The function of oestradiol and its receptor in the rat testis

Proefschrift

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aan mijn ouders

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Paper I

Wilma M.O. van Beurden-Lamers, Albert O. Brinkmann, Eppo Mulder and Henk van der Molen (1974)
Biochem. J. 140, 495
High affinity binding of oestradiol-17β by cytosols from testis interstitial tissue, pituitary, adrenal, liver and accessory sex glands of the male rat.

Paper II

Wilma M.O. van Beurden, Eppo Mulder, Frank H. de jong and Henk J. van der Molen (1977)
Endocrinology in press
The effect of estrogens on luteinizing hormone plasma levels and on testosterone production in intact and hypophysectomized rats.

Paper III

Wilma M.O. van Beurden, Bep Roodnat, Frank H. de Jong, Eppo Mulder and Henk J. van der Molen (1976)
Steroids 28, 847
Hormonal regulation of LH stimulation of testosterone production in isolated Leydig cells from immature rats: the effect of hypophysectomy, FSH and estradiol-17β.

1 General introduction

1.1. Hormones acting on the testis

The testis consists of 2 different tissues i.e. the interstitial tissue, which is the site of steroidogenesis, and seminiferous tubules where spermatogenesis occurs.

Testosterone production takes place in the Leydig cells of the interstitial tissue and is under the influence of the tropic hormone LH (1,2). The action of LH is initiated by interaction with specific receptors located on the cell membrane of the interstitial cells (3). Binding to the receptor leads to a stimulation of cAMP production (4,5) and this in turn leads to an activation of cAMP dependent protein kinases (6). The obligatory function of cAMP in the stimulation of testosterone production can be questioned since cAMP production is undetectable with doses of LH which result in maximum steroidogenesis (7). The role of protein kinase seems more clear since a correlation exists between protein kinase activation and stimulation of testosterone production (6). The involvement of protein synthesis in the mechanism of action of LH was confirmed by the discovery of the synthesis of a specific protein after LH stimulation (8) and the inhibition of testosterone production by protein synthesis inhibitors (9).

Whereas LH acts on the Leydig cell, FSH evokes responses in the seminiferous tubules. It has been shown, that FSH specifically stimulates protein kinase activity in seminiferous tubules of immature rats or hypophysectomized adult rats (10) and this stimulation is accompanied by a corresponding increase in cAMP production. Also testicular protein synthesis is stimulated by FSH. A specific androgen binding protein is induced in the Sertoli cells of the tubuli of immature hypophysectomized rats after FSH administration (11). This androgen binding protein (ABP) is secreted in the testicular fluid and it has been suggested that the function of ABP is the accumulation of androgen within the seminiferous tubules (11).In addition to this androgen binding protein

a specific androgen receptor can be demonstrated in the cytosol and nuclear fraction of seminiferous tubules from hypophysectomized immature and adult rats (11,12,13). This receptor can be distinguished from ABP by differences in dissociation constants, steroid specificity and temperature sensitivity (11,13). The androgen receptor is predominantly present in Sertoli cells.The demonstration of androgen-receptor complexes in cytoplasmic and nuclear fractions of seminiferous tubules might support the concept that spermatogenesis is an androgen dependent process.

1.2 Scope of this thesis

A specific oestradiol binding protein is present in the cytosol fraction of rat testicular tissue. This binding protein has a sedimentation coefficient of 8S and fulfils the criteria of a true steroid receptor, i.e. a saturable amount of receptor is present and the receptor has a high affinity for oestradiol (15). The hormone-receptor complex can be translocated to the nuclei when nuclei isolated from testis are incubated with cytosol and oestradiol (16). The oestradiol receptor is localized in the Leydig cells of the interstitial compartment of the testis (14). In the seminiferous tubules no oestradiol receptor can be detected.

In addition to the oestradiol receptor, oestradiol (17) is also present in rat testicular tissue but not much is known about the possible effect of oestrogens in the testis. In intact and hypophysectomized mice oestrogens can have an inhibiting effect on some steroidogenic enzymes (19). Administration of oestrogens to intact male rats results in a decrease of testosterone levels in testis and plasma (19-24). Some authors did not observe a decrease in LH plasma levels concomitant with the decrease in testosterone levels (20-22). Therefore it has been suggested that the oestrogen effect is a direct effect on the testis. Other authors, however, have reported a negative feedback action of

oestrogens on LH secretion (23,24).

We have investigated several aspects of the steroid and tissue specificity of the oestradiol receptor in the rat. Since it seems likely that steroid hormones exert their effects via RNA synthesis, we have investigated whether oestradiol has an effect on RNA synthesis in rat testicular tissue (chapter 3).

In order to detect a possible final effect of oestrogen in the male rat, which could be mediated via the oestrogen receptor, we have studied the effect of oestrogen administration on testosterone production. To exclude the possibility that the effect was caused by a negative feedback action on LH secretion we have also studied LH levels. The results of this study are presented in chapter 4.

The testicular oestradiol receptor is present not only in adult but also in immature rats (25). In the immature rat part of the oestradiol receptor molecules are present in the nucleus. Since it is conceivable that the possible response of testicular tissue to oestradiol is restricted to a certain period of life we have investigated for the pubertal rat the effect of oestradiol on LH stimulated testicular testosterone production in isolated Leydig cells (chapter 5).

2 Summary of the litterature on the mechanism of action of steroid hormones

2.1 Steroid receptors

Studies concerning the mechanism of action of steroid hormones have revealed that target cells for steroid hormones contain specific hormone binding proteins, called receptors. The long retention of oestrogens by target tissues for oestrogens was the first indication for the existence of steroid receptors (26). After incubation of a high speed supernatant (cytosol) of uterine tissue the oestradiol receptor could be identified as a protein oestradiol complex sedimenting with a sedimentation coefficient of 9.5S on a sucrose gradient during ultracentrifugation (27). Under hypotonic conditions the receptor is present as an 8S molecule (28,29,30) which can be reversible transformed to a 4S complex after addition of salt at a concentration of 0.4M (29,31,32). An intermediate 6S form may occur in the presence of ionic conditions in the presumed physiological range (33,34,35,36). Whether this 6S form is the physiological form of the receptor in the cellular milieu can still be questioned, because the sedimentation behaviour can vary depending on the experimental conditions used (37,38).

Several properties distinguish the specific oestradiol receptor from nonspecific binding proteins i.e.: 1. the receptor has a high affinity for oestrogens with dissociation constants in the range of 10^{-9}-$10^{-10}M^{-1}$ (39,40,41,42). 2. only a limited number of receptor molecules per cell is present (43,44). 3.the receptor is steroid specific; it only binds compounds chemically similar to oestradiol (33,43 44). 4. receptors are tissue specific; only target tissues contain receptors whereas they are absent in non-target tissues like lung, spleen etc. (33).

The general occurrence of oestrogen receptors in target tissues for oestrogens has been amply demonstrated, for example in uterus, vagina (33), mammary gland (45),

hypothalamus and pituitary (46,47,48).

Since the discovery of the oestrogen receptor the presence of receptors for other steroids in their respective target tissues has also been demonstrated: e.g. receptors for androgens (49,50), progesterone (51,52,53), glucocorticoids (54,55,56) and mineralocorticoids (57,58) have been reported.

2.2 Transfer of the hormone-receptor complex to the nucleus

In the absence of endogenous oestradiol the larger part of the uterine oestradiol receptors can be demonstrated in the cytoplasm of the uterus. In vivo injection of female rats or in vitro incubation of uterine tissue with oestradiol leads to accumulation of steroid-receptor complex in the nuclei of uterine tissue (59,60,61). Concomitant with this nuclear accumulation a disappearance of cytosol receptor can be observed (32,59,62). These observations have lead to the hypothesis that nuclear retention of steroid and receptor is dependent on the prior formation of steroid-receptor complexes in the cytoplasm (43,59). Indeed no receptor-steroid complex can be found in nuclei when uterine nuclei are directly incubated with ^{3}H-oestradiol, whereas after incubation with cytosol the steroid-receptor complex can be detected in the nuclei. It seems that a temperature-dependent step is involved in the transfer of the hormone-receptor complex to the nucleus. Whereas binding to nuclei is observed after incubation of nuclei with cytosol at 22°-37°C (63,64), no nuclear binding can be detected after incubation of nuclei with cytosol or whole uterine tissue slices at 0°C (63,64). In contrast, when oestrogen receptors were first incubated at 25°C in the absence of nuclei and subsequently incubated at 0°C with nuclei, steroid-receptor complex did accumulate in the nuclei. This so-called 'activation' of the receptor has also been reported for the dihydrotestosterone receptor in prostate (65,66), glucocorticoid receptor in thymus (67), aldosterone receptor in kidney (68) and progesterone receptor in oviduct (69).

The temperature-dependent activation of the oestrogen receptor probably results in a conformational change which is detectable as a shift in sedimentation coefficient from 4 to 5S after centrifugation on high salt sucrose gradients (38,70,71).Alternatively from studies with calf uterine cytosol,evidence has been presented that an enzymatic process which requires a Ca^{2+}sensitive factor with proteolytic activity is involved in the activation of the hormone-receptor complex(72).

2.3 Hormone-receptor binding to nuclear components

Part of the specifically bound oestradiol of the oestradiol-receptor complex from uterine nuclei can be extracted by 0.4M KCl, while part remains in the residual non-KCl extractable fraction (60,61). Similar observations have been described for the nuclear binding of oestrogens in other tissues (73,74) and for other hormones (75,76). The observed correlation between hormone binding in the residual fraction and the final hormone response, as has been found in uterus (61), has lead to the conclusion that the residual binding may be one of the most important factors in specifying the hormonal response. Those nuclear sites which specifically bind steroid-receptor complexes are called 'acceptors'. The presence of nuclear acceptor sites appears to be tissue specific, since nuclei from target tissues bind cytoplasmic receptors to a greater extent than do nuclei from non-target tissues (77,78,79). The question, whether the acceptor sites are present in saturable amounts or not remains to be solved. In several studies saturation of binding of receptor-steroid complex to nuclear acceptor sites has been observed (80,81), although in other studies saturation could not be achieved (82,83,84).

Oestrogen receptors associate with nuclear chromatin. The precise chemical composition of the nuclear acceptor is not known. It has been reported that steroid receptors can interact directly with DNA (80,85,86) while in other studies bind-

ing to non-histone nuclear proteins was observed (87,88). The progesterone receptor in chick oviduct consists of two components one of which binds to DNA while the other binds to acidic nuclear proteins (89). Finally ribonucleoprotein particles have been indicated as possible nuclear acceptor sites (90,91).

2.4 Effect of steroid hormones on RNA synthesis

a. Effect of hormone administration on in vivo RNA synthesis

In vivo or in vitro administration of steroid hormones leads to the synthesis of specific proteins in the hormone target tissues, like the production of vitellogenin in the rooster liver (92) and ovalbumin in chick oviduct (93) after oestrogen administration. Not only the synthesis of specific proteins but also total protein synthesis can be enhanced as has been observed in uterus (94) and prostate (95) after steroid hormone administration.

Although there is some evidence available that the induction of proteins by steroid hormones is mediated by post-transcriptional processes (96) it is now generally accepted that the increase of protein synthesis is the result of de novo synthesis of new RNA species.

Target tissues show a great variation of responses that can occur after administration of steroid hormones, but each response that has been investigated in biochemical terms appears to be dependent on the synthesis of nuclear RNA. Administration of aldosterone (97), glucocorticosteroids (98,99), oestrogens (100,101,102), progesterone (103) and dihydrotestosterone (104,105) results in increased RNA synthesis in their respective target tissues. Prevention of the effect of steroid hormones by inhibitors of RNA synthesis and the increase of RNA synthesis after hormone administration have shown the importance of RNA production in the early stages of hormone action. Much information about the effect of steroid hormones on RNA synthesis has been obtained from studies on the effect of oestrogen on RNA synthesis in rat

uterine tissue. Oestrogen injection results within 2 minutes in an increase of the synthesis of nuclear RNA (102). This stimulation reaches a peak after 20-30 minutes and decreases until 2 hours after oestradiol administration after which time RNA synthesis increases again and remains high for at least 24 hours (106,107). The time course of the response is different for the different species of RNA. Whereas the synthesis of 45S and 32S RNA - the precursors for ribosomal RNA - is stimulated 2-4 hours after oestradiol injection, the labelling of tRNA is already increased after 1 hour (108). Preceding these changes in RNA synthesis an increase of high molecular weight RNA has been demonstrated (109), probably of heterogeneous RNA (HnRNA), a RNA species which is believed to be the precursor of mRNA (110).

The stimulation of rRNA and tRNA appears to be dependent on protein synthesis, since their stimulation can be inhibited by protein synthesis inhibitors, in contrast to the stimulation of HnRNA synthesis (108). These findings have led to the conclusion, that the synthesis of RNA species of very high molecular weight in rat uteri under the influence of oestradiol could reflect synthesis of new mRNA species and also that the translation of these RNA species leads to the formation of proteins which have an influence on rRNA synthesis. In fact a specific acidic protein which can be identified electrophoretically is formed in response to oestrogen (111). The acidic protein called 'induced protein' (IP) can be detected 30 minutes after oestradiol has entered the cell.The synthesis of IP is inhibited by actinomycin D, cordycepin and α-amanitin suggesting a de novo synthesis of mRNA for IP (112,113,114) which is confirmed by the finding that discrete RNA species appear already 15 minutes after exposure of the uterus to oestradiol (115). From these observations it has been postulated that oestradiol induced synthesis of mRNA followed by the synthesis of IP causes the subsequent stimulation of rRNA synthesis, which itself results in increased protein synthesis and uterine growth.

b. The effect of hormone administration on in vitro nuclear polymerase activity

The increase in RNA synthesis after in vivo hormone administration can be the result of several phenomena which possibly act simultaneously. A change in the availability of the chromatin as well as a change in the number or in the activity of already existing RNA polymerase molecules would result in an increased nucleotide incorporation. Several studies have been performed by which the stimulation of RNA synthesis caused by in vivo administration of the hormone was studied in vitro in isolated nuclei.

RNA synthesis in mammalian nuclei can be divided in: nucleolar RNA synthesis, which generally represents rRNA synthesis; and nucleoplasmic RNA synthesis, which represents mRNA production. Polymerase I and II are assumed to be responsible for the synthesis of rRNA and mRNA respectively. Since polymerase I is more active under low salt conditions and the presence of Mg^{2+} and polymerase II is more active under high salt conditions and the precence of Mn^{2+} and because this enzyme can be specifically inhibited by α-amanitin it is possible to distinguish between the activity of polymerase I and II. RNA polymerase activity is always measured as incorporation of UTP in RNA by isolated nuclei.

After oestradiol administration an early rise in UTP incorporation by RNA polymerase II from uterus could be demonstrated, which reached a peak 30 minutes after hormone administration and then decreased to control values before displaying a second increase over control activity from 2 to 12 hours. The first rise in polymerase II activity is followed by an increase in UTP incorporation by polymerase I (116,117,118). Also in other tissues a rise in polymerase II and I activity has been observed (119,120) after hormone administration.

The enhanced UTP incorporation by polymerases I and II could be the result of a change in template activity or in the activity of the RNA polymerase molecules itself.

Some authors did not find an increase in template activity of chromatin after oestradiol treatment in rat (121) and mouse uterus (122), but it was observed by others, that oestradiol enhanced template activity 10-30 minutes after hormone administration (116,123,124,125). Similar results have been found for other hormones in other tissues. Testosterone increased template activity of kidney chromatin (119) and according to some reports also that of prostate chromatin (126,127). However, in other studies no change in prostate chromatin template activity could be demonstrated (128) The reason for these contradictory results could be the occurrence of hydrolytic factors or the use of high salt concentrations. These conditions may affect template activity (122,129).

RNA polymerase I and II activity in uterine nuclei can be separated in 2 fractions: enzyme firmly associated with chromatin and soluble enzyme. When soluble and chromatin associated polymerase II were extracted from uterine nuclei after oestradiol treatment and were incubated with exogenous template, an increase could be found in the amount of polymerase II molecules 12-24 hours after hormone injection. The soluble polymerase I activity remained constant during the first 6 hours after injection whereas the activity of the more firmly bound enzyme increased already 1-2 hours after injection (130). Also, in rat prostate the increase in isolated polymerase I activity precedes the increase in polymerase II activity after testosterone administration (126).

From these results may be concluded, that administration of oestrogen may cause an increase in template activity of uterine chromatin. This could lead to the synthesis of specific mRNA molecules and specific proteins. These proteins in turn induce the synthesis or activation of polymerase I and II molecules, which results in an increase of total RNA production followed by an increased protein synthesis and uterine growth.

c. The effect of hormone-receptor complex on RNA synthesis in isolated nuclei

Whether the presence of a receptor is obligatory for a hormone to exert its effect on RNA synthesis is still questionable. More information about the role of the receptor in the effect of hormones on RNA synthesis has been obtained from studies in which hormone-receptor complexes were added to isolated nuclei. The ability of uterine nuclei to incorporate radioactive nucleotides into RNA is enhanced when they are first incubated with a mixture of oestradiol and uterine cytosol under low salt conditions (131,132). Since oestradiol alone or receptor alone was not effective, interaction of both the steroid and the receptor protein was considered to be responsible for the stimulatory activity. However, the stimulation was only observed when the oestradiol-receptor complex is transformed from the 4S form to the activated 5S form (131). The effect of oestradiol on RNA polymerase activity could also be demonstrated with RNA polymerase solubilized from treated nuclei and assayed with purified DNA; therefore, the effect of the steroid-receptor complex was thought to be at least in part on the RNA polymerase I enzyme itself (131). Under high salt conditions the stimulatory effect of the oestradiol-receptor complex was not always clear (132). Similar results have been found in a study on the effect of addition of receptor-DHT complex to prostate nuclei (133,134,135). A high stimulation of polymerase I activity could be seen when nuclei were incubated with DHT-receptor complex under low salt conditions. Also, incubation of chromatin with receptor-hormone complex resulted in an increased RNA synthesis (134). No stimulation of nucleotide incorporation into RNA could be observed when high salt conditions were used (135). Later experiments have revealed that high salt conditions abolished the binding of the receptor-hormone complex to the prostatic chromatin (136). When salt concentrations were chosen at which both polymerase I and II activities could be measured, addition of receptor-hormone complex resulted in an enhance-

ment of nucleotide incorporation by both polymerase I and II (136). The specificity of this effect seemed to be present in the chromatin fraction since no stimulation was observed when chromatin from other tissues were used (134, 135).

d. The effect of steroid hormones on RNA synthesis in chick oviduct

Some of the most detailed studies on the effect of steroid hormone receptors on gene transcription have been conducted on chick oviduct. In vivo oestrogen or progesterone administration to immature chicks leads to growth and differentiation of the oviduct and to an enormous increase in ovalbumin synthesis (137,138,139,140,141). Hybridization studies, using radioactive copies of the gene responsible for ovalbumin synthesis to detect new ovalbumin mRNA molecules, revealed that ovalbumin synthesis after oestrogen or progesterone administration is directly correlated with de novo synthesis of mRNA (137,142,143). Prior to the increase in ovalbumin mRNA (144,145), there is an overall increase in template activity.

It has been reported that rifampicin binds to those E.coli RNA polymerase molecules which are not involved in chain elongation. Thus it is possible to distinguish between increases in RNA synthesis due to chain elongation and to chain initiation. From such studies, it appears that the oestrogen induced increase in RNA synthesis is the result of an increase in RNA chain initiation sites while the rate of chain elongation remains unchanged (146, 147). The temporal relationship between the appearance of nuclear bound oestrogen receptor molecules and of RNA chain initiation sites is an indication for the role of the receptor in the hormonal stimulation of gene transcription (148).

The effect of the progesterone receptor on template activity in vitro can be studied by incubation of chromatin from non hormone treated oviduct nuclei with purified progesterone-receptor complex and E.coli RNA polymerase. The pro-

2.6. The oestradiol receptor in the testis

An oestradiol receptor is present in the interstitial tissue of the testis. Until now no final response after oestradiol administration is known. No defect exists between interaction of oestradiol with the receptor and binding of the steroid-receptor complex in the nucleus. After binding of oestradiol the receptor-hormone complex is translocated to the nucleus (152) and can be extracted from the nucleus as a 5S receptor (25,153).

Thus, till this stage every condition has been fulfilled for the expression of a hormone response. Whether the binding of oestradiol-receptor complexes in the nuclei of testis interstitial tissue will result in a final hormone response, mediated via RNA synthesis, has been the subject of several investigations reported in this thesis.

3 Properties of the oestradiol receptor in rat testis and the effect of oestradiol on RNA synthesis in testicular tissues

3.1. Properties of the specific oestradiol binding protein in rat testicular tissue.

In the cytosol fraction of rat testicular tissue an oestradiol binding protein is present. This binding protein is localized in the interstitial compartment of the testis and cannot be demonstrated in the seminiferous tubules. This binding protein fulfils the first two criteria for a true receptor protein (see chapter 1). It is present in a limited amount in a concentration of 140 fmole/mg of cytosol protein of interstitial tissue. The affinity constant is $10^{10} M^{-1}$ (154) which is high compared to the affinity constants of a non-specific binding protein like serum albumin or of the more specific sex steroid binding globulin in plasma which have affinity constants of 10^5 and $10^9 M^{-1}$ respectively (155).

In order to investigate whether the testicular oestrogen binding protein also possesses the last two properties characteristic for a receptor protein, the steroid specificity and the tissue specificity of the oestradiol binding protein were studied. Also several methods for quantitative determination of specific oestradiol binding were compared and the protein character of the oestradiol binding was established. The results of this study are presented in appendix paper I and can be summarized as follows:

The oestradiol binding protein of testicular cytosol had a high affinity for oestradiol-17β and for diethylstilboestrol; a moderate affinity for oestrone, oestriol and oestradiol-17α and a low affinity for dihydrotestosterone and testosterone. Thus the binding protein is steroid specific.

In order to establish whether the oestradiol binding protein is tissue specific it was necessary first to establish which method could be used for the quantitative determination of specific oestradiol binding in cytosol fractions. For the quantitative determination of specific binding of steroids in cytosol several methods have been described which

are all based on the following principle: cytosol is incubated with ^{3}H-steroid (incubation A). In a parallel incubation cytosol is incubated with the same amount of ^{3}H-steroid plus a 100-fold cold steroid (incubation B). After incubation of the cytosols free steroid and bound steroid are separated. The difference between bound steroid A and bound steroid B is called 'specific binding'. For the separation of bound from free steroid several methods can be used. For example: charcoal adsorption, agar electrophoresis,sucrose density gradient centrifugation and Sephadex chromatography. No difference in the amount of specific oestradiol binding was found with these techniques. For practical reasons Sephadex chromatography and agar electrophoresis were chosen as techniques for quantitative determination of specific binding of oestradiol in the cytosol fractions of several tissues.

Specific binding of oestradiol could be demonstrated in the cytosol fractions of the following tissues: liver, adrenal and pituitary glands, prostate, seminal vesicle, epididymis and testis interstitial tissue. No specific binding could be found in: plasma, hypothalamus and seminiferous tubules.

It is difficult to conclude whether the occurrence of the specific high affinity oestradiol binding in so many different tissues does support the hypothesis that receptors are only present in target tissues and not in nontarget tissues, particularly because in the male rat the function of oestradiol is not known. Therefore until now it has been impossible to conclude whether these tissues, including interstitial tissue, are real oestrogen target tissues.

Even if the tissue specificity of the oestradiol binding proteins in the male rat remains to be questioned, the oestradiol binding protein in rat testicular cytosol can be regarded as a true receptor protein for the following reasons: the binding protein has a high affinity for oestradiol and not for other steroids; it is present in limited amounts; it has a sedimentation coefficient of 8S; it is heat labile and sensitive for pronase and not for RNAse and DNAse. All these characteristics are the same as the characteristics of the

oestradiol receptor which is present in uterine tissue, a well-known oestrogen target tissue.

3.2 The effect of oestradiol on RNA synthesis in testis interstitial tissue and seminiferous tubules.

3.2.1 Introduction

The oestradiol-17β receptor in rat testis interstitial tissue moves into the nucleus after binding of oestradiol (152). The binding of steroid hormones to a cytoplasmic receptor protein has been indicated as a prerequisite for their biological activity (chapter 2). The formation of hormone-receptor complex has been shown to precede the transport of the hormone to the nucleus and the subsequent stimulation of RNA synthesis (see chapter 2). In uterine tissue oestradiol-17β stimulates the incorporation of radioactive precursors into RNA, due to both an increase in the rate of RNA synthesis and to an increase in specific activity of the precursor pool of ribonucleotides (156,157,158,159, 160). In this respect we have considered the possibility that an effect of oestradiol in the testis should be preceded by stimulation of RNA synthesis.

In the present study we have examined changes occurring in the uptake of ^{3}H-uridine into the acid soluble nucleotide pool and into RNA from testis interstitial tissue and seminiferous tubules following in vivo administration of oestradiol to rats.

3.2.2 Materials and methods

Materials

(5,6^{3}H) Uridine (40Ci/mmol) was purchased from Radiochemical Centre Amersham, U.K. HCG was obtained from N.V. Organon, Oss, The Netherlands. Oestradiol was obtained from Steraloids, Pawling, New York, U.S.A..

Animals and incubation procedures

Three month old male rats of the R-Amsterdam strain were used. The animals received a subcutaneous injection of 100 µg oestradiol-17β in 0.1 ml propylene glycol.In some experiments hypophysectomized rats were used, which received a daily injection of 0.5 IU HCG for 8 days. At various times after administration of oestradiol the animals were killed by cervical dislocation. One testis was removed, decapsulated and immediately incubated with 4 ml Krebs Ringer glucose buffer, containing 50 µC (5,6,^{3}H) uridine, for 60 min at 32^{o}C in an atmosphere of 95% O_2-5% CO_2. After incubation interstitial tissue and tubules were obtained by wet dissection at 0^{o}C (161).

Determination of radioactivity in acid soluble and acid insoluble fractions

Testis interstitial tissue was homogenized at 0^{o}C with an all glass potter homogenizer in 6 ml distilled water per gram of tissue. Cold 1M perchloric acid (PCA) (2 ml) was added to the homogenate. After standing in ice for 10 min the mixture was centrifuged 10 min at 3,000xg. The resulting precipitate was washed 3 times with 3 ml 1N PCA to give the 'acid insoluble fraction'. The supernatant fluid plus washings were combined to give the 'acid soluble fraction'. The precipitate was washed with 3 ml ethanol and RNA was extracted by hydrolysis for 60 min at 37^{o}C in NaOH. After alkaline hydrolysis the samples were cooled and acidified by addition of 0.5 ml 3M PCA and allowed to stand for 15 min. The samples were centrifuged at 3,000xg for 10 min. The amounts of radioactivity and RNA in the supernatant were determined by liquid scintillation counting and by the orcinol procedure (162) respectively.

