	.014	.017	.017	.016	.015	.013	.012	.015	.023	.015	.018	.016	.014	.017	.016	.017	.016	.014
BLANK	Mean	1.067	1.379	1.537	1.717	1.965	2.115	2.219	2.239	2.307	2.351	2.374	2.400	2.411	2.444	2.473	2.466	2.471	2.478
	SE	.017	.014	.008	.021	.015	.012	.011	.012	.011	.009	.010	.011	.009	.009	.010	.010	.009	.009
INJECTION																			
TP	Mean	1.137	1.455	1.659	1.831	2.044	2.163	2.258	2.257	2.320	2.378	2.402	2.432	2.445	2.476	2.512	2.495	2.508	2.512
	SE	.020	.016	.014	.016	.012	.009	.007	.010	.012	.004	.004	.015	.007	.010	.009	.007	.010	.010
OIL	Mean	1.133	1.455	1.658	1.788	2.011	2.140	2.237	2.247	2.303	2.354	2.367	2.394	2.418	2.443	2.479	2.465	2.474	2.478
	SE	.016	.014	.007	.027	.009	.011	.010	.015	.012	.010	.011	.010	.009	.015	.010	.009	.010	.013
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																			
"F"		2.81	2.98	7.00	6.08	6.83	3.22	2.15	1.51	0.85	1.27	1.69	1.87	1.84	0.45	1.35	1.27	1.46	1.14
		*	**	***	***	***	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*** p < 0.001; ** 0.001 < p < 0.01; * 0.01 < p < 0.05; ns p > 0.05

TABLE XVIII

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 5

BODY WEIGHT (log base 10) FOR AGES 3 TO 19 WEEKS: GROUP MEANS AND STANDARD ERRORS

Group	Week	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
4 HOUR IMPLANT																		
TP	Mean	1.607	1.845	2.018	2.139	2.217	2.267	2.301	2.340	2.365	2.382	2.391	2.414	2.435	2.475	2.500	2.483	2.486
	SE	.015	.016	.012	.009	.008	.007	.007	.012	.012	.013	.014	.014	.011	.015	.016	.018	.018
BLANK	Mean	1.597	1.849	2.026	2.139	2.215	2.259	2.298	2.331	2.354	2.370	2.384	2.398	2.421	2.454	2.477	2.464	2.469
	SE	.024	.018	.014	.011	.010	.009	.009	.008	.011	.010	.010	.010	.009	.009	.010	..010	.010
8 HOUR IMPLANT																		
TP	Mean	1.620	1.857	2.025	2.139	2.212	2.265	2.306	2.344	2.367	2.390	2.408	2.428	2.452	2.486	2.514	2.496	2.501
	SE	.013	.013	.013	.011	.011	.012	.012	.011	.011	.011	.012	.012	.011	.011	.010	.011	.010
BLANK	Mean	1.574	1.818	1.994	2.117	2.203	2.258	2.302	2.342	2.366	2.383	2.400	2.422	2.441	2.475	2.497	2.480	2.488
	SE	.025	.020	.017	.015	.015	.013	.013	.011	.011	.010	.010	.010	.008	.007	.008	.010	.009
24 HOUR IMPLANT																		
TP	Mean	1.636	1.845	2.048	2.155	2.238	2.290	2.340	2.375	2.402	2.421	2.440	2.462	2.482	2.514	2.532	2.523	2.529
	SE	.011	.018	.023	.010	.010	.008	.007	.008	.008	.007	.007	.007	.007	.006	.007	.007	.008
BLANK	Mean	1.619	1.827	2.004	2.134	2.213	2.262	2.307	2.345	2.369	2.387	2.409	2.423	2.445	2.478	2.496	2.493	2.505
	SE	.012	.016	.013	.013	.015	.012	.012	.013	.014	.014	.013	.012	.012	.012	.014	.012	.014
48 HOUR IMPLANT																		
TP	Mean	1.551	1.776	1.961	2.097	2.185	2.265	2.285	2.320	2.343	2.362	2.378	2.396	2.419	2.449	2.476	2.461	2.462
	SE	.036	.021	.021	.021	.021	.015	.021	.021	.020	.019	.021	.018	.017	.017	.017	.016	.014
BLANK	Mean	1.570	1.775	1.957	2.096	2.173	2.227	2.272	2.297	2.328	2.347	2.361	2.380	2.402	2.434	2.459	2.443	2.441
	SE	.014	.013	.013	.013	.010	.009	.011	.010	.010	.010	.009	.010	.009	.010	.011	.013	.013
INJECTION																		
TP	Mean	1.623	1.811	2.001	2.135	2.217	2.273	2.317	2.355	2.381	2.397	2.411	2.442	2.466	2.493	2.523	2.507	2.510
	SE	.034	.021	.018	.017	.019	.018	.012	.017	.017	.016	.014	.015	..015	.016	.015	.013	.014
OIL	Mean	1.645	1.813	1.984	2.118	2.204	2.250	2.289	2.325	2.352	2.370	2.385	2.400	2.424	2.456	2.483	2.466	2.470
	SE	.027	.013	.012	.009	.009	.011	.011	.011	.010	.011	.011	.010	.0012	.013	.013	.014	.011
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																		
"F"		1.96	3.06	3.58	2.27	1.89	1.46	2.60	2.92	2.77	2.85	3.14	4.13	4.57	4.02	3.60	3.68	4.23
		ns	**	***	**	ns	ns	*	**	**	**	**	***	***	***	***	***	***

