Studies on Estrogen Uptake and Estrogen Receptors in the Male Genital Organs

Possible Physiological Role of Estrogen in the Accessory Sex Organs

(SUMMARY)

THESIS SUBMITTED TO THE
ALIGARH MUSLIM UNIVERSITY, ALIGARH
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CHEMISTRY

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SUMMARY

It has long been recognized that the male accessory genital organs are dependent upon androgenic hormones for maintenance of their cellular integrity and complex functions. It is also known for some years that measurable levels of circulating estrogens are present in male mammals which suggests the possibility that estrogen(s) also may have a role in regulating physiological processes in the male, either directly or by modulating the action of androgen. Thus, the physiological effects of sex hormones in the male genital organs may reflect the combined effects of the estrogenic and androgenic hormones. One of the significant developments made during the last fifteen years in the study of molecular mechanism(s) of steroid hormone action is the discovery of proteins that can selectively bind biologically active steroids in the target cells and these proteins have been designated as steroid receptors. In experimental animals, injected radioactive estrogen has been observed to accumulate selectively in target tissues and it has been speculated that estrogen responsive target tissues contain a specific binding protein to account for such observations. This specific binding protein has been defined as estrogen receptor.

In recent years mammalian epididymis is receiving concerted attention of the investigators. Understanding of the molecular control of epididymal function is essential to identify new approaches to fertility regulation in male. The present studies
are concerned with the demonstration of presence of estrogen receptors and the physiological role of estrogen in the epididymis of rat. A preliminary study on these aspects was carried out on the epididymis of rhesus monkey as well. In both these studies prostate was also investigated for the purpose of comparison. The estrogen induced biochemical changes in the epididymis were compared with those observed after androgen treatment. The results of the present study are summarised below.

In the first series of experiments the uptake and retention of $^{3}H$ estradiol by the epididymis (caput, corpus and cauda portions) and vas deferens was investigated, and for comparison two other accessory genital tissues viz. seminal vesicles and ventral prostate were also investigated. The distribution of the labelled hormone in the various accessory sex organs was examined in adult orchidectomised (castrated for 48 hr) rats following intravenous administration of the steroid. At 30 min. the epididymis and vas deferens showed relatively higher uptake as compared to other organs and at all intervals examined (15, 30, 60 min. and 6 hr) the corpus epididymis and vas deferens retained maximum radioactivity. The accumulation of estrogen in the accessory sex organs was against a concentration gradient. There was a marked reduction in the uptake of labelled estradiol by all the accessory genital organs following long-term (21 days) castration and androgen treatment for seven days prior to autopsy restored the uptake to varying
degrees in different organs.

In the second series of experiments, studies were carried out to demonstrate the presence of estrogen receptors in the immature rat epididymis and ventral prostate and also the biological response of the epididymis to estrogen during prepubertal period was investigated. A receptor protein that selectively binds estrogens was demonstrated in the cytosol of the epididymis and ventral prostate of prepubertal (21 day old) rats. Dihydrotestosterone and cyproterone acetate did not compete with (H) estradiol-17/3 for the estrogen receptors, but estradiol and diethylstilbestrol were effective competitors. Scatchard plot analyses were linear, suggesting a single class of high affinity binding sites for estradiol with a Kd of 5.45 x 10^-11 mol/l and a concentration of binding sites of 20 fmole per mg cytosol protein in the epididymis. In the cytosol of the ventral prostate, the concentration of the receptors was 10.2 fmole per mg protein with a Kd of 5.43 x 10^-11 mol/l.

In intact prepubertal rats a seven-day treatment (28-day-old at autopsy) with estradiol (1.0/µg) caused a slight reduction in the weight of the epididymis but the concentration of glycerylphosphorylcholine (GPC) and sialic acid (indices of epididymal secretory function) remained unaltered. In prepubertal rats castrated for 7 days, treatment with estradiol (1.0/µg) for 7 days (35-day-old at autopsy) resulted in a marked increase
in the weight and content of GPC and sialic acid in the epi-
didymis; estradiol and dihydrotestosterone were equipotent in
causing an increase in sialic acid concentration. The study
further revealed that estradiol has some synergistic role in
influencing the androgen action on the secretory function of the
prepubertal rat epididymis (and growth of the prostate).

In the third series of experiments, an estrogen binding
protein was demonstrated in the cytosol of the epididymis and
caudal prostate of immature rhesus monkey. Scatchard plot
analyses were linear, suggesting a single class of high affinity
binding sites for the synthetic estrogen, R-2858 (moxestrol)
with a Kd of 5.88 x 10^{-10} \text{ mol/l} and a concentration of binding
sites of 17.0 \pm 4.4 \text{ fmol/mg cytosol protein} in the epididymis.
In the prostate the concentration of binding sites was 19.7 \pm 3.6
\text{ fmol/mg cytosol protein} with a Kd of 5.42 \times 10^{-10} \text{ mol/l}. In
immature castrated monkeys, administration of estradiol dipro-
pionate caused a significant increase in the weight, total
protein and sialic acid content of the epididymis.

In undertaking the present investigations on the role of
estrogen and estrogen receptors in the male accessory genital
organs, it was assumed that the two receptor proteins (androgen
and estrogen) are distinct. If estrogen promotes the secretory
activity of the epididymis, the question that follows is why
there is need for two different hormones (estrogen and androgen)
to perform the same function (stimulation of secretion). While
working with estrogen receptor system in the male accessory genital organs, one encounters practical difficulties in choosing an experimental design. The current dogma is that the structural and functional integrity of the male accessories is androgen dependent. If estrogens do influence the functioning of male accessories through an independent estrogen receptor system (as progesterone does in the estrogen primed uterus), one has to take into consideration the possibility that estrogen receptor system may also be dependent on androgen action (as progesterone receptor system is dependent on estrogen action in the uterus). It follows then that if androgens regulate the estrogen receptor level in the male genital organs, the estrogen action in the functioning of male accessories may not be autonomous to estrogen.

Since the precise role of estrogen in the male is not clearly understood, the studies on estrogen receptors in the male genital organs are likely to be criticised on the grounds that estrogen receptors only bind the hormone and that by measuring the receptors, one is not measuring the effect of the estrogen upon cells of male sex accessories. That is to say that one is measuring what is available to bind estrogen in the "androgen target" organs rather than the function of estrogen itself. The only explanation that can be offered to answer this criticism is that if we accept that the "receptor" is a machinery, a mechanism between the hormone and its effect,
determination of its "concentration" in the cystosolic fraction gives an index of the potential of the cells to respond to the specific hormone. In the absence of specific end response that could be used as an index of estrogen action, the growth and the secretory activity of the epididymis (and prostate) were used as a measure of estrogen action. On the basis of the results of the present study it is suggested that estrogen may have a physiological role in androgen target organs. Additionally, a synergistic relationship may exist between estrogen and androgen that is secondary to their interaction with their respective receptors in the accessory genital organs of the male.
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With Profound Regards
Dedicated to
my Grand Father
Honble Hafiz Mohd. Ibrahim
Whose memory is my treasure
This is to certify that the investigations reported in this thesis entitled "Studies on Estrogen Uptake and Estrogen Receptors in the Male Genital Organs: Possible Physiological Role of Estrogen in the Accessory Sex Organs" were carried out by Mrs. Nuzhat Kamal (Nee: Nuzhat Pervaze Rehman), M.Sc., M.Phil., under my supervision. She fulfills the requirements laid down by the Aligarh Muslim University, Aligarh for the Ph.D. degree in Chemistry.

The work included in this thesis is original and has not been submitted for any other degree.

B. S. Setty
(B.S. SETTY) 15.9.83
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CHAPTER FOUR

Biological Action of Estrogen on the Epididymis of Prepubertal Rhesus Monkey - A Preliminary Study on Estrogen Binding Protein in the Epididymis and Prostate

Introduction

Materials and Methods

Studies on Estrogen Binding Protein

Studies on Estrogen Induced Response

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The present studies are concerned with the physiological role of estrogen in the accessory genital organs of the male rat and rhesus monkey. The investigations were mainly concentrated on the epididymis of rat and rhesus monkey.

Objectives:
(a) To study the uptake of (3H) estradiol-17β by the accessory sex organs of rat
(b) To demonstrate presence of estrogen receptors in the epididymis and to establish their identity as distinct from androgen receptors
(c) To study the relative affinity of some steroidal and nonsteroidal compounds
(d) To study hormonal regulation of estrogen uptake and estrogen receptors in the epididymis and
(e) To study the biological response of epididymis to estrogen.

The thesis is divided into four chapters.

In the first chapter a brief survey of pertinent literature is presented. Special attention is paid on the biological actions of estrogen in the male accessory genital organs.
The remaining three chapters are devoted to results of the present study.

The second chapter deals with the studies on the uptake and retention of (3H)-estradiol-17β by the accessory genital organs of the male rat. Effect of long term castration and androgen replacement therapy constitutes a part of the study.

The third chapter is concerned with the demonstration of estrogen receptors in the epididymis and ventral prostate of prepubertal rat and biological response of the epididymis to estrogen and androgen.

The fourth chapter deals with the results of a preliminary study on estrogen binding protein in the epididymis and prostate of prepubertal rhesus monkey. The results of a cognate study on the biological action of estrogen and androgen on the epididymis of immature castrated monkey are also included in this chapter.
"Most investigators who have speculated about the mode of action of steroids whether they believe the effect is by activating an enzyme, by altering the permeability of a membrane, or by serving as a coenzyme in a given reaction have emphasized the physical binding of the steroid to a protein as an essential part of the mechanism of action or a preliminary step to that action. They have in this way explained the specificities, synergisms, and antagonisms of the several steroids in terms of the formation of specific steroid-protein complexes. The differences between different target organs, e.g., those respond to androgens and those respond to estrogens, can be attributed to differences in the distribution of the specific proteins involved in these binding reactions"

Villee (1961)

The major testicular androgen, testosterone serves as a circulating precursor for the formation of 5α-dihydrotestosterone which is the active hormone in the target organs. In the male, there is also evidence that the testis of many species, including human, synthesizes estrogens (Fig. 1).

Characteristic steroid secretory functions of the adrenal, testis and ovary reflect differences in their histogenesis and the trophic hormones to which they respond. Qualitatively their steroidogenic potentials are similar. Ryan (1972) has summarised the evidence for a "unified concept of steroid
FIG. 1 PRINCIPAL SEX STEROIDS
formation" in these tissues (see Fig. 2). Biosynthetic pathways from acetate to estrogen have been mapped in each case. The final enzymatic sequence entails the conversion of 3-oxo-4-unsaturated C\textsubscript{19} steroids (testosterone and androstenedione) to phenolic C\textsubscript{18} steroids (estradiol and estrone). This step is catalysed by estrogen synthetase (aromatase) enzymes. In addition, circulating androgens are aromatised to estrogens in the peripheral tissues.

A. Concept of Hormone Receptor

Investigations on the mode of action of sex steroids can be divided into two types of approach: (1) Examination of the nature and chronology of the biochemical and biosynthetic responses induced by the hormones and (2) study of the fate of the steroid hormone in the organism and particularly in 'responsive' or target tissues. One of the significant developments made during the last fifteen years in the study of molecular mechanism(s) of steroid hormone action is the discovery of proteins that can selectively bind biologically active steroids in the target cells and these proteins have been designated as steroid receptors. Steroid hormone action is restricted to those cells that contain appropriate intracellular receptor proteins. The available evidence indicates that despite the remarkable diversity of effects of hormones, the major features of both the primary interaction of steroids
FIG. 2  THE UNIFIED CONCEPT OF STEROID FORMATION
with the target cells and the onset of response may be similar in a variety of tissues.

B. Hormonal Regulation of Male Sex Accessory Organ Function

The structure and physiology of the accessory sex glands of the male depend in a precise and delicate fashion on the level of testosterone or its metabolites. Many recent studies have indicated that male reproductive organs contain both cytoplasmic and intranuclear macromolecules that specifically and very strongly bind dihydrotestosterone (a metabolite of testosterone) by noncovalent forces. These macromolecules are the "receptor" proteins mentioned above. It is known that estrogens are directly and indirectly antagonistic to androgen effects on the accessory sex organs. Evidence is now available to suggest that estrogens may have also a direct stimulatory influence on the male genital organs.

C. Presence of similar Hormone receptors in different target tissues

Though the basic concept of steroid-receptor interaction and its physiological significance have been investigated extensively only in rat uterus and ventral prostate, numerous studies have been carried out during the last few years on steroid receptors in other genital tissues of the male. Along with the prostate, the seminal vesicles and epididymis have
long been known to be androgen dependent tissues. It is also known that testosterone is essential for normal spermatogenic activity. Enough evidence has accumulated indicating that seminiferous tubules (Hansson et al., 1976), epididymis (Ritzen et al., 1975), seminal vesicles (Mainwaring, 1977) and prostate (Liao, 1977), contain intracellular receptors, characteristic of androgen responsive tissues.

D. Presence of androgen receptors in the female genital system and estrogen receptors in male genital system

The physiological significance of estrogen synthesis in the testis and androgen synthesis in the ovary is not clearly understood. Similarly, the role of hormone receptors in the genital tissues of opposite sexes, e.g. androgen receptors in the rat uterus (Ruh et al., 1973) and estrogen receptors in rat prostate (Dubois et al., 1980) remains enigmatic. The fact that estradiol can induce progesterone receptor suggests that uterine cells which synthesise it contain also an estradiol receptor (Baulieu, 1975). Admittedly, this is relatively a simple situation in the uterus since it has been known for a number of years that estrogen priming is essential for progesterone to act on the uterus. A similar explanation is not readily available to appreciate the importance of estrogen receptors in the regulation of male genital organ function. Recently, Jehan and Setty (1977) have demonstrated that rat
epididymal secretory function can be stimulated by administering estrogen to castrated rats. This interesting observation prompted the investigator to undertake the present study with a view to examine if the epididymis is indeed a target organ for estrogen.

"the physiological consequences of circulating testosterone represent the sum total of the combined effects of the estrogen and androgen metabolites of the parent molecule"

Wilson (1975)
REFERENCES


REVIEW OF PERTINENT LITERATURE
Section A: The Male Genital System

The genital system of male mammals consists of three essential components (Fig. 1). (1) The testis, the primary sex organ which performs a dual function - gametogenic and endocrine. (2) The sex accessory tract comprising a series of organs viz. the epididymis, vas deferens, seminal vesicles and prostate. The spermatozoa are transported from the testes and are stored in the epididymis. The secretions of seminal vesicles and prostate provide the vehicle, seminal plasma for carrying spermatozoa at the time of ejaculation. (3) The external genitalia include penis, the copulatory organ and scrotum which accommodates the testes. All these organs share a sensitivity to the sex steroids, for differentiation and maintenance of their structural and functional properties. Removal of androgenic stimulus by castration produces marked structural alterations and depression of functional activities and most of these changes can be reversed by administration of exogenous androgenic steroids.

Accessory genital organs used in the present study:

In recent years the mammalian epididymis is receiving concerted attention of the investigators. Understanding of the molecular control of epididymal function is essential to identify new approaches to fertility regulation in male.
FIG. I: MALE GENITAL SYSTEM OF A NORMAL ADULT RAT.
The epididymis, a single, highly convoluted duct, is divisible on morphological grounds into three parts viz. caput, corpus and cauda. Caput (head) is the enlarged proximal portion connected to the testis by a number of efferent ducts, corpus (body) the middle narrower portion extending down along the posterolateral border of the testis and distal or cauda (tail) region which in turn opens into the narrow tube, the vas deferens. The growth, differentiation and functional maturation of the mammalian epididymis are androgen dependent events (Orgebin-Crist et al., 1975; Dhar and Setty, 1976; Setty and Jehan, 1977; Riar et al., 1977). Androgen is essential for normal maturation of spermatozoa within the epididymis (Blaquier et al., 1972; Dyson and Orgebin-Crist, 1973). The epididymis is lined with a pseudo-stratified epithelium and 'principal' cells carry out all the important functions. In sexually mature animals the epididymis is a dynamic structure, exhibits regional differences in histological features, secretory and absorptive capacities and quantitative differences in biochemical characteristics (Glover and Nicander, 1971; Hamilton, 1975; Setty, 1979).

Epididymis has four major functions: transport, concentration, maturation and storage of the spermatozoa. The function of the epididymal epithelium is considered to be in part absorption and in part secretion. The sperms are transported from the rete testis to the efferent ducts by the fluid
pressure in testis. Their passage through the efferent ducts is aided by the active onward beating cilia of the ciliated cells and in the epididymal duct by the peristaltic movements of musculature in the wall. From the dilute sperm suspension originating in the testis, water is absorbed into the epithelial cells, during the passage through the epididymis, especially in caput, and a highly concentrated sperm suspension is left in the tail of the epididymis. The sperm mature in the epididymis i.e. they acquire motility and fertilizing ability (Setty, 1979). Maturation is probably achieved as a result of certain secretions from the epithelial cells stimulated by androgen. The tail of the epididymis is the sperm depot.

The prostate:

The rat prostate consists of three distinct lobes. Owing to the relative locations in reference to the urethra, they are designated as ventral, dorsal and lateral lobes; cranial or anterior lobes are referred to as coagulating glands which are bound to the seminal vesicles; its fructose content serves as a useful marker of androgenic status (Mann, 1964). Biochemical and physiologic differences between the dorsal, lateral and ventral prostatic lobes have been observed that indicate that the lateral lobes or the dorsal lobes are more sensitive to prolactin, have a higher citric acid content and are rich in zinc (Price and Williams-Ashman, 1961; Grayhack and Lebowitz, 1967; Negro-Vilar et al., 1977). Ventral
prostate is very sensitive to androgen stimulation and its
growth response is commonly used in bioassay of androgenic
activity (Dorfman and Shipley, 1956). The ventral prostate of
rat is often used as a means of studying the basic aspects of
prostate physiology (Coffey, 1974; Tushimaa and Niemi, 1974;
Rajfer and Coffey, 1978). It consists of two discrete lobes
that are attached to the urethra by a layer of connective tissue
and by a series of ducts lined with mainly cuboidal epithelium
that drain into the urethra. The organ is rich in alkaline
phosphatase activity (Price and Williams-Ashman, 1961).

The human prostate is considered to be composed of four
separate regions (McNeal, 1981). (1) The peripheral zone which
constitutes over 70% of the glandular prostate. Almost all
carcinomas arise here. (2) The central zone constitutes 25%
of the glandular prostate. (3) Preprostatic region and (4) the
anterior fibromuscular stroma. The prostate of the rhesus
monkey consists of 2 anatomically discrete lobes, a cranial and
a caudal, the cranial lobe equating with the central zone in the
human and the caudal lobe with the peripheral zone (Ghanadian
et al., 1977). The surface of the cranial lobe is deeply
furrowed, resembling that of the seminal vesicles while the
caudal lobe is more closely applied to the urethra and has a
smooth surface. The caudal lobe shows a significantly higher
uptake of \(^{3}\text{H}\)-testosterone (Ghanadian et al., 1977) and shows
a high concentration of zinc (Setty et al. unpublished results).
Prostatic secretion is rich in acid phosphatase activity (Mann, 1964). Epididymis and prostate (Ventral lobe in rat) have been chosen by the present investigator to study the possible role of estrogen in the physiology of accessory genital organs of the male.
Section B: Steroid hormones of the testis:

The function of the testis is concerned with the production of spermatozoa and hormones. The tubular apparatus (spermatic epithelium) is responsible for the manufacture of spermatozoa, and Leydig cells of the interstitial tissue gives rise to the hormones. The mammalian testis produces androgens and estrogens. They are biosynthesized from cholesterol, which in turn, is made in vivo from acetyl coenzyme A. The two main steroid hormones secreted by the testis with androgenic properties are testosterone and \( \Delta^4 \)-androstenedione. The third androgen, 5\( \alpha \)-dihydrotestosterone (DHT) is secreted in small amounts but is mainly formed by the peripheral conversion of testosterone. Estrogens are also secreted by the testis and estradiol-17\( \beta \) synthesis has been demonstrated in the testes of several species (van der Molen et al., 1981) (Fig. 2). There are two pathways for biosynthesis of testosterone (Fig. 3). It may be synthesized from cholesterol via "\( \Delta^5 \)-pathway" involving pregnenolone, 17\( \alpha \)-hydroxy pregnenolone, dehydroepiandrosterone and 5-androstene 3/5, 17/3 diol, as intermediates or via a "\( \Delta^4 \)-pathway" involving pregnenolone, progesterone, 17\( \alpha \)-hydroxyprogesterone and androstenedione.

