TESTICULAR OESTRADIOL-17 β

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE

AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM,

OP GEZAG VAN DE RECTOR MAGNIFICUS

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EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.

DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP

WOENSDAG 22 MEI 1974,

DES NAMIDDAGS TE 4.15 UUR

DOOR

FRANK HOTZE DE JONG

GEBOREN TE OUDENRIJN

Promotor	:	Prof.Dr. H.J. van der Molen
Coreferenten	:	Prof.Dr. J.C. Birkenhäger
		Dr. R.V. Short

Voor Maartje,
om in te kleuren

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VOORWOORD

Onderzoeken is een arbeid, die moeilijk anders dan in teamverband verricht kan worden. Daarom geloof ik dat het juist is dit voorwoord te beginnen door alle medewerkers van de afdeling Biochemie (Chemische Endocrinologie) en ook hen, met wie ik buiten deze afdeling samengewerkt heb, te danken voor gegevens, raadgevingen, diskussies en meningsverschillen. Vervolgens wil ik hen, die wel een zeer duidelijk stempel op dit proefschrift drukten, persoonlijk noemen.

In de eerste plaats de promotor, Prof.Dr. H.J. van der Molen. Beste Henk, mede dankzij jouw pragmatisme en soms wat agressieve redeneertrant is het onderzoek niet op allerlei interessante zijsporen gederailleerd. Ik waardeer je vrijgevigheid, die duidelijk wordt in je wetenschappelijke openheid, je bestuurlijke opstelling binnen de afdeling en de set ups bij het volleybal, bijzonder.

Prof.Dr. J.C. Birkenhäger, beste Jan, nog meer dan voor je bemoeienissen als coreferent zou ik je willen danken voor de wijze waarop je me de vrijheid gelaten hebt in de Hoogbouw bezig te zijn met zaken, die niet direkt met patiënten te maken hebben.

Dr. R.V. Short, I thank you for your willingness to read this thesis as "coreferent". I am sure that there will be ample time to discuss the significance of testicular oestradiol synthesis in the future.

Dear Yolanda, you were the first one to encounter the difficulties in the estimation of oestradiol. I admire your "never-give-up" mentality very much.

Beste Anne, jij bent erin geslaagd de radioimmunologische bepaling van oestradiol van de grond te krijgen en van de grond te houden. Het is duidelijk dat dit boekje zonder jouw bezigheden en administratieve vaardigheid nog niet geschreven zou zijn.

Beste Wil, Wytske, Sarah en Tilly, alleen het feit dat jullie de klinische "routine"-kastanjes uit het vuur haalden, maakte het voor mij mogelijk "research" te bedrijven. Daarvoor, en voor de vele in dit boekje verwerkte testosterongetallen, ben ik jullie zeer dankbaar.

Beste Marja, het feit dat onze ruzies in het verleden zelden hoger opliepen dan door Van Dale beslecht kon worden, pleit voor ons beider inkasseringsvermogen. Dat dit ook in de periode waarin jij dit proefschrift typte het geval was, moet waarschijnlijk alleen op jouw conto geschreven worden.

Drs. P.M. Frederik, ofwel Peter, wist wederom op verbluffende wijze de dingen die in mijn hoofd nog geen vorm hadden aangenomen, terug te brengen tot een tweedimensionaal omslag.

Ik dank Pim voor zijn niet aflatende vrolijke hulpvaardigheid, Brian voor zijn kommentaar op het Engels, Binding Boven voor de gastvrijheid en het TDT voor zijn bijdrage, vooral tijdens de grote proeven.

De medewerkers van de Audiovisuele Dienst, in het bijzonder de heer D.M. Simons, dank ik voor het geduld dat zij met mij en mijn figuren gehad hebben.

Beste Focko, het is triest je van zo'n afstand te moeten herinneren aan wekelijkse maaltijden bij de Chinees, maandelijkse ontmoetingen bij de brandweer en dagelijkse verhitte gesprekken over van alles en statistiek. We moesten als je terug bent de blussingsmethodieken nog maar eens doornemen.

Tenslotte wil ik mijn ouders nog eens expliciet danken voor het feit dat zij mijn studie, en daardoor dit boekje mogelijk maakten. En om Arjane te danken, zijn er gelukkig nog andere mogelijkheden dan dit voorwoord.

LIST OF TRIVIAL NAMES

androstenedione	- 4-androstene-3,17-dione
androsterone	- 3α-hydroxy-5α-androstan-17-one
cholesterol	- 5-cholesten-3β-ol
cortisol	- 11β,17,21-trihydroxy-4-pregnene-3,20-dione
corticosterone	- 11β,21-dihydroxy-4-pregnene-3,20-dione
cyanoketone	- 2α-cyano-4,4',17α-trimethyl-17β-hydroxy-5-androsten-3-one
dehydroepiandrosterone	- 3β-hydroxy-5-androsten-17-one
dehydroepiandrosterone sulphate	- 17-oxo-5-androsten-3β-yl sulphate
desoxycorticosterone	- 21-hydroxy-4-pregnene-3,20-dione
dexamethasone	- 9α-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione
diethylstilboestrol	- trans-3,4-bis(4-hydroxyphenyl)-3-hexene
epitestosterone	- 17α-hydroxy-4-androsten-3-one
epitestosterone chloroacetate	- 3-oxo-4-androsten-17α-yl monochloroacetate
etiocholanolone	- 3α-hydroxy-5β-androstan-17-one
19-hydroxyandrostenedione	- 19-hydroxy-4-androstene-3,17-dione
17α-hydroxypregnenolone	- 3β,17-dihydroxy-5-pregnen-20-one