Ion exchange chromatography of the acid soluble fractions

The acid soluble fraction was neutralized with KOH and the precipitate of $KClO_4$ was removed by filtration. The acid so-

luble fraction was applied to a column (5 cm x 0.6 cm) of Dowex X 8 (Cl^-) ion exchange resin, 200-400 mesh. The column was washed with water (20 ml) to remove the nucleosides. The total free nucleotides were then eluted with 1M NaCl as a single fraction. The radioactivity and $E_{260\ nm}$ of the NaCl eluate were measured.

Calculations for determination of the specific activities of the nucleotide pools and RNA fraction

The specific activity (SA) of the nucleotide pool was expressed as dpm/E_{260}, the SA of RNA as dpm/µgRNA. The SA of the RNA fraction was corrected for changes in the SA of the nucleotide pool by the following calculation:

$$SA_{RNA} = \frac{\text{dpm RNA}}{\mu\text{gRNA}} : \frac{\text{dpm nucleotide pool}}{E_{260}\ \text{nucleotide pool}}$$

3.2.3 Results

Experiments with intact rats

In order to investigate the effect of E_2 on ^{3}H-uridine incorporation in the nucleotide pool and in RNA of rat testicular tissues rats were injected with 100 µg oestradiol. Control rats received vehicle only. After 6 and 24 hours the ^{3}H-uridine incorporation into RNA and nucleotide pool of interstitial tissue and seminiferous tubules was determined.

The results in table 1A show that 6 and 24 hours after oestradiol administration the incorporation of ^{3}H-uridine in RNA of testis interstitial tissue was increased. The uptake of ^{3}H-uridine in the nucleotide pool remained constant 6 hours after E_2 injection, after 24 hours the specific activity of the nucleotide pool seemed to be enhanced. After correction of the incorporation of uridine into RNA for changes in the specific activity of the nucleotide pool there seemed to be no difference between RNA synthesis of control rats and rats 24 hours after E_2 administration. In the seminiferous tubules no stimulation of RNA synthesis

Table 1 The effect of oestradiol on RNA synthesis in rat testicular tissues. Rats were injected with 100 µg oestradiol, control rats received vehicle only. Six and 24 hours after injection testes were incubated with ^{3}H-uridine and the incorporation of ^{3}H-uridine into RNA and nucleotide pool was measured

treatment	Specific activities (SA)*					
	INTERSTITIAL TISSUE			SEMINIFEROUS TUBULES		
A INTACT RATS	RNA a)	pool b)	RNA corr. c)	RNA	pool	RNA corr.
control	590 ± 66 (10)	6.0 ± 0.5 (10)	100 ± 10 (10)	290 ± 50 (7)	4.6 ± 1.4 (7)	100 ± 23 (7)
6 hours E_2	930 ± 45 (10)	5.9 ± 0.4 (10)	157 ± 8 (10)	380 ± 53 (7)	7.6 ± 0.9 (7)	78 ± 22 (7)
24 hours E_2	1253,867	11.9,11.3	106,75	225,335	6.8,5.6	96,44
B HYPOX RATS						
control	239 ± 55 (4)	7.2 ± 2.5 (4)	100 ± 23 (4)	166 ± 12 (4)	5.0 ± 2.1 (4)	100 ± 30 (4)
6 hours E_2	190 ± 36 (4)	5.5 ± 1.9 (4)	92 ± 18 (4)	159 ± 1 (4)	4.8 ± 1.6 (4)	103 ± 24 (4)
24 hours E_2	190,230	8.3,4.0	113,65	219,159	10.1,5.1	91,69
C HYPOX RATS + HCG						
control	2017 ± 209 (4)	6.9 ± 1.7 (4)	100 ± 9 (4)	-	-	-
6 hours E_2	2400 ± 200 (4)	7.2 ± 2.3 (4)	113 ± 12 (4)	-	-	-
24 hours E_2	2183,1877	5.2,11.2	67,127	-	-	-

a) SA RNA: dpm/µg RNA

b) SA pool: dpm x $10^{6}/E_{260}$

c) RNA corr.: SA corrected for changes in nucleotide-pool. $SA_{RNA\ corr.}$ of control rats is 100%

*Results as $\bar{x}$ ± S.E.M. (n) or individual observations

could be found after E_2 injection.

Experiments with hypophysectomized rats

Injection of oestradiol could result in a feedback on the secretion of the gonadotropins (23,24). This in turn could induce changes in RNA synthesis. In order to investigate whether the changes in RNA synthesis after oestradiol administration were due to a change in gonadotropin secretion or to a direct effect of oestradiol on the testis, hypophysectomized rats were used. One day after hypophysectomy rats were injected with 100 µg oestradiol. After 6 and 24 hours the incorporation of uridine into the nucleotide pool and RNA fraction was determined. No effect of oestradiol on uridine incorporation into acid precipitable material could be observed in interstitial tissue and seminiferous tubules. Also the specific activity of the nucleotide pool did not change significantly after oestradiol administration (table 1B).

Experiments with hypophysectomized rats treated with HCG

In contrast to experiments with intact rats no effect of oestradiol on the incorporation of uridine could be observed in hypophysectomized rats. This could reflect either, that the effect of oestradiol on RNA synthesis in intact rats is caused by an indirect effect via pituitary hormones or that in hypophysectomized rats one of the gonadotropic hormones, which is essential for a direct effect of oestradiol on RNA synthesis is absent. The hormone responsible for the absence of the effect on RNA synthesis could very well be LH, because this is the gonadotropin which acts on Leydig cells. Therefore we have repeated the experiments with hypophysectomized rats treated with 0.5 IU HCG per day for 8 days. This dose of HCG is known to restore testosterone levels in plasma and testicular fluid to levels close to those seen in intact rats (163). Administration of 100 µg oestradiol to HCG treated hypophysectomized rats did not result in an increase of

uridine incorporation into RNA 6 and 24 hours after oestradiol injection. Also no change in the specific activity of the nucleotide pool could be observed after oestradiol injection (table 1C).

3.2.4 Discussion

Administration of oestradiol to female rats leads to an increased incorporation of radioactive nucleosides into RNA from uterine tissue (156,157,158,159,160). According to several authors this increase during the first 4-6 hours reflects primarily the increased incorporation of administered nucleosides into nucleotide pools rather than an increased RNA synthesis (156,157,158,160). Only after 6-8 hours the increase in the specific activity of the RNA fraction could be explained by an increased RNA synthesis (156,157,160). In mice an effect of oestrogen treatment on testicular RNA synthesis has been reported (164).

In the present experiments oestradiol injection to intact male rats resulted after 6 and 24 hours in increased incorporation of ^{3}H-uridine into RNA of testis interstitial tissue. Whereas the increase in specific activity of the RNA fraction 6 hours after injection is probably due to increased RNA synthesis, the increase after 24 hours could probably be explained by an increase in the specific activity of the nucleotide pool. Oestradiol had no effect on uridine incorporation into RNA or into the nucleotide pool of seminiferous tubules.

Besides the possibility that oestradiol has a direct effect via the E_2 receptor in interstitial tissue, the possibility exists that oestradiol has an influence on gonadotropin secretion (23,24)and that the changes in RNA synthesis are merely caused by changes in gonadotropin levels. When the effect of oestradiol was studied in rats 1 day after hypophysectomy no effect on RNA synthesis could be observed. In order to exclude the possibility that oestradiol might exhibit its effect only when LH is present, the same experi-

ments were performed with HCG treated hypophysectomized rats. Also in these animals no effect of oestradiol on uridine incorporation into nucleotide pool and RNA could be found.

From the present experiments it is difficult to derive final conclusions about the effect of oestradiol on RNA synthesis. In testis, however, the effects of hypophysectomy and HCG treatment on uridine incorporation are clear. Comparison of the uridine incorporation into RNA of control intact, hypophysectomized and HCG treated hypophysectomized rats reveals that hypophysectomy results in a decreased uridine incorporation into RNA of both tubules and interstitial tissue. Daily treatment with HCG of hypophysectomized rats enhanced uridine incorporation into RNA of interstitial tissue. No significant differences were observed between the specific activities of the nucleotide pools of intact, hypophysectomized and HCG treated rats. Therefore it can be concluded that hypophysectomy results in a decrease of RNA synthesis in interstitial tissue and seminiferous tubules and that HCG stimulates RNA synthesis in interstitial tissue of hypophysectomized rats. Our results are in agreement with other studies, which have shown that LH or HCG stimulates RNA synthesis in the testis(165,166) Also FSH has an influence on testicular RNA synthesis(167).

The increase in uridine incorporation after oestradiol administration in testis interstitial tissue and the absence of this increase in seminiferous tubules may support the hypothesis that the oestradiol receptor from testis interstitial tissue is involved in the effect of oestradiol on RNA synthesis. Still the results do not conclusively prove a direct effect of oestradiol on the testis since no effect of oestradiol on RNA synthesis could be observed in hypophysectomized rats. This might indicate that the change in RNA synthesis after oestradiol administration to intact rats is the result of an effect of oestradiol on pituitary hormones. Whether a single injection of oestradiol indeed has a negative effect on gonadotropin secretion will be the subject of the following chapter.

4 The effect of oestrogen on testosterone production in rat testis and on LH plasma levels

From the results presented in chapter 3 it appears that an oestradiol receptor is present in rat testis interstitial tissue. However it is still not clear whether this testicular tissue can be considered as an oestrogen target tissue, since no function of oestradiol is known in the testis.

After daily administration of oestrogens to male rats for several days, testicular and plasma testosterone levels are decreased. According to some authors this is the result of a direct action of oestrogens on the testosterone synthesis in the testis and not of a negative feedback action of oestrogens on LH secretion, because it has been reported that plasma LH levels were not decreased after oestrogen administration (19-22). Other authors (23,24), however, did find lowered plasma LH levels after daily in vivo injection of oestrogen. More recently, Tcholakian et al. (168) reported that already 3 hours after a single oestrogen injection plasma and testicular testosterone levels were decreased without a concomitant decrease in plasma LH levels. In chapter 3.2 we have described an effect on uridine incorporation in testicular RNA 6 hours after a single oestrogen injection. Whether the observed effects, i.e. a lowered testosterone level and an increased RNA synthesis, are the result of a direct effect of oestrogen on the testis in which the receptor is involved, or whether the effects are the result of changes in gonadotropic hormone secretion, remains to be solved.

Therefore we have reexamined the effects of a single oestrogen injection on plasma LH levels and on testicular testosterone levels and production. The effect of oestrogen on testosterone production was also studied in hypophysectomized rats. The results of this study are described in appendix paper II. The results can be summarized as follows:

Six hours after injection of 50 µg E_2B or 100 µg oestra-

diol in intact rats testicular testosterone production and level were decreased. Together with the decrease in testosterone also plasma LH levels were lowered significantly 6 hours after injection of 100 µg oestradiol. The apparent decrease in LH levels after injection of 50 µg E_2B was not statistically significant. Twenty four hours after injection of 50 µg E_2B both testicular testosterone production and LH levels were decreased, whereas at the same time after injection of 100 µg oestradiol no inhibitory effect of oestradiol on testosterone production and LH plasma levels could be observed.

Since injection of 500 ng oestradiol causes a movement of the receptor from the cytoplasm to the nuclei of the interstitial tissue within 1 hour, this amount should be sufficient to evoke a response in the testis.Injection of 500 ng oestradiol to intact rats also caused a decrease in testicular testosterone production within 1 and 3 hours. This decrease in testosterone production was accompagnied by a significant decrease in plasma LH levels 3 hours after injection. Also 1 hour after injection LH levels appeared to be lower but this effect was not statistically significant. So in nearly all experiments the decrease in testosterone levels and production was accompanied by a decrease in LH levels.

Although a direct effect of oestradiol on the testis cannot be excluded, it is very likely that the observed effects are at least partly the result of a negative feedback action of oestrogens on LH secretion. In order to test this hypothesis we have studied the effect of oestrogens on testosterone production in hypophysectomized rats. Since after hypophysectomy testosterone production in testicular tissue decreases due to lack of LH, the rats were injected with sufficient amounts of LH to maintain testosterone production at the level of that in intact rats. In these LH treated hypophysectomized rats injection with 50 µg E_2B did not result in a reduction of testosterone production.

These results could reflect either that all the effects of oestrogens in intact rats are due to a feedback effect on

gonadotropin secretion or that a possible direct effect of oestradiol on testicular testosterone production is dependent on the presence of tropic hormones and therefore is not observed in hypophysectomized rats. This latter conclusion is not very likely since injection of 500 ng oestradiol immediately after hypophysectomy also has no effect on testosterone production after 1 and 3 hours, at which time the effect of oestradiol in intact rats was clear. It is not very likely that at this time after hypophysectomy all tropic hormones have disappeared.

Therefore on basis of the results in appendix paper II we prefer to conclude that the effects of oestrogens in intact rats are the result of a negative feedback action on LH secretion, rather than the result (wholly or in part) of a direct effect on the testis.

5 The effect of oestrogen administration on LH stimulated testosterone production of isolated Leydig cells from immature rats

5.1. Hormonal regulation of LH stimulation of testosterone production in isolated Leydig cells from immature rats: the effect of hypophysectomy, FSH and oestrogen

If oestrogens have an effect on testis interstitial tissue this effect may under physiological conditions in vivo very well be restricted to a certain life period during the development of the testis. This assumption is supported by observations on other phenomena in the Leydig cell, which are related to a certain developmental stage such as the high activity of 5α-reductase and 3β-hydroxysteroid dehydrogenase before puberty (169,170,171,172,173).

According to Döhler et al. (174) the plasma levels of oestrogens are high before puberty. It has been demonstrated by de Boer et al. (25) that part of the oestrogen receptors in the testis of the prepubertal rat is localized in the nucleus, due to endogenously present oestradiol. Therefore the possibility was considered that oestradiol could have an effect in the Leydig cell of the prepubertal rats, but not in the adult rat.

To test this possibility the effect of oestrogen on LH responsiveness of the Leydig cells from 21-25 day old rats has been studied. The results of this study are described in appendix paper III. An attempt was made to isolate Leydig cells from immature rats in the same way as has been described by Janszen et al. (2) for the isolation of Leydig cells from adult rats. The Leydig cells isolated from immature rats showed an increase in testosterone production in the presence of LH similarly to the results obtained with cells from adult rats. The amount of testosterone produced per 10^6 cells, however, was in cells from immature rats nearly 10 times lower than in cells from adult rats. This

might be due to a difference in testosterone metabolism, because the conversion of testosterone to 5α-reduced metabolites was higher in cells from immature rats when compared to the conversion in cells from adult rats. A lower capacity of cells from immature rats to respond to LH could however not be excluded.

The effect of in vivo oestradiol administration on the LH stimulation of testosterone production in isolated Leydig cells was studied in experiments with hypophysectomized rats, in order to exclude a negative feedback action on gonadotropin secretion. Hypophysectomy, however, resulted within 5 days in a complete loss of the ability of Leydig cells to respond to LH. This was in contrast to cells from adult rats, which after hypophysectomy showed a response to LH similar to the response of cells from intact adult rats. Administration of oestrogen to hypophysectomized immature rats had no influence on the response of the Leydig cells to LH. Since it was impossible to observe a possible inhibitory effect of oestrogen on LH responsiveness in cells in which LH responsiveness is already lost due to hypophysectomy, we tried to find a possibility to maintain LH responsiveness after hypophysectomy. Odell et al. (175,176) described that administration of FSH to hypophysectomized immature rats can restore LH responsiveness. Indeed, administration of FSH daily during 5 days to hypophysectomized immature rats did at least partly maintain LH responsiveness, when treatment with FSH was started both immediately and 5 days after hypophysectomy. This effect of FSH could be ascribed to FSH and not to contiminating LH.

Daily oestrogen injection in hypophysectomized immature rats which were treated daily with FSH resulted in an inhibitory effect on LH stimulated testosterone production in isolated Leydig cells. Daily administration of either 500 ng or 5 μg E_2B did abolish the FSH induced LH respopsiveness in isolated Leydig cells.

5.2. Further characterization of the effects of hypophysectomy, FSH and oestrogen on LH stimulation of testosterone production in Leydig cells isolated from immature rats

5.2.1 Introduction

The changes in LH responsiveness of isolated Leydig cell preparations after hypophysectomy and either FSH or FSH plus oestrogen administration might be due to several causes. Testosterone production measured in isolated Leydig cells after LH administration is the result of testosterone synthesis and testosterone degradation. A change in the capacity either to synthesize or to convert testosterone would be reflected in apparent changes of LH stimulated testosterone production. Also different compositions in cell population after different in vivo hormonal treatments could result in a changed LH responsiveness, when expressed as the amount of testosterone produced per 10^6 nucleated cells.

In the experiments described in this chapter we have studied testosterone conversion in isolated Leydig cells from either intact, FSH treated, hypophysectomized rats, or FSH plus oestrogen treated hypophysectomized rats. We have also determined the amount of Leydig cells in the cell preparations after different hormonal treatments of the rats using 3β-ol-dehydrogenase as a Leydig cell marker enzyme.

Binding of LH to the LH receptor of Leydig cells will initiate the activation of several steps which finally cause the increased testosterone production (chapter1). A change in any of these steps would result in a changed testosterone production. In order to investigate whether the activation of cAMP production might be influenced by the treatments, we have also studied LH stimulated cAMP production.

Finally, we studied the time courses of the effects of FSH and oestrogen on LH responsiveness in Leydig cells from hypophysectomized rats. Since an oestradiol receptor is present in Leydig cells (14), the effect of oestrogen treatment

on FSH induced LH responsiveness might be the result of binding of oestrogen to the oestradiol receptor in Leydig cells and translocation of the oestrogen-receptor complex to the nucleus. Therefore we have tried to correlate nuclear receptor binding and the effect of oestrogen on LH stimulated testosterone production.

5.2.2 Materials and methods

Materials and methods not mentioned here were the same as described by van Beurden et al. (appendix paper III).

DNA determination

In some experiments testosterone and cAMP production have not been expressed per 10^6 cells, but per 10 µg DNA. DNA was determined using the fluorimetric method of Hinegardner et al. (177).

cAMP determination

Isolated Leydig cells were preincubated for 1 hour at 33°C in an atmosphere of O_2/CO_2 (95:5, v/v). LH was added and after 2 hours incubation the reaction was stopped by addition of 2 ml acetone. cAMP was extracted and determined as previously described by Cooke et al. (4). When indicated 0.25 M 3-isobutyl- 1-methyl-xanthine was present in the incubation medium.

Assay of nuclear oestrogen receptor

After injection of oestrogen in rats the KCl extractable nuclear receptor in the testis has been determined using a nuclear exchange method described by de Boer et al. (25)

5.2.3 Results

1) Effect of hypophysectomy, FSH and oestradiol benzoate treatment on testosterone conversion in isolated Leydig cells from immature rats

The effect of hypophysectomy, FSH treatment and oestrogen treatment on testosterone conversion was investigated in rats hypophysectomized at day 23. One group of rats was injected daily with 60 µg FSH, another group was injected daily with FSH plus 500 ng oestradiol benzoate (E_2B) and a third group was injected with vehicle only. After 5 days Leydig cells were isolated and incubated with ^{3}H-testosterone and unlabelled testosterone. Also Leydig cells from intact rats were isolated and incubated. Testosterone metabolism was measured and expressed as pmoles testosterone converted per minute per 10^6 cells (Table 1). The main metabolites were dihydrotestosterone, androstanediol and androsterone.

Table 1 Testosterone conversion in isolated Leydig cells from intact rats, 5 days hypophysectomized rats (hypox), and hypox rats treated with FSH (hypox + FSH), or FSH plus E_2B treated rats (hypox + FSH + E_2B)

	pmole/10^6 cells/min	
	Exp. 1	Exp. 2
intact	133	76
hypox	0	0
hypox + FSH	0	25
hypox + FSH + E_2B	25	25

After hypophysectomy a distinct drop in 5α-reductase activity has been observed. Administration of FSH or FSH plus E_2B appears to increase testosterone conversion in hypophysectomized rats.

2) The amount of Leydig cells in the Leydig cell preparations isolated from intact, hypophysectomized, FSH and FSH plus E_2B treated rats

The number of Leydig cells in the cell preparations from intact and hypophysectomized rats treated as described above (see 1) was determined using 3β-ol-dehydrogenase as a Leydig cell marker. Results are presented in Table 2.

Table 2 The amount of 3β-ol-dehydrogenase containing cells in Leydig cell preparations of intact rats, hypophysectomized rats and hypophysectomized rats treated with FSH or FSH plus E_2B

	% 3β-ol-dehydrogenase containing cells ($\bar{x} \pm$ S.E.M. (n))
intact	46.1 ± 3.9 (14)
hypox	33.2 ± 2.0 (6)*
hypox + FSH	31.3 ± 1.5 (7)*
hypox + FSH + E_2B	33.1 ± 3.2 (9)*

*significantly different from intact rats $p \leq 0.01$
significance was tested with the two tailed Student's t-test.

Hypophysectomy results in a 25% drop of the amount of 3β-ol-dehydrogenase containing cells, but this decrease cannot explain the absence of an LH response in Leydig cell preparations of hypophysectomized rats. Administration of FSH or FSH plus E_2B to hypophysectomized rats had no influence on 3β-ol-dehydrogenase containing cells in the cell preparations.

3) The effect of hypophysectomy, FSH or FSH plus E_2B treatment on LH stimulation of cAMP production in isolated Leydig cells

The effect of hypophysectomy, FSH and oestrogen treatment on LH stimulated testosterone production could be at the level of the stimulation of cAMP production. Therefore cAMP production was measured in isolated cells from intact and hypophysectomized rats treated as described above. Cells were incubated with or without 3-isobutyl-1-methylxanthine (MIX), a phosphodiesterase inhibitor.

Table 3 cAMP production in Leydig cells from intact rats, hypophysectomized rats and hypophysectomized rats treated with FSH or FSH plus E_2B

	ng cAMP/10 µg DNA/2 hours			
	-MIX		+MIX	
ng LH/ml	0	100	0	100
intact	0.19 ± 0.04 (4)	62.3 ± 6.8 (4)	2.08	190.9
hypox	0.23 ± 0.05 (4)	8.2 ± 5.3 (4)*	1.18	32.1
hypox + FSH	0.16 ± 0.02 (4)	10.5 ± 3.6 (4)*	0.67	28.0
hypox + FSH + E_2B	0.10 ± 0.03 (4)	8.8 ± 2.4 (4)*	0.95	30.2

*significantly different from intact $p \leq 0.01$
Values as $\bar{x} \pm$ S.E.M. (n) or as means of duplicate experiments.

Hypophysectomy resulted in a decrease of LH stimulated cAMP production, either in the presence or absence of MIX in the incubation medium. Administration of FSH or FSH plus oestrogen to hypophysectomized rats did not alter the response of Leydig cells to LH in terms of cAMP as compared to untreated hypophysectomized rats.

4) Time course of the effect of FSH on LH stimulated testosterone production in isolated Leydig cells from hypophysectomized rats

Rats were hypophysectomized at day 23. One group of rats was injected daily with 60 µg FSH. Another group was injected with vehicle only. After 1,2,3 and 5 days the rats were killed and Leydig cells were prepared and incubated with LH. Leydig cells from intact rats were used as control. The LH stimulated testosterone production of intact rats was taken as the 100% value. Results are given in Figure 1.

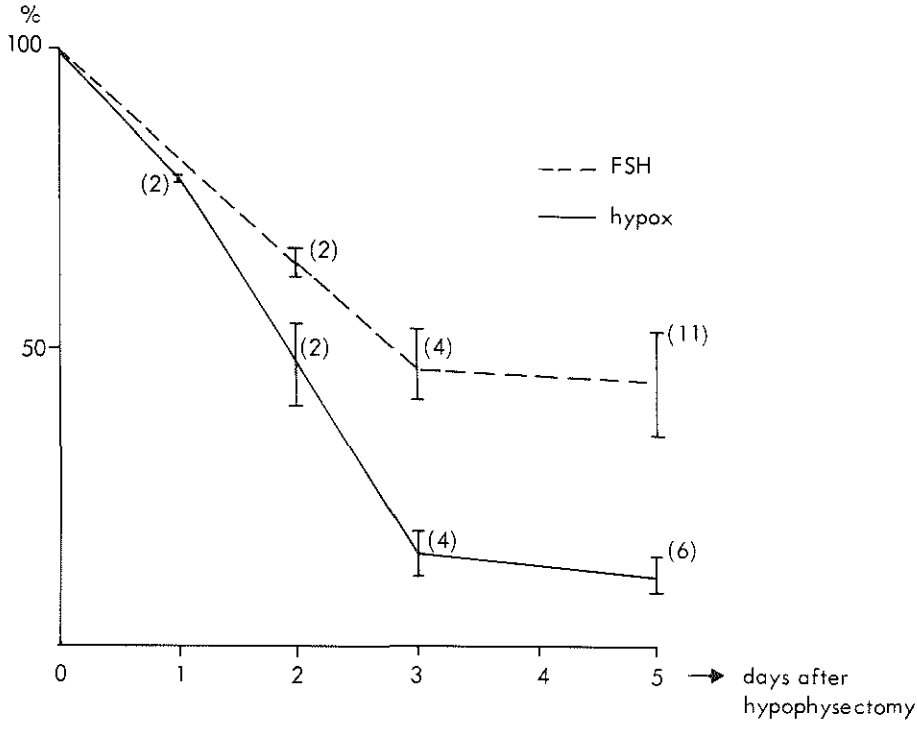

Figure 1 Hypophysectomized rats were injected daily with 60 µg FSH or with vehicle only. After 1,2,3 and 5 days Leydig cells were isolated and incubated with 0 or 100 ng LH/ml for 2 hours. LH stimulated testosterone production was expressed as percentage of the LH stimulated testosterone production of cells from intact rats of the same age as the hypophysectomized rats. Means and individual values of duplicate experiments are given or means ± S.E.M. (n)

Both in untreated and FSH treated rats the LH stimulated testosterone production decreased till 3 days after hypophysectomy. In untreated rats the LH responsiveness diminished further to a plateau level of 10% of the value of intact rats on day 5 after hypophysectomy, but in FSH treated rats from day 3 after hypophysectomy LH stimulated testosterone production remained at a constant level of 50% of the value of intact rats.

No significant difference could be observed between the LH stimulated testosterone production of Leydig cells from intact rats of 23 or 28 days old.

5) Time course of the effect of oestradiol on LH stimulated testosterone production of hypophysectomized rats

I. Experiments with hypophysectomized rats.

Rats were hypophysectomized at day 23. One group of rats was injected with E_2B (500 ng) daily. A second group was injected with vehicle only. After 1,2 and 5 days Leydig cells were isolated and LH stimulated testosterone production was measured. Testosterone production was expressed as percentage of the testosterone production of cells from intact rats (Fig. 2).