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

TABLE XIX

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 2

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 3-12)
X=reciprocal of age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TP IMPLANT 4 HOUR	8 HOUR	24 HOUR
Between weeks	9	7.5165***	4.8963***	5.0313***
Linear regression	1	7.4945***	4.6346***	4.9896***
Deviations	8	0.0220*	0.2617***	0.0416*
Within weeks	10(n-1)-m	0.1317	0.1588	0.1328
Number of rats (n)		11	8	7
Missing values (m)		0	2	3
Regression coefficient		-3.4256	-3.3523	-3.5561

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y BLANK IMPLANT 4 HOUR	8 HOUR	24 HOUR
Between weeks	9	5.9473***	2.6970***	4.4024***
Linear regression	1	5.7268***	2.5243***	4.3205***
Deviations	8	0.2205***	0.1727***	0.0820***
Within weeks	10(n-1)-m	0.1888	0.0365	0.0618
Number of rats (n)		9	5	7
Missing values (m)		0	0	9
Regression coefficient		-3.3399	-2.8678	-3.5251

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y INJECTION TP	OIL
Between weeks	9	8.0618***	7.4814***
Linear regression	1	7.9856***	7.3867***
Deviations	8	0.0762***	0.0947***
Within weeks	10 (n-1)-m	0.1048	0.0977
Number of rats (n)		11	12
Missing values (m)		0	0
Regression coefficient		-3.5395	-3.2630

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE XX

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 3

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 3-12)
X=reciprocal of age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y — TP IMPLANT 4 HOUR	8 HOUR	24 HOUR
Between weeks	9	7.1290***	6.7085***	7.3190***
Linear regression	1	7.0236***	6.0483***	7.7134***
Deviations	8	0.1053***	0.6602***	0.1852***
Within weeks	10(n-1)-m	0.1239	0.1287	0.2143
Number of rats (n)		12	10	10
Missing values (m)		1	1	0
Regression coefficient		-3.3337	-3.2443	-3.5335

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y — BLANK IMPLANT 4 HOUR	8 HOUR	24 HOUR
Between weeks	9	5.3694***	4.6977***	7.7533***
Linear regression	1	5.3395***	4.6741***	7.7108***
Deviations	8	0.0299***	0.0235	0.0425*
Within weeks	10(n-1)-m	0.0685	0.2680	0.1924
Number of rats (n)		9	7	10
Missing values (m)		0	0	0
Regression coefficient		-3.1997	-3.3945	-3.6478

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y — INJECTION TP	OIL
Between weeks	9	4.7878***	3.9364***
Linear regression	1	4.7337***	3.8676***
Deviations	8	0.0541***	0.0689***
Within weeks	10(n-1)-m	0.0529	0.0646
Number of rats (n)		8	7
Missing values (m)		0	1
Regression coefficient		-3.1955	-3.1193

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE XXI

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 2

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 13-15)
X=age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y		
		TP IMPLANT		
		4 HOUR	8 HOUR	24 HOUR
Between weeks	2	0.017500***	0.010091**	0.013220*
Linear regression	1	0.017338***	0.009927***	0.013101*
Deviations	1	0.000162	0.000164	0.000119
Within weeks	3(n-1)-m	0.012800	0.010706	0.023491
Number of rats (n)		11	7	6
Missing values (m)		0	0	0
Regression coefficient		0.02807	0.02663	0.03304