17α-hydroxyprogesterone	- 17-hydroxy-4-pregnene-3,20-dione
19-hydroxytestosterone	- 17β,19-dihydroxy-4-androsten-3-one
mevalonic acid	- 3,5-dihydroxy-3-methyl pentanoic acid
oestradiol(-17β)	- 1,3,5(10)-oestratriene-3,17β-diol
oestradiol dipropionate	- 1,3,5(10)-oestratriene-3,17β-diol dipropionate
oestradiol-17β-hemisuccinate	- 1,3,5(10)-oestratriene-3,17β-diol 17-monohemi-succinate
oestriol	- 1,3,5(10)-oestratriene-3,16α,17β-triol
oestrone	- 3-hydroxy-1,3,5(10)-oestratrien-17-one
oestrone acetate	- 17-oxo-1,3,5(10)-oestratrien-3-yl acetate
oestrone sulphate	- 17-oxo-1,3,5(10)-oestratrien-3-yl sulphate
19-oxoandrostenedione	- 4-androstene-3,17,19-trione
19-oxotestosterone	- 17β-hydroxy-4-androstene-3,19-dione
pregnanediol	- 5β-pregnane-3α,20α-diol
pregnenolone	- 3β-hydroxy-5-pregnen-20-one
progesterone	- 4-pregnene-3,20-dione
testosterone	- 17β-hydroxy-4-androsten-3-one
testosterone chloroacetate	- 3-oxo-4-androsten-17β-yl monochloroacetate
testosterone propionate	- 3-oxo-4-androsten-17β-yl propionate

LIST OF ABBREVIATIONS

ACTH	- adrenocorticotrophic hormone
BSA	- bovine serum albumin
b.w.	- body weight
C.V.	- coefficient of variation
dpm	- disintegration per minute
EDTA	- ethylenediaminetetraacetate
EFA	- essential fatty acid
FSH	- follicle-stimulating hormone
g	- relative centrifugal force
HCG	- human chorionic gonadotrophin
LH	- luteinizing hormone
MCR	- metabolic clearance rate
n	- number of estimations
NADH	- nicotinamide-adenine dinucleotide (reduced)
NADPH	- nicotinamide-adenine dinucleotide phosphate (reduced)
P	- probability
PC	- paper chromatography
PMS	- pregnant mare serum gonadotrophin
PR_B	- blood production rate
PR_U	- urinary production rate
R^*	- amount of radioactivity injected in estimation of PR_B or PR_U
$\rho, \rho_{BB}^{PRE \rightarrow PRO}$	- transfer constant for the conversion in blood of a prehormone to a product
RNA	- ribonucleic acid
S.D.	- standard deviation
S.E.M.	- standard error of the mean
TLC	- thin-layer chromatography
X	- concentration of hormone in blood
X'	- concentration of labelled hormone in blood

CHAPTER 1. OESTROGENS IN THE MALE. QUESTIONS AND SCOPE OF THIS THESIS

1.1. Introduction

Oestrogenic hormones were originally isolated from ovarian follicles and from placental tissue and were believed to occur only in female animals. Laqueur et al. (1927) observed, however, that extracts from human male urine caused vaginal cornification in spayed mice. This discovery of oestrogenic activity in urine from men was so unexpected, that the authors thought it necessary to state that there could be no doubt about the manliness of the subjects studied. One of the oestrogenic substances in human male urine was subsequently identified as oestrone (Dingemanse et al., 1938), while later on oestradiol and oestriol were also found to be present in urine from men (see: Diczfalusy & Lauritzen, 1961). Since then, the occurrence of oestrogens in the urine of male animals from several species has been described (see: Velle, 1966). However, information on the precise origin of these oestrogenic hormones and on the regulation of the production of oestrogens in the male animal is still limited. Therefore, it was decided to investigate these points with special reference to the testis as a possible source of oestradiol. In this first chapter a broad outline of some questions related to the study of production and secretion of oestradiol in the male will be given. A more detailed description of these problems can be found in the following chapters.

1.2. The origin of oestrogenic hormones in male animals

The presence of oestrogenic steroids in urine from male subjects might result from a direct secretion from endocrine glands, such as the adrenal or the testis, or from peripheral conversion of androgens. The latter possibility has been shown by Steinach & Kun (1937), who found that administration of androgens to men causes an increase in the urinary excretion of oestrogens. The results of investigations concerning the relative contribution of glandular secretion and peripheral conversion to the total amount of oestradiol produced are conflicting: some authors (MacDonald et al., 1971) concluded that all circulating oestradiol is formed peripherally, while others (Baird et al., 1968) could not exclude the possibility of oestradiol secretion from endocrine glands (see chapter 3). The first purpose of the investigation described in this thesis was to determine whether or not oestradiol is secreted by the rat testis and if it is, to estimate the relative contribution of testicular oestradiol to the total amount of oestradiol in the peripheral circulation. The results described in chapter 3 and appendix paper 1 indicate that the testis of the rat does secrete oestradiol. This testicular secretion accounts for about 20% of the circulating oestradiol.