No difference could be observed between untreated hypophysectomized rats and E_2B treated hypophysectomized rats. LH stimulated testosterone production was clearly decreased after hypophysectomy.

b) Effect of E_2B on testosterone production of Leydig cells from hypophysectomized rats treated with E_2B starting at day 3 or 5 after hypophysectomy.

Hypophysectomized rats were injected daily with 60 µg FSH. One group was treated with E_2B (5 µg or 500 ng) starting from day 3 after hypophysectomy. Another group was injected with E_2B at day 5 after hypophysectomy. On day 6 after hypophysectomy the rats were killed and testosterone production in isolated Leydig cells was measured. Results are expressed as percentage of testosterone production of cells from intact rats (Fig. 4).

Treatment with E_2B started either at day 3 or at day 5 after hypophysectomy resulted in an inhibition of the FSH induced LH responsiveness of isolated Leydig cells.

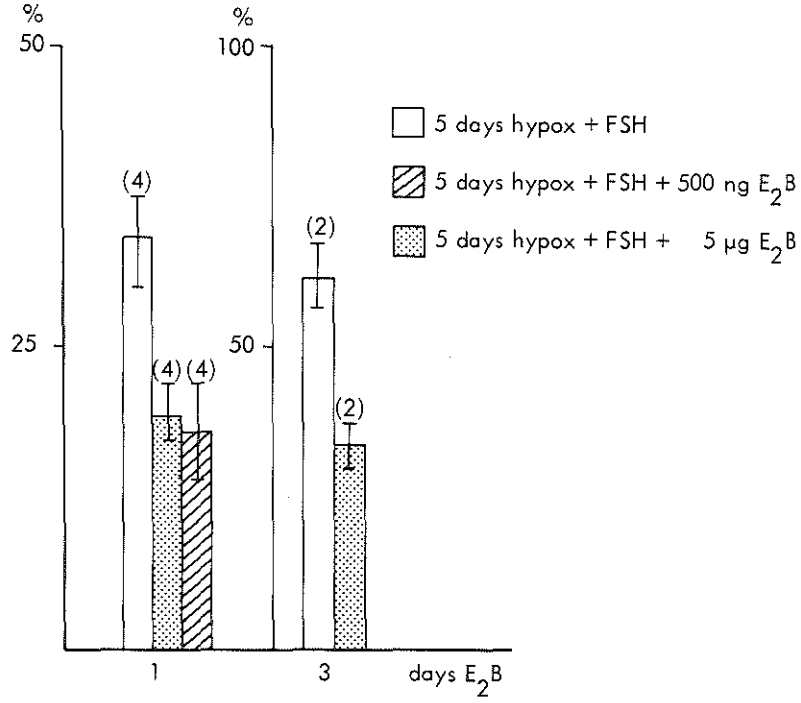

Figure 4 Hypophysectomized rats were injected for 5 days with 60 µg FSH. The last 3 or 1 days E_2B was given together with FSH. Isolated Leydig cells were incubated with 0 or 100 ng LH/ml. Testosterone production was expressed as percentage of the production of Leydig cells from intact rats. Values are given as means of duplicate experiments or means ± S.E.M. (n).

6) Correlation between the nuclear binding of the oestrogen-receptor complex and the effect of E_2B on LH stimulated testosterone production

To investigate whether the oestrogen receptor is involved in the effect of E_2B on testosterone production, 5 days hypophysectomized rats treated daily with FSH received a single dose of 5 µg E_2B. The E_2B injection was given together with the last FSH injection. Control rats received FSH only. Different times after injection the nuclear KCl extractable oestrogen receptor in the testis was measured with a nuclear exchange method. For the estimation of nuclear KCl extractable receptor 48 hours after injection of E_2B, the E_2B was given together with FSH at the fourth day after hypophysectomy and at the fifth day the rats received an injection of FSH only. LH stimulation of testosterone production was determined by incubation of isolated Leydig cells with 100 ng LH/ml at different times after injection of E_2B Results are given in Table 4.

In control rats 1 hour after injection of 500 ng oestradiol all the cytoplasmic oestradiol receptor molecules have been translocated to the nucleus and the maximal amount of KCl extractable nuclear receptor can be determined (25) with a nuclear exchange method. The maximal amount of oestradiol bound to KCl extractable nuclear receptor in 5 days hypophysectomized rats treated with FSH was 33 fmole/mg of protein of the KCl extract (mean of 2 determinations).

It can be concluded from Table 4 that the binding of oestradiol to the nuclear KCl extractable receptor parallels the inhibiting effect of oestrogen on LH stimulated testosterone production.

Table 4 Nuclear binding of oestrogen-receptor complex in the testis and the effect of E_2B on LH stimulated testosterone production.
Hypophysectomized rats were treated daily with FSH (60 µg) during 5 days. At day 4 or 5 FSH was given together with E_2B. Control rats received FSH only. Inhibition of LH stimulated testosterone production by E_2B in isolated Leydig cells was determined and compared to the number of occupied nuclear KCl extractable oestradiol binding sites in the testis.

time after injection hours	occupied nuclear oestradiol binding sites[a] fmole/mg protein	% inhibition of LH stimulated testosterone production[a]
1	7	33,34
3	19,24,27	32,56
18	n.d.	44.1 ± 8.8 (4)
24	12,11	42,0
48	4	0,0
control rats	0	0

a) individual experiments or means ± S.E.M. (n)
n.d. not determined

2.4 Discussion

From previous observations (see appendix paper III), it was known that hypophysectomy of immature rats results in a loss of the ability of isolated Leydig cells to respond to LH. Administration of FSH to hypophysectomized rats restores this LH responsiveness, whereas simultanous injection of oestradiol benzoate (E_2B) with FSH inhibits the FSH induced LH responsiveness of isolated Leydig cells.

The results in Table 1 reflect that the loss in LH re-

ponsiveness after hypophysectomy in terms of the amount of testosterone produced per cell is not due to an increased testosterone conversion. No 5α-reductase activity could be observed in isolated Leydig cells from 5 days hypophysectomized rats. This is in agreement with the results of Nayfeh et al. (178), who observed that the 5α-reductase activity in testis interstitial tissue decreased after hypophysectomy. FSH treatment of hypophysectomized rats caused a small increase in ^{3}H-testosterone conversion in one of two experiments. According to Nayfeh et al. (178) FSH can cause an increase in 5α-reductase activity in the interstitial tissue of the testis. In both experiments FSH plus E_2B treatment resulted in an increase of testosterone conversion, which could implicate that oestrogens may influence testosterone metabolism in the Leydig cells. The effect of oestrogens on testosterone metabolism in the testis is unknown. In prostatic tissue both an increase (179)and an inhibition (180) of 5α-reductase activity have been observed after oestrogen administration.

Another possible explanation for the observed effects of hypophysectomy, FSH and oestrogen treatment on LH stimulated testosterone production could be a change in the composition of the Leydig cell preparation. Using 3β-ol-dehydrogenase as a Leydig cell marker, a small decrease in the number of Leydig cells was observed after hypophysectomy. This decrease however was too small to explain the complete disappearance of LH responsiveness 5 days after hypophysectomy. Samuels and Helmrich (181) also found no great change in the concentration of this enzyme in testis following hypophysectomy.

If the effects on LH responsiveness cannot be explained by a higher conversion of testosterone or by a change in Leydig cell content of the cell preparation, a change in one or more of the steps stimulated by LH which finally lead to increased testosterone production might have been affected by hypophysectomy, FSH and E_2B treatment. Hypophysectomy results in a loss of LH receptors (182,183,184,185) in testes from adult and immature rats. Chen et al. (185)

have demonstrated that FSH treatment of hypophysectomized immature rats increases the amount of LH receptors in testis tissue. Binding of LH to its receptor appears to parallel the increase in cAMP production (7). A loss of LH receptors could therefore be accompanied by a decreased effect of LH on cAMP production. From the results in Table 3 it can be concluded that the stimulation of cAMP after hypophysectomy is indeed lower than the stimulation of cAMP in cells from intact rats. No stimulatory effect on cAMP production of FSH or FSH plus E_2B treatment could be observed in hypophysectomized rats, which would be expected if FSH increased LH receptors in hypophysectomized rats.

To gain further insight in the effect of FSH on LH stimulated testosterone production, LH stimulation of testosterone production was studied at different times after hypophysectomy in isolated Leydig cells from untreated or FSH treated hypophysectomized rats. A steep decrease in testosterone production was observed during the first 2 days after hypophysectomy irrespective of FSH treatment. From day 2 to 5 after hypophysectomy, however, there was a further drop in LH responsiveness of the Leydig cells from untreated rats, whereas in the FSH treated rats LH stimulated testosterone production remained at the level of 40-50% of the LH stimulated testosterone production of intact rats. Odell et al. (175) have also observed a change in LH responsiveness after administration of FSH to hypophysectomized rats for 5 days. The effect was not observed after 3 days FSH treatment.

The time course of the oestrogen effect on LH stimulated testosterone production in the present experiments was studied in untreated hypophysectomized rats and in rats treated with FSH after hypophysectomy. In untreated rats 1,2 or 5 days treatment with E_2B did not influence the testosterone production in isolated Leydig cells when compared with the testosterone production in cells from untreated rats.

Two different approaches have been used to investigate

the time course of the inhibiting effect of oestrogen in hypophysectomized rats treated with FSH. In the first approach hypophysectomized rats were injected with FSH with or without E_2B starting immediately after hypophysectomy. After 3 or 5 days treatment with FSH plus E_2B the LH stimulated testosterone production in isolated Leydig cells was significantly lower than after treatment with FSH alone. Administration of FSH plus E_2B to hypophysectomized rats for 2 days did not result in an inhibition of the FSH effect on LH stimulated testosterone production.

In the second series of experiments the effect of E_2B was studied in 5 days hypophysectomized rats treated daily with FSH and injected for the last 1 or 3 days with E_2B. In these rats an inhibiting effect of E_2B on LH stimulated testosterone production was already observed 1 day after injection with E_2B.

In summary it appears from the present results that an inhibiting effect of E_2B on LH stimulation of testosterone production in Leydig cells can only be observed in FSH treated hypophysectomized rats. In FSH treated rats the effect of E_2B could be observed only when the stimulating effect of FSH on LH responsiveness was clear. Therefore it can be concluded that the effect of E_2B must be due to an inhibition of the FSH induced LH responsiveness and not to an inhibition of the LH response itself.

The effect of E_2B might reflect either nonspecific effects, such as competitive inhibition of one of the steroidogenic enzymes, or the effect involves binding of oestradiol to the receptor followed by transocation to the nucleus. In order to establish the possible involvement of the oestrogen receptor in the effect of E_2B, 5 days hypophysectomized rats treated daily with FSH were injected with E_2B and the amount of nuclear receptors in the testis as well as the effect of E_2B on LH stimulated testosterone production in isolated Leydig cells were determined at different times after injection (Table 4). There appears to be a correlation between the inhibiting effect of E_2B and the binding

of oestradiol to the KCl extractable nuclear receptor. Clark and Peck (61) have suggested that longterm nuclear retention (at least 4-6 hours) of the hormone-receptor complex is a requirement for a hormone response in the uterus. In the testis part of the receptor molecules are still present in the nucleus 24 hours after injection, which implies that also in our experiments this condition for a hormone response has been fulfilled. However, according to Clark and Peck (61) binding of the receptor-hormone complex to non-KCl extractable acceptor sites may be essential for the hormone response.

Until now there is no method for the accurate determination of non-KCl extractable hormone-receptor complex in the nucleus of the testis after injection of oestrogens. Whether there is a relationship between KCl extractable and KCl non-extractable receptor sites remains to be investigated.

6 General discussion

6.1. Oestradiol receptors in the male rat

The oestradiol binding protein present in the interstitial tissue of the testis can be regarded as a true steroid receptor since many of its properties are comparable with those of the oestradiol receptor in rat uterus. Rat uterus is a recognized oestrogen target tissue and the involvement of the oestrogen receptor in the response evoked by the steroid hormone has been shown. However, a possible role of oestradiol in the male rat is still not clear. Oestradiol is produced in the male rat. Oestradiol could play a role not only in rat testis interstitial tissue but also in several other tissues of the male rat since an oestradiol receptor, obligatory for a hormone response is present in these tissues.

An 8S specific oestradiol binding protein could be demonstrated in cytosol preparations from rat prostate, epididymis, seminal vesicle, liver, pituitary and adrenal glands (chapter 3). These tissues have not been considered as important target tissues for oestradiol, but recently some indications have been obtained for a possible function of oestradiol and its receptor in these tissues. In mouse epididymis oestradiol can enhance sperm maturation (186). An effect on RNA synthesis has been observed in prostate (187) and also in liver (123) of the female rat. Oestradiol can also promote the proliferation of endothelial cells in the liver of the female rat (188). An effect on fat accumulation in the adrenal gland of the Mongolian gerbil has been observed after oestrogen administration (189). Finally the feedback action of oestradiol on LH secretion in the rat (23,24) is an example of a possible effect of oestrogens in the hypophysis, although the feedback action could also be at the level of the hypothalamus. In this thesis we tried to elu-

cidate the function of oestradiol and its receptor in the testis.

6.2 The effect of oestradiol on RNA synthesis in the testis

If rat testis interstitial tissue is an oestradiol target tissue then it might be expected that the final response evoked by oestradiol is preceded by an increase in RNA synthesis (see chapter 2). In chapter 3 is described that injection of 100 µg oestradiol in intact rats enhanced uridine incorporation in RNA of testis interstitial tissue after 6 hours. This increase is probably due to an increased RNA synthesis and not to an increased uptake of uridine in the nucleotide pool, since the specific activity of the nucleotide pool was not changed.

Oestradiol can also influence the secretion of LH (23, 24), a hormone, which also acts specifically on interstitial tissue (1,2) and has an effect on RNA synthesis (165). Therefore the observed increase in RNA synthesis after oestradiol administration might have been a reflection of changes in plasma LH levels. This assumption is supported by the observation that the effect of oestradiol on RNA synthesis was not found in hypophysectomized rats. Therefore it was further attempted to study a possible role of LH in this respect.

6.3 The effect of oestrogens on plasma LH levels and testicular testosterone production

The action of oestrogens on plasma LH levels and testicular testosterone production have been described in chapter 4. After injection of 100 µg oestradiol LH levels were significantly decreased, concomitant with a decreased testosterone production in the testis. Twenty-four hours after injection plasma LH levels as well as testosterone production were normal. The decrease in plasma LH levels and

testosterone production 6 hours after injection of 100 µg oestradiol is accompagnied by an increase in uridine incorporation in testis interstitial tissue. It would rather be expected that a decrease in plasma LH levels would cause a decrease rather than an increase in RNA synthesis (165). Therefore the observed increase in uridine incorporation must reflect either a direct effect of oestradiol on the interstitial tissue or a change in the metabolic state of the cell due to changes in testicular testosterone levels. Another possibility is that oestradiol has an influence on other tropic hormones which in turn could have an effect on RNA synthesis. Examples of an effect of oestradiol on the secretion of tropic hormones other than LH has been described for the male rat. Prolactin levels are increased (20,21,190) and FSH levels are decreased (23,24,191) after oestradiol administration.

Not only injection of 100 µg oestradiol but also administration of 50 µg E_2B or 500 ng oestradiol resulted after 24 and 3 hours respectively in decreased plasma LH levels and testicular testosterone production. No effect of oestrogen administration could be found in hypophysectomized rats so that the inhibiting effect of oestrogens on testicular testosterone production in these experiments can be explained by a negative feedback action on LH secretion.

Nevertheless there are still two observations which cannot be fully explained by a negative feedback action on LH secretion, i.e. subcutaneous injection of 500 ng oestradiol or 50 µg E_2B resulted in decreased testicular testosterone production after 1 and 6 hours respectively, whereas no significant change in LH plasma levels has been observed. This inhibitory effect on testosterone synthesis could not be observed in hypophysectomized rats. These observations might be explained by an effect of oestradiol on the secretion of other tropic hormones, which also play a regulatory role in testosterone synthesis. As suggested by some authors both FSH (192,193) and prolactin (193,194,195) have a stimulatory effect on testosterone synthesis.

Because oestradiol can have an influence on the secretion of LH, FSH and prolactin in the male rat, the intact adult male rat is a too complicated experimental model to study direct effects of oestradiol on the testis.

6.4. The effect of oestrogens on LH sensitivity of isolated Leydig cells from immature rats

The effect of oestradiol on testosterone production in the testis of adult rats seems not clear. The possibility exists that an effect of oestradiol might be restricted to a certain developmental stage of the Leydig cell. In the immature male rat oestradiol is present (174) and the oestradiol receptor in the testis is located in the nucleus due to endogenously present oestradiol (25). Therefore an attempt has been made to study whether oestradiol and its receptor have an effect on the Leydig cell of the immature rat.

In chapter 5 of this thesis the results are described of a study concerning the effect of in vivo oestrogen administration on LH stimulated testosterone production in isolated Leydig cells from immature rats. It was possible to isolate a Leydig cell preparation from immature rat testis in which testosterone production was stimulated by LH in a dose dependent way. The ability of such isolated Leydig cells to respond to LH after hypophysectomy was decreased with respect to both testosterone production and cAMP production.FSH treatment of hypophysectomized rats partly restored LH sensitivity in terms of testosterone production. This restoration cannot be due to an increase in the ability to synthesize cAMP, since LH stimulated cAMP production was not increased after treatment of hypophysectomized rats with FSH.

The restored LH responsiveness in FSH treated hypophysectomized rats was rather unexpected, since it is generally accepted that LH acts on the interstitial tissue (1,2) and FSH on the seminiferous tubules (10,11). Still there are other examples for a possible regulatory role of FSH in the

interstitial tissue of the immature animal. FSH can for example increase glucose-6-phosphate dehydrogenase in the mouse testis (196) and this enzyme is localized in the interstitial tissue (197). Also, it has been reported that 5α-reductase activity, predominantly present in interstitial tissue (198) may be influenced by FSH in the immature rat (178). Whether these effects of FSH are due to a direct interaction of FSH with the Leydig cell is not yet clear.

FSH is probably not the only hormone necessary for maintenance of LH responsiveness after hypophysectomy. In spite of FSH treatment immediately after hypophysectomy, LH responsiveness is not restored to levels of intact rats. The lack of other hormones which play a regulatory role in LH sensitivity must be responsible for the decrease in LH responsiveness. It has been suggested that prolactin (195) and corticosteroids (199) are involved in the regulation of LH sensitivity.

The effect of FSH on LH responsiveness becomes clear after 3 days treatment of hypophysectomized rats. Whereas in untreated hypophysectomized rats LH responsiveness is lower, LH responsiveness is maintained at a level of 50% of the responsiveness of intact rats by FSH treatment. Odell et al. (175) have suggested that FSH plays a role in the development of LH sensitivity in the Leydig cells: through the high FSH levels before puberty the testis becomes more sensitive to stimulation by LH. Our results are in agreement with this hypothesis.

When in addition to FSH, oestradiol benzoate was administered to hypophysectomized immature rats no induction of LH responsiveness occurred. The effect of oestradiol benzoate could already be shown after a single injection, but could only be observed at that time after hypophysectomy at which the induction of LH responsiveness by FSH was clear. At which stage of the LH response oestradiol benzoate acts is not clear yet. No difference between the LH stimulation of cAMP production in FSH treated or FSH plus oestradiol benzoate treated rats could be observed. The possibility exists

that oestradiol increases testosterone metabolism, but the results of our experiments gave no conclusive proof for an increased testosterone metabolism after oestradiol treatment of FSH treated hypophysectomized rats.

Whether in vivo oestradiol and FSH really play a regulatory role in the testis during sexual development of the male rat is not known. Nevertheless the following remarks can be made.

No great differences exist between LH levels of immature and adult rats (200,201,202,203) , but still testosterone levels remain low until day 30-40. FSH levels peak between day 20-40 (200,201,202,203,204,205) and this period is followed by a gradual increase in testosterone levels (206,207). According to Odell et al. (176) this is due to the induction of LH sensitivity by FSH. Oestradiol levels are higher before day 30 (174) than in adult rats and the role of oestradiol could be to inhibit testosterone production during the induction of LH sensitivity till maturation of the testis has been achieved. This possible mechanism of action of FSH and oestradiol in the testis is schematically presented in Figure 1.

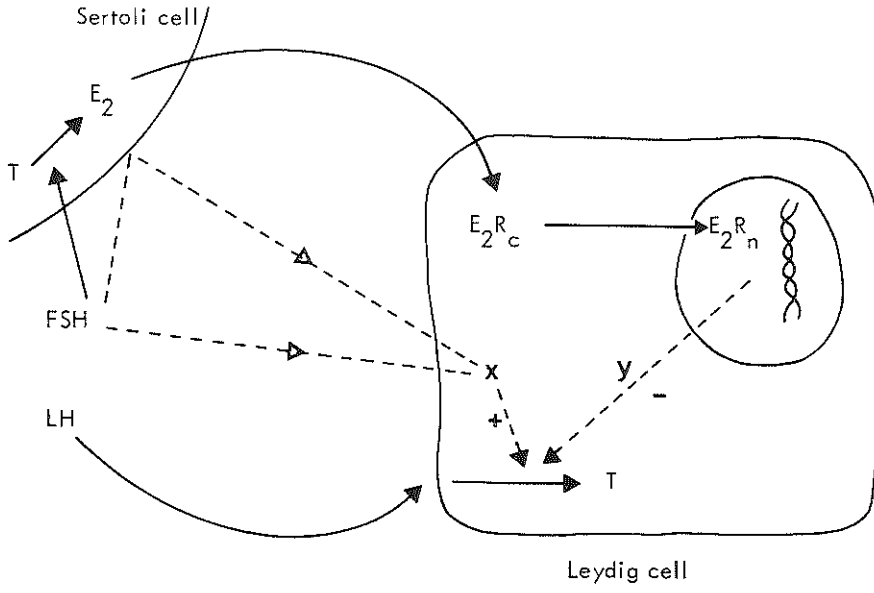

Figure 1 Possible mechanism of action of FSH and oestradiol in the testis. FSH induces either by direct interaction with the Leydig cell or indirectly via the Sertoli cell a factor X which is required for the effect of LH on testosterone (T) production. FSH also promotes the conversion of testosterone to oestradiol (E_2) in the Sertoli cell(208).
Oestradiol in turn can be bound by the oestradiol receptor in the Leydig cell and the cytoplasmic receptor steroid complex (E_2R_c) is translocated to the nucleus. The interaction of the oestradiol-receptor complex with chromatin will induce the synthesis of a factor Y, probably via induction of specific RNA molecules. This factor Y may inhibit the stimulation of testosterone production by LH.

Summary

Steroid hormone action seems to be mediated by specific binding proteins: 'receptors' which are located in the hormone target tissues. This thesis describes several results of studies on different aspects of the oestradiol receptor in the testis and the possible function of this receptor in the effect of oestradiol on testis function.

In chapter 4 the action of LH and FSH on the testis have been summarized. Some aspects of the mechanism of action of steroid hormones particularly the role of steroid receptors in the effect of steroids on RNA synthesis in target tissues are discussed in chapter 2.

In chapter 3 results of experiments on the presence of oestradiol receptors in tissues of the male rat and properties of the oestradiol receptor in rat testis are described. The cytoplasmic receptor from the testis is heat labile and sensitive to pronase treatment. The receptor has a high affinity for oestradiol-17β and diethylstilboestrol, a moderate affinity for oestrone, oestriol and oestradiol-17α and a low affinity for testosterone and dihydrotestosterone.

Agar electrophoresis, Sephadex chromatography, adsorption by dextran coated charcoal and sucrose gradient centrifugation have been compared for the quantitative determination of specific oestradiol binding in cytosol from rat testis. The four different methods gave similar results.

In the male rat an 8S saturable oestradiol binding protein could also be demonstrated in liver, prostate, seminal vesicle, epididymis, pituitary and adrenal glands.

Binding of oestradiol to the receptor in testis results in the translocation of the receptor-hormone complex to the nucleus. Since nuclear translocation of hormone-receptor complexes precedes the subsequent stimulation of RNA synthesis, the effect of oestradiol administration on testicular RNA synthesis has been studied in chapter 3.2.

Administration of 100 µg oestradiol to intact male rats resulted after 6 and 24 hours in an increased uridine uptake in RNA from interstitial tissue whereas no effect in semini-

ferous tubules has been observed. The uptake of uridine in the nucleotide pool did not change after 6 hours but after 24 hours the specific activity of the nucleotide pool in interstitial tissue seemed to be increased. The effect of oestradiol on uridine incorporation in RNA and nucleotide pool could not be demonstrated in hypophysectomized rats whether they were treated with HCG or not.

In chapter 4 the results of experiments on the effect of oestrogen on plasma LH levels and testicular testosterone production have been summarized.

Six hours after injection of 100 µg oestradiol to intact rats testicular testosterone production was significantly decreased concomitant with plasma LH levels. Twenty-four hours after injection neither testosterone nor plasma LH levels were decreased anymore. Injection of 50 µg oestradiol benzoate resulted after 24 hours in a decreased testicular testosterone production and plasma LH levels. Six hours after injection testosterone production was decreased, plasma LH levels however were not significantly lowered. Also injection of 500 ng oestradiol decreased testicular testosterone production and plasma LH levels after 3 hours. One hour after injection of 500 ng oestradiol testosterone production was decreased whereas plasma LH levels were not significantly lower. No effect of oestrogen administration on testosterone production could be observed in hypophysectomized rats.

On basis of these results it was concluded that the effect of oestrogens on testicular testosterone production was at least partly due to a negative feedback action on LH secretion.

Since a direct effect of oestrogens on the testis of the adult rat is not clear, an attempt has been made to investigate whether oestradiol has a direct effect on the testis of the immature rat.

In chapter 5 the results are presented of a study concerning the effect of oestrogens on LH stimulated testosterone production in isolated Leydig cells from 20-25 day old rats.

A cell preparation could be isolated from immature rat

testis, which contained 53% Leydig cells when 3β-hydroxysteroid dehydrogenase was taken as a marker enzyme. The characteristics of the Leydig cell preparation from immature rat testis showed marked functional differences with Leydig cell preparations from adult rat testis. Although testosterone production in cells from immature rats could be stimulated by LH in a dose-dependent way, the maximal amount of testosterone produced in cells from immature rats was 10 times lower than in cells from adult rats. Testosterone metabolism in cells from immature rats was higher due to the conversion of testosterone to 5α-reduced metabolites.

Hypophysectomy of immature rats resulted after 5 days in a loss of LH responsiveness of Leydig cells. In contrast cells from adult rats still responded to LH after hypophysectomy in the same way as cells from intact rats. LH responsiveness in cells from immature hypophysectomized rats could be partly restored by treatment with FSH for 5 days.

When oestradiol benzoate was administered together with FSH to hypophysectomized rats the induced LH responsiveness could not be observed.