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y		
		BLANK IMPLANT		
		4 HOUR	8 HOUR	24 HOUR
Between weeks	2	0.020553**	0.009709**	0.019080***
Linear regression	1	0.020517***	0.009697***	0.018945***
Deviations	1	0.000036	0.000012	0.000135
Within weeks	3(n-1)-m	0.034500	0.005416	0.003128
Number of rats (n)		9	5	6
Missing values (m)		0	0	0
Regression coefficient		0.03376	0.03114	0.03973

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y	
		INJECTION	
		TP	OIL
Between weeks	2	0.01922***	0.03033***
Linear regression	1	0.01898***	0.03028***
Deviations	1	0.00024	0.00005
Within weeks	3(n-1)-m	0.02581	0.02457
Number of rats (n)		11	12
Missing values (m)		0	0
Regression coefficient		0.02937	0.03552

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE XXII

BRIEF EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE: DAY 2

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 16-18)
X=age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y		
		TP IMPLANT		
		4 HOUR	8 HOUR	24 HOUR
Between weeks	2	0.000230	0.000730	0.000885
Linear regression	1	0.000022	0.000101	0.000564
Deviations	1	0.000208	0.000629	0.000321
Within weeks	3(n-1)-m	0.014600	0.008903	0.026597
Number of rats (n)		11	7	7
Missing values (m)		0	2	2
Regression coefficient		0.001000	-0.002791	0.006600

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y		
		BLANK IMPLANT		
		4 HOUR	8 HOUR	24 HOUR
Between weeks	2	0.000489	0.000787	0.000284
Linear regression	1	0.000025	0.000361	0.000009
Deviations	1	0.000239	0.000426	0.000275
Within weeks	3(n-1)-m	0.021058	0.007215	0.001223
Number of rats (n)		9	5	6
Missing values (m)		0	0	0
Regression coefficient		0.003727	-0.006010	0.000850

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y	
		INJECTION	
		TP	OIL
Between weeks	2	0.000282	0.000423
Linear regression	1	0.000113	0.000079
Deviations	1	0.000169	0.000344
Within weeks	3(n-1)-m	0.031248	0.022231
Number of rats (n)		11	12
Missing values (m)		0	0
Regression coefficient		-0.002262	-0.001809

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE XXIII

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

ANOGENITAL DISTANCE (mm) AT WEANING (21 days)°

TREATMENT	AGE AT WHICH TREATMENT COMMENCED								
	D2			D3			D5		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
TP IMPLANT[1]	4	10.0	0.3	6	9.3	0.4	2	7.9	0.2
TP INJECTION[2]	7	8.1	0.3	4	8.9	1.1	7	8.5	0.2
CONTROL[3]	11	9.1	0.3	8	9.6	0.5	3	7.6	0.1

TABLE XXIV

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

OVARIAN WEIGHT (mg) AT 100 DAYS°°

TREATMENT	AGE AT WHICH TREATMENT COMMENCED								
	D2			D3			D5		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
TP IMPLANT[1]	4	14.3	2.1	5	33.4	6.3	11	40.1	2.1
TP INJECTION[2]	7	49.8	3.1	4	51.8	5.4	7	41.1	4.0
CONTROL[3]	11	74.8	7.2	7	76.7	5.2	11	83.2	5.0

TABLE XXV

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

SEXUAL BEHAVIOUR (lordosis quotient) AT 19 WEEKS°°

TREATMENT	AGE AT WHICH TREATMENT COMMENCED								
	D2			D3			D5		
	n	Mean	SE	n	Mean	SE	n	Mean	SE
TP IMPLANT[1]	3	0.0	0.0	5	0.0	0.0	11	47.5	9.0
TP INJECTION[2]	7	13.6	5.6	4	15.0	6.5	5	28.0	14.7
CONTROL[3]	11	100.0	0.0	8	100.0	0.0	12	100.0	0.0

° All of the *a priori* contrasts were non-significant ($p > 0.05$)
°°Significance of differences presented in Chapter 5
[1] 2.5mm TP-filled PDS capsule plus 0.05ml oil injection
[2] 90μg TP in 0.05ml oil plus an empty capsule
[3] 0.05ml oil injection plus an empty capsule