Various workers have attempted to determine the relative importance of \( \Delta^4 \) and \( \Delta^5 \)-pathways in testis. In human fetal and canine testis the \( \Delta^5 \)-pathway appears to be the favourable
FIG. 2. PRINCIPAL ACTIVE METABOLITES FORMED FROM TESTOSTERONE BY THE TESTIS AND THE PERIPHERAL TISSUES
FIG. 3 ANDROGEN SYNTHESIS PATHWAYS
one. In contrast, the $\Delta^4$-pathway is more important in rat testis. Androstenedione and testosterone are converted to estrone and $17\beta$-estradiol, respectively, by a closely integrated series of reactions collectively referred to as aromatization. The administration of chorionic gonadotrophin (hCG) to adult men stimulates and increases the output of estrogen and androgen (van der Molen et al., 1981). The Leydig cells of the interstitial tissue of the testis are responsible for the bulk of androgen production from endogenous cholesterol and this process is regulated by ICSH, also called luteinizing hormone or LH. Sertoli cells may also elaborate steroids; they have been implicated as sites of testicular estrogen biosynthesis which is regulated by follicle stimulating hormone (FSH).

**Estrogens in plasma:**

The plasma concentration of estrogens in the adult male is distinctly lower than that of testosterone. In humans, the concentration of plasma testosterone is in the range of 500 ng per 100 ml, and that for estradiol is between 1.6 and 4.5 ng per 100 ml (Pirke and Doerr, 1973; Hawkins and Oakey, 1974; Hang et al., 1974; Kley et al., 1974). Plasma estrone concentrations generally are 1.5 to 2 times higher than for estradiol (Hawkins and Oakey, 1974; Kley et al., 1974). The daily production rates for testosterone and estrogens are as follows: testosterone, 7 mg per day; estrone 150 $\mu$g per day; and
estradiol, 50 μg per day (Baird et al., 1969). In contrast to the direct testicular secretion of testosterone, plasma estradiol and estrone are derived primarily from peripheral metabolism of androgens. In the testis of men, testosterone is converted to estradiol and androstenedione to estrone (Longcope et al., 1969), releasing estradiol (97.2-892.0 pg/ml) and estrone (30.0-234.8 pg/ml) into spermatic venous blood (Longcope et al., 1972). It is estimated that direct testicular secretion of estradiol contributes only 20 per cent, at most, of the total daily production (Siiteri and MacDonald, 1973). The conversion of androstenedione to estrone is the main source of estrone entering the body (Lipsett, 1974). Despite the rather small conversion rates of testosterone to estradiol (0.4 percent) and androstenedione to estrone (1 to 1.7 per cent) (Siiteri and MacDonald, 1973; Longcope et al., 1969), extragonadal conversion or aromatisation of androgen provides the major portion of circulating estrogen and this appears to occur in fat (Bolt and Gobel, 1972; Schindler et al., 1972), hair follicles (Schweikert et al., 1975) and neural tissue (Naftolin et al., 1971) (Fig. 4). The testicular contribution to estradiol production rate in dog and monkey (Kelch et al., 1972) is similar to that in man. It is of some interest that both adrenal and testicular androgens act as precursors for estradiol (Longcope et al., 1969; Siiteri and MacDonald, 1973). In male rat blood production rate of 51 ng per day of estradiol is reported (de Jong et al., 1973) of which 21 per cent is testicular contribution. Thus in men
GONADS

ANDROSTENEDIONE → TESTOSTERONE

ESTRONE → ESTRADIOL

ANDROSTENEDIONE + TESTOSTERONE

AROMATASE

BRAIN, FAT, LIVER,
KIDNEY, BONE,
LUNG, THYMUS,
SKIN, MUSCLE.

ESTRADIOL +
ESTRONE

CIRCULATING
ESTROGENS

FIG 4: PHYSIOLOGIC SOURCES OF ESTROGEN IN THE MALE
and commonly used laboratory animals, estradiol-17β is the main biologically active estrogen which is secreted by the testes. Since adrenal androgens can serve as precursors for estrogen formation (Longcope et al., 1969) the fact that DHT or testosterone produces a given effect in a castrated animal does not necessarily mean that it acts alone, the net result may be the synergistic effect of estrogen and androgen.

**Cellular origin of testicular estradiol:**

Testosterone and estradiol are preferentially synthesized in different compartments of the testis. de Jong et al. (1973) have isolated seminiferous tubules and interstitial tissue from adult rat testis. Prior to incubation estradiol concentrations in the interstitial tissue were considerably higher (9-15 times) than in seminiferous tubules. During incubation, the concentration of estradiol increased in the seminiferous tubules but not in interstitial tissue, whereas testosterone concentrations increased in the interstitial tissue. Interstitial tissue but not isolated seminiferous tubules, Sertoli cells are able to utilize cholesterol and pregnenolone to synthesise androgens (Hall et al., 1969; Cooke et al., 1972; Moyle and Ramachandran, 1973).

Dorrington and her colleagues (1978) demonstrated that Sertoli cells isolated from the seminiferous tubules of 20 day old rats synthesized estradiol when maintained in culture in
the presence of testosterone and FSH. It has been suggested that in the testis FSH regulates aromatase activity at a site distinct from LH action. Thus, LH influenced testosterone is produced by the Leydig cells and then transported to the lymphatic spaces surrounding the seminiferous tubules to the Sertoli cells where it is aromatised under the influence of FSH. By contrast, Cannick et al. (1979) and Valladares and Payne (1979) have reported estrogen synthesis in the interstitial tissue rather than the seminiferous tubules and this was stimulated by LH rather than by FSH (Rommerts et al., 1978). The relative contribution of Leydig cells to the total estrogen output at various stages in male is still not clear.

Aromatase activity is relatively low in Sertoli cells from rats older than 20 days (Dorrington et al., 1978) and high enzyme activities can be detected in interstitial cell fractions from adult rats after stimulation with hCG in vivo (Cannick et al., 1979; Valladares and Payne, 1979). These results suggest that the cellular distribution of aromatase activity may change during maturation of testis. More important, the capacity of the Leydig cells to synthesise estradiol suggests that Sertoli cells are not obligatory for estradiol production in adult rats. In the immature rat, however, estradiol synthesis appears to be mainly dependent on the FSH-dependent aromatase present in Sertoli cells (Rommerts et al., 1982). It is now known that aromatisation involves the loss of
the angular C-19 methyl group and cis elimination of the 1/3 and 2/3 hydrogens from the androgen precursors, androstenedione and testosterone to yield estrone and estradiol, respectively (Fig. 5).

Endocrine function of rat testis in prepubertal rat:

Since we have used immature rat epididymis and ventral prostate to demonstrate presence of estrogen receptors, it is pertinent to review endocrine environment prevailing during this period. There is a general agreement that Leydig cell number begins to increase around day 20 in the rat (Clegg, 1966; Knorr et al., 1970; Lording and deKretser, 1972; Pahnke et al., 1975). Metabolic studies using radioactive precursors have revealed that the Leydig cells of immature rat predominantly secrete steroids of the androstane and pregnane series and only relatively small quantities of testosterone until about day 45 of age (Folman et al., 1973; Matsumoto and Yamada, 1973; Strickland et al., 1970). Androsterone and 5α-androstane-3α, 17β-diol are the predominant circulating androgens in the prepubertal period (upto 7th week) of rat (Moger, 1977, 1977) and relatively small quantities of testosterone are produced. Estradiol levels in testes and in serum are also reported to be relatively high during prepubertal period (Dohler and Wuttke, 1975; Rommerts et al., 1982) as compared to adult animals.
FIG. 5: PATHWAYS OF ESTROGEN BIOSYNTHESIS IN GONADS
Section C: The Mechanism of Hormone Action

Sequence of events:

There are three types of naturally occurring proteins that have specific property of binding steroid hormones with selectively high affinity. Serum "transport" proteins, "receptor" proteins of target tissues, and steroid converting "enzyme" proteins. Sex steroid hormones, after their secretion in the testis and ovary, are transported in the plasma bound to "transport" proteins as relatively stable complexes. The initial action of a sex steroid involves entry of the "free" hormone into the cells of the target organ. In the target cells the active steroid interacts with an intracellular specific binding protein called, for convenience the "cytosol" receptor, since it is easily obtained in the soluble fraction of the tissue homogenate after high speed centrifugation of all particles, and is therefore probably originally located in the cytoplasm. The binding of the sex steroid with high affinity to the cytoplasmic receptor protein (which is specific for each individual hormone), results in the formation of a hormone-receptor-complex (step 1) and translocation of the complex to the cell's nucleus (step 2). The discovery that hormones accumulate in nuclei of target cells was an important development in our understanding of the mechanism of hormone action. This two step mechanism of the steroid receptor
interaction was independently proposed by Gorski et al. (1968) and Jensen et al. (1968). Two techniques were instrumental in our understanding of these fundamental aspects of hormone-receptor interaction: (1) gradient ultracentrifugation of low salt cytosol extracts and (2) the rational use of adsorbents (e.g. charcoal) according to the differential dissociation principle (Baulieu, 1972).

In the nucleus, the complex binds to a large number of sites on chromatin. The chromatin binding site can be classified as either acceptor site or non-acceptor site. Acceptor site is generally visualized as specific complexes of chromosomal proteins, probably non-histone proteins, which the receptor-hormone-complexes recognise and bind with high affinity. Non-acceptor sites are the secondary sites on chromatin where the receptor-hormone-complex can bind with a lower affinity. The hormone-receptor complex in the nucleus interacting with nuclear acceptors presumably triggers the main hormonal effects by modifying gene transcription. This is the "classical" model of steroid hormone action which is probably the most popular among those working in this field. It is possible to interpret this initial interaction of hormone-receptor-complex with nuclear components in more than one model of hormone action (Gorski et al., 1976). The receptors appear to reside in the cytoplasm pending availability of their respective steroids and then bind to chromatin site after formation of the complex for
binding to the nucleus.

**Mechanism of estrogen and androgen action:**

Receptors have been found for each of the five physiologically well defined steroid hormones. The story of the estrogen receptors is almost the history of steroid receptors. As was the case in the discovery of estradiol itself 50 years ago, the female hormone was the first to be studied when the time came to elucidate the mechanism of sex steroid action. The rat ventral prostate and rat uterus respond to androgen(s) and estrogen(s) respectively in convenient, consistent and specific ways. These two organs have served as excellent model tissues in studies related to mechanisms of hormone action and the present state of knowledge is briefly reviewed.

In the rat uterus, estradiol-17β, the major blood estrogen, is retained without metabolic conversion (Jensen and Jacobson, 1962). It was found that immediately after the injection of a physiological dose of radioactive (3H) estradiol-17β into immature rats, uptake of the radioactive steroid by various tissues appears to reflect blood estrogen concentrations. The radioactivity then disappears rapidly from the blood and tissues less sensitive to estrogens (kidney, liver, muscle and diaphragm), but only slowly from target tissues such as uterus and vagina.
When testosterone with high specific radioactivity became available, the high affinity of the target tissues toward androgen could be demonstrated clearly (Tveter and Attramadal, 1968; Fang et al., 1969). When rats were injected with radioactive testosterone, clear retention was observed in ventral and dorsal prostates, seminal vesicles and coagulating glands at 30 minutes to 3 hours from the time of \(^{3}H\) testosterone injection. On the other hand, the radioactivity in the blood, spleen, lung, thymus and diaphragm is rapidly cleared within the first hour (Liao, 1975). An important difference in the male genital system from that of the female is that in the former testosterone is rapidly converted to 5α-dihydrotestosterone (DHT) by a 5α-reductase and it is now well established that DHT is the active hormone at cellular level (Tymoczko and Liao, 1976). These basic observations suggested that estrogens and androgens are concentrated and selectively retained by their respective target organs. We now know that this selective uptake and retention of sex steroids by the reproductive tissues is due to the intrinsic properties of the intracellular "receptor" of the target cells.

Upon ultracentrifugation on a sucrose gradient, cytoplasmic estrogen receptor sediments as a discrete band with a sedimentation coefficient of 8S (Gorski et al., 1968). \(^{3}H\)-estradiol-17β retained by the uterine nuclear fraction which appears to be associated with a protein that can be solubilized...
by extraction with 0.3-0.4 M KCl at pH 7.4-8.5 (Jungblut et al., 1967). The solubilized estradiol-receptor complex sediments at about 5S (Jensen et al., 1969).

Analogous to the 17/β-estradiol receptor complex of the rat uterus, \(^{3}\)H DHT-protein complexes having sedimentation constants of 8 to 9S have been detected in the cytosol preparation of rat ventral prostate (Liao, 1975). Baulieu et al. (1971) reported that their 8-10S complex also binds testosterone to a significant extent, but not 17/β-estradiol. The \(^{3}\)H DHT retained by prostate cell nuclei appears to bind to a protein that can be extracted with 0.4 to 0.6 M KCl solution (Liao and Fang, 1969). The sedimentation constant of the DHT-bound protein is about 3S (Fang and Liao, 1969), which appears to be somewhat smaller than the 5S estradiol-17/β receptor complex of the uterine nucleus mentioned in the previous paragraph. Although the different values of sedimentation constant may be somewhat artifactual, they have proved useful in identifying steroid receptors from different target tissues and emphasises the close similarity of hormone receptor complexes in the two sexes, albeit for different hormones.

Role of receptors in hormone induced changes in the target tissues:

The nuclear binding of steroid receptor complex trans-located from the cytoplasm is considered to be an important step in the stimulation of nuclear mediated events that result
Several studies have shown the importance of RNA and protein production in the early stages of steroid hormone action. The receptor-hormone complex exerts its primary effect at the transcriptional level in bringing about an increase in ribonucleic acid synthesis. This aspect has been reviewed by several investigators (androgen: Williams-Ashman and Reddi, 1972; Liao, 1975; Mainwaring, 1975, 1977; estrogen: Brenner and West, 1975; Segal and Koide, 1979; Clark et al., 1978). The search for the earliest responses to sex steroids has led to the generally accepted concept that a limited number of protein species may be produced rapidly, almost certainly preceded by the synthesis of a limited number of messenger RNA species. This is followed by a larger and more general production of messenger RNA, ribosomal RNA and transfer RNA resulting in increased protein synthesis, both general and specific, structural and secretory. The tissue growth generally reflects both hypertrophy and hyperplasia.

Briefly stated, the accepted concept of steroid hormone action is that the ability and the degree to which a target tissue responds to hormone is dependent on the availability of the receptor. For hormonal response to occur a tissue must contain a certain minimal level of receptors in the tissue's cytoplasm. Upon binding hormone, this receptor-hormone-complex is translocated to the nucleus where a minimum number of receptors must be retained on acceptor sites for some critical
period of time which is necessary to stimulate both early and late responses to the hormone.

A general model of the steroid-receptor-interaction, the nuclear transfer processes and other features of the steroid receptor system are shown in Figs. 6 and 7.
FIG. 6: SCHEMATIC REPRESENTATION OF THE ESTROGEN INTERACTION PATHWAY AND BIOCHEMICAL RESPONSES IN TARGET CELLS. THE HORMONE (E) ENTERS THE CELLS AND BINDS TO AN EXTRANUCLEAR RECEPTOR PROTEIN (Re), INDUCING ITS CONVERSION TO AN ACTIVATED FORM (Rn) THAT IS TRANSLOCATED TO THE NUCLEUS WHERE THE HORMONE–RECEPTOR COMPLEX ENHANCES THE PRODUCTION OF PRERIBOSOMAL AND MESSENGER RNAs INVOLVED IN THE SYNTHESIS OF FUNCTIONAL AND/OR SECRETED PROTEINS.
FIG. 7: SCHEMATIC REPRESENTATION OF INTERACTION OF PATHWAY OF ESTRADIOL (E). DIAGRAM AT LEFT INDICATES EXTRANUCLEAR ESTRADIOL RECEPTOR COMPLEX UNDERGOING TRANSFORMATION AND ENTERING NUCLEUS TO BIND TO CHROMATIN. DIAGRAMS AT RIGHT INDICATE SEDIMENTATION PROPERTIES OF COMPLEXES EXTRACTED FROM THE CELL.
Section D: Actions of Estrogen in the Male

Research during the past 10 years has begun to elucidate the very significant functions of endogenous estrogens in the male. Estrogen mediates the action of testosterone to abolish the function of centers responsible for cyclic gonadotropin release and partially mediates the action of testosterone to effect development of male sexual behaviour (Dickson and Clark, 1981). It also partially mediates the feedback action of testosterone in the hypothalamus to decrease LHRH secretion. Estrogen contributes to the growth and function of sex accessory organ epithelium and fibromuscular stroma. It is the last aspect of estrogen action that the present study is concerned with.

Estrogen produces a number of effects in the male accessory sex organs and it is important to take into consideration if these effects are produced at normal endogenous levels of estrogen. The results of estrogen administration to rodents vary with species, age of animals, specific gland under consideration, dosage, duration of treatment, and presence or absence of androgen (Price and Williams-Ashman, 1961). Estrogen administration produces marked castration like effects on accessory genital organs in all mammals that have been studied: prostate, seminal vesicles, epididymis and other sex accessories
regress. This potent effect of estrogen is due to reduction in androgen synthesis as a result of inhibition of pituitary gonadotrophin synthesis and release. In contrast, when low doses are administered to castrated rodent, a number of growth promoting effects are observed. Furthermore, estrogen can induce squamous epithelial metaplasia and can promote the growth of the fibromuscular tissue of these organs. Possibly, the positive interactions of small amounts of estrogen with androgen in the epithelial cells and the estrogen induced fibromuscular tissue anabolism are in keeping with normal organ function. This aspect has been extensively reviewed by previous investigators (Zuckerman, 1935; Emmens and Parkes, 1947; Burrows, 1949; Bern, 1959; Price and Williams-Ashman, 1961; Ofner, 1968).

Low doses of estrogen potentiates the action of testosterone on the secretory activity of the canine prostate and bull seminal vesicles (Thomas and Knych, 1966a; Gouvelia et al., 1971). Similar positive interactions have been recorded on rat and mouse accessory sex organ weight, their biochemical composition (Korenchevsky et al., 1936; Thomas and Knych, 1966b; Saunders, 1958; Shimazaki et al., 1969; Anderson and Muntzing, 1972). In vitro studies have revealed estrone and estradiol to affect the rat prostate directly (Lasnitzki, 1974). Low concentrations of the hormone had a weak androgenic effect. The epithelium was slightly higher and the stroma less dense
than in the controls. Higher doses increased the regression seen in the control explants. In castrated rat prostate, estradiol has been shown to stimulate epithelial growth and secretory activity (Salander and Tisel, 1971). Glandular epithelium of canine prostate is recently reported to be estrogen sensitive (Leav et al., 1978).