The loss in LH responsiveness after hypophysectomy in terms of testosterone production could neither be ascribed to a change in the amount of Leydig cells present in the Leydig cell preparation nor to a higher conversion of testosterone. The LH stimulated cAMP production however was very low in cells from hypophysectomized rats compared to cells from intact rats. There was no difference between cAMP production of Leydig cells from untreated, FSH treated or FSH plus oestradiol benzoate treated hypophysectomized rats.

After hypophysectomy LH responsiveness declines for the first 2 days both in untreated and FSH treated rats in the same way. From day 2 after hypophysectomy LH responsiveness declines further in cells from untreated rats but remains at a constant level in cells from rats treated with FSH.

A single injection of oestradiol benzoate to hypophysectomized rats treated with FSH did inhibit LH responsiveness only when oestradiol was administered at that time after

hypophysectomy, when the effect of FSH on LH responsiveness was clear.

An attempt has been made to correlate the presence of the oestradiol receptor in the nucleus and the inhibiting effect of oestradiol benzoate on LH stimulated testosterone production. Three hours after injection of oestradiol benzoate in hypophysectomized rats treated with FSH nearly all the receptor molecules were translocated to the nucleus. After 24 hours part of the receptor molecules were still present in the nucleus. At these times oestradiol benzoate appeared to inhibit the LH stimulated testosterone production in isolated Leydig cells. These observations could be an indication for the involvement of the receptor in the effect of oestradiol benzoate.

Finally in chapter 6 it has been attempted to integrate the results presented in this thesis and a possible mechanism of action of FSH and oestradiol in the testis is proposed.

Samenvatting

Waarschijnlijk oefenen steroidhormonen hun werking uit door middel van specifiek bindende eiwitten zg. receptoren, die aanwezig zijn in de doelwitorganen van de hormonen. proefschrift worden de resultaten van onderzoekingen naar verschillende aspekten van de oestradiol-receptor in de testis en de mogelijke rol van de receptor in het effekt van oestradiol op de testisfunktie besproken.

In hoofdstuk 1 is een overzicht gegeven van de werking van LH en FSH op de testis. In hoofdstuk 2 zijn enige aspekten van het werkingsmechanisme van steroidhormonen besproken, waarbij de nadruk is gelegd op de rol van de steroidreceptoren in het effekt van steroiden op RNA synthese in doelwitorganen.

In hoofdstuk 3 zijn de resultaten weergegeven van een onderzoek naar de eigenschappen van de oestradiol receptor in de rattetestis. De cytoplasmatische receptor uit de testis is gevoelig voor hoge temperaturen en voor pronase behandeling. De receptor heeft een hoge affiniteit voor oestradiol-17β en diethylstilboestrol, een iets minder hoge affiniteit voor oestron, oestriol en oestradiol-17α en een lage affiniteit voor testosteron en dihydrotestosteron.

Kwantitatieve bepalingen van specifieke oestradiol binding in het cytosol van rattetestis met behulp van agar elektroforese, Sephadex chromatografie, adsorptie aan "dextran coated charcoal" en sucrose gradient centrifugering zijn met elkaar vergeleken. Er was geen verschil in de resultaten verkregen met deze vier methodes.

Behalve in het interstitium van de testis kon ook een verzadigbaar oestradiol bindend eiwit met een sedimentatiewaarde van 8S worden aangetoond in: lever, prostaat, zaadblaas, epididymis, hypofyse en bijnier.

Binding van oestradiol aan de testikulaire receptor resulteert in de verplaatsing van het receptor-hormoon-komplex naar de kern. Omdat bekend is, dat verplaatsing van hormoon-receptor-komplex naar de kern in het algemeen leidt tot sti-

mulatie van RNA synthese, is het effekt van in vivo oestradiol toediening op testikulaire RNA synthese bestudeerd (hoofdstuk 3.2).

Toediening van 100 µg oestradiol aan intakte mannelijke ratten had na 6 en 24 uur een toename in uridine inbouw in RNA van het interstitium tot gevolg, terwijl dit niet het geval was in de seminifere tubuli. De opname van uridine in de nucleotide pool was na 6 uur niet veranderd, maar er zijn aanwijzingen gevonden dat na 24 uur de specifieke aktiviteit van de nucleotide pool van het interstitium was toegenomen. In gehypofysektomeerde ratten, die al dan niet met HCG behandeld waren kon geen verandering van uridine inkorporatie in RNA en nucleotide pool worden aangetoond na oestradiol injektie.

In hoofdstuk 4 is een samenvatting gegeven van de resultaten van een studie over het effekt van oestradiol op plasma LH levels en testikulaire testosteronproduktie. Toediening van 100 µg oestradiol veroorzaakte na 6 uur een signifikante daling zowel in plasma LH levels als testosteronproduktie. Na 24 uur waren de plasma LH levels en de testosteronproduktie niet meer verlaagd. Injektie van 50 µg oestradiol benzoaat had na 24 uur verlaagde testikulaire testosteronproduktie en plasma LH spiegels tot gevolg. Zes uur na injectie van 50 µg oestradiol-benzoaat was de testosterone produktie ook verlaagd, de plasma LH spiegels echter waren niet signifikant lager. Injektie van 500 ng oestradiol verlaagde de testikulaire testosterone produktie en plasma LH spiegels na 3 uur. Een uur na injektie van 500 ng oestradiol was de testosteronproduktie verminderd terwijl de plasma LH spiegels op dit tijdstip niet signifikant veranderd waren. Oestrogeen toediening aan gehypofysektomeerde ratten had geen enkele verandering in testosteronproduktie tot gevolg.

Gebaseerd op deze resultaten is gekonkludeerd dat het effekt van oestrogenen op testikulaire testosteronproduktie tenminste voor een gedeelte het gevolg is van een negatieve feedback op de LH sekretie.

Omdat het effekt van oestrogenen op de testis van de vol-

wassen rat niet duidelijk is, is onderzocht of oestradiol mogelijk een direkt effekt uitoefent op de testis van de jonge rat.

In hoofdstuk 5 zijn de resultaten beschreven van een onderzoek naar het effekt van oestrogenen op de door LH gestimuleerde testosteronproduktie in geïsoleerde Leydig cellen van 20-25 dagen oude ratten. Het was mogelijk uit testes van jonge ratten een celpreparaat te isoleren dat op basis van 3β-hydroxysteroid-dehydrogenase aktiviteit 53% Leydig cellen bevatte. Het Leydig cel preparaat van jonge ratten toonde enige opvallende funktionele verschillen met die van volwassen ratten. Hoewel de testosteronproduktie in cellen van jonge ratten gestimuleerd kon worden met LH op een dosis afhankelijke wijze, was de maximale hoeveelheid geproduceerd testosteron 10x lager dan in cellen van volwassen ratten. Het metabolisme van testosteron in cellen van jonge ratten was hoog door een omzetting naar 5α-gereduceerde metabolieten. Vijf dagen na hypofysektomie bleken Leydig cellen van jonge ratten hun kapaciteit om op LH te reageren verloren te hebben. Cellen van volwassen ratten daarentegen werden na hypofysektomie nog steeds door LH gestimuleerd op dezelfde wijze als cellen van intakte ratten. Het verlies in LH gevoeligheid van cellen van jonge gehypofysektomeerde ratten kon ten dele worden hersteld door behandeling met FSH gedurende 5 dagen. Werd tegelijk met FSH oestradiol benzoaat toegediend aan gehypofysektomeerde jonge ratten dan werd de door FSH geïnduceerde LH gevoeligheid niet waargenomen.

Na hypofysektomie is noch een verandering in de hoeveelheid Leydig cellen in het celpreparaat noch een verhoogde afbraak van testosteron verantwoordelijk voor het verdwijnen van de LH stimuleerbaarheid van de testosteronproduktie. De LH gestimuleerde cAMP-produktie van Leydig cellen van gehypofysektomeerde ratten was lager dan van cellen van intakte ratten. Er was geen verschil in cAMP-produktie van Leydig cellen van onbehandelde, FSH of FSH plus oestradiol-benzoaat behandelde gehypofysektomeerde ratten.

Gedurende de 2 dagen na hypofysektomie neemt de LH gevoe-

ligheid van de Leydig cellen op dezelfde wijze af, of de ratten met FSH worden behandeld of niet. Vanaf dag 2 na de hypofysektomie neemt de LH gevoeligheid in cellen van onbehandelde ratten verder af, terwijl de de LH gevoeligheid in cellen van met FSH behandelde dieren gehandhaafd wordt op een konstant niveau.

Wanneer gehypofysektomeerde ratten, die behandeld waren met FSH éénmaal werden geïnjekteerd met oestradiol benzoaat, dan werd de stimulatie van de testosteron-synthese slechts geremd op dat tijdstip na hypofysektomie waarop het effekt van FSH op de LH gevoeligheid duidelijk is.

Gepoogd is de aanwezigheid van de oestradiol-receptor in de kern te korreleren met het remmend effekt van oestradiol benzoaat op de LH gestimuleerde testosteronproduktie. Drie uur nadat gehypofysektomeerde met FSH behandelde dieren geïnjekteerd zijn met oestradiol benzoaat zijn bijna alle in de cel aanwezige receptor molekulen in de kern aanwezig. Na 24 uur was nog steeds een gedeelte van de receptor molekulen in de kern aanwezig. Op deze tijdstippen lijkt er ook een remmend effekt van oestradiol op de testosteronproduktie in Leydig cellen te zijn. Deze waarnemingen duiden er mogelijk op dat de receptor een rol speelt in het effekt van oestradiol benzoaat.

Tenslotte is in hoofdstuk 6 een poging gedaan om de resultaten te passen in een mogelijk werkingsmechanisme van FSH en oestradiol in de testis.

References

1. Cooke, B.A., de Jong, F.H., van der Molen, H.J. and Rommerts, F.F.G. (1972) Nature New Biol. 237, 255.
2. Janszen, F.H.A., Cooke, B.A., van Driel, M.J.A. and van der Molen, H.J. (1976) J. Endocr. 70, 345.
3. Castro, A.E., Alonso, A. and Mancini, R.E. (1972) J. Endocr. 52, 129.
4. Cooke, B.A., van Beurden, W.M.O., Rommerts, F.F.G. and van der Molen, H.J. (1972) FEBS Lett. 25, 83.
5. Dorrington, J.H. and Fritz, I.B. (1974) Endocrinology 94, 395.
6. Cooke, B.A., Lindh, L.M. and Janszen, F.H.A. (1976) Biochem. J. 160, 439.
7. Catt, K.J. and Dufau, M.L. (1973) Nature New Biol. 244, 219.
8. Janszen, F.H.A., Cooke, B.A., van Driel, M.J.A. and van der Molen, H.J. (1976) FEBS Lett. 71, 269.
9. Cooke, B.A., Clotscher, W.F., de Jong, C.M.M., Renniers, A.C.H.M. and van der Molen, H.J. (1974) J. Endocr. 63, 17.
10. Means, A.R., MacDougall, E., Soderling, T.R. and Corbin, J.D. (1974) J. Biol. Chem. 249, 1231.
11. Hansson, V., Trygstad, O., French, F.S., McLean, W.V.S. Smith, A.A., Tindall, D.J., Weddington, S.C., Petrusz, P., Nayfeh, S.N. and Ritzen, E.M. (1974) Nature 250, 387.
12. Mulder, E., Peters, M.J., van Beurden, W.M.O. and van der Molen, H.J. (1974) FEBS Lett. 47, 209.
13. Mulder, E., Peters, M.J., de Vries, J. and van der Molen, H.J. (1975) Moll. and Cell. Endocrinol. 2, 171.
14. Mulder, E., Peters, M.J., van Beurden, W.M.O., Galdieri, M., Rommerts, F.F.G., Janszen, F.H.A. and van der Molen, H.J. (1976) J. Endocr. 70, 331.

15. Brinkmann, A.O., Mulder, E., Lamers-Stahlhofen, G.J.M., Mechielsen, M.J. and van der Molen, H.J. (1972) FEBS Lett. 26, 301.
16. Mulder, E., Brinkmann, A.O., Lamers-Stahlhofen, G.J.M. and van der Molen, H.J. (1973) FEBS Lett. 31, 131.
17. de Jong, F.H., Hey, A.H. and van der Molen, H.J. (1974) J. Endocr. 60, 409.
18. Samuels, L.T., Uchikawa, T. and Huseby, R.A. (1967) in Endocrinology of the Testis (Wolstenholme, G.E.W. and O'Connor, M., eds.), Churchill, London, p. 211.
19. Chowdhury, M., Tcholakian, R. and Steinberger, E. (1974) J. Endocr. 60, 375.
20. Danutra, V., Harper, M.E., Boyns, A.R., Cole, E.N., Brownsey, B.G. and Griffiths, K. (1973) J. Endocr. 57, 207.
21. Mallampati, R.S. and Johnson, D.C. (1973) Neuro-endocrinology 11, 46.
22. Moger, W.H. (1976) Biol. Reprod. 14, 222.
23. Verjans, H.L., de Jong, F.H., Cooke, B.A., van der Molen, H.J. and Eik-Nes, K.B. (1974) Acta Endocr. 77, 636.
24. de Jong, F.H., Uilenbroek, J.T.J. and van der Molen, H.J. (1975) J. Endocr. 65, 281.
25. de Boer, W., de Vries, J., Mulder, E. and van der Molen, H.J. (1977) Biochem. J. 162, 331.
26. Jensen, E.V. and Jacobson, H.I. (1962) Recent Progr. Horm. Res. 18, 387.
27. Toft, D. and Gorski, J. (1966) Proc. Nat. Acad. Sci. U.S. 55, 1574.
28. Erdos, T. (1968) Biochem. Biophys. Res. Commun. 32, 338.
29. Rochefort, H. and Baulieu, E.E. (1968) C.R. Acad. Sci. Ser. D. 267, 662.
30. Korenman, S.G. and Rao, B.R. (1968) Proc. Nat. Acad. Sci. U.S. 61, 1028.
31. Jensen, E.V., Suzuki, T., Numata, M., Smith, S., DeSombre, E.R. (1969) Steroids 13, 417.

32. Jensen, E.V., Numata, M., Smith, S., Suzuki, T., Brecher, P.F. and DeSombre, E.R. (1969) Develop. Biol. Suppl. 3, 151.
33. Baulieu, E.E., Alberga, A., Jung, I., Lebeau, M.C., Mercier-Bodard, C., Millgrom, E., Raynaud, J.P., Raynaud-Jammet, C., Rochefort, H., Truong, H. and Robel, P. (1971) Recent Progr. Horm. Res. 27, 351.
34. Reti, I. and Erdos, T. (1971) Biochimie 53, 435.
35. Rochefort, H. and Baulieu, E.E. (1971) Biochimie 53, 893.
36. Chamness, G.C. and McGuire, W.L. (1972) Biochemistry 11, 2466.
37. Stancel, G.M., Leung, K.M.T. and Gorski, J. (1973) J. Biochem. 12, 2130.
38. Notides, A.C. and Nielson, S. (1974) J. Biol. Chem. 249, 1866.
39. Feherty, P., Robertson, D.M., Waynforth, H.B. and Kellie, A.E. (1970) Biochem. J. 120, 837.
40. Giannopoulos, G. and Gorski, J. (1971) J. Biol. Chem. 246, 2530.
41. Shyamala, G. and Gorski, J. (1969) J. Biol. Chem. 244, 1097.
42. Toft, D., Shyamala, G. and Gorski, J. (1967). Proc. Nat. Acad. Sci. U.S. 57, 1740.
43. Gorski, J., Toft, D., Shyamala, G., Smith, D. and Notides, A. (1968) Recent Progr. Horm. Res. 24, 45.
44. Korenman, S.G. (1969) Steroids 13, 163.
45. Leung, B.S., Jack, W.M. and Reiney, C.G. (1976) J. Steroid Biochem. 7, 89.
46. Vertes, M. and King, R.J.B. (1971) J. Endocr. 51, 271.
47. Eisenfeld, A.J. (1970) Endocrinology 86, 1313.
48. Mowles, T.F., Ashkanazy, B., Mix, E. and Sheppard, H. (1971) Endocrinology 89, 484.
49. Mainwaring, W.I.P. and Irving, R. (1973) Biochem. J. 134, 113.
50. Blaquier, J.A. and Calandra, R.S. (1973) Endocrinology 93, 51.

51. O'Malley, B.W. and Toft, D.O. and Sherman, M.P. (1971) J. Biol. Chem. 246, 1117.
52. O'Malley, B.W., Spelsberg, T.C., Schrader, W.T. Chytil, F., Steggles, A.W. (1972) Nature 235, 141
53. Milgrom, E., Agter, M. and Baulieu, E. (1970) Steroids 16, 741.
54. Beato, M. and Feigelson, P. (1972) J. Biol. Chem. 247, 7890.
55. Wira, C. and Munck, A. (1970) J. Biol. Chem. 245, 3436.
56. Baxter, J.D. and Tomkins, G.M. (1971) Proc. Nat. Acad. Sci. U.S. 68, 932.
57. Sharp, G.W.G. and Alberti, K.G.M.M. (1971) Advanc. Biosc. 7, 281.
58. Edelman, I.S. (1971) Advanc. Biosc. 7, 267.
59. Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and DeSombre, E.R. (1968) Proc. Nat. Acad. Sci. U.S. 59, 632.
60. Mester, J. and Baulieu, E.E. (1975) Biochem. J. 146, 617.
61. Clark, J.H. and Peck, E.J. (1976) Nature 260, 635.
62. Sarff,M. and Gorski, J. (1971) Biochemistry 10, 2557.
63. Jensen, E.V., DeSombre, E.R. (1972) Ann. Rev. Biochem. 41, 203.
64. Jensen, E.V., Mohla, S., Gorell, T., Tanaka, S., DeSombre, E.R. (1972) J. Steroid Biochem. 3, 445.
65. Fang, S. and Liao, S. (1971) J. Biol. Chem. 246, 16.
66. Liao, S. and Liang, T. (1974) in Hormones and Cancer (McKerns, K.W., ed.), Academic Press, New York, p. 229.
67. Munck, A. and Wira, C. (1971) Advanc. Biosc. 7, 301.
68. Marver, D., Goodman, D., Edelman, I.S. (1972) Kidney Int. 1, 210.
69. Buller, R.E., Toft, D.O., Schrader, W.T. and O'Malley, B.W. (1975) J. Biol. Chem. 250, 801.
70. DeSombre, E.R., Mohla, S. and Jensen, E.V. (1972) Biochem. Biophys. Res. Commun. 48, 1601.

71. Yamamoto, K.R. (1974) J. Biol. Chem. 249, 7068.
72. Puca, G.A., Nola, E., Sica, V. and Bresciani, F. (1972) Biochemistry 11, 4157.
73. Best-Belpomme, M., Mester, J., Weintraub, H. and Baulieu, E.E. (1975) Eur. J. Biochem. 57, 537.
74. Gschwendt, M. (1976) Eur. J. Biochem. 67, 411.
75. Nijberg, L.M. and Wang, T.Y. (1976) J. Steroid Biochem. 7, 267.
76. Middlebrook, J.L., Wong, M.D., Ishii, D.N. and Aronow, L. (1975) Biochemistry 14, 180.
77. Gschwendt, M., Hamilton, T.H. (1972) Biochem. J. 128, 611.
78. Musliner, T.A., Chader, G.J., Villee, C.A. (1970) Biochemistry 9, 4448.
79. Steggles, A.W., Spelsberg, T.C., Glasser, S.R. O'Malley, B.W. (1971) Proc. Nat. Acad. Sci. U.S. 68, 1479.
80. King, R.J.B. and Gordon, J. (1972) Nature New Biol. 240, 185.
81. Higgins, S.J., Rousseau, G.G., Baxter, J.D. and Tomkins, G.M. (1973) J. Biol. Chem. 248, 5866.
82. Bresciani, F., Nola, E., Sica, V. and Puca, G.A. (1973) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 32, 2126.
83. Williams, P., Gorski, J. (1972) Proc. Nat. Acad. Sci. U.S. 69, 3464.
84. Chamness, G.C., Jennings, A.W., McGuire, W.L. (1974) Biochemistry 13, 327.
85. Harris, G.S. (1971) Nature New Biol. 231, 246.
86. Toft, D. (1972) J. Steroid Biochem. 3, 515.
87. Puca, G.A., Vincenzo, S. and Nola, E. (1974) Proc. Nat. Acad. Sci. U.S. 71, 979.
88. Spelsberg, T.C., Steggles, A.W. and O'Malley, B.W. (1971) J. Biol. Chem. 246, 4188.
89. O'Malley, B.W., Schrader, W.T. and Spelsberg, T.C. (1973) in Receptors for Reproductive Hormones, Adv. Exp. Med. Biol. 36, 174.
90. Liao, S., Liang, T., Tymoczko, J.L. (1973) Nature New Biol. 241, 211.

91. Liang, T. and Liao, S. (1974) Biol. Chem. 249, 4671.
92. Bergink, E.W., Kloosterboer, H.J., Gruber, M. and AB, G. (1973) Biochim. Biophys. Acta 294, 497.
93. Muller, K.R., Cox, R.F. and Carey, N.H. (1970) Biochem. J. 120, 337.
94. Hamilton, T.H. (1968) Science 161, 649.
95. Wilson, J.D. and Gloyna, R.E. (1970) Recent Progr. Horm. Res. 26, 309.
96. Tomkins, G.M., Levensin, B.B., Baxter, J.D. and Dethlefsen, L. (1972) Nature New Biol. 239, 9.
97. Edelman, I.S. and Figmognari, G.M. (1968) Recent Progr. Horm. Res. 24, 1.
98. Kenney, F.T. and Kull, E.J. (1963) Proc. Nat. Acad. Sci. U.S. 50, 493.
99. Hallahan, C., Young, D.A. and Munck, A. (1973) J. Biol. Chem. 248, 2922.
100. Miller, B.G. and Baggett, B. (1972) Biochim. Biophys. Acta 281, 353.
101. Gorski, J. and Nicolette, J.A. (1963) Archs. Biochem. Biophys. 103, 418.
102. Means, A.R. and Hamilton, T.H. (1966) Proc. Nat. Acad. Sci. U.S. 56, 1594.
103. O'Malley, B.W. and McGuire, W.L. (1968) J. Clin. Invest. 47, 654.
104. Fujii, T. and Villee, C.A. (1968) Endocrinology 82, 463.
105. Mainwaring, W.I.P., Wilce, P.A. and Smith, A.E. (1974) Biochem. J. 137, 513.
106. Hamilton, T.H., Teng, C.S. and Means, A.R. (1968) Proc. Nat. Acad. Sci. U.S. 59, 1265.
107. Hamilton, T.H., Widnell, C.C. and Tata, J.R. (1968) J. Biol. Chem. 243, 408.
108. Knowler, J.T. and Smellie, R.M.S. (1971) Biochem. J. 125, 605.
109. Knowler, J.T. and Smellie, R.M.S. (1973) Biochem. J. 131, 689.
110. Darnell, J.E., Philipson, L., Wall, R. and Adesik, M. (1971) Science 174, 507.

111. De Angelo, A.B. and Gorski, J. (1970) Proc. Nat. Acad. Sci. U.S. 66, 693.
112. Notides, A. and Gorski, J. (1966) Proc. Nat. Acad. Sci. U.S. 56, 230.
113. Borgna, J.L., Bonnafous, J.C., Mousseron-Canet, M., Mani, J.C. and Cazaubon, C. (1976) Biochimie 58, 443.
114. Baulieu, E.E., Wira, C.R., Milgrom, E. and Raynaud-Jammet, C. (1972) Acta Endocr. Suppl. 168, 396.
115. Wira, C.R. and Baulieu, E.E. (1972) C.R. Acad. Sci. Ser. D. 274, 73.
116. Glasser, S.R., Chytil, F. and Spelsberg, T.C. (1972) Biochem. J. 130, 947.
117. Borthwick, N.M. and Smellie, R.M.S. (1975) Biochem. J. 147, 91.
118. Hardin, J.W., Clark, J.H., Glasser, S.R. and Peck, E.J. (1976) Biochemistry 15, 1370.
119. Jänne, O., Bullock, L.P., Bardin, C.W. and Jacob, S.T. (1976) Biochim. Biophys. Acta 418, 330.
120. Borthwick, N.M. and Bell, P.A. (1975) FEBS Lett. 60, 396.
121. Levitz, M., Katz, J., Krone, P., Prochoroff, N.N. and Troll, W. (1974) Endocrinology 94, 633.
122. Dati, F.A. and Maurer, H.R. (1971) Biochim. Biophys. Acta 246, 589.
123. Church, R.B. and McCarthy, B.J. (1970) Biochim. Biophys. Acta 199, 103.
124. Bartke, K. and Warren, J.C. (1972) Proc. Nat. Acad. Sci. U.S. 69, 2668.
125. Teng, C.S. and Hamilton, T.H. (1968) Proc. Nat. Acad. Sci. U.S. 60, 1410.
126. Mainwaring, W.I.P., Mangan, F.R. and Peterken, B.M. (1971) Biochem. J. 123, 619.
127. Mainwaring, W.I.P. and Jones, D.M. (1975) J. Steroid Biochem. 6, 475.
128. Liao, S. and Lin, A.H. (1967) Proc. Nat. Acad. Sci. U.S. 57, 379.

129. Beato, M., Homoki, J., Doenecke, D. and Sekeris, C.E. (1970) Experienta 26, 1074.
130. Courvalin, J.C., Bouton, M.M., Baulieu, E.E., Nuret, P. and Chambon, P. (1976) J. Biol. Chem. 251, 4843.
131. Mohla, S., DeSombre, E.R. and Jensen, E.V. (1972) Biochem. Biophys. Res. Commun. 46, 661.
132. Raynaud-Jammet, C., Bouton, M.M., Carelli, M.G. and Baulieu, E.E. (1975) Methods in Enzymology, volume XXXVI, p. 319-327.
133. Davies, P., Fahmy, A.R., Pierrepoint, C.G. and Griffiths, K. (1972) Biochem. J. 129, 1167.
134. Davies, P. and Griffiths, K. (1973) Biochem. Biophys. Res. Commun. 53, 373.
135. Davies, P. and Griffiths, K. (1973) Biochem. J. 136, 611.
136. Davies, P. and Griffiths, K. (1974) J. Endocr. 62, 385.
137. Schimke, R.T., McKnight, G.S., Shapiro, D.J., Sullivan, D. and Palacios, R. (1975) Recent Progr. Horm. Res. 31, 175.
138. Palmiter, R.D., Catlin, G.H. and Cox, R.F. (1973) Cell. Differ. 2, 163.
139. O'Malley, B.W., McGuire, W.L., Kohler, P.O. and Korenman, S.G. (1969) Recent Progr. Horm. Res. 25, 105.
140. Palmiter, R.D. (1971) Biochemistry 10, 4399.
141. Palmiter, R.D. (1972) J. Biol. Chem. 247, 6450.
142. Harris, S.E., Rosen, J.M., Means, A.R. and O'Malley, B.W. (1975) Biochemistry 14, 2072.
143. McKnight, G.S., Pennequin, P. and Schimke , R.T. (1975) J. Biol. Chem. 250, 8105.
144. Means, A.R., Comstock, J.P., Rosenfeld, G.C. and O'Malley, B.W. (1972) Proc. Nat. Acad. Sci. U.S. 69, 1146.
145. Comstock, J.P., Rosenfeld, G.L., O'Malley, B.W. and Means, A.R. (1972) Proc. Nat. Acad. Sci. U.S. 69, 2377.
146. Tsai, M.J., Schwartz, R.J., Tsai, S.Y. and O'Malley, B.W. (1975) J. Biol. Chem. 250, 5165.