1.3. The regulation of the testicular production and secretion of oestradiol

Maddock & Nelson (1952) observed that the urinary excretion of oestrogens increased more steeply than that of 17-ketosteroids after administration of HCG to men. This finding started the discussion on the regulation of testicular production and secretion of oestrogens. However, in vivo studies with intact subjects cannot provide answers to questions on the role of the testis in the total production rate of oestradiol (see chapter 3). Therefore, the second aim of this study was to investigate the regulation of the testicular production and secretion of oestradiol directly. Various conditions, which are known to influence testicular function were used. Results of these experiments, which were carried out largely under in vivo conditions, are described in chapter 3 and in the appendix papers 1 and 2. It is concluded that only HCG influences testicular oestradiol secretion, when administered in short-term experiments. No effects could be shown after administration of HCG during longer periods, of FSH, or of prolactin.

1.4. The intratesticular localization of the production of oestradiol

Both tissue compartments in the testis, the seminiferous tubules and the interstitial tissue, have been considered as a possible site of testicular oestrogen biosynthesis. Maddock & Nelson (1952) suggested the Leydig cell as the source of testicular oestrogens, because after administration of HCG to men they observed hypertrophy of the interstitial tissue concomitant with an increased urinary excretion of oestrogens. Ashbel et al. (1951) concluded that

testicular oestrone was localized in the seminiferous tubules on basis of results obtained with histochemical techniques for the detection of phenolic 17-ketosteroids. From studies with "feminizing testes" or with testicular tumours either the interstitial tissue (French et al., 1965, Sharma et al., 1965) or the Sertoli cell (Berthrong et al., 1949, Teilum, 1949) was suggested as the source of testicular oestrogens.

As the third aim of the present study it was attempted, therefore, to get more insight into the cellular localization and origin of testicular oestradiol. Endogenous oestradiol was estimated during incubations of whole testis tissue or of separated interstitial tissue and seminiferous tubules (Christensen & Mason, 1965) from the rat testis. Furthermore, incubations with radioactive oestrogen precursors were performed with these tissues. The results of these experiments, as described in chapter 4 and appendix paper 2, indicate that testicular oestradiol is mainly localized in the interstitial tissue. Biosynthesis of oestradiol might, however, take place in the seminiferous tubules.

1.5. The function of testicular oestradiol

The administration of oestrogens to men or male experimental animals causes severe degeneration of the testis and accessory sex organs (see: Emmens & Parkes, 1947). This involution is thought to result from a lack of gonadotrophins through the negative feed-back action of oestrogens on the secretion of the hypophyseal gonadotrophins (see: Burger et al., 1972), although direct effects of oestrogens on the testis have also been postulated (Samuels et al., 1967). The relevancy of these possibilities is discussed in chapter 5.

1.6. Methods for the estimation of oestradiol

In order to realize the intended aims of this investigation, it was necessary to measure the endogenous concentration of oestradiol in plasma and tissues from the male rat. Methods which were sufficiently sensitive and specific to meet this goal had not been properly documented when this investigation was started. Therefore, the reliability of several methods for the estimation of oestradiol was tested for these specific requirements. The results of these methodological investigations are summarized in chapter 2, together with the reliability criteria of the methods used for the estimation of testosterone, which was estimated as a reference in the same samples.

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CHAPTER 2. METHODS USED FOR THE ESTIMATION OF STEROIDS

2.1. Introduction

Progress in endocrinological research depends for an important part on progress in the methodology for estimation of the concentrations of hormones, that are present in tissues or body fluids under physiological or pathological conditions. A main aspect of analytical methodology in this respect is the sensitivity of the methods used, and in direct connection therewith the specificity of detection. For the estimation of steroids, both sensitivity and specificity have been improved enormously during the last 15 years through the development of double isotope dilution techniques (see: Bojesen et al., 1967), gas-liquid chromatography (see: Eik-Nes & Horning, 1968) and saturation analysis (see: Diczfalusy, 1970). The older and generally less sensitive techniques of bioassay (see: Dorfman, 1969) and fluorimetry and colorimetry (see: Loraine & Bell, 1966) have gradually been replaced, although many of the principal observations have initially been made using these techniques. This development is clearly reflected in the following reviews dealing with techniques that can be used for the estimation of a single steroid or a class of steroids: androgens (van der Molen, 1971), oestrogens (Preedy et al., 1967) and progesterone (van der Molen & Aakvaag, 1967).

In order to study the endogenous production and secretion of oestradiol from the testis it was mandatory to use techniques with a sensitivity which would permit measurement of the endogenous concentrations of this steroid in

Table 2.1. Sensitivity of detection of some techniques used for the quantitative estimation of oestradiol

Technique	Sensitivity (ng)	Reference
Bioassay*		
uterine weight	12.5	Astwood (1938)
vaginal cornification	0.5	Emmens (1941)
vaginal mitosis	0.004	Martin & Claringbold (1960)
vaginal metabolic activity	0.002	Martin (1960)
Spectroscopic techniques		
infrared spectroscopy	5,000	Oertel et al. (1959)
colorimetry	100	Salokangas & Bulbrook (1961)
fluorimetry	5	Preedy (1965)
Double isotope derivative methods		
bromination with ^{82}Br	1.0	Slaunwhite & Neely (1963)
pipsylation with ^{35}S pipsyl chloride	1.0	Baird (1968)
$NaB(^{3}H)_4$ reduction	10	Kautsky et al. (1970)
Gas-liquid chromatography		
flame ionization detection	200	Adlercreutz et al. (1967)
electron capture detection	0.5	Attal & Eik-Nes (1968)
Radioligand assay		
uterine cytosol protein	0.02	Korenman et al. (1969)
sex steroid binding globulin	0.05	Dufau et al. (1970)
radioimmunoassay	0.01	Appendix paper 1