The ability of estrogen to increase mitotic activity in the epithelium and fibromuscular component of the accessory sex organs is well documented (Price and Williams-Ashman, 1961). Estrogen is reported to induce the fibromuscular growth in the seminal vesicles and prostate of rhesus monkey (van Wagenen, 1935; Zuckerman and Sandys, 1939) and rodents (Price and Williams-Ashman, 1961). Two reports mention estrogen-induced fibromuscular growth in the canine prostate gland (Gouvelis et al., 1971; Zuckerman and Groome, 1937). In castrate rats estrogen can cause a 5-fold or greater increase in anterior prostate and seminal vesicle weight attributable primarily to fibromuscular growth (Korenchevsky et al., 1936; Peyel-Cabanès et al., 1978; David et al., 1934; Greene and Thomson, 1942; Tisel, 1976; Fugii and Villee, 1968). Similarly, the mouse accessory sex organs, particularly the coagulating gland and seminal vesicle, show fibromuscular hyperplasia (Gyorkey, 1964; Burrows, 1935). In the sex accessory organs of other species such as the guinea pig, mouse and rabbit (Belis et al., 1977; Bern and Levy, 1952; Krichesky and Benjamin, 1947; Mariotti and
Mawhinney, 1981; Neubauer et al., 1981) estrogens have been shown to effect fibromuscular growth. Despite the consistency of this finding, several key points remain to be understood in order to assess the physiologic significance of estrogen action in the stroma. The relative potency and comparative nature of androgen and estrogen action on fibromuscular tissue need to be defined. Androgen seems to be effective stimulator of muscle growth (Mariotti and Mawhinney, 1981; Neubauer et al., 1981) although evidence has been offered to the contrary (Feyel-Cabanes et al., 1978). It may be that both androgen and estrogen contribute significantly to the functioning of the stroma.

Effects of estrogen on the epididymis have been given much less attention than in the testis and other sex accessories. There is some evidence of its influence on the epididymal musculature resulting in accelerated sperm transport (Meistrich et al., 1975; Das et al., 1976). Evidence supporting the physiologic significance of estrogen in male accessory organs comes from the fact that these organs contain, in relation to other tissues of the male, high concentrations of estrogen receptors.
Section E: Estrogen Receptors in Accessory Sex Organs of Male

In experimental animals, injected radioactive estrogen has been observed to accumulate selectively in target tissues and it has been speculated that estrogen-responsive target tissues contain some specific binding protein to account for such observations (Glascock and Hackstra, 1959; Jensen and Jacobson, 1960). These specific binding proteins have been defined as estrogen receptors (Jensen and Jacobson, 1960). Evidence for the presence of a specific estradiol-17β receptor in male accessory sex organs has been provided by several investigators.

In humans, studies have shown the prostate to either concentrate injected estrogen (Segal et al., 1959; Fergusson, 1961) or to contain estrophilic molecules (Bashirelahi et al., 1976; Bashirelahi and Young, 1976; Hawkins et al., 1975; Krieg et al., 1978; Wagner et al., 1975). Accessory sex organs of the baboon (Karr et al., 1978), calf and pig (Jungblut et al., 1971) and ventral prostate of rat (Armstrong and Bashirelahi, 1978; Ginsberg et al., 1980; Jung-Testas et al., 1981) also contain estrogen receptors. The canine prostate gland has recently been shown to contain abundance of estrogen receptor (Chaisiri et al., 1978; Robinette et al., 1978; Dube
et al., 1979), in fact apparently more estrogen receptor than androgen receptor (Robinette et al., 1978). Following intravenous administration of (3H) estradiol to mature male guinea pigs, the sex accessory tissues incorporated greater concentrations of the hormone than the nongenital tissues (Blume and Mawhinney, 1978). In the seminal vesicles of this species estrogen receptors are found in both epithelial and stromal cells—more numerous being present in the stroma (Belis et al., 1977; Blume and Mawhinney, 1978). In the rat, Mangan et al. (1967) reported that one hour after the intraperitoneal administration of (3H)-diethylstilbestrol the greatest concentration of radioactivity was associated with the prostate. Tveiter (1970) found that following the intramuscular administration of (3H) estradiol into three month old rats, the radioactivity associated with the prostate was higher than that with skeletal muscle.

In rat ventral prostate (Jung-Testas et al., 1981) stroma is found to have relatively more receptors than the epithelium. Recent reports have shown that the stroma of both the human (Bashirelahi et al., 1978) and canine prostate gland (Leav et al., 1978) have significant estrophilic activity. Apart from the actions of estrogen in the muscle, it is interesting to speculate about the relatively small amount of estrogen receptor in the epithelium as is reported in the seminal vesicle of guinea pig (Belis et al., 1977; Belis and Mawhinney, 1978)
and ventral prostate of rat (Jung-Testas et al., 1981). The presence of a receptor for estradiol-17β in the epididymis of man (Murphy et al., 1980), dog (Younes et al., 1979) and rabbit (Danzo et al., 1975, 1978; Danzo and Eller, 1979) has also been reported. Estrogen has been found to have a stimulatory effect on the secretory function of the epididymis of rat (Peyre and La Porte, 1966; Jehan and Setty, 1977). It still remains to be established if estrogen receptors present in the epididymis (epithelium?) are required to modulate the epididymal environment in such a way that the resulting effect influences sperm maturation.

From the above brief survey of literature it is clear that there is no compelling evidence to suggest that estrogens alone or in combination with androgens play a physiological role in the regulation of male reproductive processes. The demonstration of presence of estrogen receptors in the male genital tract implies a direct receptor-mediated function for estrogens in the regulation of these tissues.
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STUDIES ON THE UPTAKE AND RETENTION
OF (\(^3\)H) ESTRADIOL-17\(/3\) BY THE ACCESSORY
GENITAL ORGANS OF MALE RAT
INTRODUCTION

It has long been recognised that the male accessory genital organs are dependent upon androgenic hormones for maintenance of their cellular integrity and complex functions (Price and Williams-Ashman, 1961; Bishop, 1961; Risley, 1963; Orgebin-Crist et al., 1975; Cavazos, 1975; Setty, 1979). It is also known for some years that measurable levels of circulating estrogens are present in male mammals (Leach et al., 1956; Dorrington and Armstrong, 1979; Marcus and Korenman, 1976; van der Molen et al., 1981). Male genital organs are adversely affected by estrogen administration (Price and Williams-Ashman, 1961). However, this does not rule out the possibility that estrogen(s) may have a role in regulating physiological processes in the male, either directly or by modulating the action of androgen. Thus the physiological effects of sex hormones in the male genital organs may reflect the combined effects of the estrogenic and androgenic hormones. One of the approaches to study the mode of action of sex steroids is to examine the fate of the steroid hormone after it has been administered to immature or gonadectomised animals. The classical studies of Glascock and Haekstra (1959) and Jensen and Jacobson (1962) with estrogen in the female rat and those of Liao and Fang (1969) with androgen in the male rat
have revealed that immediately after the injection of a physiological dose of radioactive sex steroid into rats, uptake of the labelled hormone by various tissues reflects the concentration of the administered hormone in the blood. The radioactivity then disappears rapidly from the blood and tissues less sensitive or non-responsive to sex steroids (kidney, muscles diaphragm), but only slowly from target tissues such as uterus and ventral prostate (see Chapter I, Section C). It is now recognised that the selective uptake and retention of sex steroids by the target tissues is due to the presence of specific hormone binding receptor proteins in them (Jensen et al., 1977).

In the present study this experimental model was adapted to examine if labelled estrogen selectively accumulates in the accessory genital organs of adult castrated male rats. The non-genital tissues studied for the purpose of comparison included pituitary, liver and thigh muscle. The primary object of the study was to investigate the uptake and retention of \(^{3}H\) estradiol by the epididymis and vas deferens, and for comparison two other accessory genital tissues viz. seminal vesicles and ventral prostate were also investigated. This kinetic study involved determination of radioactivity at different intervals after the administration of \(^{3}H\) estradiol-17\(^{3}\) in vivo. The second object was to investigate the hormonal dependence of estrogen uptake by these organs. No attempt has
been made to characterise the radioactive material and only the
total radioactivity in each of the tissues was measured.

MATERIALS AND METHODS

Animals

Colony bred adult male rats (200-250 g) of the Institute
were used in the study. All the animals were maintained in
air-conditioned quarters (24 ± 1°C) under uniform husbandry
conditions. They were fed a pelleted diet and received water
ad libitum.

Radioactive steroids and chemicals

The radio labelled estrogens (2,4,6,7-^H)-estradiol-17/3
(Fig. 1) (specific activity: 91 mCi/mMol) and (4-^14C) estradiol
(Fig. 2) (specific activity: 52 mCi/mMol) used in the present
study were obtained from Radiochemical Centre, Amersham, England.
The purity was checked before use by TLC on silica gel plates in
solvent system chloroform:ethyl acetate (4:1 v/v). The steroids
were stored at 4°C as dilute solutions in benzene/ethanol
9:1 v/v).

The scintillation fluid contained PPO (2,5-diphenyl-
oxazole: 4 g/l) and POPOP (1,4-bis-5-phenyl-oxazole-2-yl)
benzene: 100 mg/l (New England Nuclear, USA) dissolved in dis-
tilled toluene. Other chemicals used were solvent ether and
FIG. 1 2,4,6,7-\textsuperscript{H}\textsuperscript{3} ESTRADIOL-17\textbeta
FIG. 2. 4-C$^{14}$ ESTRADIOL-17$\beta$
anhydrous sodium sulphate of Analar grade.

**Surgical procedure**

To eliminate interference by endogenous hormones, the studies were carried out in castrated animals.

The rats were castrated bilaterally through a scrotal incision under ether anaesthesia. Before separating the testis, the testicular blood vessels were ligated leaving the blood supply of the epididymis intact.

**Dose of labelled hormone administered**

In immature and adult spayed female rats 0.1 to 1.0 μg estradiol as a single dose is considered as physiological dose (Alberga and Baulieu, 1968; Clark et al., 1973).

Experiment I (48 hr castration):

Five groups of rats, each consisting of 4 to 5 animals were administered with tritiated estradiol 48 hr after castration and were killed at different intervals (15 min, 30 min, 60 min, 6 hr and 24 hr) to study the distribution of estrogen and its retention in various tissues.

Experiment II (21 day castration):

Two groups of rats, each consisting of 4 animals were castrated. One group of rats served as controls. The other
group of rats received testosterone propionate (TP) at the rate of 200 μg/rat daily for 7 days, last injection being given 48 hr prior to autopsy. The steroid was dissolved in sterile olive oil and 0.1 ml of the vehicle was used for daily intramuscular injection. The control rats received vehicle alone for a similar period. On day 22 after castration, i.e. 48 hr after withdrawal of hormone replacement therapy, the animals were injected with tritiated estradiol and were killed 15 or 30 min later.

Administration of labelled estrogen:

The animals in both the experiments were injected with (2,4,6,7-³H) estradiol-17β (0.1 μg, 33.5 μCi) in 0.1 ml of 4% ethanolic saline through the jugular vein under ether anaesthesia. Distribution of radioactivity was determined in the epididymis (caput, corpus and cauda portions), vas deferens, seminal vesicles (with coagulating gland), ventral prostate, pituitary, liver and thigh muscle. Blood was collected by cardiac puncture into heparinized syringes and centrifuged to obtain plasma. All the tissues were weighed accurately in a torsion balance and preserved in deep-freezer at -18°C until they were processed further. The radioactivity in blood and pituitary was determined only in 48 hr castrates. In the 21 day castrated rats, estradiol uptake by the non-genital tissues (liver and muscle) was measured only in the controls for the purpose of comparison with 48 hr castrates.
Extraction of radioactive steroid:

The tissues were homogenized in 6 ml distilled water using Ultra Turrax (Janke and Kunkel, K.G., Germany). An equal quantity of 5 N NaOH was added to each sample followed by the addition of approximately 3,000 counts of (4-14C) estradiol-17/β for calculating the extent of recovery after extraction. All the samples were left at 37°C for 48 hr and then extracted with 4 x 15 ml diethyl ether. The combined ether phase was dried over anhydrous sodium sulphate and evaporated directly in LSC vials. To each vial, 10 ml of scintillation fluid was added and the radioactivity was counted in Packard Scintillation Spectrometer (Model 3330). The counts were corrected for background radioactivity, counter efficiency and quenching; the recoveries were in the range of 60 to 75%. The radioactivity in tissues is expressed as dpm/mg of wet tissue, dpm/mg DNA and dpm/organ and in blood as dpm/5 μl of plasma. DNA in different tissues was determined according to the procedure of Burton (1956) (for procedural details of DNA estimation, see Chapter III).

Hormone replacement therapy-choice of the dose:

Twenty one day post-castration period permits disappearance of residual spermatozoa in the epididymis and vas deferens and also causes significant reduction in the weights of all the accessory sex organs (Johan et al., 1973; Jehan and
Setty, 1977). This period is sufficient to cause a marked decrease of the androgen receptor content of the epididymis and ventral prostate (Pujol and Bayard, 1979). The dose (200 µg/rat) and duration (7 days) of testosterone treatment was based on the work of Tindall et al. (1974) and Calandra et al. (1975) who have reported it to be optimal in androgen replacement therapy.

RESULTS

Experiment I:

Results presented in Fig. 3 show the distribution of radioactivity in different accessory genital organs and in blood plasma at various time intervals after i.v. injection of (2,4,6,7-³H) estradiol. Each point represents the mean of 3 or 4 determinations.

Maximum levels of (³H) estrogen were seen in all the accessory sex organs at 15 minutes after administration. There was little decrease in the radioactivity at 30 minutes though by 60 minutes it was markedly reduced. The pattern of distribution of radioactivity in blood was quite in contrast to the sex accessory organs. The highest value was present at 15 min and after that time, the values decreased exponentially. By 60 min there was a 50% reduction in the radioactivity and at 6 hr the retention was less than 20% of the initial peak value.
Fig. 3: Distribution of radioactivity in various tissues of castrated male rats after administration of $^{3}H$ estradiol-17$\beta$. Animals were sacrificed 1/4, 1/2, 1, 6 and 24 hr after the injection.

(Note: Total radioactivity is expressed as dpm/mg wet tissue, 5$\mu$l of plasma)
Amongst the accessory genital organs studied, the uptake of estrogen was relatively high at 15 min in the epididymis (Fig. 4), vas deferens (Fig. 5) and ventral prostate (Fig. 6). At 30 min a marginal rise (11%) in the radioactivity was evident in the seminal vesicles (Fig. 7). The other accessories did not exhibit any difference between 15 and 30 minutes. Appreciable fall in radioactivity became evident at 60 minutes. The reduction was relatively much less in seminal vesicles (13%) and epididymis (22%) than in ventral prostate (41%) and vas deferens (38%). A further decrease in the levels of labelled estrogen was observed in all the organs at 6 hr. By 24 hr, estrogen level was reduced to 25% of its initial value (15 min) in the vas deferens while in the other three organs (epididymis, ventral prostate and seminal vesicles) the retention continued to be about 40% of the original level (15 min). Further analysis of the data revealed that while there was a significant fall in the radioactivity in the vas deferens between 6 and 24 hr (55%), the reduction in the epididymis, seminal vesicles and ventral prostate was moderate (6 hr vs 24 hr: 13-22%).

When the three segments of the epididymis were examined separately, corpus epididymis always showed relatively higher uptake than caput and cauda regions (Fig. 8); vas deferens resembled the corpus in this respect.
FIG. 4: UPTAKE AND RETENTION OF (2, 4, 6,7-3 H) ESTRADIOL-17β AT 15, 30, 60 min, 6 hr AND 24 hr BY TOTAL EPIDIDYMIS (dpm/mg wet tissue wt).
FIG. 5: UPTAKE AND RETENTION OF (2, 4, 6, 7$^3$H) ESTRADIOL-17$\beta$
AT 15, 30 AND 60 min, 6 hr AND 24 hr BY VAS DEFERENS
(dpm/mg wet tissue wt).
FIG. 6: UPTAKE AND RETENTION OF (2, 4, 6, 7-³H) ESTRADIOL-17β AT 15, 30, 60 min, 6 hr AND 24 hr BY VENTRAL PROSTATE (dpm/mg wet tissue wt).
FIG. 7: UPTAKE AND RETENTION OF (2,4,6,7-³H) ESTRADIOL-17β AT 15, 30, 60 min, 6 hr, 24 hr BY SEMINAL VESICLES (dpm/mg wet tissue wt).
FIG. B. UPTAKE AND RETENTION OF (2,4,6,7-3H) ESTRADIOL-17β AT 15, 30 AND 60 min BY BLQOD PLASMA, CAPUT, CORPUS, CAUDA AND VAS DEFERENS OF RAT
The distribution of radioactivity in non-genital tissues at various intervals is shown in Fig. 9. The uptake by the liver and pituitary was much higher than that by the muscle and plasma. About 40% reduction in the radioactivity in muscle was noticed by 60 min and at 6 hr about 65% of initial radioactivity had disappeared both in liver and muscle. A rather constant level of radioactivity was present in the pituitary between 15 min and 60 min after administration. In general, the concentration of radioactivity at all intervals examined was higher in the liver and pituitary than in any other organ examined in the present study.

Experiment II:

With a view to study the effect of long-term castration on the uptake of labelled estrogen by the accessory genital organs and a non-genital tissue, thigh muscle, a preliminary study was carried in adult rats that remained castrated for 21 days. The rats received (³H) estradiol-17β as in Experiment I and the animals were killed 15 min later - the time interval when maximum radioactivity was present in most of the organs examined (see results of Experiment I, Fig. 1). Results presented in Table I show that in the three segments of the epididymis, vas deferens, seminal vesicles and ventral prostate, there was a marked decrease in the uptake of estrogen while it remained unaffected in the muscle.
FIG. 9: UPTAKE AND RETENTION OF (2, 4, 6, 7-3H) ESTRADIOL-17β AT 15, 30, min, 1hr, 6 hr AND 24 hr BY PLASMA, LIVER AND PITUITARY.
TABLE I. Uptake of $^{3}$H estradiol-17$eta$ by rat accessory genital organs after short - (48 hr) and long-term (21 day) castration (dpm/mg wet weight)*

<table>
<thead>
<tr>
<th>Group</th>
<th>Caput epididymis</th>
<th>Corpus epididymis</th>
<th>Cauda epididymis</th>
<th>Vas deferens</th>
<th>Seminal vesicles</th>
<th>Ventral prostate</th>
<th>Thigh muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hr castration</td>
<td>186 ±11** (4)</td>
<td>239 ± 6 (4)</td>
<td>136 ± 5 (4)</td>
<td>260 ± 8 (4)</td>
<td>120 ±11 (4)</td>
<td>201 ±15 (4)</td>
<td>174 ± 6.6 (4)</td>
</tr>
<tr>
<td>21 days castration</td>
<td>116 ± 3  (4)</td>
<td>181 ±23 (4)</td>
<td>91 ± 2  (4)</td>
<td>107 ± 3 (4)</td>
<td>69 ± 5  (4)</td>
<td>137 ± 11 (4)</td>
<td>186 ± 23 (4)</td>
</tr>
<tr>
<td>% reduction</td>
<td>-38%</td>
<td>-24%</td>
<td>-33%</td>
<td>-59%</td>
<td>-43%</td>
<td>-39%</td>
<td>---</td>
</tr>
</tbody>
</table>

* In both the groups the uptake was determined 15 min after administration
** Mean ± S.E. followed by number of determinations in parentheses
Results presented in Table II show that following 21 days of castration, there was a significant reduction in the uptake of \((^3H)\) estradiol-17\(^\beta\) by all the accessory sex organs excepting ventral prostate. Androgen treatment promoted estrogen uptake by all the accessory organs and it was comparable to short term castrates in the seminal vesicles and corpus epididymis; the uptake by the ventral prostate exceeded that of short-term controls. The trend was similar when the uptake values are expressed in the different organs on equal amount of DNA (Table III). The extent of accessory sex organ involution following castration and recovery after androgen treatment are shown in Table IV along with DNA content. When the uptake of estrogen is expressed as dpm per organ (data not shown) the trend gets altered since this method of comparison includes a substantial component attributable to differences in organ size. Moreover, such comparisons give erratic picture and is due to the fact that castration causes pronounced decrease in cell number and loss of cytoplasmic component in seminal vesicles and ventral prostate (Higgins and Parker, 1980). In contrast, the involution of the epididymis following castration appears to be due to cytoplasmic shrinkage and not due to reduction in cell number (Brooks, 1977). This aspect is discussed in detail in Chapter III. Suffice to add that the extent of recovery by different accessory genital organs is not uniform following androgen replacement.
TABLE II. Uptake of \( ^3 \)H) estradiol-17β by rat accessory genital organs after castration and androgen replacement therapy (dpm/mg wet weight)*