147. Schwartz, R.J., Tsai, M.J., Tsai, S.Y. and O'Malley, B.W. (1975) J. Biol. Chem. 250, 5175.
148. Kalimi, M. , Tsai, S.Y., Tsai, M.J., Clark, J.H. and O'Malley, B.W. (1976) J. Biol. Chem. 251, 516.
149. Schwartz, R.J., Kuhn, R.N., Buller, R.E., Schrader, W.T. and O'Malley, B.W. (1976) J. Biol. Chem. 251, 5166.
150. Buller, R.E., Schwartz, R.J., Schrader, W.T. and O'Malley, B.W. (1976) J. Biol. Chem. 251, 5178.
151. Yamamoto, K.R., Gehring, U., Stampfer, M.R. and Sibley, C.H. (1976) Recent Progr. Horm. Res. 34, 3.
152. de Boer, W., Mulder, E. and van der Molen, H.J. (1976) J. Endocr. 70, 397.
153. de Boer, W., de Vries, J., Mulder, E. and van der Molen, H.J., J. Steroid Biochem., in press.
154. Brinkmann, A.O. (1972) Ph.D. Thesis, Erasmus University Rotterdam.
155. Westphal, U. (1970) in Biochemical Actions of Hormones (Litwack, G., ed.), Academic Press, New York, vol. 1, p. 209.
156. Billing, R.J., Barbiroli, B. and Smellie, R.M.S. (1969) Biochim. Biophys. Acta 190, 52.
157. Billing, R.J., Barbiroli, B. and Smellie, R.M.S. (1969) Biochim. Biophys. Acta 190, 60.
158. Greenman, D.L. (1971) Steroids 17, 17.
159. Munns, T.W. and Katzman, P.A. (1971) Biochemistry 10, 4941.
160. Miller, B.G. and Baggett, B. (1972) Endocrinology 90, 645.
161. Rommerts, F.F.G., van Doorn, L.G., Galjaard, H., Cooke, B.A. and van der Molen, H.J. (1973) J. Histochem. Cytochem. 21, 572.
162. Mijbaum, W. (1939) Z. Physiol. Chem. 258, 117.
163. Harris, M.E. and Bartke, A. (1974) Endocrinology 95, 701.

164. Kotoh, K., Huseby, R.A., Baldi, A. and Samuels, L.T. (1973) Cancer Res. 33, 1247.
165. Reddy, P.R.K. and Villee, C.A. (1975) Biochem. Biophys. Res. Commun. 63, 1063.
166. Chiu, J.F., Thomson, J. and Hnilica, L.S. (1976) Biochim. Biophys. Acta 435, 1.
167. Means, A.R. (1971) Endocrinology 89, 981.
168. Tcholakian, R.K., Chowdhury, M. and Steinberger, E. (1974) J. Endocr. 63, 411.
169. Sowell, J.G., Folman, Y. and Eik-Nes, K.B. (1974) Endocrinology 94, 346.
170. Podesta, E.J. and Rivarola, M.A. (1974) Endocrinology 95, 455.
171. Ficher, M. and Steinberger, E. (1971) Acta Endocr. 68, 285.
172. Steinberger, E. and Ficher, M. (1971) Endocrinology 89, 679.
173. Wiebe, J.P. (1976) Endocrinology 98, 505.
174. Döhler, K.D. and Wuttke, W. (1975) Endocrinology 97, 898.
175. Odell, W.D., Swerdloff, R.S., Jacobs, H.S. and Hescox, M.A. (1973) Endocrinology 92, 160.
176. Odell, W.D. and Swerdloff, R.S. (1975) J. Steroid Biochem. 6, 853.
177. Hinegardner, R.T. (1971) Analyt. Biochem. 39, 197.
178. Nayfeh, S.N., Coffey, J.C., Kotite, N.J. and French, F.S. (1975) in Hormonal Regulation of Spermatogenesis (French, F.S., Hansson, V., Ritzen, E.M. and Nayfeh, S.N., eds.), Plenum Press, New York and London, p. 53.
179. Danutra, V., Harper, M.E. and Griffiths, K. (1973) J. Endocr. 59, 539.
180. Jenkins, J.S. and McCaffery, V.M. (1974) J. Endocr. 63, 517.
181. Samuels, L.T. and Helmreich, M.L. (1956) Endocrinology 58, 435.

182. Hsueh, A.J.W., Dufau, M.L., Katz, S.I. and Catt, K.J. (1976) Nature 261, 710.
183. Frowein, J. and Engel, W. (1975) J. Endocr. 64, 59.
184. Thanki, K.H. and Steinberger, A. (1976) Endocr. Res. Commun. 3, 49.
185. Chen, Y.D.I., Payne, A.H. and Kelch, R.P. (1977) Proc. Soc. Exp. Biol. Med., in press.
186. Meistrich, M.L., Hughes, T.J. and Bruce, W.R. (1975) Nature 358, 145.
187. Bashirelahi, N., McRoberts, P. and Villee, C.A. (1975) Biochem. Med. 12, 194.
188. Widman, J.J. and Dariush Fahimi, H. (1976) Lab. Inv. 34, 141.
189. Nickerson, P.A. (1975) Path. Europ. 10, 287.
190. Gugten, A.A. v.d., Sala, M. and Kwa, H.G. (1970) Acta Endocr. 64, 265.
191. Swerdloff, R.S., Grover, P.K., Jacobs, H.S. and Bain, J. (1973) Steroids 21, 703.
192. Johnson, B.H. and Ewing, L.L. (1971) Science 173, 635.
193. El Safoury, S. and Bartke, A. (1974) J. Endocr. 61, 193.
194. Hafiez, A.A., Lloyd, C.W. and Bartke, A. (1972) J. Endocr. 52, 327.
195. Bartke, A. and Dalterio, S. (1976) Biol. Reprod. 15, 90.
196. Hitzeman, S.J.W. (1971) Exp. Zool. 178, 369.
197. Blackshow, A.W. and Elkington, J.S.H. (1970) J. Reprod. Fert. 22, 69.
198. Molen, H.J. van der, Grootegoed, J.A., de Greef-Bijleveld, M.J., Rommerts, F.F.G. and van der Vusse, G.J. (1975) in Hormonal Regulation of Spermatogenesis (French, F.S., Hansson, V., Ritzen, E.M. and Nayfeh, S.N., eds.), Plenum Press, New York and London, p. 3.
199. Engel, W. and Frowein, J. (1974) Nature 251, 146.
200. Negro-Vilar, A., Krulich, L. and McCann, S.M. (1973) Endocrinology 93, 660.

201. Gupta, D., Rager, K., Zarzycki, J. and Eichner, M. (1975) J. Endocr. 66, 183.
202. Swerdloff, R.S., Walsh, P.C., Jacobs, H.S. and Odell, W.D. (1971) Endocrinology 88, 120.
203. Odell, W.D. and Swerdloff, R.S. (1974) in The Control of the Onset of Puberty (Grumbach, M.M., Grave, G.D. and Mayer, F.E., eds.), John Wiley, New York, p. 313.
204. Miyachi, Y., Nieschlag, E. and Lipsett, M. (1973) Endocrinology 92, 1.
205. Payne, A.H., Kelch, R.P., Murono, E.P. and Kerlan, J.T. (1977) J. Endocr. 72, 17.
206. Pahnke, V.G., Leidenberger, F.A. and Künzig, H.J. (1975) Acta Endocr. 79, 610.
207. Knorr, D.W., Vanha-Perttula, T. and Lipsett, M.B. (1970) Endocrinology 86, 1298.
208. Armstrong, D.T., Moon, Y.S., Fritz, I.B. and Dorrington, J.H. (1975) in Hormonal Regulation of Spermatogenesis (French, F.S., Hansson, V., Ritzen, E.M. and Nayfeh, S.N., eds.), Plenum Press, New York and London, p. 85.

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Wilma van Beurden.

Curriculum vitae

Op 7 februari 1947 ben ik geboren in Nijmegen. Nadat ik in 1966 het getuigschrift gymnasium-β aan het Lyceum Mater Dei te Nijmegen heb behaald, ben ik in datzelfde jaar de studie biologie aan de Katholieke Universiteit te Nijmegen begonnen. In 1969 behaalde ik het kandidaatsexamen en in 1972 het doctoraalexamen met als hoofdvak chemische cytologie en als bijvakken humane genetica en dierfysiologie.

Vanaf 1972 ben ik als wetenschappelijk medewerkster verbonden aan de afdeling Chemische Endocrinologie (Instituut Biochemie II) van de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam.

List of abbreviations

ABP	- androgen binding protein
cAMP	- cyclic adenosine-3':5'-monophosphoric acid
DHT	- dihydrotestosterone
DNA	- deoxyribonucleic acid
dpm	- disintegrations per minute
E_2	- oestradiol
E_2B	- oestradiol-3-benzoate
HCG	- human chorionic gonadotropin (human chorio-gonadotropin)
HnRNA	- heterogeneous nuclear RNA
hypox	- hypophysectomized
FSH	- follicle stimulating hormone (follitropin)
IU	- international unit
IP	- induced protein
LH	- luteinizing hormone (lutropin)
M	- moles per litre
MIX	- 3 isobutyl-1-methylxanthine
mRNA	- messenger RNA
n	- number of determinations
PCA	- perchloric acid
RNA	- ribonucleic acid
rRNA	- ribosomal RNA
S	- Svedberg unit
S.E.M.	- standard error of the mean
tRNA	- transfer RNA
UTP	- uridine 5'-triphosphate

List of trivial names and enzymes

Trivial names used in this work	Systematic names
aldosterone	- 4-pregnen-11β,21-diol-3,18-trione
diethylstilboestrol	- 3,4-bis(4-hydroxyphenyl-3-hexane)
dihydrotestosterone	- 17β-hydroxy-5α-androstan-3-one
oestradiol	- 1,3,5(10)-oestratriene-3,17β-diol
oestradiol-17α	- 1,3,5(10)-oestratriene-3,17α-diol
oestriol	- 1,3,5(10)-oestratriene-3,16α,17β-triol
oestrone	- 1,3,5(10)-oestratrien -3-ol-17-one
progesterone	- 4-pregnene-3,20-dione
testosterone	- 17β-hydroxy-4-androsten-3-one
glucose-6-phosphate dehydrogenase	- D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase (EC 1.1.1.49)
cholesterol side-chain cleaving enzyme	- cytochrome P-450 containing enzyme complex catalyzing the conversion of cholesterol to pregnenolone and isocaproic acid (NADPH dependent)
3β-hydroxysteroid dehydrogenase	- 3(or 17)β-hydroxysteroid: $NAD(P)^+$ oxidoreductase (EC 1.1.1.51) or 3β-hydroxy-Δ^5-steroid:NAD^+ 3-oxidoreductase
protein kinase	- ATP:protein phosphotransferase (EC 2.7.1.37)
3β-ol dehydrogenase	- 3β-hydroxysteroid dehydrogenase

5α-reductase	- 5α-steroid:NAD(P)$^{+}$ Δ^{4} oxidoreductase (EC 1.3.1)
RNA polymerase	- nucleosidetriphosphate:RNA nucleotidyltransferase (EC 2.7.7.6)

Appendix papers

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High-Affinity Binding of Oestradiol-17β by Cytosols from Testis Interstitial Tissue, Pituitary, Adrenal, Liver and Accessory Sex Glands of the Male Rat

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The specificity of the binding of oestradiol-17β by cytoplasmic fractions of several tissues of the male rat was investigated. 1. Agar-gel electrophoresis, Sephadex chromatography, adsorption by dextran-coated charcoal and sucrose-gradient centrifugation were used to estimate the binding capacity and specificity. The four different methods all gave similar results for the capacity of the specific oestradiol-17β-binding macromolecules in the testis. 2. The presence of a specific saturable binding protein with a sedimentation coefficient of 8S was demonstrated in liver, adrenal, pituitary, prostate, epididymis and testis interstitial tissue. The highest concentration of oestradiol-17β-binding macromolecules was found in testis interstitial tissue (0.12pmol/mg of protein) and in the pituitary (0.075pmol/mg of protein). 3. The oestradiol-17β receptor in the testis cytosol showed the characteristics of a protein with respect to Pronase treatment and temperature sensitivity. In competition experiments with different steroids the receptor showed a high affinity for oestradiol-17β, a moderate affinity for diethylstilboestrol and oestradiol-17α and a low affinity for oestrone, oestriol, testosterone and 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one). 4. The wide distribution of oestradiol-17β receptors in the male rat is in apparent contradiction to the current concept of the specificity of steroid-hormone action. Further research is required to investigate a possible physiological meaning of the presence of specific receptors in the different tissues.

The action of sex steroid hormones on target tissues may be mediated through specific receptors. The presence of an oestradiol-17β receptor has previously been demonstrated in the interstitial tissue of the rat testis (Brinkmann *et al.*, 1972). A nuclear form of receptor has also been found and it appears that the cytoplasmic receptor can be transferred to the nuclei of testis interstitial tissue (Mulder *et al.*, 1973).

Baulieu *et al.* (1971) formulated the criteria for a true steroid receptor, i.e. a receptor should show high affinity, saturability, steroid specificity and tissue specificity. It was previously shown that the cytoplasmic oestradiol-17β receptor in testis interstitial tissue has a high affinity for oestradiol-17β (K_a is 10^{10}M^{-1}) and that only a limited number of binding sites are present (Brinkmann *et al.*, 1972). In the present paper we present the results of an investigation on tissue specificity and steroid specificity of cytoplasmic oestradiol-17β binding.

Materials and Methods

Materials

[2,4,6,7-^{3}H]Oestradiol-17β (specific radioactivity 105Ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). The radiochemical purity was verified by paper chromatography and t.l.c.

Deoxyribonuclease, ribonuclease and Pronase were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Preparation of subcellular fraction and incubation procedures

Male Wistar rats (200–250g) were killed by decapitation. The isolated tissues were homogenized in 1vol. of 10mM-Tris–HCl buffer, pH7.4, at 0°C with three strokes of a Potter–Elvehjem homogenizer at 1100rev./min. The homogenate was centrifuged at 105000*g* for 60min at 0°C. The 105000*g* supernatant (cytosol) was incubated with steroid for 2h at 0°C.

Isolated interstitial tissue and seminiferous tubules were obtained by wet dissection of decapsulated whole testis tissue (Christensen & Mason, 1965).

Measurement of steroid binding

When cytosol is incubated with oestradiol-17β the steroid may bind to specific and non-specific

binding proteins. The cytosol was incubated with [^{3}H]oestradiol-17β to determine total binding and with [^{3}H]oestradiol-17β plus a 100-fold excess of unlabelled oestradiol-17β to determine non-specific binding (Williams & Gorski, 1973). The quantity of specifically bound hormone was calculated by subtracting the value for [^{3}H]oestradiol-17β bound in the presence of a 100-fold excess of unlabelled oestradiol-17β (non-specifically bound oestradiol-17β) from the value for total [^{3}H]oestradiol-17β binding.

After incubation of cytosol with steroids bound and unbound steroid were separated by one of the following techniques. (In control experiments buffer with labelled steroid was used instead of cytosol.)

(*a*) *Gradient centrifugation.* After incubation with steroid 200μl of cytosol was layered on 5ml of a 5–20% (w/v) sucrose gradient prepared in 10mM-Tris–HCl buffer, pH7.4. After centrifugation in a Beckman L2-65B centrifuge at 0°C for 16h at 150000$g_{av.}$ in a SW65 rotor the bottom of the tube was pierced and 30 fractions were collected. Radioactivity was measured in each fraction.

(*b*) *Dextran-coated charcoal adsorption method.* A 0.25% charcoal suspension (200μl), containing 0.025% dextran, was added to 100μl of cytosol after incubation with steroid. After mixing the suspension was kept at 0°C for 15min. The samples were then centrifuged for 10min at 1200g to separate bound from free steroid. A 200μl portion of the supernatant was taken for measurement of radioactivity in the bound-steroid fraction.

(*c*) *Agar-gel electrophoresis.* Agar-gel electrophoresis was performed essentially as described by Wagner 1972). A 50μl portion of incubated cytosol was layered on an agar plate (100mm×85mm×5mm thick) kept at 0°C (agar Noble; Difco, Detroit, Mich., U.S.A.). It was possible to apply ten samples on one plate. After electrophoresis for 90min at 130mA per plate (200–250V) at 0°C, the plate was cut into ten strips, each containing one sample, and each strip was divided in 20 fractions of 4mm. For counting of radioactivity, steroid from the individual agar fractions was dissolved by shaking for 12h at room temperature in 10ml of Triton-containing scintillation fluid (see under 'Measurement of radioactivity').

(*d*) *Sephadex chromatography.* Sephadex chromatography was performed as described by Williams & Gorski (1973). A 50μl portion of incubated cytosol was layered on a column (8cm×0.6cm) of Sephadex G-25 (superfine grade). The column was eluted with 10mM-Tris–HCl buffer, pH7.4, at 0°C and the excluded volume (bound radioactivity) was collected in a vial and radioactivity was measured.

Pretreatment with charcoal

When indicated excess of unbound steroid was removed by adding 0.5mg of dextran-coated charcoal to 200μl of incubated cytosol. After mixing, the suspensions were incubated for 15min at 0°C. Charcoal was removed by centrifugation for 10min at 1200g.

Protein determination

The protein content of the isolated cytosols was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Generally the cytosols contained 20–25mg of protein/ml. Cytosols of interstitial tissue, pituitary, hypothalamus and uterus, however, contained 4–10mg/ml of cytosol.

DNA determination

DNA content was measured as described by Giles & Myers (1965).

Measurement of radioactivity

Radioactivity was measured in a Packard model 3375 liquid-scintillation spectrometer. The scintillation fluid consisted of a mixture of Triton X-100 (Rohm and Haas, Philadelphia, Pa., U.S.A.) and toluene (1:2, v/v) containing 0.1g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/l and 4.8g of PPO (2,5-diphenyloxazole)/l (Packard Instrument S.A. Benelux, Brussels, Belgium).

Results

Properties of the oestradiol-17β receptor in the testis cytosol

Steroid specificity of the oestradiol-17β receptor in the testis. Relative affinities of a number of steroids for the oestradiol-17β receptor in the testis are presented in Fig. 1. These experiments were carried out by

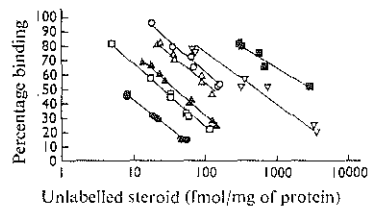

Fig. 1. *Binding affinity of various steroids for the testis cytosol oestradiol-17β receptor*

Testis cytosol was incubated with 0.07nM-[^{3}H]oestradiol-17β and increasing amounts of unlabelled steroids. After incubation (2h) the percentage binding was measured by the charcoal technique. ●, Oestradiol-17β; □, diethylstilboestrol; ▲, oestradiol-17α; △, oestrone; ○, oestriol; ▽, 5α-dihydrotestosterone; ■, testosterone.

Table 1. *Comparative binding affinity of various steroids for the testis cytosol oestradiol-17β receptor*

Testis cytosol was incubated with 0.07 nM-[^{3}H]oestradiol-17β and increasing amounts of unlabelled steroids. Percentage binding was measured by the charcoal technique. Comparative binding affinity was calculated as the amount of steroid that will decrease the initial percentage binding of the labelled oestradiol-17β to 50%.

Steroid	Comparative binding affinities (fmol/mg of protein)
Oestradiol-17β	6.3
Diethylstilboestrol	25
Oestradiol-17α	38
Oestrone	127
Oestriol	190
Dihydrotestosterone	500
Testosterone	3000

incubating 200μl of cytosol with 3500 d.p.m. (0.07 nM of [^{3}H]oestradiol-17β and increasing amounts of the different unlabelled steroids. The percentage binding was measured by using the charcoal technique. In the competition experiments with testosterone and dihydrotestosterone it was necessary to decrease the concentration of endogenous steroids by hypophysectomy of the animals 8 days before the experiment. Comparative binding affinities were determined by the method of Korenman (1969). The results are given in Table 1. The highest competition for the binding sites was achieved with oestradiol-17β, diethylstilboestrol and oestradiol-17α. The decrease in percentage binding after incubation with increasing amounts of unlabelled oestradiol-17β was the same whether the testis cytosol was prepared from normal or hypophysectomized animals.

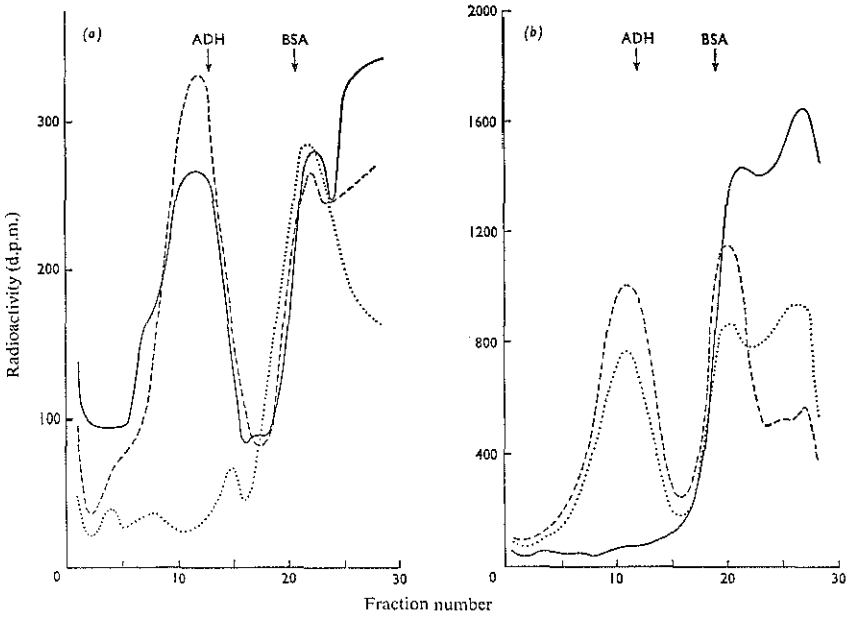

Fig. 2. *Effect of enzyme digestion and preincubation at different temperatures on oestradiol-17β binding by cytosol of rat testis*

(*a*) Testis cytosol was preincubated with 2μg of Pronase, 1 mg of deoxyribonuclease or ribonuclease for 30 min at 12°C. After 2 h incubation with 0.08 nM-[^{3}H]oestradiol-17β a 200μl portion was layered on a sucrose gradient. Alcohol dehydrogenase (ADH, 7.4S) and bovine serum albumin (BSA, 4.6S) were used as sedimentation markers. —— Ribonuclease treatment; – – – –, deoxyribonuclease treatment; · · · ·, Pronase treatment. (*b*) Sucrose-gradient analysis was performed after labelling *in vitro* of testicular cytosols preincubated at 0°C, 30°C and 37°C for 30 min. ——, Preincubation at 37°C; · · · ·, preincubation at 30°C; – – – –, preincubation at 0°C.

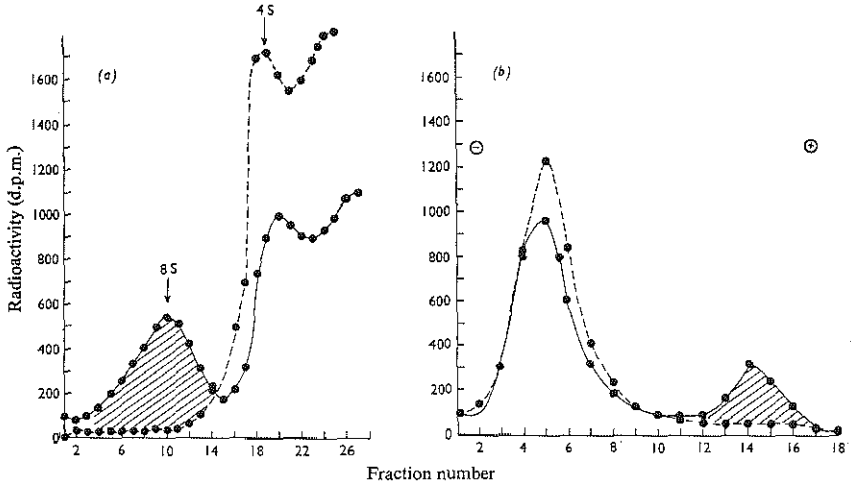

Fig. 3. *Gradient centrifugation and agar-gel electrophoresis of testis cytosol labelled with [^{3}H]oestradiol-17β*

Testicular cytosol was incubated with 0.4nM-[^{3}H]oestradiol-17β (●——●) or with 0.4nM-[^{3}H]oestradiol-17β plus 0.04μM-unlabelled oestradiol-17β (●- - - -●). After incubation binding was determined by gradient centrifugation (*a*) or agar-gel electrophoresis (*b*). Shading indicates specific binding. Alcohol dehydrogenase and bovine serum albumin were run in a parallel gradient to determine the sedimentation values.

Protein character of the oestradiol-17β receptor in the testis. Testis cytosol (1.0ml) was preincubated with Pronase, deoxyribonuclease or ribonuclease. It was shown by gradient centrifugation that the 8S receptor was absent only after Pronase treatment, as shown in Fig. 2(*a*). Preincubation of cytosols at 30° and 37°C affected the amount of receptor-bound steroid. After preincubation at 30°C a decrease in steroid bound to 8S receptor was observed and after preincubation at 37°C no 8S steroid–receptor complex was found (Fig. 2*b*).

Comparison of different techniques for quantitative analysis of hormone binding

In four series of experiments cytosol of total testis tissue was incubated with different amounts of labelled oestradiol-17β ranging from 0.3 to 3.9nM. Analysis of oestradiol-17β binding by agar-gel electrophoresis and gradient centrifugation is illustrated in Fig. 3. Non-specific and specific binding were calculated as described in the Materials and Methods section.

The results are presented in Table 2. When the results for each concentration were compared by the rank-correlation test of Wilcoxon (1945) it appeared that the values for non-specific binding calculated from the gradient-centrifugation curves were significantly lower ($P = 0.05$) than the values obtained with the other techniques. Highest values for non-specific binding were calculated from the results obtained with the charcoal techniques ($P < 0.05$).