* Lowest detectable levels in bioassay are calculated from data, concerning groups of animals; the amount of steroid used per assay is much higher than the values given.

male experimental animals. The only data on the level of oestradiol in male animals available at the start of this investigation was the concentration of oestradiol in peripheral plasma from men (see Table 3.6.). In order to measure oestradiol in the small amounts of plasma that can be obtained from the rat, it was deemed necessary to be able to measure at least 25 pg. The limits of detection of a number of methods for the estimation of oestradiol are summarized in Table 2.1.: only competitive protein binding with the use of receptor proteins or radioimmunoassay appeared to be sensitive enough for estimation of oestradiol in male rats. These techniques had hardly been introduced at the start of this investigation and have therefore been evaluated in detail (section 2.3.).

The sensitivity of gas-liquid chromatography with electron capture detection is sufficient to measure concentrations of testosterone in peripheral and testicular venous blood from rats, which are much higher than those of oestradiol (Bardin & Peterson, 1967, appendix paper 1). Nevertheless, the high practicability of radioimmunoassay made it worthwhile to investigate this technique as well. The merits of these two methods for the estimation of testosterone are discussed in section 2.2.

2.2. The estimation of testosterone

2.2.1. Gas-liquid chromatography

2.2.1.1. Method

Testosterone was estimated using the method as described by Brownie et al. (1964) with minor modifications (Table 2.2.). Solvents were purified as described by van der Molen & de Jong (1974). Gas-liquid chromatography was performed on a model 104 Pye gas-liquid chromatograph,

Table 2.2. Procedure for the assay of testosterone using gas-liquid chromatography with electron capture detection (Brownie et al., 1964)

- Add 40,000 dpm ^{3}H-testosterone to alkaline plasma
- Extract plasma with ether
- Wash ether extracts and evaporate solvent
- Purify residue by thin-layer chromatography on silica gel using the solvent system toluene:ethyl acetate - 2:1 (v/v)
- Extract testosterone from the appropriate silica gel fraction with toluene/water
- Evaporate the combined toluene layers
- Chloroacetylate residue
- Extract testosterone from the chloroacetylation mixture with ethyl acetate
- Evaporate the combined ethyl acetate layers
- Purify testosterone chloroacetate by thin-layer chromatography on silica gel using the solvent system toluene:ethyl acetate - 9:1 (v/v)
- Extract testosterone chloroacetate from the appropriate silica gel fraction with toluene/water
- Evaporate the combined toluene layers
- Inject suitable aliquot onto the gas-liquid chromatographic column and estimate amount of testosterone present in total sample
- Add suitable amount of second internal standard
- Take suitable aliquot of sample for recovery estimation
- Inject suitable aliquot of sample onto the gas-liquid chromatographic column for final gas-liquid chromatography

equipped with an automatic solid injection system. The ^{63}Ni electron capture detector was operated in the pulsed mode (pulse interval: 150 µsec, pulse height: 60V, pulse width: 0.75 µsec). The 3 ft coiled column was packed with a stationary phase of 1% QF-1 on gaschrom Q; nitrogen was used as carrier and as purge gas. The internal standard for gas-liquid chromatography was 3-oxo-4-pregnen-20β-yl chloroacetate instead of 5-cholesten-3β-yl chloroacetate, as used in the original method.

Table 2.3. R_f values during thin-layer chromatography and relative retention times (RRT) during gas-liquid chromatography in the systems used during the gas-liquid chromatographic estimation of testosterone (partly from van der Molen & de Jong, 1974)

Steroid	Thin-layer chromatography		Gas-liquid chromatography
	R_f in toluene:ethyl acetate-2:1	R_f after chloroacetylation in toluene:ethyl acetate-6:1	RRT* of chloroacetates on 1% QF-1
oestriol	0.05		
cortisol	0.05	0.08	
corticosterone	0.06	0.08	
pregnanediol	0.18	0.91	27.9
desoxycorticosterone	0.22	0.35	-
testosterone	0.26	0.42	17.1
epitestosterone	0.26	0.42	15.0
20α-hydroxy-4-pregnen-3-one	0.32	0.47	28.9
20β-hydroxy-4-pregnen-3-one	0.32	0.52	25.0
17α-hydroxyprogesterone	0.33	0.12	-
etiocholanolone	0.35	0.56	7.9
androsterone	0.40	0.55	7.4
pregnenolone	0.43	0.70	11.6
dehydroepiandrosterone	0.44	0.59	8.6
androstenedione	0.45	0.18	-
3α-hydroxy-5β-pregnan-20-one	0.46	0.71	9.9
oestradiol	0.57	0.82	25.7
progesterone	0.59	0.25	-
oestrone	0.88	0.56	10.8

* Retention time relative to that of 5α-cholestane = 1.