<table>
<thead>
<tr>
<th>Treatment (Group)</th>
<th>Caput epididymis</th>
<th>Corpus epididymis</th>
<th>Cauda epididymis</th>
<th>Vas deferens</th>
<th>Seminal vesicles</th>
<th>Ventral prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hr castration (I)</td>
<td>174±11 (4)**</td>
<td>237±12 (4)</td>
<td>154± 5 (4)</td>
<td>240±11 (4)</td>
<td>131±11 (4)</td>
<td>177±14 (4)</td>
</tr>
<tr>
<td>21 days castration (II)</td>
<td>99± 6 (4)</td>
<td>162±12 (4)</td>
<td>94± 6 (4)</td>
<td>142±15 (4)</td>
<td>75± 2 (4)</td>
<td>153±12 (4)</td>
</tr>
<tr>
<td>P value, I vs II</td>
<td>( \triangleq 0.005 )</td>
<td>( \triangleq 0.005 )</td>
<td>( \triangleq 0.001 )</td>
<td>( \triangleq 0.005 )</td>
<td>( \triangleq 0.005 )</td>
<td>NS</td>
</tr>
<tr>
<td>TP treated† (III)</td>
<td>139± 3 (4)</td>
<td>259±17 (4)</td>
<td>121± 7 (4)</td>
<td>183±24 (4)</td>
<td>131±10 (4)</td>
<td>247± 8 (4)</td>
</tr>
<tr>
<td>P value, II vs III</td>
<td>( \triangleq 0.005 )</td>
<td>NS</td>
<td>( \triangleq 0.005 )</td>
<td>NS</td>
<td>( \triangleq 0.005 )</td>
<td>( \triangleq 0.001 )</td>
</tr>
</tbody>
</table>

* The uptake was determined 30 min after administration
** Mean ± SE followed by number of determinations in parentheses; NS = not significant
† Testosterone propionate was administered @ 200 μg/rat daily for 7 days, last injection was given 48 hr prior to \( ^3 \)H) estradiol administration.
TABLE III. Uptake of (³H) estradiol-17β by rat accessory genital organs after castration and androgen replacement therapy (dpm/mg DNA)*

<table>
<thead>
<tr>
<th>Treatment (Group)</th>
<th>Caput epididymis</th>
<th>Corpus epididymis</th>
<th>Cauda epididymis</th>
<th>Vas deferens</th>
<th>Seminal vesicles</th>
<th>Ventral prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hr castration (I)</td>
<td>32680±2001 (4) *</td>
<td>53719±5289 (4)</td>
<td>34718±2493 (4)</td>
<td>51997±2430 (4)</td>
<td>31930±4299 (4)</td>
<td>37888±7633 (4)</td>
</tr>
<tr>
<td>21 day castration (II)</td>
<td>10431±406 (4)</td>
<td>15715±2298 (4)</td>
<td>15012±1539 (4)</td>
<td>11424±520 (4)</td>
<td>4339±508 (4)</td>
<td>9907±866 (4)</td>
</tr>
<tr>
<td>P value, I vs II</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>TP treated†(III)</td>
<td>17520±471 (4)</td>
<td>40562±4055 (4)</td>
<td>29872±3190 (4)</td>
<td>33630±5790 (4)</td>
<td>35564±3125 (4)</td>
<td>30848±1637 (4)</td>
</tr>
<tr>
<td>P value, II vs III</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

The uptake was determined 30 min after administration

Mean ± SE followed by number of determinations in parentheses; NS = not significant

Testosterone propionate was administered @ 200 μg daily for 7 days, last injection was given 48 hr prior to (³H) estradiol administration.
TABLE IV. Weight and DNA content of the accessory genital organs of male rat following castration and androgen replacement.

<table>
<thead>
<tr>
<th>Treatment (Group)</th>
<th>Wet weight (mg)</th>
<th>DNA (mg/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epididymis</td>
<td>Vas deferens</td>
</tr>
<tr>
<td>48 hr castration (I)</td>
<td>426.5±14.8</td>
<td>81.0±3.1</td>
</tr>
<tr>
<td>21 day castration (II)</td>
<td>80.8±0.9</td>
<td>29.5±2.7</td>
</tr>
<tr>
<td>P value, I vs II</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TP treatment (III)</td>
<td>162.0±5.8</td>
<td>56.3±3.6</td>
</tr>
<tr>
<td>P value, II vs III</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: The apparent fall in DNA content (Group II) is at the face of increase in its concentration (µg/mg tissue) in epididymis and vas deferens and the weight reduction is due to drastic reduction in cytoplasmic component.

The apparent lack of effect of TP on DNA content (Group III) in epididymis and vas deferens is at the face of increase in weight and fall in concentration (µg/mg tissue); the incomplete recovery of the organ weight is due to incomplete restoration of cytoplasmic component.

* Mean ± SE of four observations (other details as under Tables II and III).
DISCUSSION

The results of the first experiment demonstrate that estrogen is accumulated in the accessory genital organs of castrated male rats against a concentration gradient and that these organs may have estrogen responsive cells. It must, however, be pointed out that the uptake and retention of tritiated estradiol-17β by the epididymis, vas deferens, seminal vesicles and prostate is not as marked as that reported in the literature for testosterone (Liao and Liang, 1975; Tveter et al., 1975). Moreover, two other organs viz. liver and pituitary which are not target tissues for estrogen in the conventional sense show much higher uptake of estrogen. In view of the fact that estrogen may have important regulatory influence on the physiology of the androgen target tissue viz. epididymis (Jehan and Setty, 1977), vas deferens (Kumari et al., 1980), seminal vesicles (Mariotti and Mawhinney, 1981) and ventral prostate (Bouton et al., 1981), it became necessary to study the pattern of distribution of radioactivity after administration of labelled estradiol and analyse its uptake and retention in the various accessory genital organs. A comparison with the results reported by previous investigators with testosterone would reveal the relative affinity of the male accessory genital organs to the two sex steroids.
It has been reported that the amount of radioactivity in the ventral prostate increases following injection of ($^3$H)-testosterone to reach maximum levels after 1-2 hr with a concomitant fall of radioactivity in blood and skeletal muscle (Tveter, 1967). It has also been shown that androgen is retained in the prostate (Tveter, 1967), epididymis and vas deferens (Hansson and Tveter, 1971) for a prolonged period of time while the radioactivity in the muscle showed a continued decrease after administration. In the present study no marked difference was evident between the sex accessory organs (especially the seminal vesicles and ventral prostate) and the muscle at 15, 30, 60 min intervals when the radioactivity was relatively high in these organs. In contrast, the vas deferens showed relatively higher uptake of estradiol followed by epididymis. Hansson and Tveter (1971) have reported that the uptake of ($^3$H)-testosterone in corpus portion of the epididymis was higher than in caput and cauda. A similar pattern is seen in the uptake of ($^3$H)-estradiol at all time intervals examined. The significance of differential uptake of labelled estrogen by the three epididymal regions is not clear. Likewise, the high uptake of ($^3$H) testosterone (Appelgren, 1969; Hansson and Tveter, 1971) and estrogen (present study) by the vas deferens is not understood. It is pertinent to mention that the rat epididymis and vas deferens are potential sites of testosterone and estrogen synthesis (Hamilton and Fawcett, 1970; Kumari
et al., 1980). The results of the present study are in agreement with those of Das et al. (1976) who have reported relatively high uptake of labelled estrogen by the epididymis and vas deferens at 60 min after administration. In the rat ventral prostate about 50% of the radioactivity disappeared in about 6 hr from the time of injection of $^{3}H$-testosterone (Fang et al., 1969). A similar pattern of clearance is seen in the present study with $^{3}H$ estradiol in the four accessory sex organs.

Mangan et al. (1967) reported that in rat one hour after the intraperitoneal administration of $^{3}H$ diethylstilbestrol the greatest concentration of radioactivity was associated with the prostate gland. Eisenfeld and Axelrod (1966) observed that following the intravenous administration of $^{3}H$ estradiol-17β, the steroid concentration within the rat ventral prostate and epididymis was quite small in comparison with the tritium recovered from the anterior pituitary and adrenal. Tvetet (1970) has reported that a selective accumulation of $^{3}H$-estradiol by different lobes of the prostate and seminal vesicles of rat could not be demonstrated but the uptake by these organs was reported to be higher than that by skeletal muscle. These results suggest that the rat accessory sex organs do have ability to accumulate estrogen in low quantity though not, very selectively. The reason for this is that the accessory genital organs contain only small amounts of estrogen.
receptors (see Chapter I, Section E for details). Another factor which must be recognised is the presence of heterogenous cell population in these organs and estrogen receptors may be restricted to only some specific cell type (epithelial vs muscular). A third factor which has to be taken into consideration is the possibility of significant reduction in receptor content following 48 hr castration period. Such reduction in the accessory organs has been reported for both estrogen (Ginsburg et al., 1980) and androgen receptors (Baulieu and Jung, 1970; Pujol and Bayard, 1979) in the ventral prostate and epididymis.

In recent years a number of studies have indicated that the organs which were considered earlier as non-target or non-responsive to sex steroids also have specific estradiol binding proteins (kidney: de Vries et al., 1972; pancreas: Rosenthal and Sandberg, 1978; liver: Aten et al., 1978). Dionne et al. (1979) have demonstrated unequivocally the presence of cytosol estrogen binding proteins in the thigh muscles of 24 hr castrated male rat. Apparently this is the reason why we found that estrogen uptake by the muscle was as high as in some of the accessory sex organs. A high and prolonged uptake of radioactivity in the pituitary and liver may be attributed to specific estrogen receptors (Mercier et al., 1976; Le Guellec et al., 1978; Aten et al., 1978).
From the results of the estrogen uptake study it can be concluded that the low estrogen binding by the androgen target organs is due to their low estrogen receptor level and this is in consonance with low levels of estrogen production in the male. It is not unlikely that this balance among endocrine regulatory factors is a physiological necessity and changes in the level of these factors might create abnormal conditions (see Bouton et al., 1981) either by increasing androgen binding sites - the increase being mediated by the estrogen receptor present (e.g. rat ventral prostate; Bouton et al., 1981) or by acting as an antiandrogen (e.g. epididymis: Setty, 1979; Tindall et al., 1981).

The results have further demonstrated that estrogen uptake by the accessory genital organs is androgen dependent. Tveter (1970) has reported that androgen does not influence the uptake of estrogen by the ventral prostate. His conclusion was based on studies carried out in animals castrated for 3 days and in the present study animals were used 21 days after castration. Androgen treatment restored the ability of seminal vesicles and ventral prostate to normal level while the uptake by the caput and vas deferens was restored only partially. It is now well recognised that in castrated rat, exogenous testosterone in dose levels at which seminal vesicles and ventral prostate recover completely, epididymis and vas deferens do not recover completely (Prasad et al., 1973; Rajalakshmi and
Moreover, there exists a differential threshold of androgen requirement between caput and cauda portions of the epididymis in rat (Prasad et al., 1973; Rajalakshmi and Prasad, 1976; Setty, 1979). On the whole, it appears that estrogen binding component in the male genital organs is androgen dependent. The accumulation, retention and androgen dependence of estrogen uptake in the male sex accessory organs may be attributed to the presence of some specific binding protein in these organs and the specific binding proteins for estrogen have been designated as estrogen receptors (Jensen et al., 1977). The investigator was conscious of the fact that measurement of tritium incorporation into the accessory sex organs is not synonymous with the specific binding of estrogen to receptor protein in these organs. This aspect of the study is covered in Chapter III.

SUMMARY

1. The uptake and distribution of \( \text{estradiol-17\beta} \) in various accessory sex organs (caput, corpus and cauda portions of the epididymis, vas deferens, seminal vesicles and ventral prostate), pituitary, liver and thigh muscle was examined in adult castrated rats.

2. At 30 min the epididymis and vas deferens showed relatively higher uptake as compared to other organs and at all intervals examined (15, 30, 60 min and 6 hr) the corpus epididymis...
dimis and vas deferens retained maximum radioactivity. The accumulation of estrogen in the accessory sex organs was against a concentration gradient.

3. The highest radioactivity (expressed as dpm/mg wet tissue weight) was found in liver and pituitary; thigh muscle showed uptake equivalent to some of the accessory genital organs.

4. There was a marked reduction in the uptake of labelled estradiol by all the accessory genital organs following long-term (21 days castration) and androgen treatment for seven days prior to autopsy restored the uptake to varying degrees in different organs.

5. The uptake and retention of (\(^3\)H) estradiol by the accessory genital organs of male rat is not as conspicuous as that reported for (\(^3\)H) testosterone and this may be due to low amounts of estrogen binding component in these organs.
REFERENCES


STUDIES ON ESTROGEN RECEPTORS IN THE EPIDIDYMIS OF PREPUBERTAL RAT AND BIOLOGICAL RESPONSE OF THE EPIDIDYMIS TO ESTROGEN
INTRODUCTION

It is well established that testicular hormones, principally, testosterone, regulate the growth, differentiation and function of male accessory sex organs. Testosterone undergoes irreversible reduction to 5α-reduced steroids in the accessory sex organs of the male and dihydrotestosterone is the principal active hormone that is formed (Wilson, 1972). Another 5α-reduced metabolite which is considered to be a very potent androgen is 5α-androstane-3α, 17β-diol (Moore and Wilson, 1973). In rat and in man, estradiol-17β is the main biologically active estrogen secreted by the testis (van der Molen et al., 1981). It is also known that circulating androgens are aromatised in the peripheral tissues of male to estrogen (Dickson and Clark, 1981; MacDonald, 1976). It is, therefore, reasonable to assume that androgens and estrogens may exert their actions independently on separate target tissues, or they may act on the same target tissue, either synergistically or antagonistically. Prepubertal rat testis produces 5α-androstane-3α, 17β-diol and estradiol-17β (Moger, 1977). Evidence is still lacking for the possible role of estrogen in the attainment of sexual maturity.

A number of recent studies have given support to the concept of dual effects of androgen and estrogen on mammalian
prostate (see Chapter I, Section E). During the course of studies in this laboratory on the hormonal regulation of epididymal function, it was observed that estrogen promotes the secretory function of the epididymis in adult castrated rat (Jehan and Setty, 1977). Since steroid binding proteins have been implicated in the regulation of the physiological processes, intensive investigations have been carried out in different laboratories on the interaction of sex steroids with binding proteins in the epididymis (Blaquier, 1971; Blaquier and Calandra, 1973; Hansson et al., 1975; Danzo et al., 1976). Van Beurden-Lamers et al. (1974) have reported that epididymis is one of the organs which binds estradiol in adult male rat. Several investigators have presented evidence for estradiol binding also in rat ventral prostate (Armstrong and Bashirelahi, 1978). Specific estrogen binding proteins in the target tissues are defined as estrogen receptors (Jensen et al., 1977).

It is currently held that a protein molecule to qualify as a receptor it has to fulfil the following criteria:

1. The binding should be of high affinity and of reversible nature.

2. Since the target tissues have a limited number of receptors per cell, the binding should be saturable.

3. The binding should be hormone specific.

4. The binding should be confined to those organs which show response to the hormone.
5. The binding leading to the production of hormone-receptor complexes should initiate the biological response specific for the tissue.

The object of the present study was two-fold: (1) to demonstrate presence of estrogen receptors in the immature rat epididymis and ventral prostate and to establish them to be as distinct from androgen receptors and (2) to examine if estrogen has a biological role in influencing the epididymis during prepubertal period. In addition, in a preliminary study the influence of the two sex steroids viz. estrogen and androgen on the regulation of estrogen receptor in the prepubertal animal and also on the biological response of the epididymis in adult castrated rats was carried out.

In general, estrogens and androgens are considered as growth stimulators, particularly with regard to their action on the accessory sex organs and display very complex mechanisms by which they stimulate cellular biosynthetic activity. Androgens do not have a mitogenic action on all target cells, particularly the adult epididymis (Tusaimaa and Niemi, 1974). In the absence of definite estrogen-sensitive parameters which could be used as biological markers of response in the accessory sex organs of the male, an attempt has been made in the present study to examine the influence of exogenous estrogen, administered at physiological dose level, on the secretory function of the
epididymis and growth of the prostate. The levels of glycerylphosphorylcholine and sialic acid are generally used as secretory indices of epididymis (Setty, 1979) and their sensitivity in adult rat to estrogen treatment is already reported (Jehan and Setty, 1977).

MATERIALS AND METHODS

For the sake of convenience, this section is divided into two parts.

PART I: STUDIES ON ESTROGEN RECEPTORS

Animals

Male rats of Charles Foster strain (20-23) days old weighing 30-35 gm of the Institute rodent colony were used throughout the present studies. They were fed standard rodent chews and received water ad libitum.

Chemicals

(2,4,6,7-³H) estradiol-17β (S.A. 109 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. Dimethyl formamide (DMF), Tris-buffer, Ethylenediaminetetra-acetic acid (EDTA), Sodium-azide (Na₃N), toluene, dioxane and methanol were of analytical grade. Naphthalene (IDFL, India) was of scintillation grade. 2,5-Diphenyloxazole (PPO) and
((p-bis) 2-(-phenyl oxazole)) benzene (POPOP) were obtained from New England Nuclear Corporation, U.S.A. Norit A (activated charcoal) and dextran (mol. wt. 70,000), bovine serum albumin (BSA) fraction were obtained from Sigma Chemicals, U.S.A.

The different compounds which were used in the study are shown in Fig. 1.

dl-Centchroman is a new nonsteroidal orally active postcoital contraceptive (Kamboj et al., 1977) synthesised in this Institute. It is estrogenic and antiestrogenic in immature rat uterine weight assay and shows affinity for rat uterine estrogen receptors. Only trans-Centchroman is biologically active and cis-isomer is inert (Durani et al., 1979).

Cyproterone acetate is a progestational steroid with potent antiandrogenic activity (Neumann, 1977). It inhibits binding of dihydrotestosterone to cytoplasmic and nuclear receptors in the epididymis of rat and rabbit (Hansson et al., 1975; Danzo and Eller, 1975, 1976).

Purity of the radioactive steroid

The purity of the radioactive steroid was checked by thin layer chromatography (TLC) on silica gel-G plates using chloroform, ethyl acetate (3:1 v/v) as the solvent system.

Storage of radioactive estradiol

Radioactive estradiol was stored in dilute solution in
(2,4,6,7-\textsuperscript{3}H\textsubscript{3}) \textsc{estradiol-17\beta}
(1,3,5(10) \textsc{estratriene-3,17\beta-diol})

\textsc{diethylstilbestrol}

\textsc{progesterone}
(4-pregnene-3,20-dione)

\textsc{dihydrotestosterone}
(17\beta-hydroxy-5\alpha-androstan-3-one)

\textbf{FIG. 1}

\textit{CONTD.}
CYPROTERONE ACETATE
(6α-chloro-17α-acetoxy 16α,20α-methylene-4β,6α-pregnadiene-3,20-dione)

(TRANS) CENTCHROMAN
(dl-3,4-trans-2,2-dimethyl-3-phenyl-4-\(\beta\)-(3-pyrolidinomethoxy)phenyl-7-methoxy chroman)

(CIS) CENTCHROMAN
(dl-3,4-cis-2,2-dimethyl-3-phenyl-4-\(\beta\)-(3-pyrolidinomethoxy)phenyl-7-methoxy chroman)

FIG. 1
toluene:ethanol (95:5, v/v) at -18°C. Before use the solvent was evaporated under a stream of nitrogen, replaced by appropriate amount of absolute alcohol and diluted with appropriate buffers. The stock solutions were stored in refrigerator and used within a week's time.