The apparent increase in non-specific binding with increasing amounts of oestradiol-17β was not caused by an incomplete separation of free and bound oestradiol-17β. When 4nM-[^{3}H]oestradiol-17β was incubated with buffer only, none of the four techniques showed the presence of radioactivity in the fractions which normally contain the macromolecular bound oestradiol-17β. A comparison by using Wilcoxon's (1945) test for the values of the amount of specifically bound oestradiol-17β showed no significant difference between the results obtained with the four different techniques. This may reflect that after sucrose-gradient centrifugation the specifically bound oestradiol-17β is completely present in the 8S area, which was used for estimation of specific binding. In all further experiments agar-gel electrophoresis and Sephadex chromatography were used for quantitative analysis of specific binding. Sucrose-gradient analysis was not used because in tissues other than testis, specific binding proteins might

Table 2. *Analysis by different techniques of oestradiol-17β binding by testis cytosol*

Testis cytosols from different rats were incubated in four series of experiments with increasing amounts of labelled oestradiol-17β (0.3–3.9 nM). Non-specific binding and specific binding were determined by using agar-gel electrophoresis, the charcoal technique, Sephadex chromatography and sucrose-gradient centrifugation. Determinations of non-specific and specific binding after incubation with [^{3}H]oestradiol-17β are presented.

Expt. no.	Concn. of [^{3}H]-oestradiol (nM)	Non-specific binding (fmol/mg of protein)				Specific binding (fmol/mg of protein)			
		Agar	Charcoal	Sephadex	Gradient	Agar	Charcoal	Sephadex	Gradient
I	1.4	1.8	5.0	1.9	—	6.4	8.0	6.0	—
	3.8	5.0	11.0	2.9	—	7.7	7.8	9.0	—
II	0.3	1.2	0.9	0.7	0.6	2.9	2.9	2.9	4.8
	1.6	3.5	3.7	2.1	1.4	6.2	5.4	5.6	6.3
	3.9	4.5	10.0	3.0	2.1	8.4	7.0	7.4	5.7
III	0.4	1.7	1.6	0.8	1.3	6.1	6.9	5.7	5.8
	1.5	3.7	4.7	3.5	1.5	12.2	14.3	15.2	14.2
	3.0	6.6	10.0	9.0	—	12.4	13.7	11.0	—
IV	2.2	4.3	15.1	—	3.9	9.3	10.2	—	8.3

Table 3. *Specific binding of oestradiol-17β by different tissues of the male rat*

Cytosols of different tissues of the male rat were incubated with 4 nM-[^{3}H]oestradiol-17β and with 4 nM-labelled plus 0.4 μM-unlabelled oestradiol-17β. Specific binding was determined by both agar-gel electrophoresis and Sephadex chromatography. Specific binding is expressed as fmol/mg of protein of the cytosol and as fmol/mg of DNA of the homogenate. Each value is the mean of at least two determinations. The presence or absence of a binding protein with a sedimentation value of 8S is indicated with a plus or minus mark. For the sake of comparison the value of the specific binding in female rat uterus is also included.

Tissue	Specific binding (fmol/mg of protein)			Specific binding (fmol/mg of DNA)			8S
	Agar gel		Sephadex	Agar gel		Sephadex	
Liver	2.3	–	2.1	32.2	–	29.4	+
Kidney	1.4	–	20.0	6.6	–	94.0	–
Adrenal	22.2	–	—	121.3	–	—	+
Plasma	<0.5	–	<0.5	<2.5	–	<2.5	–
Skeletal muscle	<0.5	–	<0.5	<2.5	–	<2.5	–
Pituitary	75.6	–	75.6	68.7	–	68.7	+
Hypothalamus	2.6	–	1.5	13.8	–	8.0	–
Prostate*	11.4	–	8.5	69.1	–	51.5	+
Epididymis	8.7	–	7.9	46.1	–	41.9	+
Seminal vesicle	0.1	–	0.7	0.7	–	4.9	–
Total testis tissue	9.8	–	9.6	35.2	–	34.6	+
Seminiferous tubules	<0.5	–	<0.5	<2.5	–	<2.5	–
Testis interstitial tissue	140	–	100	518	–	370	+
Uterus	240	–	—	—	–	—	+

* The 4S peak also contained specifically bound oestradiol-17β.

occur in the 4S area of the gradients and the separation between bound steroid in the 4S area and free steroid on top of the gradient is less accurate. Also the gradient centrifugation is more time-consuming. With the charcoal technique the high percentage of non-specific binding makes the estimation of specific binding less accurate, particularly in tissues containing a low amount of specific binding proteins.

Occurrence of oestradiol-17β receptors in different tissues of the male rat

Cytosols of 13 different tissues of the male rat were incubated with 4 nM-[^{3}H]oestradiol-17β. Specific binding was determined by using both agar-gel electrophoresis and Sephadex chromatography. The presence of an 8S receptor in each tissue was deter-

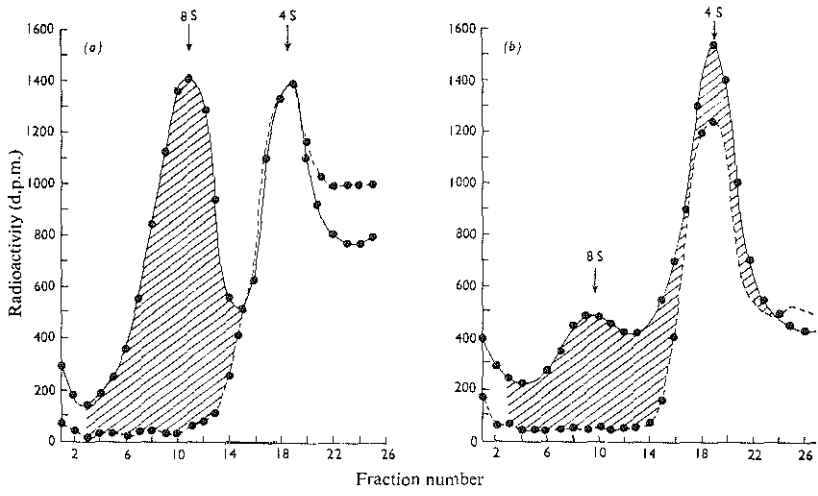

Fig. 4. *Sucrose-gradient analysis of oestradiol-17β binding in cytosol of testis and prostate*

Cytosols of testis (*a*) and prostate (*b*) were incubated with 2 nM-[^{3}H]oestradiol-17β (●——●) or with 2 nM-labelled plus 0.2 μM-unlabelled oestradiol-17β (●– – –●). After charcoal pretreatment specific binding was determined by gradient centrifugation. Shading indicates specific binding. Sedimentation values have been determined as described in Fig. 2.

mined by sucrose-gradient centrifugation. The results are given in Table 3. The uterus of the female rat was used as a control tissue. All values are the mean of at least two determinations. The results show that a specific binding protein with sedimentation coefficient of 8S was present in liver, adrenal, pituitary, testis interstitial tissue, prostate and epididymis. Such a protein, however, could not be found in plasma, muscle, hypothalamus, seminal vesicle and seminiferous tubules. The amount of specific binding in the uterus was 0.24pmol/mg of protein. This is in agreement with the values obtained by other authors (Truong & Baulieu, 1971; Toft *et al.*, 1967). The largest amount of oestradiol-17β-binding macromolecules in the male rat was found in testis interstitial tissue and in the pituitary. In the experiments with prostate the value for specific binding of oestradiol-17β to receptors in the 8S area obtained after gradient centrifugation was lower than the specific binding determined by Sephadex chromatography. Gradient centrifugation after addition of excess of unlabelled oestradiol-17β to prostate cytosol showed, however, the presence of specific low-capacity oestradiol-17β binding in the 4S area (Fig. 4). In this respect specific receptors for oestradiol-17β in testis cytosol, which occurred only in the 8S area, may be different from receptors in prostate cytosol, which occurred in both the 8S and 4S areas.

In the kidney specific binding was demonstrated by using Sephadex chromatography and the charcoal technique. After gradient centrifugation it appeared that the specific binding was completely located in the 4S peak. However, it was not possible to find any specific binding in the kidney by using agar-gel electrophoresis. A possible explanation could be that there is a labile saturable protein with a 4S sedimentation coefficient.

Discussion

Various methods can be used for the quantitative measurement of the amount of steroid specifically bound by macromolecules. In the present study the binding of oestradiol-17β by the cytoplasmic receptor in interstitial cells of the testis was analysed by using agar-gel electrophoresis, sucrose-gradient centrifugation, Sephadex chromatography and a charcoal-binding assay. The amounts of receptor-bound steroid found by these four methods did not differ significantly from each other. Jungblut *et al.* (1972) observed a similar agreement between agar-gel electrophoresis and Sephadex chromatography for the analysis of oestradiol-17β binding by cytosols of uterine tissue. However, with gradient centrifugation Jungblut *et al.* (1972) observed a much lower specific binding in uterine tissue. It was established

that all the non-specifically bound steroid dissociates during the agar-gel electrophoresis, whereas in our study some non-specific binding was always present. In uterine tissue the concentration of specific binding proteins is much higher than in testis tissue and therefore small amounts of non-specifically bound steroid might not have been detected. Another reason for the observed difference could be that Jungblut *et al.* (1972) determined specific binding as the difference between the binding before and after heating. In our study the amount of specifically bound steroid was estimated by subtracting the amount of bound steroid after incubation with labelled and unlabelled oestradiol-17β from the amount after incubation of cytosol with labelled hormone only.

The present results, showing some further characteristics of the receptor in the interstitial cells of the testis, established the protein nature and temperature-sensitivity of the oestradiol-17β-binding macromolecule. They were similar to results found for uterus receptor (Toft & Gorski, 1966). Previous studies (Brinkmann *et al.*, 1972) showed that the testis receptor has a high affinity for oestradiol-17β (K_a $10^{10}M^{-1}$). The steroid specificity of the receptor was high; oestriol, oestrone, testosterone and dihydrotestosterone showed a low degree of competition for the binding sites, whereas diethylstilboestrol and oestradiol-17α demonstrated relatively high competitive activities.

In the male rat specific binding of oestradiol-17β appears to occur in several tissues (Table 3). In addition to the testis interstitial tissue specific binding was observed in liver, adrenal, pituitary, prostate and epididymis, but not in seminiferous tubules, plasma, kidney, skeletal muscle, seminal vesicle and hypothalamus.

The occurrence of an oestradiol-17β receptor in the adrenal was not observed in other studies (Jungblut *et al.*, 1967; Stumpf, 1969). Chobanian *et al.* (1968), however, showed a high oestradiol-17β uptake in dog adrenal. In the liver of calf and rat no specific uptake of oestradiol-17β could be demonstrated by Jungblut *et al.* (1967) and Stumpf (1969). Rao & Talwar (1969) on the other hand showed retention of oestradiol-17β in the liver of the female rat, which might suggest the presence of an oestradiol-17β receptor. The existence of an oestradiol-17β receptor in the rat epididymis has not previously been reported. The presence of an androgen receptor in the epididymis has been reported (Blaquier & Calandra, 1973), so that the epididymis could be another example of the simultaneous occurrence of different oestrogen and androgen receptors in a single tissue. Jungblut *et al.* (1971) have already demonstrated very clearly the existence of individual androgen and oestrogen receptors in calf prostate and seminal vesicle. We also observed an 8S oestradiol-17β receptor in the prostate. However, in the seminal vesicle we were not able to find any specific oestradiol-17β binding. In the kidney indications were obtained for the existence of a labile saturable protein with a sedimentation coefficient of 4S, that dissociates during agar-gel electrophoresis.

The occurrence of an 8S oestradiol-17β receptor in the pituitary of the female rat is well known (Vertes & King, 1971; Eisenfeld, 1970; Mowles *et al.*, 1971). In the male rat Clark *et al.* (1972) demonstrated a nuclear oestradiol-17β receptor in the pituitary but not in the hypothalamus. This is in agreement with the results of our present study. Oestradiol-17β can be bound by cytosol of the hypothalamus of the ovariectomized female rat (Vertes & King, 1971; Eisenfeld, 1970; Mowles *et al.*, 1971). Oestradiol-17β uptake by the hypothalamus of the castrated male rat was established by radioautography (Attramadal, 1970). Injections of testosterone (Vertes & King, 1971; Tuohimaa & Johansson, 1971) lower the oestradiol-17β binding in the hypothalamus of the female rat. Therefore the endogenous high concentrations of androgens in the male rat could be a possible explanation for the fact that it was impossible to demonstrate a receptor in the hypothalamus. Another explanation might be that the presence of the aromatizing system (Massa *et al.*, 1972) in the hypothalamus of the male rat causes a higher endogenous oestradiol-17β concentration that could mask the presence of an oestradiol-17β receptor.

The physiological meaning of the oestradiol-17β receptors in the different tissues of the male rat is not yet clear. It has been suggested that the presence of specific steroid-binding macromolecules in the cytoplasm is a prerequisite for steroid-hormone action (Jensen & de Sombre, 1972). To what extent the occurrence of a receptor in these different tissues implies a steroid-induced transformation of the cytoplasmic receptor and a transfer of steroid to specific receptor sites on the chromatin in the nucleus remains to be investigated.

Oestradiol-17β is present in the male rat. The concentration in testis tissue is 44.6pg/g of testis (de Jong *et al.*, 1974). It is not known whether this concentration is sufficiently high to initiate any biological action, nor is anything known about the concentration of oestradiol-17β in the other tissues containing specific receptors for this steroid.

Under certain conditions there might be a co-operative effect of several steroids on a target tissue. Palmiter & Haines (1973) have reported the effect of oestradiol-17β, progesterone and dihydrotestosterone and their receptors on protein synthesis in one cell type of the chick oviduct. Apparently all three steroids need to be present for maximal stimulation of the synthesis of some proteins in the oviduct. Hence the possibility may be considered that the oestradiol-17β receptors observed in different tissues of the male rat might have a similar positive or

negative co-operative effect with androgen receptors on induced protein synthesis.

References

Attramadal, A. (1970) *Z. Zellforsch. Mikrosk. Anat.* **104**, 572–581

Baulieu, E.-E., Alberga, A., Jung, I., Lebeau, M. C., Mercier-Bodard, C., Milgrom, E., Raynaud, J. P., Raynaud-Jammet, C., Rochefort, H., Truong, H. & Robel, P. (1971) *Recent Progr. Horm. Res.* **27**, 345–419

Blaquier, J. A. & Calandra, R. S. (1973) *Endocrinology* **93**, 51–60

Brinkmann, A. O., Mulder, E., Lamers-Stahlhofen, G. J. M., Mechielsen, M. J. & van der Molen, H. J. (1972) *FEBS Lett.* **26**, 301–305

Chobanian, A. V., Brecher, P. I., Lillie, R. D. & Wotiz, H. W. (1968) *J. Lipid Res.* **9**, 701–706

Christensen, A. K. & Mason, N. R. (1965) *Endocrinology* **76**, 646–656

Clark, J. H., Campbell, P. S. & Peck, E. J. (1972) *Neuroendocrinology* **77**, 218–228

de Jong, F. H., Hey, A. H. & van der Molen, H. J. (1974) *J. Endocrinol.* in the press

Eisenfeld, A. J. (1970) *Endocrinology* **86**, 1313–1318

Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93

Jensen, E. V. & de Sombre, E. R. (1972) *Annu. Rev. Biochem.* **41**, 203–230

Jungblut, P. W., Hätzel, I., deSombre, E. R. & Jensen, E. V. (1967) in *Wirkungsmechanismen der Hormone* (Karlson, P., ed.), pp. 58–86, Springer-Verlag, Berlin

Jungblut, P. W., Hughes, S. F., Göhrlich, L., Gowers, U. & Wagner, R. K. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1603–1610

Jungblut, P. W., Hughes, S. F., Hughes, A. & Wagner, R. K. (1972) *Acta Endocrinol. (Copenhagen)* **70**, 185–195

Korenman, S. G. (1969) *Steroids* **13**, 163–177

Lowry, O. H., Rosebrough, N. J., Farr, N. J. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Massa, R., Stupnicka, E., Kniewald, Z. & Martini, L. (1972) *J. Steroid Biochem.* **3**, 385–399

Mowles, F. F., Ashkanazy, B., Mix, E. & Sheppard, H. (1971) *Endocrinology* **89**, 484–491

Mulder, E., Brinkmann, A. O., Lamers-Stahlhofen, G. J. M. & van der Molen, H. J. (1973) *FEBS Lett.* **31**, 131–136

Palmiter, R. D. & Haines, M. E. (1973) *J. Biol. Chem.* **248**, 2107–2116

Rao, K. N. & Talwar, G. P. (1969) *Indian J. Biochem.* **6** 71–73

Stumpf, W. E. (1969) *Endocrinology* **85**, 31–37

Toft, D. & Gorski, J. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 1574–1581

Toft, D., Shyamala, G. & Gorski, J. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 1740–1743

Truong, H. & Baulieu, E.-E. (1971) *Biochim. Biophys. Acta* **237**, 167–172

Tuohimaa, P. & Johansson, R. (1971) *Endocrinology* **88**, 1159–1164

Vertes, M. & King, R. J. B. (1971) *J. Endocrinol.* **51**, 271–282

Wagner, R. K. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1235–1245

Wilcoxon, F. (1945) *Biom. Bull.* **1**, 80–83

Williams, D. & Gorski, J. (1973) *Biochemistry* **12**, 297–306

The effect of estrogens on luteinizing hormone plasma levels and on testosterone production in intact and hypophysectomized rats

Endocrinology in press

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Abstract

Intact male rats were injected with 50 µg E_2B (estradiol benzoate) or with 100 µg E_2 (estradiol). Six hours after injection plasma and testicular testosterone levels and production were significantly decreased. Concomitant with this change a lowered LH plasma level could be observed in the estrogen treated animals. Twenty four hours after injection of 50 µg E_2B LH levels, testosterone levels and testicular testosterone production were still reduced, whereas 24 hours after E_2 administration both testosterone and LH levels were raised again. One and 3 hours after injection of 500 ng estradiol, plasma and tissue testosterone levels as well as testicular testosterone production were significantly decreased. However, this low dose of E_2 also caused a decrease in LH plasma levels. In order to investigate whether estrogens would inhibit testicular testosterone synthesis or release by mechanisms other than inhibition of LH secretion, estrogen or vehicle only were injected into hypophysectomized animals given exogenous LH. No effects of estrogens on testosterone levels or production were observed in such animals.

These findings support the view that the observed effect of administered estrogens on testosterone production in rat

testicular tissue reflect primarily extra testicular estrogens actions such as the negative feedback effect on LH secretion.

Introduction

Binding of steroid hormones to cytoplasmic receptor proteins has been indicated as a prerequisite for their biological activity (1). Estradiol-17β (E_2) is produced (2) in the testis and a high concentration of specific E_2 receptors is present in rat testis interstitial tissue (3,4,5), but little is known about the possible physiological function of estradiol in the male rat. Several authors (6,7,8,9) have demonstrated that after daily injection of estrogens to intact rats testosterone levels in plasma and testicular tissue were decreased, while no change in plasma LH levels was observed. In contrast Verjans et al. (10) and de Jong et al. (11) observed reduced LH levels after estrogen injections. Tcholakian et al. (12) showed that a single injection of 50 µg E_2B (estradiol benzoate) resulted already within 3 hours in a decrease of plasma and testicular testosterone levels, while no change in plasma LH levels was observed. This could mean, that although LH inhibition occurs (10,11) inhibition of testicular testosterone secretion might precede LH inhibition (12).

In the present study we have re-examined the effects of a single injection of estrogens on plasma LH levels and on plasma and testicular testosterone levels in intact male rats. The effect of estrogens on testosterone production in hypox rats was also investigated. We found no evidence of effects of estrogens which were not accompanied by effects on LH levels.

Materials and methods

Ovine LH (NIH-LH-S18, 1.03 units/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, Maryland, USA. (1,2,6,7)^{3}H-testosterone was purchased from Radiochemical Centre, Amersham, U.K. and was purified using paper chromatography. Adult male Wistar rats substrain R-Amsterdam (14-16 weeks old, 200-250 g) were used. The animals were injected subcutaneously with estradiol or estradiol benzoate using sesame oil as vehicle. Control animals received 0.1 ml vehicle. Ovine LH was dissolved in polyvinyl pyrrolidone as described by Morishige et al. (13) for prolactin and injected subcutaneously. The animals were killed by decapitation. Blood was collected from the trunk in heparinized glass tubes. Plasma was stored at -20^{o}C until assayed. Testes were removed and chilled in ice-cold 0.25 M sucrose, containing 1 mM EDTA. After removing the tunica albuginea one testis was homogenized in 10 ml 0.25 M sucrose, EDTA (1 mM) using a Potter Elvehjem homogenizer.

Estimation of testosterone and LH

Testosterone was estimated by a radioimmunoassay technique described by Verjans et al. (14). This method is essentially a modification of the method of Furuyama et al. (15). LH was determined by radioimmunoassay using the antibody described by Welschen et al. (16). All assays were performed in duplicate. LH values were expressed as ng NIAMD - rat LH-RP-1 per ml plasma.

Estimation of testosterone production

The production of testosterone from endogenous substrate in testis homogenate was estimated by incubating 0.5 ml homogenate for 30' at 33^{o}C in an O_2/CO_2 (95:5, v/v) atmosphere in 2 ml medium as described by van der Vusse et al. (17). The reaction was stopped by adding 3.5 ml ethylacetate containing 20,000 dpm ^{3}H-testosterone as internal standard. The

reaction mixture was extracted two times with 3.5 ml ethylacetate. Testosterone was measured in the combined fractions. Endogenous steroid production was calculated from the difference in the amount of testosterone present in incubation mixtures after 30 minutes and at zero time.

Protein determination

Protein was measured by the method of Lowry et al. (18) using bovine serum albumin as standard. The protein content of the tissue homogenate was in the order of 10 mg protein per ml.

Measurement of radioactivity

Counting of radioactivity was done in a scintillation fluid prepared by dissolving 80 g naphtalene and 5 g Permablend II in a mixture of 500 ml toluene and 500 ml methoxyethanol.

Statistics

LH levels of estrogen treated animals were compared with LH levels of control animals using the Wilcoxon's test. The significance of the difference in testosterone levels of estrogen treated animals and control animals was determined with the two tailed Student t-test.

Results

The effect of estrogen injection on testosterone synthesis and plasma LH levels of intact rats

To study the effect of estradiol on testosterone synthesis and LH secretion in intact rats three series of experiments were performed.

1. Effect of 50 µg estradiol benzoate

Sixteen animals were injected with 50 µg E_2B and 16 animals received vehicle only to serve as control. Six and 24 hours after injection 8 control rats and 8 E_2B treated rats were killed. Testosterone levels in plasma and in testicular tissue and testosterone production from endogenous precursors in testis homogenate were determined. Results are given in Figure 1.

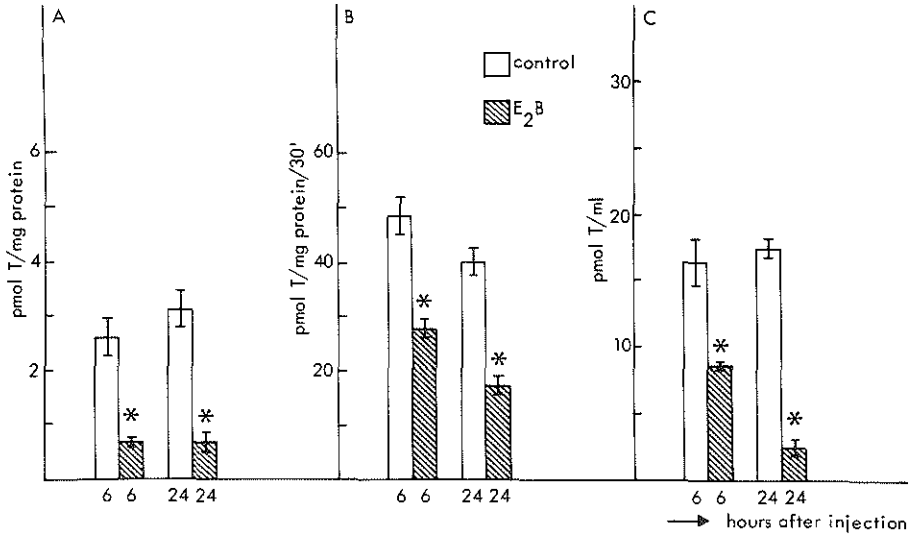

Figure 1 Effect of 50 µg E_2B on testosterone levels in plasma and testicular tissue and on testicular testosterone production.
Intact male rats were injected with 50 µg E_2B. Samples were taken 6 and 24 hours after injection. Values are expressed as means ± S.E.M. (n=8).
A: Testosterone levels in tissue expressed as pmol T/mg protein.
B: Testosterone production in testis homogenate. Data expressed as pmol T/mg protein/30'.
C: Testosterone plasma levels as pmol T/ml plasma.
* significantly different from control ($p \leq 0.01$).

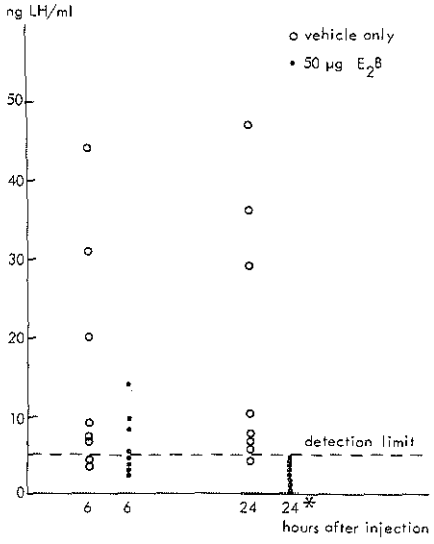

Figure 2 Effect of 50 µg E_2B on plasma LH levels. Intact male rats were injected with 50 µg E_2B. After 6 and 24 hours LH levels in plasma were determined. Data expressed as ng NIAMD-rat-LH-RP-1/ml plasma (individual values).
*significantly different from control group ($p \leq 0.01$).

LH levels in plasma were determined and are represented in Figure 2.

A significant decrease of testosterone levels in tissue and plasma was observed both at 6 and at 24 hours after injection. Testicular testosterone production was also significantly lower 6 and 24 hours after E_2B injection. Plasma LH levels were significantly lower 24 hours after E_2B administration. At 6 hours after E_2B injection the LH levels tended to be lower but this change was not statistically significant.

2. Effect of 100 µg estradiol

In the second experiment 16 rats were injected with 100 µg E_2 (estradiol-17β) and 16 rats were used as controls.

Six and 24 hours after injection testosterone and LH were measured as described in experiment 1. Testosterone concentrations in plasma and testicular tissue and testicular testosterone production are given in Figure 3, plasma LH levels in Figure 4. Six hours after injection of 100 µg estradiol a significant decrease in testosterone levels and production could be observed. Concomitant with the decrease in testosterone synthesis LH levels were also significantly decreased. However, 24 hours after E_2 injection testosterone levels and production were not different from values in control animals, whereas LH levels in the E_2 treated animals were significantly increased.