2.2.1.2. Results

The specificity of the method is achieved as a result of the three chromatographic steps, the conversion of testosterone and the use of the electron capture detector. R_f values and relative retention times for testosterone and related steroids in the systems used in the present method are summarized in Table 2.3. Epitestosterone is the only steroid likely to interfere with the estimation of testosterone using this method. This steroid is present in bovine testicular venous plasma (Lindner, 1959) and in testis tissue from the same species (Neher & Wettstein, 1960) and is not separated from testosterone during thin-layer chromatography of the free steroids or the chloroacetates. However, the retention times of epitestosterone chloroacetate and testosterone chloroacetate are different, and peaks with the retention time of epitestosterone chloroacetate have never been observed during gas-liquid chromatography of extracts of rat testicular venous plasma or testis tissue. Data on accuracy and precision of the method are summarized in Table 2.4.

Table 2.4. Accuracy and precision of the estimation of testosterone using gas-liquid chromatography with electron capture detection of testosterone chloroacetate

Assay of	n	found	S.D.	C.V.
0 ng	216	0.61 ng	0.76 ng	-
10 ng	118	9.6	2.1	21.8%
100 ng	134	98.5	13.7	13.9

n = number of estimations
S.D. = standard deviation
C.V. = coefficient of variation: $\frac{\text{S.D.}}{\text{amount found}} \times 100\%$

The sensitivity of the method, calculated from the values obtained after assay of testosterone-free blanks, was in the order of 2 ng/sample (van der Molen, 1971). Recoveries of tritiated testosterone added to plasma samples before extraction, were 58.9 $\pm$ 12.6 (S.D.) % (n=185).

Results obtained using this method were compared with those of testosterone estimations in the same samples using competitive protein binding assay (Benraad et al., 1972) and radioimmunoassay (Verjans et al., 1973). Correlation coefficients for the concentrations of testosterone in human male plasma were 0.87 and 0.87, when results of radioimmunoassay and competitive protein binding assay were compared with the gas-liquid chromatographic method. Similar experiments for human female plasma gave correlation coefficients of 0.80 and 0.71.

2.2.2. Radioimmunoassay

In some of the experiments described in chapters 3 and 4 testosterone concentrations were measured by radioimmunoassay, using the method as described by Verjans et al. (1973) (see Table 2.5.). It was deemed necessary to guarantee the specificity of the method and to measure one well-defined compound, i.e. testosterone, for the different physiological conditions used in this study. Therefore, alumina column chromatography was used for plasma and tissue samples, although no significant differences were found between results of testosterone concentrations in testicular tissue obtained with gas-liquid chromatography or radioimmunoassay without chromatography (Verjans et al., 1973).

Standard curves, specificity of the antibody, accuracy, precision and sensitivity of the method have been described in detail by Verjans et al. (1973).

Table 2.5. Procedure for the estimation of testosterone in plasma or tissue using radioimmunoassay (from: Verjans et al., 1973)

- Add 20,000 dpm ^{3}H-testosterone to plasma	- Add 20,000 dpm ^{3}H-testosterone to tissue
- Extract plasma with 3 ml hexane:ether - 8:2 (v/v)	- Sonify tissue in water - Precipitate proteins with acetone - Evaporate supernatant until only water remains - Extract water layer with 3 ml hexane:ether - 8:2 (v/v)
- Transfer organic layer to pre-eluted alumina column	
- Elute column with 1.6 ml hexane:ether - 8:2 (v/v) 6 x 1.6 ml ethanol (0.45%) in hexane 2 x 1.6 ml ethanol (0.95%) in hexane	
- Divide last eluates over two tubes	
- Evaporate solvents in each of the tubes	
- Add 250 µl antibody solution and incubate at 4°C for 16 h	
- Take 50 µl for recovery estimation	
- Add 500 µl dextran-coated charcoal suspension	
- Incubate for 5 min at 4°C	
- Centrifuge for 10 min	
- Take 500 µl supernatant for counting of bound radioactivity	

2.2.3. Discussion

The sensitivity of the radioimmunoassay of testosterone is much higher than that of the gas-liquid chromatographic procedure (Verjans et al., 1973). Accuracy and precision of both methods are acceptable, but it is difficult to compare these factors because of the different levels of sensitivity. Finally, the specificity of the gas-liquid chromatographic method is easily observed from the tracings obtained for each individual sample and offers advantages over that of the radioimmunoassay procedure. The column chromatography used in the latter technique reduces this

problem, however.

It was concluded from these results (Verjans et al., 1973) that both methods yield reliable estimations of testosterone concentrations in tissue and plasma. When the sensitivity of the method is not that important, as was the case in this study, the choice between the two techniques will depend on criteria of practicability rather than of reliability.

2.3. The estimation of oestradiol

2.3.1. Competitive protein binding

2.3.1.1. Method

Oestradiol was initially assayed in a competitive protein binding system, using the receptor from rabbit uterine cytosol as the binding principle. Uterine cytosol, buffer solutions and charcoal suspension were prepared as described by Korenman (1968), with the exception that non-pregnant rabbits were used. Solvents for extraction and chromatography of oestradiol were purified as described by van der Molen & de Jong (1974). The chromatographic step, which is necessary for the separation of oestradiol from cross-reacting compounds, was performed on Sephadex LH-20 columns according to Mikhail et al. (1970). A flow sheet of the actual method is shown in Table 2.6.

2.3.1.2. Results

A standard curve, obtained after addition of known amounts of oestradiol to radioactive steroid, evaporation of the solvent and addition of the diluted rabbit uterine cytosol is shown in panel A of Fig. 2.1.