Preparation of reagents

TEA-buffer: of pH 7.4 was prepared by dissolving 10 mM tris (1.21 g), 1.5 mM EDTA (0.55836 g) and 0.02% sodium azide (0.2 g) in 1000 ml of double distilled water. Its pH was adjusted to 7.4 by 0.1 N-hydrochloric acid. The buffer was stored in the refrigerator and used within a week's time.

Preparation of dextran coated charcoal (DCC):

Dextran 0.3% and Norit 3% were suspended in 10 ml of TEA buffer pH 7.4 and the mixture was agitated for some time. The DCC slurry thus obtained was stored in refrigerated condition, and used within a week.

Preparation of scintillation fluid:

Radioactivity was counted in the scintillation fluid containing the following ingredients:

- PPO 3.25 g
- POPOP 0.065 g
- Naphthalene 52 g
- Toluene 250 ml
- Dioxane 250 ml
- Methanol 150 ml
Preparation of the stock solution of the test compounds:

Stock solutions of $10^{-3}$M were prepared in DMF solution because of their low solubility in buffer. Further serial dilutions were done with TEA buffer containing 7% DMF v/v starting from $10^{-4}$ to $10^{-12}$M concentrations.

CHARACTERIZATION OF CYTOPLASMIC RECEPTORS

Basic principles of steroid receptor

Assay procedure:

The physical properties of the steroid receptor are considered in terms of (i) its binding affinity ($K_a$ or $1/K_d$); (ii) the number of molecules or binding sites (per mg protein or per cell); (iii) its steroid specificity; and (iv) a saturable phenomenon where the steroid receptors represent a finite number of binding sites.

The affinity is measured as the $K_d$ which is the equilibrium dissociation constant of the protein-hormone interaction. It is numerically equal to the rate of dissociation of the hormone from its binding protein divided by its rate of association. Therefore a slow rate of dissociation of hormone from its binding protein with a fast rate of association is indicative of high affinity binding. Thus the lower the $K_d$ the higher the affinity. Affinity is generally measured by the method of Scatchard (1949). A fixed concentration of a
receptor preparation is incubated with increasing concentrations of labelled hormone until equilibrium is established. The free hormone is then separated from receptor bound hormone and the ratio of bound to free is plotted as the ordinate and the concentration of bound hormone as the abscissa. A straight line is indicative of a single class of binding sites, the reciprocal of the negative slope is the $K_d$ and the intercept on the abscissa is the number of binding sites.

The specificity is measured by comparing how well various unlabeled hormones (ligands) compete with a particular labeled hormone for its specific receptor. This specificity enables a given target cell to respond to a specific hormonal signal; hormones of the same class would compete effectively for their receptors. Thus, for instance, DES very effectively inhibits the binding of $^3$H estradiol to the estrogen receptor, whereas progesterone and dihydrotestosterone do not inhibit even at relatively high concentrations.

The binding of a steroid hormone is a saturable phenomenon. Since the formation of a receptor-steroid complex is considered to be obligatory for the production of a biological response, then the quantity of steroid receptor should be limited, hence a finite number of binding sites. This criterion should be met by the demonstration that the binding can be saturated by its specific hormonal steroid. This can be accomplished by exposing the receptor to various concentrations of labeled
steroid under equilibrium conditions and subsequently examining
the amount of bound $^{3}$H steroid.

**PRESENT STUDIES**

(i) **Preparation of the cytosol:**

Immature male rats were killed by cervical dislocation
and the total epididymides of the two sides and ventral pros-
tate were removed free of fat and connective tissue and kept
in ice cold saline (0.9% sodium chloride). The tissues were
pooled from 30 rats and after washing each pool was homogenized
in 0.5 ml of the same buffer in all glass homogenizer in motor
driven pestle maintained at 0-4°C with intermittent cooling.
The homogenates of epididymides and ventral prostate were
centrifuged in ultra centrifuge at 105,000 g for 60 min and the
supernatant obtained was used as cytosol fraction.

(ii) **Saturation analysis:**

Incubation mixture consisted of 200 μl of the cytosol
alongwith the different concentrations of $^{3}$H E$_2$ in 50 μl
of TEA-buffer (series A). In a parallel incubation in addition
to $^{3}$H E$_2$, a 100 fold excess of DES was added to determine
the non-specific binding (series B). Mixture was vortexed and
incubated at 0-4°C for 22-24 hr. After incubation, 0.1 ml of
DCC was added, briefly vortexed followed by further incubation
for 10 minutes. It was centrifuged at 800 g for 10 min and
the supernatant obtained after centrifugation was decanted directly into the scintillation vials containing 10 ml of scintillation fluid. The difference between (A-B) gave the estimate of specific binding.

(iii) **Scatchard analysis**

The data obtained from the above experiment was also analysed by the method of Scatchard (1949) in order to determine the binding constant.

(iv) **Steroid specificity**

Ligand specificity for estrogen receptors was determined by incubating fixed amount of \(^3\)H-E\(_2\) (60,000 cpm) with varying concentrations of unlabeled ligands. The different compounds which were used for this competitive study were (1) E\(_2\)-17/5, (2) DES, (3) Centchroman (cis and trans), (4) DHT, (5) Cyproterone acetate, (6) Progesterone (see Fig. 1).

**Assay procedure** (see Fig. 2. Flow sheet)

Each of the test compounds was incubated with a fixed amount of cytosol receptors (200 µl) with different concentration of test compounds (30 µl).

At the end of the incubation (22-24 hr) at 0-4°C, DCC (0.1 ml) was added to remove the unbound radioactivity.

Affinity of the test compound was computed by plotting a logarithm graph between percent binding and concentration (M).
INCUBATION MIXTURE

200 μl of the cytosol +
30 μl of the test compound +
30 μl of 60,000 cpm (3H) - E₂
in the assay buffer

VORTEXED

INCUBATED FOR 22 hrs at 0-4°C

AFTER INCUBATION, 0.1 ml of DCC was added

VORTEXED AND INCUBATED FOR 10 min at 4°C

CENTRIFUGED AT 800 g FOR 10 min

SUPERNATANT WAS TAKEN FOR COUNTING IN 10 ml
OF AQUEOUS SCINTILLATION FLUID.

FIG. 2 - FLOW SHEET FOR DETERMINATION OF
AFFINITY FOR CYTOSOL RECEPTORS
At 50% inhibition log doses of the compounds give the relative affinity of the compound.

**Determination of protein**

Protein content in the cytosol was measured by Lowry's method (Lowry et al., 1951) (Details are given in Part II of this section).

**Regulation of estrogen receptors by estradiol and dihydrotestosterone**

Estimation of the cytoplasmic receptors under the influence of E₂ and DHT was determined by injecting a single dose of 10 μg of E₂ or 250 μg DHT in 0.1 ml of olive oil per rat by s.c. route. These rats were killed 6 and 24 hr after the hormone injection. Estrogen receptor level was estimated by Scatchard analysis and expressed as femtomoles of (³H) estradiol bound/mg of cytosol protein.
PART -II: STUDIES ON ESTROGEN INDUCED RESPONSE

Colony bred male rats 21 and 120 days old were used in three different experiments.

Experiment I: The rats were categorised into four groups each comprising of 18 immature (21 days old) animals. Rats of the first group (Group I) served as controls; animals of groups II and III were injected subcutaneously with estradiol-17\beta (1.0 µg/rat, daily for 7 days) and dihydrotestosterone (150 µg/rat, daily for 7 days) respectively. Animals of Group IV received combination treatment of estradiol and dihydrotestosterone; the injections were given at two different sites, the dose and duration of treatment were as in Groups II and III. All the animals were killed by cervical dislocation 24 hr after last injection along with the controls. Epididymis of the two sides and ventral prostate were dissected free of adipose tissue and weighed on a torsion balance. Tissues of 3 animals were pooled for biochemical investigations.

Experiment II: Fortyeight immature (21 days old) rats were bilaterally castrated and after a rest period of 7 days they were randomly assigned into four groups of 12 animals each. The groupings and hormonal treatments were as under Experiment I.
Following a 7-day treatment period with hormones, the animals were killed by cervical dislocation when the rats were 36 days-old. Tissues of four animals were pooled for biochemical investigations.

Experiment III: Adult rats were castrated bilaterally through scrotal incision. After allowing a period of 2 weeks for the accessories to undergo involution, the animals were distributed into 3 groups which received the following treatments:

Group I - Intact controls - no treatment
Group II - Castrated controls - sterile olive oil (0.1 ml)
Group III - Castrated - treated with estradiol-17β (1 μg/rat/day)
Group IV - Castrated - treated with testosterone propionate (200 μg/rat/day)

The steroids were dissolved in sterile olive oil and injected through intramuscular route daily for 7 days.

The estrogen dose (1 μg/rat - which is approximately 5 μg/kg in adult rat) was decided arbitrarily taking into consideration the studies carried out in adult female rat. A dose of 0.1 to 1.0 μg estradiol is considered to be physiological dose in immature and in adult ovariectomized rat (Alberga and Baulieu, 1968; Clark et al., 1973). The dose of testosterone propionate (150 μg/rat) in immature animals was decided on the basis of previous investigations (Setty and Jehan, 1977) (for details on adult dose: 200 μg/rat - see Chapter II).
At the autopsy, the epididymis, seminal vesicles and ventral prostate were dissected out carefully and their weights were recorded accurately. In adult rats the epididymis was divided into caput, corpus and cauda portions and only total epididymal weight was recorded in immature rats.

Glycerylphosphorylcholine (GPC) was estimated by the procedure of White (1959) and sialic acid by Aminoff's (1961) thiobarbituric acid method. DNA was determined according to the procedure of Burton (1956), RNA by the technique of Schneider (1946) and protein by the method of Lowry et al. (1951).

**Estimation of Deoxyribonucleic Acid (DNA)**

**Principle:** The procedure for the determination of nucleic acid is based on the fact that nucleic acids can be separated from other tissue components by their preferential solubility in acid (perchloric acid or trichloroacetic acid). The isolated nucleic acids are then quantitated by means of colorimetric reaction involving the pentose component of the nucleic acid. The diphenylamine used in the determination of deoxyribonucleic acid appears to react with the sugar (deoxyribose) derived from the purine nucleotides of DNA, since purine deoxyribose derivatives are very labile to acid.
Reagents - All the chemicals of Analytical Grade were used.

(1) Sodium hydroxide (NaOH): (a) 5 mM, (b) 0.3N
(2) Perchloric acid (PCA): (1) 0.4N, (b) 1N, (c) 1.6N and (d) 10%.
(3) Diphenylamine reagent: Diphenylamine (4%) in glacial acetic acid.
(4) Acetaldehyde solution: 1.6 mg/ml of aqueous acetaldehyde.
(5) Standard DNA solution: Highly polymerised sodium salt of Calf thymus DNA (Sigma) was used. DNA (0.4 mg/ml) was prepared in 5 mM NaOH. Two ml of the stock solution (0.8 mg) was mixed with 2.5 ml of 1.6N PCA, heated at 70°C for 10 min, cooled and the volume was made upto 5.0 ml with 1.6N PCA. This solution containing 160μg/ml DNA was further diluted to obtain a working standard of 100μg/ml with 10% PCA. Different concentrations (4μg to 100μg) were used in preparing a standard graph.

Extraction of DNA from tissue: DNA was extracted from tissue according to the method of Mills et al. (1977).

Tissue samples were weighed immediately after removal from the animal and homogenised in 4.0 ml of 0.3N NaOH at 4°C. From this 0.5 ml was set aside for protein determination. The remaining 3.5 ml homogenate was placed in water bath maintained at 37°C for 3 hr rendering the ribonucleic acid (RNA) soluble by alkaline hydrolysis. An equal volume of 1.0N PCA was added
to acidify the alkaline hydrolysate and to precipitate protein and DNA. The acidified solution was placed in ice for 30 min to allow complete precipitation. The acid precipitable material was pelleted by centrifugation at 1500 g for 15 min. The supernatant was preserved for RNA analysis. The DNA in pellet was hydrolysed in 0.4N PCA by incubating at 85°C for 30 min and then cooled. After centrifugation the supernatant was removed for colorimetric determination of DNA by Burton's Method (1956).

Aliquots of 2 ml and a blank consisting of 10% PCA were taken and 2.0 ml of 4% diphenylamine reagent (DPA), freshly prepared in glacial acetic acid, was added to each tube, followed by the addition of 0.1 ml of 1.6 mg/ml of acetaldehyde solution. The reaction mixtures were incubated at 30°C overnight and the colour intensity was read at 595 m/μ.

Estimation of ribonucleic acid (RNA)

Principle: Ribonucleotides which remain in the supernatant after removal of DNA are determined on the basis of their ribose content. For this the orcinol reaction is used to measure ribonucleic acid or pentose nucleic acid (RNA or PNA). Pentoses react with concentrated acid (hydrochloric acid) in the hot to give furfural derivatives. The latter in presence of ferric ions condenses with orcinol to give a brilliant green coloured complex which indicates pentose sugar concentration.
Reagents: The chemicals were of Analytical Grade.

(1) Orcinol reagent: 1 gm orcinol was dissolved in 100 ml concentrated hydrochloric acid (HCl) containing 0.5 gm of ferric chloride (FeCl$_3$) solution. The reagents used for extraction of RNA from tissue are common with that of DNA extraction.

(2) Acetate buffer: Acetic acid (0.2M, 8.8 ml) was added to sodium acetate (0.2M, 41.2 ml).

(3) Standard RNA solution: 100 mg of RNA (Sisco) was dissolved in 25 ml of acetate buffer, pH 5.5. A working standard solution of RNA containing 400 µg/ml was prepared which was further diluted and used in the range of 4 µg - 28 µg.

Procedure: The supernatant (1.5 ml) obtained after precipitating the DNA 1N perchloric acid (PCA) was heated with 1.5 ml of orcinol reagent for 20 min in boiling water bath. The tubes were cooled and the colour intensity was read at 660 m/µ.

Estimation of Protein

Principle: The final colour obtained in the Folin-ciocalteu method is the result of (a) biuret reaction of protein with copper and (b) reduction of phosphomolybdic acid reagent by the tyrosine and tryptophan present in the protein.
Reagents: All the chemicals of Analytical quality were used.

(1) Sodium carbonate: 2% in 0.1N sodium hydroxide (NaOH).
(2) Copper sulphate: 0.5% in 1% sodium potassium tartarate (Na, K, Tartarate).
(3) Alkaline copper sulphate solution: 50 ml of sodium carbonate solution (2% in 0.1N NaOH) were added with 1 ml of 0.5% copper sulphate solution (in 1% Na, K tartarate).
(4) Folin's reagent (Sisco): 1N
(5) Standard protein solution: Bovine serum albumin (Sigma - fraction V) was prepared in 0.1N NaOH at a concentration of 100/μg/ml. Different aliquots of the above solution were used ranging from 10/μg to 100/μg.

Procedure: Tissue homogenates (10 mg/ml) were precipitated with equal volumes of 10% Trichloro acetic acid (TCA) and left in cold for 30 minutes. The mixture was centrifuged and precipitate was rewashed with 5% TCA and distilled water. The precipitate was then dissolved in 1 ml of 1N NaOH. Different aliquots (0.1 to 1.0 ml) from the above solution were taken and the volume was made up to 1.0 ml with distilled water. A blank consisting of 1.0 ml distilled water was also run. To each tube 4.5 ml of alkaline copper sulphate solution was added and the mixture was allowed to stand for 10 min. This was followed by the addition of 0.5 ml of 1N Folin's reagent and the tubes were vortexed immediately and kept for 30 min. The colour intensity was read at 625 μm.
Estimation of Glyceryl phosphoryl choline

**Principle:** Glyceryl phosphoryl choline (GPC) is determined as glycerol, which is oxidized to formaldehyde by periodate and the excess of periodate is removed by arsenite. The formaldehyde is then estimated colorimetrically by the formation of a complex compound with chromotropic acid.

**Reagents:** The chemicals used were of Analytical Grade.

1. Sodium hydroxide (NaOH): 0.1N
2. Zinc sulphate (ZnSO\(_4\cdot7\)H\(_2\)O): 0.5% w/v
3. Sulphuric acid (H\(_2\)SO\(_4\)): 2N
4. Sodium meta periodate (NaIO\(_4\)): 0.1N
5. Sodium arsenite (NaASO\(_2\)): 13.13%
6. Chromotropic acid reagent: 1.08 gm of the sodium salt of chromotropic acid in 100 ml of distilled water was mixed with 450 ml of 65% sulphuric acid.
7. Copper sulphate (CuSO\(_4\)): 20%
8. Calcium hydroxide (Ca(OH)\(_2\))

**Standard:** Stock standard solution of glycerol in distilled water at a concentration of 4 mg/ml was used.

**Working standard solution:** 2.5 ml from the standard stock solution was diluted to 50 ml (200 \(\mu\)g/ml), from this standard solution different aliquots were taken ranging from 50 \(\mu\)g to
200 μg and in each case the final volume was adjusted to 3.0 ml.

Procedure: Tissue homogenate was prepared in distilled water (20 mg/ml) and 3 ml of the homogenate was transferred to a test tube to which 0.5 ml of 5% ZnSO₄ and 0.5 ml of 1N NaOH was added. Mixture was vortexed and cooled, left for 10 sec and centrifuged. From this, 2.7 ml of the supernatant was taken and heated with 0.3 ml CuSO₄ (20%) to which was added 0.42 gm Ca(OH)₂.

The mixture was vortexed, left for 30 min and then centrifuged for 10 min. From this 1.5 ml of the supernatant was taken and volume was made up to 2 ml with distilled water. To this, 0.5 ml of 2N H₂SO₄ and 10.5 ml of 1N sodium metaperiodate were added, vortexed and left for 5 minutes. The excess of periodate was reduced by 13.13% sodium arsenite and left for 10 min. The volume was then made up to 10 ml with distilled water. Finally, to 0.5 ml of the above solution, 5 ml of chromotropic acid reagent was added. The mixture was placed in boiling water bath for 30 minutes, cooled and the colour was read at 580 mμ against blank.

Calculation: In order to express the results as GPC, glycerol values thus obtained from standard curve are multiplied by 5.7.

Therefore, GPC per mg tissue = \[
\frac{\text{Concentration in } \mu g \times 5.7}{3 \times \text{wt of tissue}}
\]
Estimation of Sialic acid

Sialic acid is a group name for acylated or substituted neuraminic acids. It is estimated in hydrolysed tissue homogenate according to the procedure of Warren (1959).

Principle: The oxidation of sialic acid with periodate results in the formation of chromogen. The excess of periodate is removed by acid arsenite. The intensity of colour is obtained on heating with thiobarbituric acid (TBA). The coloured complex formed is stable and soluble in acidified butanol. The absorption of coloured material is measured at 549 m\u00b4.

Reagents:

1. Periodic acid (HIO4): 0.025M in 0.125N H2SO4 (pH 1.2)
2. Hydrochloric acid (HCl): 0.5N
3. Sodium arsenite: 2% in 0.5N HCl
4. Thiobarbituric acid: 0.1M solution in water (adjusted to pH 9.0 with NaOH).
5. Butanol containing 5% v/v 12N HCl.
6. Sulphuric acid (H2SO4): 0.125N and 0.1N
7. Sodium hydroxide (NaOH): (a) Saturated solution and (b) 0.1N

Procedure: Tissue homogenate (10 mg/ml) in 0.1N H2SO4 was heated at 80°C for 1 hr to release the bound sialic acid into its free form. From this 0.5 ml of the homogenate was oxidized
with 0.25 ml of periodate reagent for 30 min at 37°C, followed by the addition of 0.2 ml of 2% sodium arsenite to reduce the excess of periodate as soon as the yellow colour of the liberated iodine has disappeared (1-2 min); 2 ml of TBA (pH 9.0) was added and the test samples were covered and heated in boiling water for 7.5 min. The coloured solutions were cooled in ice water and then shaken vigorously with 50 ml of acid butanol. Separation of the two phases was facilitated by a short, rapid centrifugation and the intensity of the colour (due to the coloured complex formed with N-acetyl neuraminic acid) in the butanol layer was read at 549 m\(\mu\). Since 2-deoxyribose gives a strong red colour in this reaction, a correction for its absorption was made by taking reading also at 532 m\(\mu\).