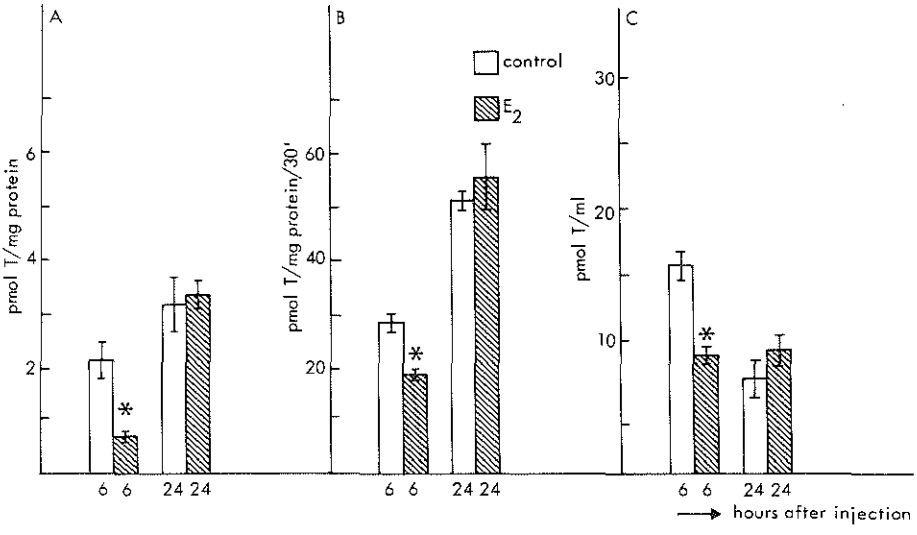

Figure 3 Effect of 100 µg E_2 on testosterone levels in plasma and testicular tissue and on testicular testosterone production.
Intact animals were injected with 100 µg E_2. Testosterone was measured 6 and 24 hours after injection. Values given as means ± S.E.M. (n=8). A,B,C see Figure 1.

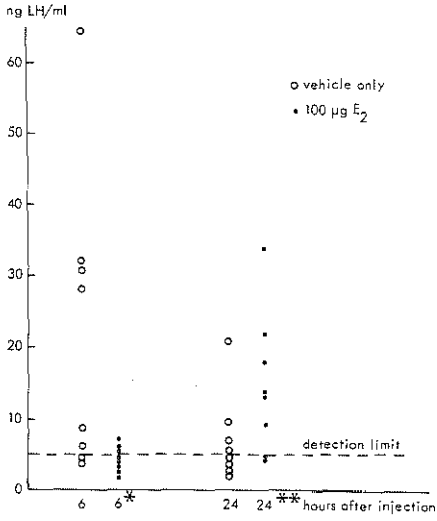

Figure 4 Effect of 100 µg E_2 on plasma LH levels. Intact male rats were injected with 100 µg E_2. Six and 24 hours after injection LH levels in plasma were measured. Data expressed as ng NIAMD-rat-LH-RP-1/ml plasma (individual values).

* $p \leq 0.05$. Significantly lower than control group.

** $p \leq 0.05$. Significantly higher than control group.

3. Effect of 500 ng estradiol

In this experiment 20 rats were used. Ten control rats received 0.1 ml sesame oil only, 10 rats received 500 ng estradiol. One and three hours after injection 5 control rats and 5 estradiol treated rats were killed and testosterone levels and production (Fig. 5) and LH levels (Fig. 6) were estimated.

Testosterone levels and production 1 and 3 hours after E_2 injection were significantly decreased. The LH levels 1 hour after injection were diminished. However, this change was not significant. Three hours after injection the LH levels were significantly lower in E_2 treated animals.

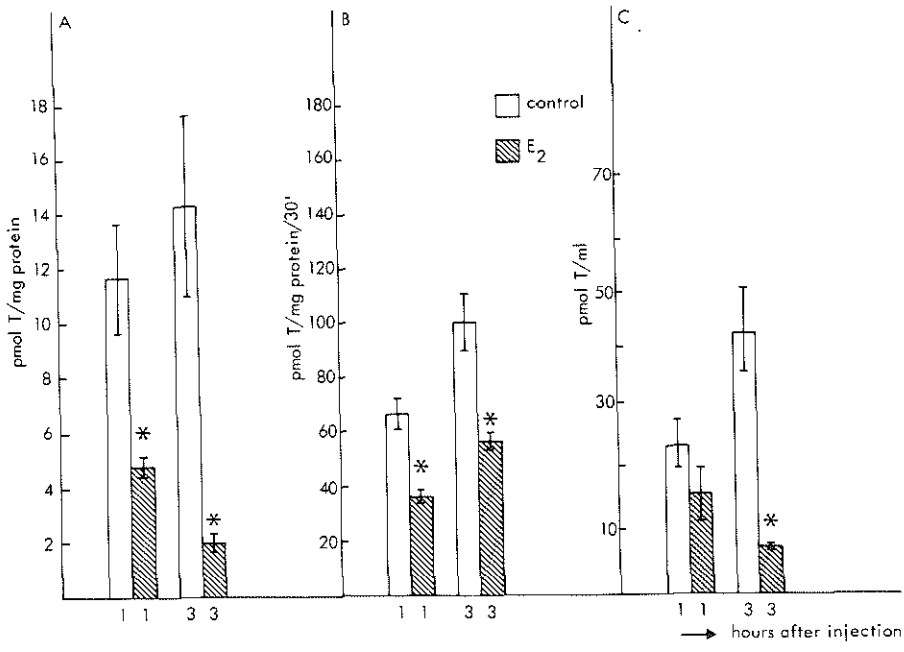

Figure 5 Effect of 500 ng E_2 on testosterone levels in plasma and testicular tissue and on testicular testosterone production.
Intact male rats were injected with 500 ng E_2. Testosterone was measured 1 and 3 hours after injection. Data expressed as means ± S.E.M. (n=5). A,B,C see Figure 1.

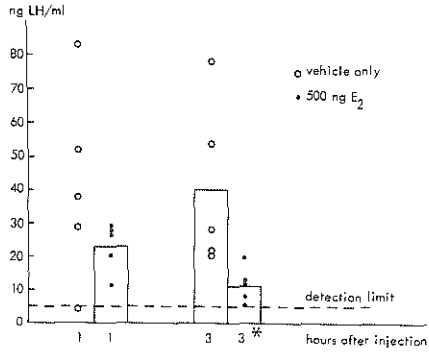

Figure 6 Effect of 500 ng E_2 on LH plasma levels. Rats were injected with 500 ng E_2. LH plasma levels were measured after 1 and 3 hours. Data given as ng NIAMD-rat-LH-RP-1 (individual values).

$^{*}p \leq 0.02$.

Effect of estrogens on testosterone synthesis in hypophysectomized rats

In order to investigate whether estradiol can have a direct effect on testicular testosterone synthesis different from the effect caused by a negative feedback on LH secretion, hypophysectomized animals were used. Two series of experiments were performed.

1. Effect of estrogen 1 day after hypophysectomy

In the first experiment animals were hypophysectomized and immediately injected with 10 µg ovine-LH dissolved in polyvinyl pyrrolidone for maintenance of testosterone synthesis. The LH was dissolved in polyvinyl pyrrolidone in order to obtain a constant LH level (12). After 15 hours the animals received a second injection of LH (10 µg) in polyvinyl pyrrolidone and 2 hours later 8 animals were injected with 50 µg E_2B in sesame oil and 8 animals with vehicle only. Six hours after E_2B administration both groups of animals were killed and plasma and testicular testoste-

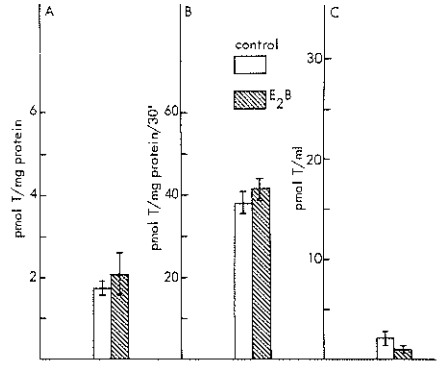

Figure 7 The effect of 50 µg E_2B on testosterone levels in plasma and testicular tissue and on testicular testosterone production in hypophysectomized rats.
One day hypophysectomized animals, injected with LH, were injected with 50 µg E_2B. Six hours after administration of E_2B testosterone values were measured. Values expressed as means ± S.E.M. (n=8). A,B,C see Figure 1.

rone levels and testicular testosterone production were measured.

From the results in Figure 7 it can be concluded that E_2B had no effect on testosterone levels in plasma and testicular tissue and on testosterone production in hypophysectomized rats.

2. Effect of estradiol immediately after hypophysectomy

In the second experiment 14 rats were hypophysectomized and immediately injected with 50 µg LH dissolved in polyvinyl pyrrolidone. One group of animals were injected with 500 ng E_2 and the other animals received vehicle only to serve as control animals. One hour after estradiol injection the animals were killed and testosterone was measured. The results are given in Figure 8. There was no significant effect of estradiol on testosterone synthesis and on testicular and plasma testosterone levels.

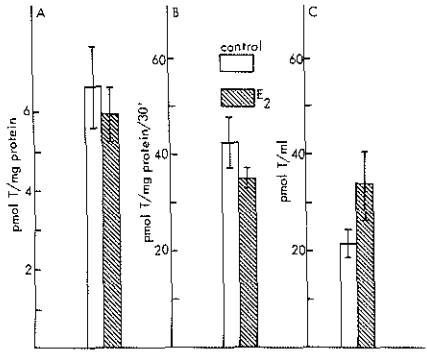

Figure 8 Effect of 500 ng E_2 on testosterone levels in plasma and testicular tissue and on testicular testosterone production in hypophysectomized rats.
Immediately after hypophysectomy rats were injected with 50 µg LH and with 500 ng E_2. One hour after injection testosterone values were measured. Data expressed as means ± S.E.M. (n=7). A,B,C see Figure 1.

Discussion

Our results of the present investigation on estrogen administration in male rats support the generally accepted belief that a reduction of testicular testosterone in intact animals is always accompanied by a decline in circulating LH. This is clearly demonstrated by the experiments with intact rats where injection of 50 µg E_2B or 100 µg E_2 caused a decrease in testosterone synthesis with a concomitant decrease in plasma LH levels in estrogen treated animals. Our results appear to contrast with the findings of Tcholakian et al. (12), who in similar experiments did not observe a diminished LH level after a single injection of 50 µg E_2B. Possible explanations for this discrepancy include differences in strains of rats or in assays. Tcholakian et al. (12) used an antiserum against rat LH and rat LH for iodination - a system designated as 'RR rat LH RIA' by Niswender et al. (19). We have used an 'OR rat LH RIA' in

which an anti-ovine LH was used as described by Welschen et al. (16). Since the sensitivity of the OR rat LH RIA is six times higher than that of the RR rat LH RIA (20), it might be possible that changes in LH levels which could be detected with the OR rat LH RIA system remained unnoticed using the RR rat LH RIA.

One hour after injection of 500 ng oestradiol the translocation of the E_2 receptor from the cytoplasm to the nucleus in rat testis interstitial tissue is completed (21). This implies that with 500 ng E_2 it should be possible to induce a direct effect in rat testicular tissue. One and three hours after administration of 500 ng E_2 a reduced testosterone production was observed. However, this amount of E_2 also caused a significant decrease in LH plasma levels. Several authors have found that daily injection for 5 days of 500 ng E_2 or E_2B did not cause a decrease in plasma LH levels in castrated and intact male rats (8,21,22), but others (24,25) did observe that plasma LH levels were reduced after 5-7 days of daily injection of 500 ng E_2B into castrated male rats. Kalra et al. (26) concluded that a single injection of 500 ng E_2B did not increase LH levels measured 16-24 hours later in castrated male rats. However, Figure 3 shows that different results can be obtained when LH levels are measured at different times after injection. Although injection of 100 µg E_2 reduced plasma LH levels 6 hours later the effect was no longer evident 24 hours after injection, at which time LH levels were even significantly higher than in the controls. These findings may be similar to those of Libertun (27), who demonstrated that administration of 500 ng E_2 to castrated female rats which were continuously perfused with LHRF, caused a decrease in LH levels already one hour after injection whereas 6 hours after injection the LH levels were raised above control levels.

The results of our experiments with intact rats reflect a variability of testosterone values in plasma and testicular tissue. There are several possible explanations for this variability. First there appears to be a seasonal variation

Table 1 Testicular testosterone (T) production and T levels and plasma T levels in different time periods of the year

Month	T level in plasma pmol/ml	testicular T production pmol/mg protein/30'	testicular T level pmol/mg protein
July	13.7 ± 0.9* (32)	45.2 ± 2.2 (32)	2.7 ± 0.2 (32)
October	30.7 ± 4.8 (10)	85.6 ± 8.6 (10)	13.4 ± 2.2 (12)
November	19.5 ± 2.8 (4)	30.1 ± 2.6 (4)	5.9 ± 1.0 (4)

*x ± S.E.M. (n)

of T levels in male rats (28,29). The experiments presented in Figures 1 and 3 were performed in June-July, whereas the experiments presented in Figure 5 were performed in October. The testosterone productions and testosterone levels in testicular tissue and plasma from control animals in the different time periods are given in Table 1. A possible influence of the time after injection might be another explanation. Krulich et al. (30) showed that there are changes in LH levels after injection of saline or after stress situations. To exclude such influences it is necessary always to take a control group which is killed at the same time after injection as the hormone treated group. The variability of testosterone values cannot be explained by differences in the radioimmunoassay system. Each assay series included several samples containing a known amount of testosterone as control values.

In order to investigate the possibility that E_2 might have a direct effect on testosterone synthesis in addition to effects via its negative feedback action on LH secretion, we used hypophysectomized animals. Hypophysectomy has no influence on the amount of E_2 receptor in rat testis interstitial tissue (21). Therefore it should be possible to see a direct effect of E_2 in hypophysectomized animals. For main-

tenance of testosterone synthesis a prolonged constant level of LH was maintained through injection of LH dissolved in polyvinyl pyrrolidone (13). Under these conditions injection of 50 μg E_2B did not influence testosterone production levels in one day hypophysectomized rats. Testosterone levels in testicular tissue and testosterone production of the control animals were in the same range as was normally found for intact animals in that period of the year (July), plasma levels were lower than the plasma levels from intact rats.

Since the results might have reflected the loss of some pituitary factors involved in an action of E_2B on the testis, we injected 500 ng E_2 together with 50 μg LH immediately after hypophysectomy. In these animals as well no effect of E_2 on testosterone synthesis could be observed. The testosterone levels of these animals should be compared with the levels of intact animals in November.

Our results are in agreement with the results of Samuels et al. (31), who observed reduced activity of steroidogenic enzymes after treatment of intact but not of hypophysectomized rats with diethylstilbestrol. The effects of administered estrogens on testicular testosterone production in the rat thus appear to be mediated via a feedback action on LH secretion. However, it is also known that rat testis interstitial tissue contains E_2 receptor in concentrations comparable to those in the rat uterus. The presence of this receptor suggests that the Leydig cell may be an estrogen target cell, but a possible direct action of estrogen on the Leydig cell, if any, remains to be defined.

Acknowledgements

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References

1. Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and de Sombre, E.R. Proc. Nat. Acad. Sci. U.S. 59: 632 (1968).
2. de Jong, F.H., Hey, A.H. and van der Molen, H.J. J. Endocr. 60: 409 (1974).
3. Brinkmann, A.O., Mulder, E., Lamers-Stahlhofen, G.J.M., Mechielsen, M.J. and van der Molen, H.J. FEBS letters 26: 301 (1972).
4. Mulder, E., Brinkmann, A.O., Lamers-Stahlhofen, G.J.M. and van der Molen, H.J. FEBS letters 31: 131 (1973).
5. van Beurden-Lamers, W.M.O., Brinkmann, A.O., Mulder, E. and van der Molen, H.J. Biochem. J. 140: 495 (1974).
6. Chowdhury, M., Tcholakian, R. and Steinberger, E. J. Endocr. 60: 375 (1974).
7. Danutra, V., Harper, M.E., Boyns, A.R., Cole, E.N., Brownsey, B.G. and Griffiths, K. J. Endocr. 57: 207 (1973).
8. Mallampati, R.S. and Johnson, D.C. Neuroendocrinology 11: 46 (1973).
9. Moger, W.H. Biol. Reprod. 14: 222 (1976).
10. Verjans, H.L., de Jong, F.H., Cooke, B.A., van der Molen, H.J. and Eik-Nes, K.B. Acta Endocrinologica 77: 636 (1974).
11. de Jong, F.H., Uilenbroek, J.Th.J. and van der Molen, H.J. J. Endocr. 65: 281 (1975).
12. Tcholakian, R.K., Chowdhury, M. and Steinberger, E. J. Endocr. 63: 411 (1974).
13. Morishige, W.K. and Rothchild, I. Endocrinology 95: 260 (1974).
14. Verjans, H.L., Cooke, B.A., de Jong, F.H., de Jong, C.M.M. and van der Molen, H.J. J. Steroid Biochem. 4: 665 (1973).
15. Furuyama, S., Maijes, D.M. and Nugent, C.A. Steroids 16: 415 (1970).

16. Welschen, R., Osman, P., Dullaart, J., de Greef, W.J., Uilenbroek, J.Th.J. and de Jong, F.J. J. Endocr. 64: 37 (1975).
17. van der Vusse, G.J., Kalkman, M.L. and van der Molen, H.J. Biochim. Biophys. Acta 380: 473 (1975).
18. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Rendal, R.J. J. Biol. Chem. 193: 265 (1951).
19. Niswender, G.O., Midgley, A.R. and Reichert, L.E. Gonadotrophins 1968, p. 299, Ed.: E. Rosemberg, Los Altos, California: Geron-X Inc.
20. Uilenbroek, J.Th.J., Thesis Erasmus University Rotterdam, p. 28 (1974).
21. de Boer, W., Mulder, E. and van der Molen, H.J. J. Endocr. (1976) in press.
22. Swerdloff, R.S., Grover, P.K., Jacobs, H.S. and Bain, J. Steroids 21: 703 (1973).
23. Swerdloff, R.S. and Walsh, P.C. Acta Endocr. (Kbh) 73: 11 (1973).
24. Gay, V.L. and Dever, N.W. Endocrinology 89: 161 (1971).
25. Verjans, H.L., Eik-Nes, K.B., Aafjes, J.H., Vels, F.J.M. and van der Molen, H.J. Acta Endocr. (Kbh) 77: 643 (1974).
26. Kalra, P.S., Fawcett, C.P., Krulich, L. and McCann, S.M. Endocrinology 92: 1256 (1973).
27. Libertun, C., Orias, R. and McCann, S.M. Endocrinology 94: 1094 (1974).
28. Kinson, G.A. and Liu, C.C. J. Endocr. 56: 337 (1973).
29. Mock, E.J., Kamel, F., Wright, W.W. and Frankel, A.I. Nature 256: 62 (1975).
30. Krulich, L., Hefco, E., Illner, P. and Read, C.B. Neuroendocrinology 16: 293 (1974).
31. Samuels, L.T., Uchikawa, T., Huxby, R.A. in: Endocrinology of the testis. Wolstenholme, G.E.W. and O'Connor, M. (Editors), Churchill, London (1967) p. 211.

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HORMONAL REGULATION OF LH STIMULATION OF TESTOSTERONE PRODUCTION IN ISOLATED LEYDIG CELLS OF IMMATURE RATS: THE EFFECT OF HYPOPHYSECTOMY, FSH, AND ESTRADIOL-17β

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ABSTRACT

Testosterone production in isolated Leydig cells from testes of immature and adult rats was stimulated by addition of LH in a dose dependent way. Hypophysectomy of adult rats had no influence on LH-stimulated testosterone production in isolated Leydig cells after 5 days. In contrast hypophysectomy of immature rats resulted after 5 days in an almost complete loss of LH sensitivity of isolated Leydig cells. Daily administration of FSH during 5 days starting immediately after hypophysectomy maintained LH responsiveness of isolated Leydig cells of immature rats. Also FSH administration starting on day 5 after hypophysectomy resulted in a restoration of LH responsiveness. Estradiol benzoate, injected simultaneously with FSH, abolished the FSH-induced LH responsiveness.

INTRODUCTION

In testicular tissue of adult rats both estradiol and an estradiol receptor are present (1,2), but no clear effect of estradiol in testicular tissue has been demonstrated. Some reports have shown that estradiol administration to intact adult rats is followed by a decrease in plasma testosterone but not in LH levels, which could indicate that estradiol directly influences testicular testosterone production (3,4, 5,6). However, in a number of other studies (7,8,9) it seemed not possible to suppress plasma and testicular testosterone levels after estradiol administration without a concomitant decrease in plasma LH levels.

Not only in adult but also in immature rats an estradiol receptor is present (10). It has been demonstrated that part of the receptor is located in the nuclei of immature rat testes presumably due to endogenously present estradiol (11).

In immature rats testicular Leydig cell function and development are under the influence of several controlling factors. It has been shown that LH administration causes an increase in the number of Leydig cells (12) and a rise in plasma testosterone levels (13,14). Hypophysectomy of immature rats appears to destroy the steroid responsiveness of the testis to LH, while administration of FSH can restore the ability to respond to LH (15,16,17).

In experiments described in the present report we have studied the effect of LH on testosterone production in isolated Leydig cells. A comparison has been made of isolated Leydig cells from immature and adult rats. It is known that the rate of testosterone metabolism in testicular tissue of immature rats is higher than in testes from adult rats (18, 19,20,21,22,23). Therefore we also studied to which extent such metabolism of testosterone may influence the estimated amounts of testosterone which were used as a parameter for LH responsiveness. Finally we have examined the influence of in vivo pretreatment of hypophysectomized immature rats with FSH and estradiol on the LH stimulation of testosterone production in isolated Leydig cells. This use of isolated Leydig cells from hypophysectomized rats makes it possible

to study a possible direct action of estradiol on testicular testosterone synthesis without interfering with the secretion of pituitary gonadotrophins.

MATERIALS AND METHODS

Materials

Rat follicle stimulating hormone (NIAMD rat FSH-B1) was a gift from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, U.S.A. Ovine follicle stimulating hormone (ovine, NIH S-11) and luteinizing hormone (ovine, NIH S-18) was a gift from the National Institute of Health, Bethesda, Maryland, U.S.A. The collagenase type 1 was purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Ficoll 400 from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. Lima bean trypsin inhibitor from Boehringer Mannheim GmbH, Germany and bovine albumin fraction V from Fluka, Bucho, Switzerland.

Animals

All rats used were rats from the Wistar strain, substrain R-Amsterdam. Immature rats were hypophysectomized on day 21-25. Rats were killed 5 or 10 days later. Control intact animals were from the same age as the hypophysectomized rats. Adult rats were 3-5 months old.
FSH was injected subcutaneously (60 µg in 100 µl saline per day). LH dissolved in 0.06% bovine albumin in saline was injected subcutaneously. Estradiol benzoate was dissolved in sesame oil and injected subcutaneously. Control animals received vehicle only.

Isolation of Leydig cells

Rats were decapitated and the testes were removed and decapsulated. Two testes from adult rats or 4-6 testes from immature rats were incubated with 7 ml collagenase (1 mg/ml) according to the method described by Janszen et al. (24). After centrifugation of cells through 13% Ficoll solution the sediment was washed with Krebs Ringer bicarbonate buffer (KRBG) pH 7.4, containing 0.1 mg/ml lima bean trypsin inhibitor. After centrifugation for 10 min at 100 g at room temperature the cells were resuspended in KRBG pH 7.4, containing 0.1 mg/ml trypsin inhibitor. Cell densities of these suspensions were determined by counting of the nucleated cells in a hemocytometer.

Incubation of cell suspension and estimation of testosterone

The cells were preincubated for one hour at 32°C under an

atmosphere of O_2/CO_2 (95:5 v/v). The incubations were carried out in duplicate in volumes of 200 µl in plastic tubes with a cell density of 3-4 x 10^6 cells/ml at 32°C under O_2/CO_2 (95:5 v/v). The tubes were continuously shaken at 100 cycles/min. LH was added in 200 µl KRBG 0.1% bovine albumin. After 2 hours incubation 3.5 ml ethylacetate was added and steroids were extracted. Each incubation was performed in duplicate. Testosterone was estimated using the radioimmunoassay system described by Verjans et al. (25).

Histochemical demonstration of 3ß-hydroxysteroid dehydrogenase activity

3ß-Hydroxysteroid dehydrogenase activity was determined according to Janszen et al. (24).

Estimation of testosterone metabolism in isolated Leydig cells

After 1 hour preincubation 10^6 dpm ^{3}H-testosterone (s.a. 400 dpm/pmole) in 200 µl KRBG 0.1% bovine albumin was added to 200 µl cell suspension. Reaction was stopped after 10 min, by adding 3-5 ml ethylacetate. During this time period the formation of metabolites was linear. The ethylacetate extract was separated by thin layer chromatography on silicagel in dichloromethane : diethylether, 85 : 15. After development, the plate was dried at room temperature and was developed a second time. The areas corresponding with the R_f values of the following steroids were scraped off the plates and were transferred to counting vials: R_f 0-0.15: polar steroids; R_f 0.20-0.25: 5α-androstane-3α, 17ß-diol/5α-androstane-3ß, 17ß-diol; R_f 0.30: testosterone; R_f 0.35-0.40: androsterone (3α-hydroxy-5α-androstane-17-one); R_f 0.40-0.45: dihydrotestosterone; R_f 0.50-0.55: 4-androstene-3,17-dione; R_f 0.70: 5α-androstane-3,17-dione. To the isolated silicagel fractions 10 ml scintillation fluid was added and after shaking the amount of radioactivity was determined. The scintillation fluid consisted of a mixture of Triton X-100 and toluene (2:1 v/v) containing 0.1 g of POPOP 1,4-bis-(5-phenyloxazol-2-yl)benzene/l and 4.8 g of PPO (2,5-dipnehyl-oxazole)/l.

RESULTS

Characterization of Leydig cells isolated from testes of immature rats

1. Purity of the Leydig cell preparation

Part of the cell preparations made by collagenase treatment of testes of immature rats, was centrifuged through a Ficoll

solution and another part was used immediately after incubation with collagenase. The cells were then incubated with 0, 10 or 100 ng LH/ml. After 2 hours testosterone concentrations were determined (Table 1). The Leydig cell content of the cell preparation was estimated histochemically by 3β-hydroxysteroid dehydrogenase activity. From the results in Table 1 it can be concluded that after centrifugation of the unpurified cell preparation through Ficoll the testosterone production per 10^6 cells is increased and that the Ficoll purified cell preparation contains in the order of 53% Leydig cells.