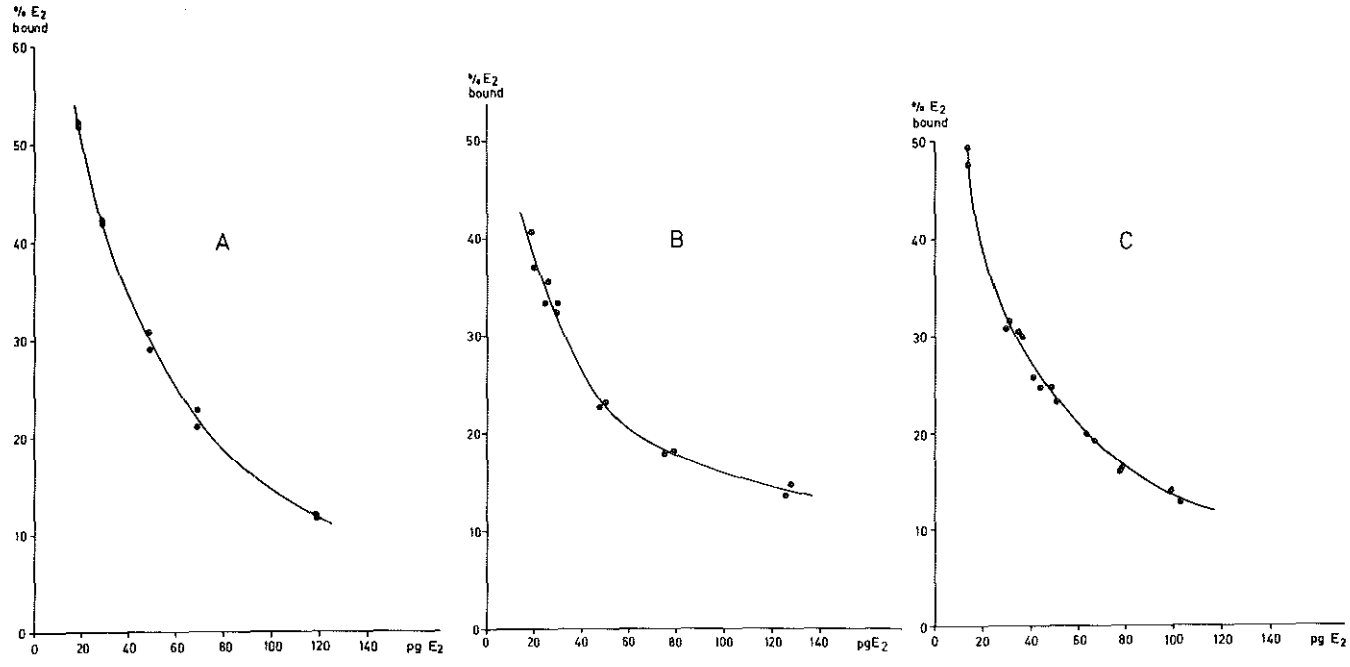

Fig. 2.1. Standard curves obtained for known amounts of oestradiol using

A. the competitive protein binding method.

B. the double competitive protein binding method.

C. radioimmunoassay.

Table 2.6. Procedure for the assay of oestradiol using competitive protein binding

- Add 20,000 dpm ^{3}H-oestradiol to plasma
- Extract plasma with ether
- Wash ether extracts and evaporate ether
- Purify residue by column chromatography (Sephadex LH-20, toluene:methanol - 85:15, v/v)
- Collect oestradiol fraction
- Take aliquot of the solution for recovery estimation and evaporate solvent
- Add 100 µl of diluted protein preparation to residue
- Incubate steroid-protein mixture for 17 h at 4°C
- Add 500 µl dextran-coated charcoal suspension
- Mix and leave for 10 min at 4°C
- Centrifuge for 10 min at 4°C
- Take 500 µl of supernatant for counting of bound radioactivity

The specificity of the binding protein to bind oestradiol is limited (Korenman, 1968). Therefore, it is necessary to separate oestradiol from other steroids prior to the binding assay. Sephadex LH-20 columns were chosen because the recovery of steroids is high, an efficient separation between oestradiol and other oestrogens can be obtained (Mikhail et al., 1970) and blank values in the binding assay are low. Furthermore, decomposition of picogram amounts of oestradiol does not occur during chromatography on Sephadex LH-20 in contrast to the results obtained during thin-layer chromatography on silicagel (Coyotupa et al., 1970). The influence of the chromatographic step on blank values is shown in Table 2.7. Since blank values are high and variable, the smallest amount of oestradiol which can be discerned from zero is 40 pg.

Table 2.7. Blank values (means ± S.D.) obtained after extraction and/or chromatography of oestradiol (E_2) using different techniques

	pg E_2 found after correction for mass of added ^3H-E_2		
	competitive protein binding	double competitive protein binding	radioimmunoassay
^3H-E_2 added to water and purified without chromatography	24.7 ± 3.6 (4)	-	33.6 ± 2.0 (4)
^3H-E_2 added to column effluent after chromatography	15.9 ± 11.3 (116)	20.5 ± 14.0 (16)	-2.9 ± 4.4 (14)
^3H-E_2 added to water and purified with chromatography	23.1 ± 10.4 (36)	24.6 ± 14.9 (10)	1.2 ± 5.6 (267)

The number of estimations is given in parentheses.