The readings (optical density values) obtained at 532 and 549 m\(\mu\) were inserted into equation.

\[
\mu\text{moles N-acetyl neuraminic acid} = \frac{0.090 \times \text{OD} \, 549 - 0.033 \times \text{OD} \, 532}{\text{mg of tissue used}}
\]

\[
= \mu\text{moles of sialic acid/mg tissue}
\]
RESULTS

PART I: STUDIES ON ESTROGEN RECEPTORS

(a) Saturation Analysis:

The results of the saturation analysis of epididymis and ventral prostate cytosol receptors reveal that with the increasing concentration of the radioactive estradiol specific binding increases and then plateau off showing the limited binding capacity of the receptors (Figs. 3 and 4). On the other hand, non-specific binding increases with the increasing concentration of the steroid indicative of the non-saturability and unlimited binding capacity. Referring to the Figs. 5 and 6, where bound steroid (B) vs B/F are plotted, gave the estimate of the Kd values.

(b) Scatchard Analysis:

These results reveal the presence of specific and saturable estrogen binders in the epididymis and ventral prostate of immature rat. The dissociation constant (Kd) was $5.45 \times 10^{-11}$ mol/l for epididymis and $5.43 \times 10^{-11}$ mol/l for ventral prostate. The mean number of binding sites were 19.5 fm/mg protein and 10.2 fm/mg protein for epididymis and ventral prostate respectively.
**FIG. 3** SATURATION ANALYSIS FOR CYTOPLASMIC ESTROGEN RECEPTOR IN THE IMMATURE RAT EPIDIDYMIS, SHOWING TOTAL, NON-SPECIFIC AND SPECIFIC BINDING SITES.

- • TOTAL BINDING
- X• X NON SPECIFIC BINDING
- ○○ SPECIFIC BINDING
**FIG. 4**: SATURATION ANALYSIS FOR CYTOPLASMIC ESTROGEN RECEPTOR IN THE IMMATURE RAT PROSTATE, SHOWING TOTAL, NON-SPECIFIC AND SPECIFIC BINDING SITES. ○—○ TOTAL BINDING ×—× NON SPECIFIC BINDING ●—● SPECIFIC BINDING.
FIG. 5: SCATCHARD ANALYSIS OF $[^{3}H]$-ESTRADIOL-17$\beta$ BOUND (fm/mg PROTEIN)

$[^{3}H]$-ESTRADIOL-17$\beta$ BINDING TO IMMATURE RAT EPIDIDYMIS CYTOPLASMIC ESTROGEN RECEPTOR.
FIG. 6: SCATCHARD ANALYSIS OF $[^3H]$-ESTRADIOL-17$\beta$ BINDING TO IMMATURE RAT PROSTATE CYTOPLASMIC ESTROGEN RECEPTOR.
(c) Steroid specificity:

The steroid specificity of estradiol binding to epididymis and ventral prostate cytosol reveal that when $^{3}$H estradiol was competed with excess of unlabelled estradiol for 20-24 hrs at 0-4°C, it was able to inhibit the binding of $^{3}$H estradiol (Figs. 7 and 8).

To further evaluate the specificity of $E_2$ binding to epididymis and prostate cytosol, competition experiments carried out reveal that when incubated with two ligands which bind to androgen receptors viz. DHT and cyproterone acetate and also progesterone could not significantly inhibit the binding $^{3}$H-$E_2$ at $10^{-4}M$ in either of the tissues (Figs. 9 and 10). On the other hand DES, a potent binder of estrogen receptor was able to inhibit the binding of $^{3}$H estradiol and was almost equipotent to estradiol binding (Figs. 11 and 12). The binding of triphenyl-ethane class of compounds viz. trans-Centchroman which has a fairly good receptor affinity for rat uterine $E_2$-receptors further confirm the presence of estrogen receptors (Figs. 13 and 14) (Table I). However the binding was negligible for cis-Centchroman (a geometrical isomer). These data indicate that the binding is highly estrogen specific.

In a preliminary experiment, estrogen receptors were determined by Scatchard analysis after administration of $E_2$ or DHT (Table II). Following administration of 10 $\mu g$ of $E_2$ there was no change in the level of cytoplasmic receptors in the
**FIG. 7** INHIBITION OF $^{[3]H}$ ESTRADIOL-17 $\beta$ BINDING BY ESTRADIOL-17 $\beta$ FOR CYTOPLASMIC ESTROGEN RECEPTOR OBTAINED FROM IMMATURE RAT EPIDIDYMIS. SHOWN ARE EXPERIMENTAL VALUES OF TWO DIFFERENT EXPERIMENTS.
FIG. 8: INHIBITION OF $[^3\text{H}]$ ESTRADIOL-17\(\beta\) BINDING BY ESTRADIOL-17\(\beta\) FOR CYTOPLASMIC ESTROGEN RECEPTOR OBTAINED FROM IMMATURE RAT PROSTATE. SHOWN ARE EXPERIMENTAL VALUES OF TWO DIFFERENT EXPERIMENTS.
FIG. 9  STEROID SPECIFICITY OF CYTOPLASMIC ESTROGEN RECEPTOR IN IMMATURE RAT EPIDIDYMIS. ESTRADIOL-17β AND DES SHOW SIGNIFICANT INHIBITION AT 10 nM CONCENTRATION; NO INHIBITION OBSERVED WITH DHT AND CYPROTERONE ACETATE (CA) AT THE SAME DOSE; (MEAN OF TWO EXPERIMENTS)
FIG. 10: STEROID SPECIFICITY OF CYTOPLASMIC ESTROGEN RECEPTOR IN IMMATURE RAT PROSTATE. ESTRADIOL 17β AND DES SHOW SIGNIFICANT INHIBITION AT 10 nM CONCENTRATION; NO INHIBITION OBSERVED WITH DHT AND CYPROTERONE ACETATE (CA) AT THE SAME DOSE (MEAN OF TWO EXPERIMENTS).
FIG. 11: INHIBITION OF $[^3H]E$STRADIOL-17$\beta$ BINDING BY DES FOR CYTOPLASMIC ESTROGEN RECEPTOR OBTAINED FROM IMMATURE RAT EPIDIDYMIS. SHOWN ARE EXPERIMENTAL VALUES OF TWO DIFFERENT EXPERIMENTS.
FIG. 12: INHIBITION OF $[^3H]$ESTRADIOL-17 $\beta$ BINDING BY DES FOR CYTOPLASMIC ESTROGEN RECEPTOR OBTAINED FROM IMMATURE RAT PROSTATE. SHOWN ARE EXPERIMENTAL VALUES OF TWO DIFFERENT EXPERIMENTS.
FIG. 13: INHIBITION OF [3H] ESTRADIOL-17β BINDING BY CENTCHROMAN FOR CYTOPLASMIC ESTROGEN RECEPTOR OBTAINED FROM IMMATURE RAT EPIDIDYMIS. SHOWN ARE EXPERIMENTAL VALUES OF TWO DIFFERENT EXPERIMENTS.
FIG. 14: INHIBITION OF $[^{3}H]$ ESTRADIOL-17$\beta$ BINDING BY CENTCHROMAN FOR CYTOPLASMIC ESTROGEN RECEPTOR OBTAINED FROM IMMATURE RAT PROSTATE. SHOWN ARE EXPERIMENTAL VALUES OF TWO DIFFERENT EXPERIMENTS.
<table>
<thead>
<tr>
<th>Organ</th>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymis</td>
<td>(E_2)</td>
<td>16 pm</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>30 pm</td>
</tr>
<tr>
<td></td>
<td>Centchroman (trans)</td>
<td>117 (\mu)M</td>
</tr>
<tr>
<td>Prostate</td>
<td>(E_2)</td>
<td>80 pm</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>295 pm</td>
</tr>
<tr>
<td></td>
<td>Centchroman (trans)</td>
<td>750 nM</td>
</tr>
</tbody>
</table>

**TABLE I.** Concentration of \(E_2\), DES and Centchroman required to inhibit the binding of \((^3H)E_2\) by 50% in the epididymis and ventral prostate of immature rat.
<table>
<thead>
<tr>
<th>Organ</th>
<th>6 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_2/10 \mu g ) Epididymis</td>
<td>21.5</td>
<td>17.0</td>
</tr>
<tr>
<td>( DHT/250 \mu g ) &quot;</td>
<td>12.5</td>
<td>20.6</td>
</tr>
</tbody>
</table>

**TABLE II. Effect of \( E_2 \) and DHT on cytoplasmic estrogen receptors (fmol/mg protein)**
epididymis at 6 hrs but was followed by a small decrease in estrogen receptors at 24 hrs. However, a significant decrease in receptor control was observed at 6 hrs after administration of 250μg of DHT which is restored by 24 hr.

Since only limited number of experiments were carried out no clear explanation can be offered to these observations.
Results of the Experiment I (Table III) reveal that a 7-day estrogen treatment caused a small but significant reduction in the weight of the epididymis. In contrast DHT treatment resulted in a significant increase in the weight of the epididymis and ventral prostate (Table IV) and a similar response was evident in animals that received the combination treatment. Alterations in DNA content in the two organs closely reflected the change in their weight. Estrogen treatment caused a significant reduction in the concentration of RNA and a fall was also evident in the concentration of protein in the two accessory organs examined. DHT and combination treatments were equipotent in causing increase in the RNA and protein level in both the organs. GPC concentration in the epididymis remained unaffected under the influence of DHT or combination treatments while the sialic acid level appeared to be lowered (Table III). Estrogen treatment had no significant effect on the concentration of GPC and sialic acid.

In castrated prepubertal rat a significant increase in the weight of the epididymis was evident following estrogen or androgen treatment (Table V). Androgen was relatively more effective in this regard and a synergistic effect was seen in the combination group. A similar trend of effect was seen in the level of DNA - which is indicative of some cell proliferation. A marked increase in the concentration of protein in the
TABLE III. Weight and biochemical changes in the epididymis of intact immature rat following estradiol, dihydrotestosterone and their combination treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(Group)</th>
<th>Weight (mg)</th>
<th>DNA Concentration µg/mg</th>
<th>DNA Content µg/Organ</th>
<th>RNA Concentration µg/mg</th>
<th>RNA Content µg/Organ</th>
<th>Protein Concentration mg/gm</th>
<th>Protein Content µg/Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact control</td>
<td>(I)</td>
<td>34.68±2.03*</td>
<td>10.19±0.12</td>
<td>353.0±19.62</td>
<td>4.86±0.05</td>
<td>169.08±11.43</td>
<td>124.08±7.2</td>
<td>4.37±0.49</td>
</tr>
<tr>
<td>E₂ Treatment</td>
<td>(II)</td>
<td>28.92±1.06</td>
<td>9.56±0.16</td>
<td>276.75±12.49</td>
<td>3.81±0.04</td>
<td>110.17±3.59</td>
<td>105.72±5.32</td>
<td>3.08±0.26</td>
</tr>
<tr>
<td>P value I vs II</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>DHT Treatment</td>
<td>(III)</td>
<td>54.02±2.35</td>
<td>10.99±0.07</td>
<td>593.94±25.62</td>
<td>4.91±0.04</td>
<td>264.93±9.82</td>
<td>133.69±6.93</td>
<td>7.298±0.66</td>
</tr>
<tr>
<td>P value I vs III</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>E₂+DHT Treatment</td>
<td>(IV)</td>
<td>48.50±1.62</td>
<td>11.20±0.10</td>
<td>543.11±17.05</td>
<td>5.11±0.03</td>
<td>247.88±8.78</td>
<td>133.13±1.27</td>
<td>6.458±0.23</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SE; NS = Not significant

Tissues of 3 rats (6 epididymides) were pooled and each of the biochemical values represents mean of 6 estimations (6 pools).

E₂: 1 µg/rat daily for 7 days; DHT: 150 µg/rat for 7 days; combination: as in E₂ and DHT groups.

(GPC, Sialic acid levels are given on next page)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (mg)</th>
<th>GPC</th>
<th>Sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Group)</td>
<td>Conc-</td>
<td>Conc-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tration</td>
<td>tration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg/gm</td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>(I)</td>
<td>34.68±2.03*</td>
<td>15.27±0.83</td>
</tr>
<tr>
<td>E2 Treatment</td>
<td>(II)</td>
<td>28.92±1.06</td>
<td>12.65±0.90</td>
</tr>
<tr>
<td>P value I vs II</td>
<td></td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>DHT Treatment</td>
<td>(III)</td>
<td>54.02±2.35</td>
<td>15.16±0.18</td>
</tr>
<tr>
<td>P value I vs III</td>
<td></td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>E2+DHT Treatment</td>
<td>(IV)</td>
<td>48.50±1.62</td>
<td>15.86±0.44</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td></td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Mean ± SE; NS = Not significant

Tissues of 3 rats (6 epididymides) were pooled and each of the biochemical values represents mean of 6 estimations (6 pools).

E2: 1 μg/rat daily for 7 days; DHT: 150 μg/rat for 7 days; combination: as in E2 and DHT groups.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Weight (mg)</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Concentration (μg/mg)</td>
<td>Content (μg/organ)</td>
<td>Concentration (μg/mg)</td>
</tr>
<tr>
<td>Intact control (I)</td>
<td></td>
<td>36.94 ± 3.8*</td>
<td>5.06 ± 0.13</td>
<td>190.53 ± 25.10</td>
<td>5.47 ± 0.13</td>
</tr>
<tr>
<td>E₂ treatment (II)</td>
<td></td>
<td>34.37 ± 1.22</td>
<td>5.11 ± 0.09</td>
<td>177.36 ± 7.48</td>
<td>4.61 ± 0.02</td>
</tr>
<tr>
<td>P value, I vs II</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>DHT treatment (III)</td>
<td></td>
<td>73.83 ± 6.10</td>
<td>5.17 ± 0.23</td>
<td>384.49 ± 40.51</td>
<td>6.48 ± 0.15</td>
</tr>
<tr>
<td>P value, I vs III</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E₂ + DHT treatment (IV)</td>
<td></td>
<td>76.83 ± 2.65</td>
<td>5.19 ± 0.02</td>
<td>387.02 ± 19.79</td>
<td>6.36 ± 0.18</td>
</tr>
<tr>
<td>P value, I vs IV</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Mean ± SE; NS = Not significant

Tissues of 3 rats were pooled and each of the biochemical values represents mean of 6 estimations (six pools)
E₂: 1 μg/rat daily for 7 days; DHT: 150 μg/rat daily for 7 days; combination: as in E₂ and DHT groups.
TABLE V. Weight and biochemical changes in the epididymis of castrated immature rat following estradiol, dihydrotestosterone and their combination treatment.

<table>
<thead>
<tr>
<th>Treatment (Group)</th>
<th>Weight (mg)</th>
<th>DNA (µg/mg)</th>
<th>Protein (mg/gm)</th>
<th>GPC Concentration (mg/gm)</th>
<th>GPC Content (mg/organ)</th>
<th>Sialic acid Concentration (µmol/gm)</th>
<th>Sialic acid Content (µmol/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrated control (I)</td>
<td>24.28±0.36*</td>
<td>3.47±0.332</td>
<td>145.18±4.64</td>
<td>12.42±0.209</td>
<td>0.301±0.007</td>
<td>1.52±0.037</td>
<td>0.036±0.001</td>
</tr>
<tr>
<td>Castrated, E₂ (II)</td>
<td>29.54±0.996</td>
<td>3.84±0.066</td>
<td>150.14±9.86</td>
<td>12.75±0.353</td>
<td>0.377±0.022</td>
<td>2.36±0.178</td>
<td>0.069±0.007</td>
</tr>
<tr>
<td>P value, I vs II</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Castrated, DHT treated (III)</td>
<td>35.41±0.220</td>
<td>4.70±0.316</td>
<td>184.64±23.75</td>
<td>14.09±0.417</td>
<td>0.498±0.018</td>
<td>2.33±0.247</td>
<td>0.082±0.009</td>
</tr>
<tr>
<td>P value, I vs III</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Castrated, E₂+DHT treated (IV)</td>
<td>37.50±0.382</td>
<td>4.40±0.111</td>
<td>176.13±8.10</td>
<td>15.11±0.646</td>
<td>0.566±0.099</td>
<td>2.57±0.131</td>
<td>0.096±0.006</td>
</tr>
<tr>
<td>P value, I vs IV</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.02</td>
<td>&lt;0.001</td>
<td>&lt;0.005</td>
<td>&lt;0.007</td>
</tr>
<tr>
<td>P value, III vs IV</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Mean ± SE; NS = Not significant

Tissues of 4 rats (8 epididymides) were pooled and each of the biochemical values represents mean of 3 estimations (3 pools).

E₂ : 1/µg/rat daily for 7 days; DHT: 150/µg/rat daily for 7 days; combination: as in E₂ and DHT groups; treatment commenced 7 days after castration.
androgen treated rats suggests that IHT was relatively more effective than estrogen and synergistic effect was also evident. Increase in the weight of epididymis and protein content in estrogen treated rats is suggestive of increase in cell size without hyperplasia. Both estrogen and androgen caused an increase in GPC content of the epididymis and androgen treatment, either alone or in combination with estrogen, induced a better response. Estrogen and androgen were equipotent in causing an increase in sialic acid concentration and again combination treatment appeared to be more effective than either hormone alone.

Estrogen treatment was without any effect on the weight of the ventral prostate while androgen caused a four-fold increase (Table VI); a synergistic effect was seen in animals receiving combination treatment. The concentration of DNA and of protein also revealed a significant increase in animals treated with androgen; combination treatment caused a further increase in DNA level.

Castration of adult rats for 3 weeks caused a significant reduction in the weight of all the accessory sex organs (Table VII). Estrogen treatment had no effect on the weight of the epididymis and vas deferens of castrated rats but resulted in an increase in the weight of seminal vesicles and ventral prostate. However, the accessory organ weight remained significantly lower as compared to intact controls (P <0.01). Following
TABLE VI. Weight and biochemical changes in the ventral prostate of castrated immature rat following estradiol, dihydrotestosterone and their combination treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(Group)</th>
<th>Weight (mg)</th>
<th>DNA (µg/mg)</th>
<th>Protein (mg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrated Control</td>
<td>(I)</td>
<td>5.66±0.363*</td>
<td>2.01±0.073</td>
<td>55.12±3.88</td>
</tr>
<tr>
<td>Castrated, E₂ treated (II)</td>
<td></td>
<td>5.16±0.220</td>
<td>2.03±0.026</td>
<td>66.77±13.22</td>
</tr>
<tr>
<td>P value I vs II</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Castrated, DHT treated(III)</td>
<td></td>
<td>19.33±0.441</td>
<td>2.72±0.079</td>
<td>128.17±4.48</td>
</tr>
<tr>
<td>P value I vs III</td>
<td>&lt;0.001</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td>Castrated, E₂+ DHT treated(IV)</td>
<td></td>
<td>28.16±1.45</td>
<td>3.28±0.033</td>
<td>116.52±7.17</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td>&lt;0.001</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>NS</td>
</tr>
<tr>
<td>P value III vs IV</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE; NS = Not significant

Tissues of 4 rats were pooled and each of the biochemical values represent mean of 3 estimations (3 pools).