Table 1

Basal and LH-stimulated testosterone production in isolated unpurified and Ficoll purified Leydig cells of immature rats (means of duplicate experiments) and percentage of 3β-hydroxysteroid dehydrogenase containing cells.
(x ± S.E.M. (n))

	ng testosterone/ 10^6 cells/2 h			3β-hydroxysteroid dehydrogenase containing cells
ng LH/ml	0	10	100	
unpurified cells	0.2	1.25	1.85	28.2 ± 3.5% (n = 3)
Ficoll purified cells	0.3	2.65	3.5	52.9 ± 5.3% (n = 11)

2. Dose response curve and time curve of LH-stimulated testosterone production

The time course of the LH-stimulated testosterone production in Ficoll purified cells from immature rats was studied by incubating the cells with 100 ng LH/ml. After 0, 30, 60 and 120 min testosterone concentrations were determined.

A linear increase in the LH-stimulated testosterone production was observed during these 2 hours (Figure 1). In the following series of experiments Ficoll purified cells and an incubation time of 2 hours were used as a routine.

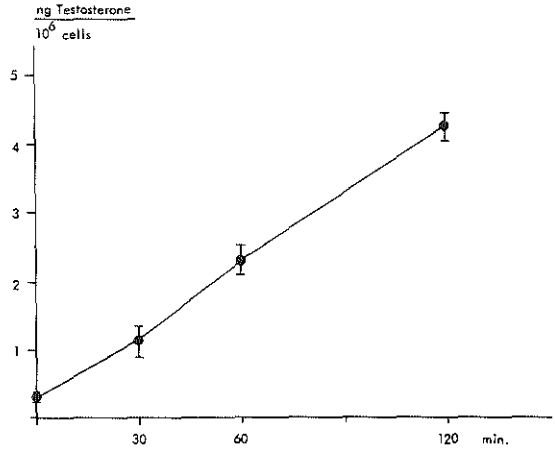

Fig. 1. Time course of testosterone production in the presence of 100 ng LH/ml by isolated Leydig cells prepared from testes of 21-25 day old rats. Cells were preincubated for 60 minutes at 32°C. Means and individual values of duplicate observations are given.

Testosterone production in response to different doses of LH was determined in Leydig cells from adult and immature rats (Figure 2). LH stimulated the testosterone production by cells from adult as well as from immature rats, although the testosterone production was 10 times lower in Leydig cells from immature rats as compared to cells from adult rats for each dose of LH used.

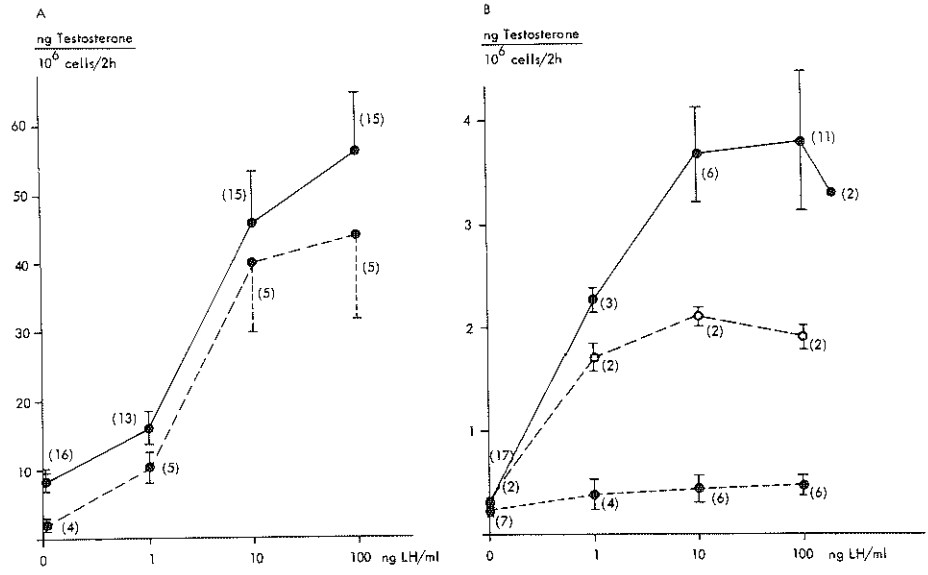

Fig. 2. Dose response curves for the effect of LH on testosterone production by Leydig cell preparations from intact and hypophysectomized rats.
Values are means ± S.E.M. (n = number of experiments).
A: adult rats; B: immature rats.

●——● cells from intact rats
●---● cells from rats 5 days after hypophysectomy
○---○ cells from rats 3 days after hypophysectomy

3. Metabolism of ^{3}H-testosterone by isolated Leydig cells

In order to investigate whether the lower testosterone production in response to LH in cells from immature rats was the result of a higher rate of metabolism, cells of adult and immature rats were incubated with ^{3}H-testosterone. The amount of formed metabolites was expressed as pmol/10^{6} cells/min. In cells from immature rats much more testosterone was converted (146 pmol/10^{6} cells/min) than in cells from mature rats (25.0 pmol/10^{6} cells/min). The main meta-

bolites in cells from immature rats were 5α-androstane-3α/3β,17β-diol, dihydrotestosterone and androsterone whereas in adult rats the main metabolite was androstenedione (Table 2).

Table 2

Metabolism of ^{3}H-testosterone by isolated Leydig cells from adult and immature rats.
Leydig cells from adult and immature rats were incubated with 10^{6} dpm ^{3}H-testosterone for 10 min. Cells from immature and adult rats metabolized 146 and 25 pmol/10^{6} cells/min respectively. Metabolites were characterized by TLC.

steroid	pmole/10^{6} cells/min immature	adult
androstenedione	36.5	23.5
dihydrotestosterone	35.8	n.d.
3α/3β-androstanediol	45.0	n.d.
androsterone	37.9	n.d.
androstanedione	1.3	n.d.
total	156.5	23.5

n.d. = not detectable

The influence of hypophysectomy on LH stimulation of testosterone production in Leydig cells from immature and adult rats

Adult rats and 21-25 day old rats were hypophysectomized. At day 3 and 5 after hypophysectomy testis Leydig cells were isolated. Testosterone production was measured after incubation of the isolated Leydig cells with different amounts of LH. Cells of intact rats of the same age as the hypophysectomized rats were used for comparison (Fig. 2). Leydig cells obtained from immature rats 5 days after hypophysectomy did no longer respond to LH in contrast to cells obtained from mature rats 5 days after hypophysectomy.

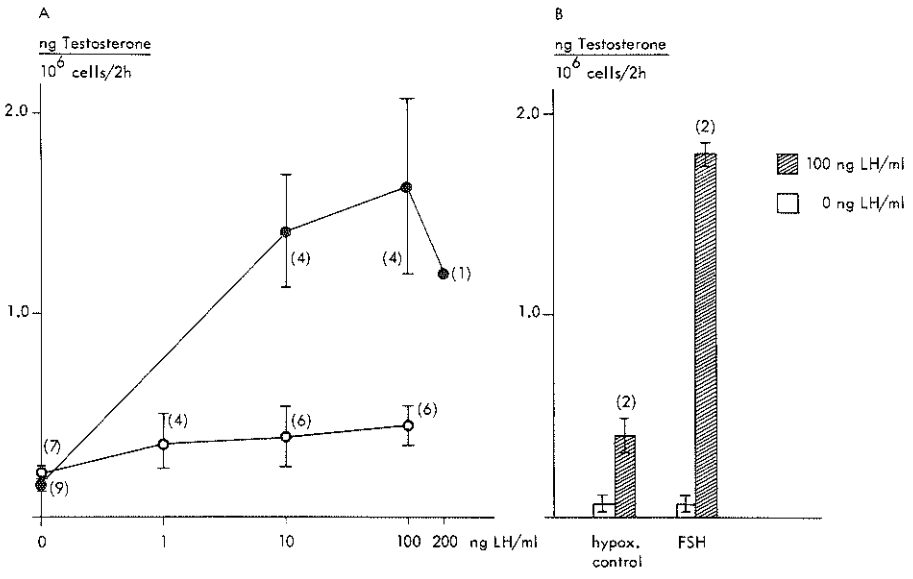

Fig. 3. The effect of in vivo administration of FSH in hypophysectomized rats on testosterone production of isolated Leydig cells in the presence of LH. Immature hypophysectomized rats received daily injections of 60 µg ovine-FSH for 5 days. Control rats were injected with saline.
A: ●——● cells from rats treated with FSH starting immediately after hypophysectomy; O——O cells from control hypox rats. Values are expressed as means ± S.E.M. (n = number of experiments).
B: FSH: cells from rats treated with FSH from day 5 after hypophysectomy. Hypox control: cells from control hypox rats. Means and individual values of duplicate observations are given.

The influence of in vivo administration of FSH to hypophysectomized immature rats on LH stimulation of testosterone production in isolated Leydig cells

Hypophysectomized immature (21-25 days old) rats received a daily injection of 60 µg ovine FSH. Control rats received vehicle only. After 5 days Leydig cells were isolated and incubated with different amounts of LH. Testosterone production was measured. Results of testosterone production are

given in Figure 3A and testes weights are presented in Table 3. After administration of o-FSH the LH responsiveness of the Leydig cells from these hypophysectomized immature rats was maintained, although production was lower than in control rats. In another series of experiments rats received daily injections of o-FSH (60 µg/day) starting from day 5 after hypophysectomy. Leydig cells isolated on day 10 after hypophysectomy were incubated with 100 ng LH/ml. From the results in Figure 3B it can be concluded that FSH can also restore the ability of the Leydig cells isolated from hypophysectomized rats to respond to LH.

Table 3

Testes weights of hypophysectomized rats treated with FSH or FSH + E_2 or without treatment.

Rats were hypophysectomized at day 21-25. One group of rats was injected for 5 days with ovine FSH (60 µg daily), another group received a daily injection of 60 µg FSH plus estradiol benzoate (500 ng or 5 µg) and a third group was injected with vehicle only for 5 days. For comparison the testes weights of intact animals of the age of 21-25 days and 26-30 days are given.

animals	testes weights (g/2 testes)
intact 21-25 days	0.20 ± 0.02 (5)*
intact 26-30 days	0.39 ± 0.01 (4)
hypox	0.12 ± 0.01 (5)
hypox; FSH	0.21 ± 0.01 (8)
hypox; FSH + 500 ng E_2B	0.18 ± 0.01 (7)
hypox; FSH + 5 µg E_2B	0.22 ± 0.01 (3)

* x ± S.E.M. (n)

In order to investigate whether the observed effects of ovine FSH on the Leydig cells were due to contamination with LH (the specifications indicate a contamination < 1%) the

experiments were repeated using highly purified rat-FSH with a certified LH impurity < 0.1%. One group of rats received daily injections of 60 µg rat-FSH, another group was injected with 600 ng ovine LH per day and a third group received 60 µg rat FSH plus 600 ng ovine-LH per day. Control rats received vehicle only. After 5 days isolated Leydig cells were incubated with 0 or 100 ng LH/ml in order to assess the stimulation of testosterone production (Fig. 4). Testes weights are given in Table 4. Results with rat-FSH were similar to those obtained with ovine-FSH. No significant difference could be observed between testosterone production in Leydig cells from hypox control rats or from hypox rats treated with LH.

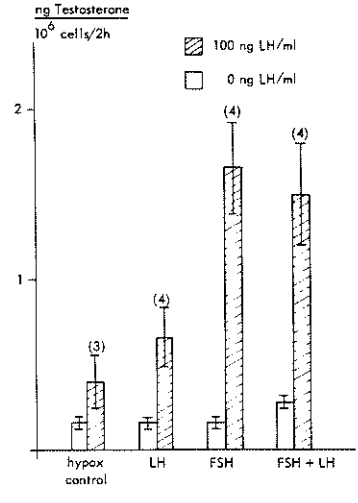

Fig. 4. The effect of in vivo administration of FSH and/or LH to hypophysectomized immature rats on LH stimulation of testosterone production in isolated Leydig cells. After 5 days hypophysectomy different groups of rats were injected daily for 5 days with either: 60 µg rat-FSH per day (FSH), 600 ng ovine LH per day (LH), or 600 ng ovine LH plus 60 µg FSH per day (LH + FSH). Control rats received saline (hypox control). After 5 days Leydig cells were isolated and incubated with 0 or 100 ng LH/ml.
Values are expressed as means ± S.E.M. (n).

The influence of in vivo administration of estradiol and FSH in hypophysectomized rats on LH stimulation of testosterone production in isolated Leydig cells

Three groups of hypophysectomized immature (21-25 day old) rats received respectively o-FSH (60 µg/day), estradiol benzoate (E_2B) (500 ng or 5 µg/day) or FSH (60 µg/day) plus E_2B (500 ng or 5 µg). Control rats were injected with vehicle only. After 5 days Leydig cells were isolated and incubated with 0 or 100 ng LH/ml. Testosterone production rates are presented in Figure 5 and testes weights in Table 4. Estradiol benzoate alone had no effect on LH-stimulated testosterone production in Leydig cells of hypophysectomized rats at any of the concentrations used. The FSH-induced stimulation of LH responsiveness was greatly reduced when FSH was administered in combination with either 500 ng or 5 µg estradiol benzoate.

Table 4

Testes weights of rats after hypophysectomy followed by treatment with FSH, LH or LH + FSH treatment.

Rats were hypophysectomized at day 21-25; starting 5 days after hypophysectomy rats were injected daily for 5 days with 60 µg rat FSH, or with 600 ng ovine LH or with 600 ng ovine LH plus 60 µg rat FSH. One group received vehicle only. Testes weights of intact rats of the same age, i.e. 31-35 days are given for comparison.

animals	testes weights (g/2 testes)
intact (31-35 days)	0.51 ± 0.07 (6)*
hypox	0.07 (2)
hypox + LH	0.06 ± 0.01 (3)
hypox + FSH	0.17 ± 0.01 (3)
hypox + FSH + LH	0.15 ± 0.005 (4)

* x ± S.E.M. (n)

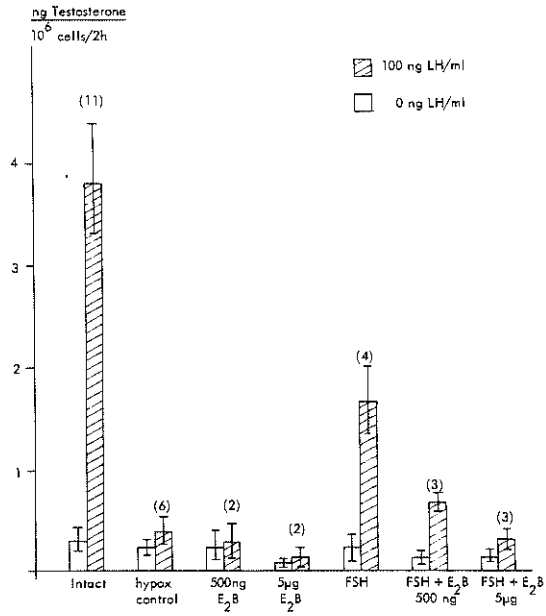

Fig. 5. Estradiol benzoate suppression of the effect of FSH on LH stimulation of testosterone production in Leydig cells. Rats were hypophysectomized at day 21-25 and received 5 daily injections of either FSH 60 µg (FSH); estradiol benzoate 500 ng (E_2B 500 ng) or 5 µg (E_2B 5 µg); 60 µg FSH plus 500 ng estradiol benzoate (FSH + E_2B 500 ng) or 60 µg FSH plus 5 µg E_2B (FSH + E_2B 5 µg). Control rats were injected with vehicle only (control hypox). Intact rats of the same age as the hypox rats were used for comparison. Values are expressed as means ± S.E.M. (n).

DISCUSSION

The present study confirms previous observations (24) that isolated Leydig cells from immature and mature rats can be used as a model for studying the effect of LH on testicular Leydig cell testosterone production. The results of the present study reflect that hypophysectomy of immature rats results in a loss of LH responsiveness in isolated Leydig

cells. In vivo administration of FSH to hypophysectomized immature rats can restore the ability to respond to LH. The effect of FSH was prevented by simultaneous administration of estradiol benzoate.

The isolated Leydig cells were obtained by collagenase treatment of testes from immature rats followed by centrifugation through Ficoll according to the method of Janszen et al. (24). The cell preparation contained 53% Leydig cells, when 3ß-hydroxysteroid dehydrogenase was used as a Leydig cell marker enzyme (Table 1) (26,27). Cell preparations isolated in the same way from testes of adult rats contained 40% Leydig cells (24). The ability of Leydig cells of immature rats to respond to LH has been questioned (28,29). However, the present results, especially the dose response curve (Fig. 2) of the LH stimulation of testosterone production, indicate that isolated Leydig cells from immature rats can respond to LH. This is in agreement with several observations on increased plasma testosterone concentration in immature rats after LH injection (13,14). It has also been reported that testosterone production occurs during incubation of whole testes of immature rats with LH (30). The response of Leydig cells from immature rats in terms of absolute mass of testosterone produced per 10^6 cells was approximately 10 times lower than the response in cells from adult rats. Differences in purity of the cells as a possible explanation for the lower response can be excluded, since the Leydig cell preparation of immature rat testes contains

more Leydig cells than the cell preparation from adult rat testes.

The estimated testosterone production is the result of simultaneous synthesis and degradation of testosterone. Therefore the lower testosterone production in cells from immature rats may be the result of either a lower capacity to respond to LH and/or a higher metabolism of the formed testosterone. With respect to the former possibility it has been described that the activity of the cholesterol side-chain cleavage enzyme is lower in the 20 day old rat when compared to adult rats (31). Incubation of Leydig cells with a saturating amount of ^{3}H-testosterone (Table 2) showed that cells from immature rats metabolize testosterone at a rate of 146 pmol/min/10^{6} cells into dihydrotestosterone, 3α/3β-androstanediol and androsterone. In cells from adult rats testosterone was metabolized at a rate of 25 pmole/10^{6} cells/min to androstenedione. The higher concentration of 5α-reductase and 5α-reduced metabolites has been previously reported for testes of rats between day 20 and day 40 (18, 19,20,21,22,23). It cannot be concluded from the present experiments that the lower response to LH in terms of testosterone production is due to the higher metabolism of testosterone.

The response of Leydig cells from immature rats to LH appeared to be more dependent on the presence of pituitary hormones than the response in cells from adult rats. LH stimulation was hardly changed in cells from adult rats 5 days

after hypophysectomy (Fig. 2A), but no or little response to LH was observed in cells from immature 5 days hypophysectomized rats (Fig. 2B). Similar results were obtained by Odell et al. (15,16), who have reported that LH administration to hypophysectomized rats did not result in growth of sex accessory glands (15), nor in a rise of plasma testosterone levels (16). When FSH was administered daily to hypophysectomized rats for 5 days the ability of isolated Leydig cells to respond to LH was partly maintained (Fig. 3A) and testicular weight was higher in these animals than testicular weights from non-treated hypophysectomized rats (Table 3), probably due to an increase in tubular protein synthesis (31). FSH also restored LH responsiveness in immature rats when administration was started 5 days after hypophysectomy (Fig. 3B). Our results are in agreement with the results of Odell et al. (15,16), who observed prostate growth and increased plasma testosterone levels after LH administration to immature hypophysectomized FSH-treated rats. Administration of LH alone for 5 days did not result in a significant restoration of the LH response (Fig. 4). Administration of LH in combination with FSH caused no higher response to LH in isolated cells than after treatment with FSH alone. Thus no synergistic effect of FSH and LH, as was suggested by Lostroh (33), could be observed in our study. Some authors described higher FSH levels in 20-40 day old rats than in adult rats (34,35,36,37,38). Our results therefore tend to support the hypothesis that FSH could play a role in

regulation of Leydig cell function during the onset of puberty.

In rat testicular interstitial tissue an estradiol receptor is located in the Leydig cells (39). This receptor can be demonstrated from day 4 onwards (10) and part of the estradiol receptors are present in the nuclear fraction of the Leydig cells of immature rats (11). It has recently been reported that FSH stimulates estradiol production from testosterone in Sertoli cells from young rats (40). Therefore we have considered the possibility that FSH exerts its effect via estradiol formation. Treatment of hypophysectomized rats with varying doses of estradiol benzoate, however, did not maintain responsiveness to LH in the Leydig cell. When different amounts of estradiol were administered together with FSH, the response to LH induced by FSH was reduced in a dose dependent way (Fig. 5). Estradiol had no effect on testicular weight (Table 3). Similar results have been obtained by Moger (6), who observed a lower response to LH in terms of plasma testosterone levels in intact adult rats after estradiol treatment. However, in these experiments the influence of feedback effects of estradiol on pituitary hormones could not be excluded. Whether the effects of hypophysectomy, FSH and estrogen treatment on LH responsiveness is due to a change in testosterone synthesis or a change in T metabolism remains to be investigated. However, it is not likely that a higher metabolism of testosterone in hypophysectomized immature animals compared to

intact animals could account for the lower response to LH, because enzymes involved in testosterone metabolism, especially 5α-reductases, are dependent on gonadotrophic hormones (41).

The stimulating effect of FSH on LH responsiveness of Leydig cells and the inhibitory effect of estradiol on the effect of FSH could reflect that these hormones are involved in a mechanism which controls the response to LH in the Leydig cell of the immature rat.

REFERENCES

1. de Jong, F.H., Hey, A.H. and van der Molen, H.J., J. ENDOCR. 60, 409 (1974).
2. van Beurden-Lamers, W.M.O., Brinkmann, A.O., Mulder, E. and van der Molen, H.J., BIOCHEM. J. 140, 495 (1974).
3. Chowdhury, M., Tcholakian, R. and Steinberger, E., J. ENDOCR. 60, 375 (1974).
4. Danutra, V., Harper, M.E., Boyns, A.R., Cole, E.N., Brownsey, B.G. and Griffiths, K.J., J. ENDOCR. 57, 207 (1973).
5. Mallampati, R.S. and Johnson, P.C., NEUROENDOCRINOLOGY 11, 46 (1973).
6. Moger, W.H., BIOL. REPROD. 14, 222 (1976).
7. van Beurden, W.M.O., Mulder, E., de Jong, F.H. and van der Molen, H.J. (submitted for publication).
8. Verjans, H.L., de Jong, F.H., Cooke, B.A., van der Molen, H.J. and Eik-Nes, K.B., ACTA ENDOCR. 77, 636 (1974).
9. de Jong, F.H., Uilenbroek, J.Th.J. and van der Molen, H.J., J. ENDOCR. 65, 281 (1975).
10. de Boer, W., Mulder, E. and van der Molen, H.J., J. ENDOCR. in press (1976).
11. de Boer, W., de Vries, J., Mulder, E. and van der Molen, H.J. (submitted for publication).
12. Chemes, H.E., Rivarola, M.A. and Bergada, C., J. REPROD. FERT. 46, 279 (1976).
13. Odell, W.D., Swerdloff, R.S., Bain, J., Wollesen, F. and Grover, P.K., ENDOCRINOLOGY 95, 1380 (1974).
14. Parlow, A.F., Coyotupa, J. and Kovacic, N., J. REPROD. FERT. 32, 163 (1973).
15. Odell, W.D., Swerdloff, R.S., Jacobs, H.S. and Hescox, M.A., ENDOCRINOLOGY 92, 160 (1973).
16. Odell, W.D. and Swerdloff, R.S., J. STER. BIOCHEM. 5, 853 (1975).
17. Swerdloff, R.S. and Odell, W.D., Postgraduate Medical Journal 51, 200 (1975).

18. van der Molen, H.J., Grootegoed, J.A., de Greef-Bijleveld, M.J., Rommerts, F.F.G. and van der Vusse, G.J., in: Hormonal regulation of spermatogenesis. Ed.: French, F.S., Hansson, V., Ritzén, E.M. and Nayfeh, S.N., Plenum Press, New York and London (1975) p. 3.
19. Sowell, J.G., Folman, Y. and Eik-Nes, K.B., ENDOCRINOLOGY 94, 346 (1974).
20. Podesta, E.J. and Rivarola, M.A., ENDOCRINOLOGY 95, 455 (1974).
21. Ficher, M. and Steinberger, E., ACTA ENDOCR. 68, 285 (1971).
22. Steinberger, E. and Ficher, M., ENDOCRINOLOGY 89, 679 (1971).
23. Wiebe, J.P., ENDOCRINOLOGY 98, 505 (1976).
24. Janszen, F.H.A., Cooke, B.A., van Driel, M.J.A. and van der Molen, H.J., J. ENDOCR. in press (1976).
25. Verjans, H.L., Cooke, B.A., de Jong, F.H., de Jong, C.C.M. and van der Molen, H.J., J. STER. BIOCHEM. 4, 665 (1973).
26. Niemi, M. and Ikonen, M., ENDOCRINOLOGY 72, 443 (1963).
27. Levy, H., Deane, H.W. and Rubin, B.L., ENDOCRINOLOGY 65, 933 (1959).
28. Hashimoto, I. and Suzuki, Y., ENDOCR. JAP. 13, 326 (1966).
29. Steinberger, E. and Steinberger, A. (1972), The Testis: growth versus function. On regulation of organ and tissue growth, pp. 299, Ed.: R.J. Goss. Ac. Press, New York.
30. Payne, A.H., Kelch, R.P., Murono, E.P. and Kerlan, J.T., J. ENDOCR. in press (1976).
31. Kobayashi, S. and Ichii, S., ENDOCR. JAPON. 14, 134 (1967).
32. Fakunding, J.L., Tindall, P.J., Bedman, J.R., Mena, C.R. and Means, A.R., ENDOCRINOLOGY 98, 392 (1976).
33. Lostroh, A.J., ENDOCRINOLOGY 85, 438 (1969).
34. Miyachi, Y., Nieschlag, E. and Lipsett, M., ENDOCRINOLOGY 92, 1 (1973).
35. Swerdloff, R.S., Walsh, P.C., Jacobs, H.S. and Odell, W.D., ENDOCRINOLOGY 88, 120 (1971).
36. Odell, W.D. and Swerdloff, R.S., in: The control of the onset of puberty. Ed. by Grunbach, M.M., Grave, G.D. and Mayer, F.E., John Wiley, New York, p. 313 (1974).
37. Negro-Vilar, A., Krulich, L. and McCann, S.M., ENDOCRINOLOGY 93, 660 (1973).
38. Gupta, D., Rager, K., Zarzycki, J. and Eichner, M., J. ENDOCR. 66, 183 (1975).
39. Mulder, E., Peters, M.J., van Beurden, W.M.O., Galdieri, M., Rommerts, F.F.G., Janszen, F.H.A. and van der Molen, H.J., J. ENDOCR. (in press).
40. Armstrong, D.R., Moon, Y.S., Fritz, I.B. and Dorrington, J.F., in: Hormonal regulation of spermatogenesis, p. 85. Ed: French, F.S., Hansson, V., Ritzén, E.M., Nayfeh, S.N., Plenum Press, New York and London (1975).

41. Nayfeh, S.N., Coffey, J.C., Kotitz, N.J. and French, F.S., in: Hormonal regulation of spermatogenesis, p. 53, Ed.: French, F.S., Hansson, V., Ritzén, E.M., Nayfeh, S.N., Plenum Press, New York and London (1975).