Table 2.8. Accuracy and precision of the estimation of oestradiol (E_2) by competitive protein binding. Known amounts of oestradiol were added to water and the solution was processed by the method as outlined in Table 2.6.

Assay of	n	E_2 found*	S.D.	C.V.
30 pg E_2	3	35.3 pg	5.1 pg	14.4%
50 pg E_2	7	69.1 pg	14.6 pg	21.1%
human male plasma I	5	15.5 pg/ml	4.2 pg/ml	27.1%
human male plasma II	6	21.4 pg/ml	5.8 pg/ml	27.1%

* Values are corrected by subtraction of blanks.

n = number of estimations
S.D. = standard deviation
C.V. = coefficient of variation: $\frac{\text{S.D.}}{\text{amount found}} \times 100\%$

Data on accuracy and precision of the method are summarized in Table 2.8. The oestradiol concentrations measured in human male plasma using this competitive protein binding technique agree well with values reported in the literature (Baird, 1968). Recoveries of oestradiol after extraction and chromatography were 86.2 $\pm$ 10.7 (S.D.) % (n=44).

Estimation of oestradiol in peripheral plasma from male rats in volumes between 1 and 5 ml resulted in percentages binding which varied between 0.5 and 5.0, thus indicating the presence of unlikely high concentrations (in the order of >200 pg/ml) of "oestradiol-like" substances. This observation is in agreement with data published by Labhsetwar (1972), who estimated "oestradiol-like" substances in peripheral plasma from rats using radioimmunoassay and by Weisz & Gunsalus (1973), who found similar results in immature female rats. These observations will be discussed in section 2.3.3.2.

2.3.2. Double competitive protein binding

2.3.2.1. Method

Since the specificity of the single competitive protein binding technique was not sufficient for the estimation of oestradiol in peripheral plasma from male rats, it was decided to evaluate the double competitive protein binding system as described originally by Robertson et al. (1971). The procedure for this double competitive protein binding system is summarized in Table 2.9.

Table 2.9. Procedure for the double competitive protein binding assay of oestradiol

- Add 20,000 dpm ^{3}H-oestradiol to plasma
- Extract plasma sample with ether
- Wash ether extracts and evaporate ether
- Purify residue by column chromatography (Sephadex LH-20, toluene:methanol - 85:15, v/v)
- Collect oestradiol fraction and evaporate solvent
- Add 200 µl of concentrated protein preparation to residue
- Incubate for 30 min at 30°C
- Add 50 µl concentrated dextran-coated charcoal suspension
- Incubate for 30 min at 30°C, centrifuge 5 min
- Take 200 µl supernatant and incubate for 3 min at 70°C. Cool to 30°C
- Add 100 µl diluted protein preparation
- Incubate steroid protein mixture for 30 min at 30°C
- Take 100 µl for recovery estimation
- Add 500 µl diluted dextran-coated charcoal suspension
- Incubate for 30 min at 30°C
- Centrifuge for 10 min
- Take 500 µl supernatant for counting of bound radioactivity

2.3.2.2. Results

A standard curve, obtained after addition of the concentrated cytosol preparation to known amounts of oestradiol, incubation, denaturation of the protein and competitive protein binding, is shown in panel B of Fig. 2.1. Blank values for the assay of column effluent or water, processed through the procedure, are summarized in Table 2.7. These values were not different from those obtained using the single binding assay. Recoveries for the total procedure, including extraction and purification, were 57.8 $\pm$ 11.0 (S.D.) % (n=24).

Estimation of oestradiol in peripheral plasma from male rats (5 ml aliquots) gave a result of 2.0 $\pm$ 0.7 (S.D.) pg/ml (n=3) after subtraction of the appropriate blank values. The difference between these results and those obtained with the single competitive protein binding assay (>200 pg/ml, see 2.3.1.2.) suggests that additional substances, different from oestradiol, are measured as oestradiol in the single competitive protein binding assay.

2.3.3. Radioimmunoassay

2.3.3.1. Method

The method used for the estimation of oestradiol in plasma samples using radioimmunoassay is described in appendix paper 1. Additional procedures for the estimation of oestradiol in testicular tissue are described in appendix paper 2.

2.3.3.2. Results

A standard curve, obtained after addition of known amounts of oestradiol to radioactive steroid, evaporation of solvent and addition of antibody is shown in panel C of Fig. 2.1.

The specificity of the antibody used for the estimation of oestradiol has been described by Exley et al. (1971). The specificity for estimation of oestradiol is high, but incorporation in the radioimmunoassay method of the chromatographic procedure, using Sephadex LH-20 microcolumns, reduced blank values appreciably as shown in Table 2.7. Results of accuracy and precision of the method are given in Table 1 from appendix paper 1. The sensitivity of the method, calculated from a blank value of 1.2 $\pm$ 5.6 (S.D.) pg (n=267), is 10.7 pg.