TABLE XXVI

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

BODY WEIGHT (log base 10) FOR AGES 3 TO 20 WEEKS: GROUP MEANS AND STANDARD ERRORS

Group	Week	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DAY 2																			
IMPLANT[1]	Mean	1.559	1.745	1.928	2.042	2.079	2.110	2.152	2.162	2.184	2.203	2.218	2.243	2.236	2.246	2.250	2.264	2.265	2.256
	SE	.030	.033	.018	.010	.007	.008	.010	.006	.008	.006	.008	.008	.009	.008	.010	.009	.010	.012
INJECTN[2]	Mean	1.589	1.819	1.981	2.127	2.195	2.264	2.329	2.346	2.376	2.415	2.438	2.451	2.463	2.489	2.512	2.513	2.499	2.499
	SE	.023	.015	.029	.016	.019	.017	.016	.015	.016	.014	.014	.014	.014	.014	.014	.013	.012	.014
CONTROL[3]	Mean	1.574	1.795	2.042	2.134	2.179	2.235	2.301	2.317	2.344	2.387	2.403	2.419	2.431	2.455	2.478	2.485	2.477	2.472
	SE	.021	.018	.087	.016	.019	.015	.013	.033	.010	.011	.009	.010	.011	.012	.011	.013	.013	.013
DAY 3																			
IMPLANT	Mean	1.587	1.811	2.019	2.125	2.194	2.252	2.298	2.326	2.352	2.375	2.395	2.413	2.372	2.411	2.414	2.418	2.412	2.402
	SE	.038	.046	.027	.022	.026	.030	.031	.033	.036	.037	.038	.042	.035	.049	.051	.051	.049	.050
INJECTN	Mean	1.592	1.836	2.012	2.138	2.207	2.275	2.333	2.367	2.404	2.452	2.459	2.470	2.485	2.502	2.514	2.529	2.518	2.516
	SE	.075	.064	.045	.049	.028	.035	.040	.032	.033	.035	.035	.035	.034	.030	.027	.022	.023	.023
CONTROL	Mean	1.642	1.849	1.982	2.172	2.215	2.273	2.333	2.350	2.371	2.409	2.421	2.438	2.448	2.480	2.505	2.524	2.505	2.508
	SE	.030	.029	.017	.026	.018	.018	.021	.019	.018	.024	.022	.021	.021	.018	.017	.023	.023	.023
DAY 5																			
IMPLANT	Mean	1.682	1.869	1.979	2.145	2.220	2.290	2.340	2.378	2.397	2.435	2.459	2.473	2.489	2.492	2.511	2.496	2.506	2.515
	SE	.028	.248	.096	.014	.015	.015	.017	.017	.017	.018	.018	.018	.020	.019	.020	.020	.022	.024
INJECTN	Mean	1.570	1.819	1.952	2.098	2.195	2.262	2.317	2.352	2.381	2.405	2.442	2.456	2.463	2.493	2.515	2.529	2.537	2.539
	SE	.039	.027	.032	.023	.019	.013	.010	.010	.008	.015	.013	.008	.010	.011	.011	.009	.007	.005
CONTROL	Mean	1.670	1.857	1.925	2.129	2.212	2.268	2.312	2.339	2.361	2.382	2.402	2.421	2.435	2.458	2.489	2.464	2.476	2.487
	SE	.028	.020	.012	.013	.011	.011	.009	.010	.013	.011	.011	.011	.011	.011	.010	.009	.010	.010
SIGNIFICANCE OF DIFFERENCES BETWEEN GROUP MEANS																			
"F"		2.26 *	1.54 ns	0.43 ns	2.27 *	3.41 **	5.78 ***	6.23 ***	8.52 ***	7.93 ***	8.27 ***	9.29 ***	8.17 ***	11.41 ***	10.85 ***	12.43 ***	9.29 ***	8.90 ***	9.32 ***

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$; ns $p > 0.05$

[1] 2.5mm TP-filled PDS capsule plus 0.05ml oil injection
[2] 90µg TP in 0.05ml oil plus an empty capsule
[3] 0.05ml oil injection plus an empty capsule

TABLE XXVII

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 3-14)
X=reciprocal of age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TREATMENT ON DAY 2 IMPLANT	 INJECTION	 CONTROL
Between weeks	11	1.9318***	5.6623***	7.8670***
Linear regression	1	1.9115***	5.6428***	7.7853***
Deviations	10	0.0203*	0.0195	0.0817**
Within weeks	12(n-1)-m	0.0358	0.1586	0.2906
Number of rats (n)		4	7	10
Missing values (m)		0	0	0
Regression coefficient		-2.6080	-3.3855	-3.3341