E₂: 1µg/rat daily for 7 days; DHT: 150µg/rat daily for 7 days; combination as in E₂ and DHT groups.

Treatment commenced 7 days after castration.
TABLE VII. Changes in the weight (mg/100 gm body weight) of accessory genital organs in adult castrated
male rats treated with testosterone propionate (TP) and estradiol (E₂).

<table>
<thead>
<tr>
<th>Treatment (Group)</th>
<th>Epididymal segments</th>
<th>Total epididymis</th>
<th>Vas deferens</th>
<th>Seminal vesicles</th>
<th>Ventral prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caput</td>
<td>Corpus</td>
<td>Cauda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control (I)</td>
<td>111.5±5.9*</td>
<td>25.3±1.9</td>
<td>100.3±1.9</td>
<td>237.0±7.2</td>
<td>42.3±1.7</td>
</tr>
<tr>
<td>Castrated control (II)</td>
<td>15.8±1.0</td>
<td>8.3±1.3</td>
<td>19.3±1.0</td>
<td>43.3±2.4</td>
<td>14.8±1.5</td>
</tr>
<tr>
<td>P value I vs II</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Castrated, E₂ treated (III)</td>
<td>16.3±0.5</td>
<td>7.8±0.8</td>
<td>22.0±0.8</td>
<td>46.0±1.1</td>
<td>16.3±1.9</td>
</tr>
<tr>
<td>P value II vs III</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Castrated, TP treated (IV)</td>
<td>29.5±2.1</td>
<td>8.5±0.5</td>
<td>30.5±1.1</td>
<td>68.5±3.5</td>
<td>23.3±1.5</td>
</tr>
<tr>
<td>P value II vs IV</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean ± SE; NS = Not significant

E₂: 1µg/rat, TP: 200/µg/rat daily for 7 days; treatment commenced from 15th day after castration
androgen therapy there was a marked increase in the weight of all the accessory organs but their weight remained lower than that in intact controls; seminal vesicles and ventral prostate responded better than epididymis and vas deferens.

In castrated rats there was a two to three-fold increase in the concentration of DNA in the epididymal segments and ventral prostate (Table VIII) which reflects the relative decrease of cytoplasmic component. Estrogen treatment showed no significant effect on the elevated levels of DNA. Androgen treatment reversed the trend but the DNA concentration continued to be relatively higher than its level in intact controls. Protein level did not show any significant alterations in the accessory sex organs of different experimental groups (Table IX).

DISCUSSION

The growth and differentiation of the male sex accessory glands is dependent on testicular hormones. In the rat, the rapid growth of the seminal vesicles, ventral prostate, and epididymis parallels the rise in circulating androgen levels and occurs between 35 and 60 days of age (Brooks, 1976; Setty and Jehan, 1977; Gupta et al., 1975). However, changes do occur in these organs prior to the onset of rapid growth. In the rat, rapid differentiation of the cells of the seminal vesicles, ventral prostate, epididymis, and vas deferens occurs
TABLE VIII. Changes in DNA level (μg/mg) in the different segments of the epididymis, vas deferens and ventral prostate of adult rats: effect of castration and hormone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(Group)</th>
<th>Epididymis</th>
<th>Vventral prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caput</td>
<td>Corpus</td>
</tr>
<tr>
<td>Intact control</td>
<td>(I)</td>
<td>4.16±0.19*</td>
<td>4.40±0.27</td>
</tr>
<tr>
<td>Castrated control</td>
<td>(II)</td>
<td>9.42±0.72</td>
<td>10.70±1.17</td>
</tr>
<tr>
<td>P value I vs II</td>
<td></td>
<td>Λ0.001</td>
<td>Λ0.005</td>
</tr>
<tr>
<td>Castrated, E₂ treated</td>
<td>(III)</td>
<td>10.42±0.12</td>
<td>8.99±0.17</td>
</tr>
<tr>
<td>P value II vs III</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Castrated, TP treated</td>
<td>(IV)</td>
<td>6.41±0.50</td>
<td>6.56±0.79</td>
</tr>
<tr>
<td>P value II vs IV</td>
<td></td>
<td>Λ0.001</td>
<td>Λ0.05</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td></td>
<td>Λ0.02</td>
<td>Λ0.05</td>
</tr>
</tbody>
</table>

* Mean ± SE; NS = Not significant

E₂: 1μg/rat; TP: 200μg/rat daily for 7 days; treatment commenced from 16th day of castration.
TABLE IX: Changes in protein level (mg/gm) in the different segments of the epididymis, vas deferens and ventral prostate of adult rats: effect of castration and hormone replacement.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(Group)</th>
<th>Epididymis</th>
<th>Ventral prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caput</td>
<td>Corpus</td>
</tr>
<tr>
<td>Intact control</td>
<td>(I)</td>
<td>96.01±6.02</td>
<td>134.87±7.06</td>
</tr>
<tr>
<td>Castrated control</td>
<td>(II)</td>
<td>109.88±5.31</td>
<td>111.34±11.05</td>
</tr>
<tr>
<td>P value I vs II</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Castrated, E₂ treated</td>
<td>(III)</td>
<td>93.93±9.33</td>
<td>101.97±4.00</td>
</tr>
<tr>
<td>P value II vs III</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Castrated, TP treated</td>
<td>(IV)</td>
<td>107.35±9.09</td>
<td>114.33±7.77</td>
</tr>
<tr>
<td>P value II vs IV</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Mean ± SE; NS = Not significant
E₂: 1 μg/rat, TP: 200 μg/rat daily for 7 days; treatment commenced from 15th day after castration
between 10 and 21 days after birth (Flickinger, 1969, 1971; Dahnke, 1970; Sun and Flickinger, 1979). Serum estradiol levels increase between the 9th and 19th days to levels higher than those observed in adults (Dohler and Wuttke, 1975). The major changes in the accessory organs include proliferation of the endoplasmic reticulum, growth of the Golgi apparatus, appearance in the prostate and seminal vesicles of secretory vacuoles, stereocilia in the epididymis. The presence of appreciable quantities of GPC and sialic acid in the epididymis of 3-week old animals (Setty and Jehan, 1977) suggests that epididymal synthetic and secretory potential in the prepubertal period may be under the influence of estrogen. It is pertinent to mention that a 7-day testosterone treatment commencing from the fourteenth day increases mitotic activity but not the levels and of GPC/sialic acid. Presumably the stimulatory influence of estrogen on the epididymis and other accessory sex organs is mediated through estrogen receptor (see below).

In rat testis cytoplasmic receptors for estrogen appear on day 1 after birth and attain adult levels by day 22 (Eisenfeld, 1972). Recent studies of Bouton et al. (1981) have suggested that estrogen treatment in adult rats increases androgen receptor concentration and the effect appears to be mediated by estrogen. Since cytoplasmic receptors for androgen appear in the epididymis of rat around 20 days (Calandra et al., 1974) when the epididymis is histologically undifferentiated (Setty and Jehan, 1977), it is tempting to speculate that during
the prepubertal period estrogen prepares the epididymis to respond to rising levels of androgen between 3rd and 5th weeks of pubertal life (Moger, 1977). In order to prove the validity of this suggestion, it was necessary to demonstrate presence of estrogen receptors in prepubertal rat and present evidence of a quantitative correlation with an identifiable biological response.

In this study we have demonstrated that the epididymis and ventral prostate of immature rats contain cytoplasmic estradiol binding molecules that exhibit the characteristics of estrogen receptors. Our experiments have revealed that there exists a saturable estrogen binding component in the cytosol of the epididymis and ventral prostate. The specificity of estrogen binder was indicated by the inability of progesterone and dihydrotestosterone to compete with labelled estradiol. The relative binding affinities of three different estrogens for the 'receptor' protein with the following order was observed: estradiol-17β, diethylstilbestrol, centchroman (trans). Centchroman (cis) failed to compete with the binding of (3H) estradiol-17β to estrogen receptor sites in epididymis and ventral prostate. These results indicate that those male accessory sex organs that respond to estrogen possess estrogen receptors and they also suggest that estrogen uptake and retention by these organs (see Chapter II) is due to the presence of receptors specific for estrogen. However, the
estrogen receptor concentration of the cytosol from immature rat epididymis (approximately 20 fmol/mg protein) and ventral prostate (10 fmol/mg protein) is low. Comparable value has been reported in young adult rat prostate by Jung-Testas et al. (1981).

Ponderal and biochemical changes observed in intact immature rats suggest that the epididymal growth promoted by androgen is primarily due to cell proliferation. This is the period when the epididymal epithelium exhibits high mitotic activity and androgen treatment further increases it (Setty and Jehan, 1977). Presumably it is for this reason that the rise in GPC and sialic acid content of the epididymis is not accompanied by an increase in their concentration in androgen treated animals. Estrogen at the dose level used appears to interfere with the normal growth process of the epididymis in the growing rats while at the same dose level it had no effect on the growth of ventral prostate. This suggestion is supported by the observation that the epididymis of estrogen treated animals contained proportionately less DNA as compared to the controls. However, the secretory function of the epididymis appears to be insensitive to estrogen treatment in intact prepubertal rats as is revealed by near normal levels of GPC and sialic acid. When their content is calculated on DNA basis, it becomes clear that the epididymal cells of estrogen treated rats are still as active in the synthesis of secretory products as normal cells. It is
reasonable to suggest that in prepubertal rats an optimal ratio of estrogen to androgen is essential to obtain a stimulatory response in the epididymis and if the dose of exogenous estradiol administered to intact immature rats is hyperphysiological, it will interfere with the action of endogenous androgen at the target organ level (see Tindall et al., 1981). To verify if this suggestion is valid, a second series of experiments were carried out in prepubertal castrated animals. The results of these experiments clearly demonstrated that estrogen, in physiological dose range has a direct stimulatory effect on the secretory function of the epithelium.

A physiological role for estrogen in pubertal and adult male rats cannot be ruled out since estradiol formation continues through adult life (van der Molen et al., 1981) and epididymis and ventral prostate demonstrate ability to concentrate and retain labelled estradiol (Chapter II). In adult castrated rats estrogen failed to promote the growth of involuted epididymis and this is in contrast to the results observed in prepubertal castrated animals. The loss of weight of adult castrated rat epididymis is mainly due to shrinkage of cytoplasmic component as revealed by elevated DNA concentration (Brooks, 1977). Exogenously administered androgen restores the epididymal weight only partially. Since the differentiated epithelium of the epididymis consists of a stable population of cells (Clermont and Flannery, 1970; Tushimaa and Niemi, 1974), the epididymal growth in androgen treated castrated rats is due
to restoration of cytoplasmic component and estrogen per se lacks this ability in adult rats. Results reported previously from this laboratory suggest that the epididymis of adult castrated rat responds to estrogenic stimulus as revealed by increase in sialic acid and GPC levels (Jehan and Setty, 1977). It, therefore, appears that epididymal epithelium of adult castrated rat is as sensitive to estrogen as that of immature rat and this may be indicative of a direct receptor-mediated function of estrogen in the regulation of epididymal function. It is relevant to note that estrogen receptors are reported to be present in the epididymis of adult animals (Danzo and Eller, 1979).

The physiological significance of estrogen receptors in rat ventral prostate and other mammals has remained speculative and is believed to be involved in mediating the action of estrogen in the growth of fibro-muscular tissue (Jung-Testas et al., 1981; Dickson and Clark, 1981; Neubauer et al., 1981; Bouton et al., 1981). In rat relatively high levels of estrogen receptors exist in the stromal compartment and lower levels in the epithelium (Jung-Testas et al., 1981). In contrast to other species, the rat ventral prostate does not contain significant amounts of stroma and is, therefore, relatively less sensitive to estrogen (see Mariotti et al., 1981). Nevertheless, estrogen has some growth promoting effect on the ventral prostate in adult castrated rat and it appears to influence both stroma and
epithelium (Corrales et al., 1981). If estrogen is administered with androgen to prostate cultures it potentiates the proliferative response (Tisell, 1971). The importance of stromal-epithelial interactions in the regulation of functional activity of accessory sex organs is now receiving the attention of investigators (Cunha et al., 1980).

An interesting observation of the present study (castrated prepubertal rat study) is that estrogen appears to have some synergistic role in influencing the androgen action on the secretory function of the prepubertal rat epididymis and growth of the prostate. Some investigators (Mariotti et al., 1981) have reported that no positive androgen-estrogen interaction is seen in rodent accessory sex organs while others have shown that in low doses estrogen enhances the action of androgen. Low doses of estrogen potentiates the action of testosterone on the secretory activity (fructose concentration) of mouse seminal vesicle (Thomas and Knynch, 1966), rat accessory sex organ weight (Saunders, 1958) and the levels of acid/alkaline phosphatases and citric acid (Shimazaki et al., 1969; Anderson and Muntzing, 1972; Grayhack and Kretchmar, 1963). Though it is not known if these interactions represent the sum of androgen and estrogen action on separate cell types (e.g. epithelium and stroma) or reflect an interaction within a given cell type within the epithelium, it is pertinent to note that within a given glandular epithelial cell of canine prostate, estrogen-sensitive and androgen-sensitive secretory granules can coexist.
(Leav et al., 1978). It is, therefore, tempting to suggest that a synergistic relationship may exist between estrogen and androgen that is secondary to their interaction with the respective receptors. The precise nature of cooperative action is not clearly understood.

In conclusion, the demonstration of presence of specific estrogen receptors in the rat epididymis is not a novel finding in that specific estrogen binding proteins have been demonstrated in the prostate and seminal vesicles of guinea pig (Blume and Mawhinney, 1978), prostate of dog (Younes et al., 1979) and of man (Bashirelahi and Young, 1976) and epididymis of dog (Younes and Pierrepoint, 1981) and rabbit (Danzo et al., 1975, 1978; Danzo and Eller, 1979). From the survey of the literature (Chapter I, Section B) and from the results of the present study it is clear that the male accessory sex organs are exposed to estrogen and that they possess the biochemical machinery to respond to estrogenic stimuli. The duality of epididymal response to the sex hormones is apparent from the observation that estrogen stimulates the secretory activity of regressed epididymal epithelium which had undergone differentiation before castration. The ability of the epididymal epithelium to respond to either sex hormones (estrogen and androgen) suggests that these steroids may be acting in concert at physiological levels as regulators of epididymal secretory function. The question that arises from such a conclusion is whether two
different sex steroids are necessary to perform the same function. Future studies should, therefore, be directed toward identifying estrogen-specific biochemical responses in the epididymis and the specific cell types responsible. Information on the possible direct action of estrogen on the primate epididymis will be of particular interest.
SUMMARY

1. A receptor protein that selectively binds estrogens has been demonstrated in the cytosol of the epididymis and ventral prostate of prepubertal (21 day old) rats.

2. Dihydrotestosterone and cyproterone acetate did not compete with \(^{3}H\) estradiol-17\(\beta\) for the estrogen receptors, but estradiol and diethylstilbestrol were effective competitors.

3. Scatchard plot analyses were linear, suggesting a single class of high affinity binding sites for estradiol with a \(K_d\) of \(5.45 \times 10^{-11}\) mol/l and a concentration of binding sites of 20 fmole per mg cytosol protein in the epididymis. In the cytosol of the ventral prostate the concentration of the receptor was 10.20 fmole per mg protein with a \(K_d\) of \(5.43 \times 10^{-11}\) mol/l.

4. In intact prepubertal rats a seven-day treatment (28-day old at autopsy) with estradiol (1.0 \(\mu\)g) caused a slight reduction in the weight of the epididymis but the concentration of GPC and sialic acid remained unaltered.

5. In prepubertal rats castrated for 7 days, treatment with estradiol (1.0 \(\mu\)g) for 7 days (35-day-old at autopsy) resulted in a marked increase in the weight and content of GPC and sialic acid in the epididymis; estradiol and dihydrotestosterone were equipotent in causing an increase in sialic acid concentration.
6. Estradiol appears to have some synergistic role in influencing the androgen action on the secretory function of the prepubertal rat epididymis (and growth of the prostate).

7. It is concluded that a synergistic relationship may exist between estrogen and androgen that is secondary to their interaction with their respective receptors in the accessory genital organs of the male. The precise mechanism of cooperative action is not known.
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BIOLOGICAL ACTION OF ESTROGEN ON THE EPIDIDYMIS OF PREPUBERTAL Rhesus Monkey - A PRELIMINARY STUDY ON ESTROGEN BINDING PROTEIN IN THE EPIDIDYMIS AND PROSTATE
INTRODUCTION

In the previous chapter evidence was presented to demonstrate the presence of a receptor protein that selectively binds estrogen in the cytosol of the epididymis and ventral prostate of prepubertal rats. The studies also revealed that estrogen, in physiological dose range has a direct stimulatory effect on the secretory function of the epididymis. These findings suggest that the epididymal response to estrogen is a direct receptor-mediated event.

The idea that estrogens may be contributors to the hormonal regulation of prostatic function has been considered for many years and most researchers, studying a variety of species, have concluded that estrogens are capable of producing two direct effects in the accessory sex organs, squamous metaplasia of the epithelium and growth of the fibromuscular tissue (Emmens and Parkes, 1947; Price and Williams-Ashman, 1961). Regression of the prostate following estrogen administration has generally been assumed to be mediated indirectly via hypothalamic-pituitary-gonadal axis (Scott et al., 1957), though some direct effects of estrogen on the prostate have been reported (Sandberg and Gaunt, 1976). Because treatment with estrogens is as effective as castration in the therapy of prostatic cancer (Chisholm and O'Donoghue, 1975), much effort
has been made to identify and quantitate an estrogen receptor in the human prostate.

The results obtained by different investigators on estrogen receptor level in the human prostate have been conflicting (Wagner et al., 1975; Hawkins et al., 1975; Bashirelahi et al., 1976; Menon et al., 1977; deVoogt et al., 1978; Shain et al., 1978; Edman et al., 1979; Raynaud et al., 1980) and are presumably due to methodological differences followed by different investigators. The major obstacle in most of these assays has been tissue contamination with plasma sex steroid binding protein and some investigators have overcome this problem by using a synthetic estrogen, R-2858 (moxestrol, 11β-methoxy-17α(-ethynyl-1,3,5(10)-estratriene 3,17β-diol) which shows no affinity to plasma sex steroid binding protein (Raynaud et al., 1977, 1980). The assay system using moxestrol was carefully characterised by Raynaud et al. (1977, 1980) and has been used to measure estrogen receptor in sex accessory tissues of human male (Murphy et al., 1980).

At present little is known about the presence and physiological significance of estrogen receptors in the male accessory sex organs of non-human primates. Recently, Sufrin et al. (1975) have reported the existence of a binding protein for estradiol-17β in the baboon caudal prostate. In the present preliminary study an attempt has been made to demonstrate the presence of an estrogen binding protein in cytosol
prepared from epididymis and caudal lobe of the prostate of immature rhesus monkey using moxestrol. Further, the ponderal and secretory response of the epididymis to exogenous estrogen was investigated in castrated immature monkey.

MATERIALS AND METHODS

This section is divided into two parts.

PART I: STUDIES ON ESTROGEN BINDING PROTEIN

Animals

Immature male rhesus monkeys (3 kg, 12-15 months old) of the Institute primate colony were used in the present study.

Chemicals

\((^3\text{H}) \text{R-2858 (moxestrol, (11/3-methoxy-17-o( ethynyl-1,3,5 (10)-estratriene-3,17/3-diol} \) (Fig. I) (sp. act. 87 \(^\text{Ci/mmole}\) was obtained from New England Nuclear Corporation, U.S.A. along with a sample of non radioactive R-2858. Tris-buffer, toluene, dioxane, glycerol and methanol were of analytical grade. Dithiothreitol was from Sigma Chemicals, U.S.A. and Naphthalene was of scintillation grade (IDPL, India). 2,5 diphenyl oxazole (PPO) and (p-bis)2-(5 phenyl-oxazole) benzene (POPOP) were obtained from New England Nuclear Corporation, U.S.A.; Norit A (activated charcoal), Dextran (M.W.70,000) and bovine serum albumin (fraction V) were supplied by Sigma Chemicals, U.S.A.
FIG. 1  MOXESTROL, [11β-Methoxy-^3H]-----
(R-2858)
Radiochemical purity

The purity of the radioactive steroid was checked by thin layer chromatography (TLC) on silica gel G plates using benzene:ethyl acetate (7:3) v/v as the solvent system.