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TREATMENT ON DAY 3 IMPLANT	 INJECTION	 CONTROL
Between weeks	11	4.3512***	3.3431***	5.6543***
Linear regression	1	4.3405***	3.3348***	5.6147***
Deviations	10	0.0107	0.0083	0.0396
Within weeks	12(n-1)-m	0.4292	0.2709	0.3358
Number of rats (n)		6	4	8
Missing values (m)		0	0	0
Regression coefficient		-3.2071	-3.4427	-3.1624

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TREATMENT ON DAY 5 IMPLANT	 INJECTION	 CONTROL
Between weeks	11	7.3377***	6.1088***	7.1289***
Linear regression	1	7.0532***	6.0735***	7.0238***
Deviations	10	0.2845***	0.0353	0.1051*
Within weeks	12(n-1)-m	0.4399	0.2318	0.2921
Number of rats (n)		11	7	11
Missing values (m)		10	0	1
Regression coefficient		-3.1570	-3.5757	-2.9312

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE XXVIII

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 15-17)
X=age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y — TREATMENT ON DAY 2		
		IMPLANT	INJECTION	CONTROL
Between weeks	2	0.000425	0.008675	0.011315*
Linear regression	1	0.000403	0.008665*	0.011314**
Deviations	1	0.000022	0.000010	0.000001
Within weeks	3(n-1)-m	0.003168	0.025319	0.034563
Number of rats (n)		4	7	10
Missing values (m)		0	0	0
Regression coefficient		0.00710	0.02488	0.02379

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y — TREATMENT ON DAY 3		
		IMPLANT	INJECTION	CONTROL
Between weeks	2	0.005410	0.001750	0.012947
Linear regression	1	0.004306	0.001726	0.012871*
Deviations	1	0.011040	0.000024	0.000076
Within weeks	3(n-1)-m	0.124604	0.033668	0.058204
Number of rats (n)		5	4	8
Missing values (m)		0	0	0
Regression coefficient		0.02075	0.01469	0.02836

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y — TREATMENT ON DAY 5		
		IMPLANT	INJECTION	CONTROL
Between weeks	2	0.003256	0.009667**	0.018172**
Linear regression	1	0.002693	0.009584**	0.018046**
Deviations	1	0.000563	0.000083	0.000126
Within weeks	3(n-1)-m	0.128785	0.013690	0.045895
Number of rats (n)		11	7	12
Missing values (m)		0	0	0
Regression coefficient		0.01106	0.02616	0.02742

*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$

TABLE XXIX

PROLONGED EXPOSURE OF NEONATAL RATS TO TESTOSTERONE PROPIONATE

SUMMARY OF REGRESSION ANALYSES OF BODY WEIGHT AND AGE (WEEK 18-20)
X=age; Y=log(base 10) body weight

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TREATMENT ON DAY 2		
		IMPLANT	INJECTION	CONTROL
Between weeks	2	0.000145	0.000968	0.000891
Linear regression	1	0.000099	0.000739	0.000863
Deviations	1	0.000046	0.000229	0.000028
Within weeks	3(n-1)-m	0.001859	0.021724	0.043894
Number of rats (n)		3	7	10
Missing values (m)		0	0	0
Regression coefficient		-0.004066	-0.007264	-0.006570

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TREATMENT ON DAY 3		
		IMPLANT	INJECTION	CONTROL
Between weeks	2	0.000654	0.000388	0.000167
Linear regression	1	0.000642	0.000326	0.001029
Deviations	1	0.000012	0.000062	0.000862
Within weeks	3(n-1)-m	0.149777	0.018963	0.088426
Number of rats (n)		5	4	8
Missing values (m)		0	0	0
Regression coefficient		-0.008010	-0.006387	-0.008018

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES OF Y TREATMENT ON DAY 5		
		IMPLANT	INJECTION	CONTROL
Between weeks	2	0.003698	0.000448	0.002983
Linear regression	1	0.003575	0.000400	0.002977
Deviations	1	0.000123	0.000048	0.000006
Within weeks	3(n-1)-m	0.135760	0.006105	0.375920
Number of rats (n)		10	7	12
Missing values (m)		0	0	0
Regression coefficient		0.01377	0.005342	0.011137

All of the regression coefficients were non-significant ($p > .05$)

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