Storage of radioactive material

$(^3\text{H})$ R-2858 was stored in toluene:ethanol (9:1) at 2 to 4°C. Before use the solvent was evaporated under a stream of nitrogen, replaced by appropriate amount of absolute alcohol and diluted with appropriate buffers. The stock solution was stored in a refrigerator and used within a week's time.

Preparation of Reagents

TED buffer (pH 7.4) was prepared by dissolving 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol in 1000 ml of double distilled water. Its pH was adjusted to 7.4, stored in the refrigerator and was used within a week.

Preparation of Dextran coated charcoal (DCC)

Dextran 0.25% and Norit A 2.5% were suspended in TED buffer of pH 7.4 and the mixture was agitated for some time. The DCC slurry thus obtained was stored in refrigerated condition and used within a week.

Preparation of scintillation fluid

Preparation of scintillation fluid is given in Chapter III.
Assay Procedure

The monkeys were killed by administering a lethal dose of Nembutal (Abbott Laboratories, India). Total epididymides and caudal lobe of prostate were removed free of connective tissue and were then weighed accurately on a torsion balance. The pooled tissues of 2 animals (4 epididymides weighing in the range of 1.2 to 1.5 gm and 2 prostates weighing 700 to 800 mg) were washed thrice with TED buffer (pH 7.4), minced and homogenized in 10 ml of the same buffer using Ultra-Turrax with intermittent cooling. The homogenates were centrifuged at 105,000 x g for 60 min and the supernatant thus obtained was used as the cytosol fraction. The experiments were carried out with three pools of tissues obtained from 6 animals.

Saturation analysis

Incubation mixture consisted of 200 μl of the cytosol along with the increasing concentration of R-2858 in 50 μl of TED buffer (series A). In a parallel incubation, in addition to (3H) R-2858 a hundred fold excess of unlabelled R-2858 was added to determine the non specific binding (series B). Mixture was vortexed and incubated at 0-4°C for 20 hr. After incubation, 0.1 ml of DCC was added, briefly vortexed and further incubated for 10 minutes. It was centrifuged at 800 g for 10 minutes and the supernatant obtained after centrifugation was decanted directly into LSC vials containing 10 ml of
scintillation fluid. The difference between (A-B) gave the estimate of the specific binding.

Scatchard Analysis

The data obtained from the above experiment was also analysed by the method of Scatchard (1949) to determine the binding constant.

Determination of protein

The protein concentration in the cytosol samples was determined by the method of Lowry et al. (1951).

PART II: STUDIES ON ESTROGEN INDUCED RESPONSE

Eighteen immature rhesus monkeys (4.0-4.5 kg, 30-36 months old) of the Institute's primate colony were used in the present study. Bilateral castration was achieved under aseptic conditions by the scrotal route; Nembutol was used as the anaesthetic. Testes of all the animals were examined histologically and they presented immature state. The animals were subjected to post-operative antibiotic therapy for a period of 5 days and were maintained under uniform husbandry conditions. Thirty days after the surgery the animals were distributed into 4 groups. Monkeys of group I served as controls; animals of group II and III were injected with estradiol dipropionate (50 μg/animal and 10 μg/animal respectively). Animals of group IV received testosterone propionate (2 mg/animal). The
steroids were dissolved in sterile olive oil and administered through intramuscular route. The animals of control group were injected with vehicle alone (1 ml sterile olive oil). Monkeys of all the groups (excepting those of group II) were treated daily for 30 days and were sacrificed 24 hr after last injection. In animals treated with 50 μg dose of estrogen (group II), there was extensive edema and swelling of the circum anal region, scrotum and penis. The treatment in this group was terminated after 12 days and the animals were sacrificed a day later.

At the time of autopsy the accessory genital organs (epididymis, seminal vesicles and caudal lobe of prostate) were carefully dissected and weighed accurately on a torsion balance. The epididymis was divided into proximal and distal segments, the former comprising of caput and corpus portions and the latter the cauda portion. The division was made on the basis of macroscopic appearance. Epididymal tissue samples were homogenized using Ultra-Turrax and were used for determination of protein and sialic acid by the methods of Lowry et al. (1951) and Aminoff (1961) respectively. Procedural details are given in chapter III. The data were analysed by student's 't' test.
RESULTS

PART I: ESTROGEN BINDING PROTEIN (ESTROGEN RECEPTOR)

(a) **Saturation Analysis**

The results of the saturation analysis of epididymis and prostate reveal that with increasing concentration of the radioactive R-2858, specific binding increases and after reaching maximum binding it plateaus off showing the limited binding capacity of the receptors. On the other hand, non specific binding increases with increasing concentration of the steroid which indicates the unsaturability and unlimited binding capacity (Figs. 2 and 3).

(b) **Scatchard Analysis**

The results of the scatchard analysis reveal the presence of saturable estrogen binding protein in the epididymis and prostate of immature rhesus monkey. The dissociation constant was $5.88 \times 10^{-10}$ mol/l for epididymis and $5.42 \times 10^{-10}$ mol/l for prostate. The mean number of binding sites were $17.0 \pm 4.4$ fmol/mg protein for epididymis and $19.7 \pm 3.6$ fmol/mg protein for prostate (Figs. 4 and 5).

(c) **Protein concentration in cytosol**

The protein concentration was 2.5 to 3.5 mg/ml cytosol of the epididymis and 2.5 to 3.0 mg/ml cytosol in prostate.
FIG. 2 SATURATION ANALYSIS FOR CYTOPLASMIC ESTROGEN RECEPTOR IN THE IMMATURE Rhesus Monkey Epididymis SHOWING TOTAL O--O, NON SPECIFIC X--X AND SPECIFIC ●● BINDING SITES.
FIG. 3 SATURATION ANALYSIS FOR CYTOPLASMIC ESTROGEN RECEPTOR IN THE IMMATURE RHESUS MONKEY PROSTATE SHOWING TOTAL ○○, NON-SPECIFIC ×× AND SPECIFIC •• BINDING SITES.
FIG. 4 SCATCHARD ANALYSIS OF $[^3H]R-2858$ BINDING TO IMMATURE RHESUS MONKEY EPIDIDYMIS CYTOPLASMIC ESTROGEN RECEPTOR. ($B/F =$Bound hormone/Free hormone)
FIG. 5 SCATCHARD ANALYSIS OF $[^3H]R-2858$ BINDING TO IMMATURE RHESUS MONKEY PROSTATE CYTOPLASMIC ESTROGEN RECEPTOR. (B/F = Bound hormone/Free hormone)
There was a significant increase in the weight of all the accessory sex organs following androgen and estrogen treatment and, in general, the response was relatively much higher in androgen treated animals (Table I). Maximum weight rise was evident in the seminal vesicles and prostate. The tissue growth was accompanied by a significant increase in concentration (mg/g) and content (mg/organ) of protein in the epididymis (Table II). Of the two segments of the epididymis, distal part showed relatively higher concentration of protein. Estrogen treatment, at both the dose levels caused a significant increase in the concentration of sialic acid in the distal epididymis. Androgen treatment, on the other hand, induced a significant rise in sialic acid level in both the segments of the epididymis (Table III). Expressed as total protein or sialic acid/organ, there was a marked increase in the content of these two constituents under the influence of both estrogen and androgen; androgen, however, was relatively more potent in this regard. To obtain content of sialic acid and protein per organ, the mean value of concentration of these constituents in the proximal and distal epididymides was calculated and was multiplied by the total organ weight.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Epididymis (mg)</th>
<th>Seminal vesicles (g)</th>
<th>Prostate (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrated Control</td>
<td>(I)</td>
<td>270.5 ± 1.84 (8)*</td>
<td>0.73 ± 0.22 (4)</td>
<td>0.51 ± 0.09 (4)</td>
</tr>
<tr>
<td>Castrated + EDP (50 µg)</td>
<td>(II)</td>
<td>641.3 ±15.59 (8)</td>
<td>2.77 ± 0.22 (4)</td>
<td>0.62 ± 0.09 (4)</td>
</tr>
<tr>
<td>P value I vs II</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Castrated + EDP (10 µg)</td>
<td>(III)</td>
<td>428.8 ±12.02 (10)</td>
<td>3.88 ± 0.42 (5)</td>
<td>1.06 ± 0.15 (5)</td>
</tr>
<tr>
<td>P value I vs III</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Castrated + TP (2 mg)</td>
<td>(IV)</td>
<td>867.2 ±77.74 (10)</td>
<td>8.82 ± 1.03 (5)</td>
<td>1.68 ± 0.13 (5)</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.E., no. of tissues in parentheses. NS = not significant

Group II: EDP, 50 µg daily for 12 days; Group III: EDP, 10 µg daily for 30 days; Group IV: TP, 2 mg daily for 30 days.
TABLE II - Changes in protein level in the epididymis of immature castrated rhesus monkeys treated with estradiol dipropionate (EDP) and testosterone propionate (TP)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Protein concentration (mg/gm)</th>
<th>Protein content (mg/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proximal epididymis</td>
<td>Distal epididymis</td>
</tr>
<tr>
<td>Castrated control     (I)</td>
<td>35.0 ± 3.51 (8)*</td>
<td>55.9 ± 0.83 (8)</td>
<td>12.3 ± 0.51 (8)</td>
</tr>
<tr>
<td>Castrated + EDP (50 μg) (II)</td>
<td>62.4 ± 4.38 (8)</td>
<td>111.4 ± 0.53 (8)</td>
<td>55.8 ± 2.21 (8)</td>
</tr>
<tr>
<td>P value I vs II</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Castrated + EDP (10 μg) (III)</td>
<td>72.8 ± 5.05 (10)</td>
<td>113.1 ± 2.35 (10)</td>
<td>40.6 ± 1.10 (10)</td>
</tr>
<tr>
<td>P value I vs III</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Castrated + TP (2 mg) (IV)</td>
<td>90.6 ± 2.52 (10)</td>
<td>118.4 ± 2.70 (10)</td>
<td>91.3 ± 10.01 (10)</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.E., no. of estimations in parentheses.
TABLE III - Changes in sialic acid level in the epididymis of immature castrated rhesus monkeys treated with estradiol dipropionate (EDP) and testosterone propionate (TP).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Sialic acid concentration (µmol/g)</th>
<th>Sialic acid content (µmol/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proximal epididymis</td>
<td>Distal epididymis</td>
</tr>
<tr>
<td>Castrated control</td>
<td>(I)</td>
<td>1.01± 0.015 (8)*</td>
<td>1.18± 0.035 (8)</td>
</tr>
<tr>
<td>Castrated + EDP (50 µg) (II)</td>
<td></td>
<td>1.10± 0.021 (8)</td>
<td>1.54± 0.090 (8)</td>
</tr>
<tr>
<td>P value I vs II</td>
<td>NS</td>
<td>£0.005</td>
<td>£0.001</td>
</tr>
<tr>
<td>Castrated + EDP (10 µg) (III)</td>
<td></td>
<td>1.08± 0.033 (10)</td>
<td>1.41± 0.033 (10)</td>
</tr>
<tr>
<td>P value I vs III</td>
<td>NS</td>
<td>£0.001</td>
<td>£0.001</td>
</tr>
<tr>
<td>Castrated + TP (2 mg) (IV)</td>
<td></td>
<td>1.43± 0.092 (10)</td>
<td>1.74± 0.027 (10)</td>
</tr>
<tr>
<td>P value I vs IV</td>
<td>£0.001</td>
<td>£0.001</td>
<td>£0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.E., no. of estimations in parentheses; NS = not significant
DISCUSSION

Having demonstrated that the epididymis and ventral prostate of immature rats contain cytoplasmic estradiol binding protein satisfying the criteria of estrogen receptors, we considered it worthwhile to examine if a similar protein is present in the epididymis and prostate of immature rhesus monkey. In rhesus monkey, secretion of estrogen from the testis appears to be low and peripheral aromatization seems to contribute significantly to circulating levels (Franz and Longscope, 1979). Circulating levels of estradiol in this species are reported to be higher than those found in men and baboons (Aso et al., 1976). Significant amounts of estradiol are also present in monkey rete testis fluid (Waites and Einer-Jensen, 1974).

In the present preliminary study we have been able to identify a single class of high affinity, low-capacity estrogen binding protein which is presumably estrogen receptor. Its concentration in the epididymal cytosol is $17.0 \pm 4.0$ fmol/mg protein and in the cytosol from prostate its concentration is $19.7 \pm 3.6$ fmol/mg protein. A similar value has been reported in the cytosol of human epididymis by Murphy et al. (1980). Though steroid specificity was not determined in the present study, use of the synthetic estrogen, moxestrol in our 'receptor' assay has enhanced the specificity of the technique. Published results have clearly indicated that moxestrol, which is a
potent estrogen (approximately 5-10 times as potent as estradiol in mouse uterine weight assay) has affinity only for estrogen receptor (comparable to that of estradiol) and is not bound by sex-steroid binding protein (Ojasoo and Raynaud, 1978) and is considered an ideal ligand in estrogen receptor studies. Estradiol, R-2858 and diethylstilbestrol are reported to be equally effective in competing for the specific binding of \(^{3}H\) R-2858 or \(^{3}H\) estradiol by the estrogen receptor while dihydrotestosterone and progesterone are ineffective competitors (Murphy et al., 1980). It is, therefore, reasonable to assume that R-2858 binding protein demonstrated in the present study in epididymis and prostate is estrogen receptor.

To examine if estrogen has a role in influencing the growth of sex accessories and secretory function of the epididymis, a second study was carried out in immature castrated monkey. Estrogen treatment induced a significant increase in the weight of all the three accessory sex organs examined viz., seminal vesicles, prostate and epididymis. The increase in the concentration of total protein in the epididymis following estrogen treatment indicated that the weight rise reflected a true tissue growth.

Determination of sialic acid level in the epididymis is often used as an index of epididymal secretory function (reviewed by Setty, 1979) and in lower mammals like rat, it is an androgen sensitive parameter. In our studies in rat
(Chapter III) we have noted that estrogen also causes an increase in the epididymal sialic acid level. Estrogen treatment in monkey caused an increase in the sialic acid content in the epididymis and only the distal epididymis showed an increase in its concentration. Danzo and Eller (1979) have reported that in the rabbit epididymis estrogen receptor concentration is maximum in the cauda portion and this may be of physiological significance. It is interesting to note that in adult rhesus monkey, castration and androgen replacement therapy are reported not to influence the sialic acid level in the epididymis (Bose and Kar, 1968) while in the immature castrated animals androgen has a stimulatory influence on epididymal sialic acid level (present study). It is possible that in immature and in adult monkeys, there may be a synergistic relationship between estrogen and androgen in regulating epididymal sialic acid level.

One of the problems that the investigator working in this area encounters is the lack of a estrogen specific biochemical marker in the accessory sex organs of the males. If one accepts that sialic acid level is a reasonably good end point in determining the epididymal response to estrogen, it may be considered that this response is mediated via the estrophilic molecule. At present it is difficult to perceive
accurately the intracellular significance of estrogen binding molecules in androgen dependent organs. With continued analysis of the estrogenic and androgenic effect in male sex accessory organs, and mechanisms of estrogen and androgen action in these organs, the physiological role of the two sex steroids in these organs will be more clearly defined. The present study is a first step in that direction.

SUMMARY

An estrogen binding protein has been demonstrated in the cytosol of the epididymis and caudal prostate of immature rhesus monkey. Scatchard plot analyses were linear, suggesting a single class of high affinity binding sites for the synthetic estrogen, R-2858 with a Kd of $5.88 \times 10^{-10}$ mol/l and a concentration of binding sites of $17.0 \pm 4.4$ fmol/mg cytosol protein in the epididymis. In the prostate, the concentration of binding sites was $19.7 \pm 3.6$ fmol/mg cytosol protein with a Kd of $5.42 \times 10^{-10}$ mol/l.

In immature castrated monkeys, administration of estradiol dipropionate caused a significant increase in the weight, total protein and sialic acid content of the epididymis. This biological action of estrogen on the epididymis is presumably mediated via estrogen receptor.
REFERENCES


When one is working with estrogen receptor system in the male accessory genital organs, one encounters practical difficulties in choosing an experimental design and this is peculiar with male genital organs. The current dogma is that the structural and functional integrity of the male accessories is androgen dependent. If estrogens do influence the functioning of male accessories through an independent estrogen receptor system (as progesterone does in the estrogen primed uterus), one has to take into consideration the possibility that estrogen receptor system may also be dependent on androgen action (as progesterone receptor system is dependent on estrogen action in the uterus). It follows then that if androgens influence the estrogen receptor level in the male genital organs, the estrogen action in the functioning of male accessories may not be autonomous to estrogen. In an adult male animal, estrogens and androgens are present simultaneously and are therefore available to the target organs but the nature of their interaction in the target tissues is not known.

For this reason, experiments carried out in adult castrate animals in the absence of exogenous androgen and/or estrogen may not reflect strictly the physiological situation. Since the precise role of estrogen in the male is not clearly understood, the studies on estrogen receptors in the male
genital organs are likely to be criticised on the grounds that estrogen receptors only bind the hormones and that by measuring the receptors, one is not measuring the effect of the estrogen upon cells of male sex accessories. That is to say that one is measuring what is available to bind estrogen in the androgen 'target' organs rather than the function of estrogen itself. The only explanation that can be offered to answer this criticism is that if we accept that the "receptor" is a machinery, a mechanism between the hormone and its effect, determination of its "concentration" in the cytosolic fraction gives an index of the potential of the cells to respond to the specific hormone. It must also be remembered that if estrogen and androgen receptors are distinct, we cannot expect estrogen to interact with androgen receptor and conversely androgen to interact with the estrogen receptor when the hormones are present in physiological level.

In undertaking the present investigations on the role of estrogen and estrogen receptors in the male accessory genital organs, the investigator assumed that the two receptor (androgen and estrogen) proteins are distinct, that the androgen being the male sex hormone, estrogen action in the male organs may only be subservient to androgen action and that estrogen receptors may have 'some' unidentified regulatory role. With a view to obtain experimental evidence to these assumptions, the author followed the classical physiological
approach to visualise that presence of estrogen receptors will be revealed by selective uptake and retention of estrogen by the target tissues and this in turn will be under androgenic control. Appropriate experiments were carried out to demonstrate the presence of receptors specific for estrogen in the epididymis (and prostate) of rat. A preliminary study was carried out on the biological response of the immature monkey epididymis to estrogen and the study also revealed the presence of an estrogen binding protein in this organ. In the absence of specific and response that could be used as an index of estrogen action, the growth and secretory activity of the two organs were used as a measure of estrogen action. If estrogen promotes the secretory activity of the epididymis, the question that follows is why there is need for two different hormones (estrogen and androgen) to perform the same function (stimulation of secretion). One possibility is that under physiological conditions, the two hormones have a synergistic role. The precise mechanism of synergism is, however, not clear. There is some evidence to suggest that estrogen stimulates the epididymal contractility thus aiding in sperm transport. There is also the suggestion that estrogen acts in cooperation with androgen to maintain a normal rate of sperm transport through the male genital tract.

The investigator has succeeded in demonstrating the presence of estrogen receptor in the epididymis, and estrogen
induced stimulation of the secretory function of the organ. It is, however, premature to assign a definite role to estrogen receptor in the regulation of the physiology of the epididymis. There is, therefore, need for further studies and the present integrated approach is only a first step in that direction.