For peripheral plasma samples from male rats the low percentages binding, as observed in the competitive protein binding assay of oestradiol were never observed with this radioimmunoassay using the antibody raised against the oestradiol-6-(O-carboxymethyl)oxime-B.S.A. complex. This is at variance with data published by Labhsetwar (1972) and Weisz & Gunsalus (1973), who used antibodies against oestradiol-17β-hemisuccinate-B.S.A. for the estimation of oestradiol in peripheral plasma from male or immature female rats (see 2.3.1.2.). These authors suggested that an "oestradiol-like" substance, probably of adrenal origin, competes with oestradiol in their radioimmunoassay systems. Therefore, experiments on the concentration of oestradiol in peripheral plasma of adrenalectomized male rats might have been performed in order to ensure that the "oestradiol-like" substance measured in the present study is different from that described by Labhsetwar and Weisz & Gunsalus. Since the concentration of circulating oestradiol in male rats is very low, it was decided to perform such experi-

ments in immature female rats, where high oestradiol levels are measured around the age of 15 days (Meijs-Roelofs et al., 1973). As an additional advantage in this experimental procedure uterine weight can be recorded as a bioassay estimate of circulating oestrogens in these animals.

Table 2.10. summarizes the results of experiments with immature female rats which underwent either castration, or adrenalectomy or both operations at the age of 13 days, and were killed at the age of 15 or 17 days. Changes in oestradiol levels were correlated with the changes in uterine weight. It was concluded that the oestradiol measured was not of adrenal origin. These data provide evidence for the specificity of the estimation of oestradiol in the rat, using this radioimmunological technique.

2.3.4. Discussion

The comparison of the competitive protein binding method and the radioimmunological technique used for the estimation of oestradiol shows that the techniques have comparable accuracy and precision at the level of 30 and 50 pg (see Table 2.8. and appendix paper 1, Table 1). Theoretically, it might be expected that the sensitivity of the two methods would also be comparable, since the association constants for the protein-steroid complex, one of the factors determining the lowest level of detection (Ekins & Newman, 1970), are comparable for the rabbit uterine cytosol receptor (1-5 x 10^{10} l/mole at 4^{o}C, Mester et al., 1971) and the antibody against oestradiol (4 x 10^{9} l/mole, Exley et al., 1971). The similarity of the standard curves, obtained with each of the three different methods, also reflects that the three techniques have a comparable sensitivity of detection. However, the higher specificity of the antibody causes an important increase in sensitivity when extracts of water or biological materials are assayed. This

Table 2.10. Effect of ovariectomy (OVX), adrenalectomy (ADRX) or OVX combined with ADRX on day 13 post partum on the plasma oestradiol concentrations and uterine weights of immature female rats on days 15 and 17 (means ± S.D.) (from: Meijs-Roelofs et al., 1973)

Treatment on day 13	age at autopsy	number of animals	uterine weight (mg/100 g b.w.)	oestradiol (pg/ml plasma)
-	15	3	81.5 ± 5.9	46 (52.0, 39.0)
OVX	15	9	63.6 ± 7.2	13 (15.0, 11.2)
ADRX	15	14	75.2 ± 10.8	54 (58.0, 50.0)
OVX + ADRX	15	12	39.6 ± 4.2	0 (0, 0)
-	17	5	76.6 ± 3.8	29 (31.9, 25.6)
OVX	17	8	49.9 ± 3.7	4 (0.4, 7.8)
ADRX	17	9	73.2 ± 4.5	22 (30.6, 12.5)
OVX + ADRX	17	7	43.5 ± 4.2	4 (0, 7.3)

problem of specificity is even more important because of the presence of "oestradiol-like" material in peripheral plasma from the rat. The double competitive protein binding method, which might be used to overcome this problem, still lacks the specificity which is necessary to obtain a high sensitivity. Therefore, only the radioimmunological technique can be used to estimate oestradiol in plasma or tissue from male rats, since it combines an acceptable accuracy and precision with the required sensitivity.

For levels of oestradiol in peripheral plasma from male rats it is still difficult, if not impossible, to compare the specificity obtained with the radioimmunological technique with the specificity obtained with other methods. This is a consequence of the extremely low levels present: although gas-liquid chromatographic methods (Exley & Dutton, 1969) and bioassay procedures (Martin & Claringbold, 1960) for the estimation of picogram amounts of oestrogens have been described, it has not been possible to use these methods for the measurement of oestrogens in extracts from biological samples (Wotiz et al., 1967).

Summarizing, it was concluded that radioimmunoassay is the only technique which has the required sensitivity, accuracy and precision to estimate oestradiol levels in plasma and tissue of male rats. It is difficult to obtain an independent assessment of the specificity of the radioimmunoassay method for the levels estimated, but several observations (see section 2.3.3.2.) support the validity of the estimation of oestradiol in male rats with this particular radioimmunoassay system.

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CHAPTER 3. EXPERIMENTS IN VIVO

3.1. Introduction

Laqueur et al. (1927) described the presence of oestrogenic activity in urine from normal men. This was the first observation of the production of oestrogens in male mammals. Since then, several authors have attempted to define the source of these oestrogens, initially often by experiments in which testicular or adrenal function was stimulated or suppressed. The resulting changes in the concentration of urinary oestrogens were considered to reflect production and secretion of these steroids by gonads or adrenals. However, the concentration of oestrogens in urine increases after peripheral administration of androgens to men (Steinach & Kun, 1937), or even to adrenalectomized, gonadectomized subjects. The latter observation indicates that androgens can be converted to oestrogens in tissues, which are generally regarded as non-endocrine organs, provided that precursor steroids ("prehormones", Baird et al., 1968) are present in the circulation. This phenomenon could explain the increased urinary levels of oestrogens after trophic stimulation of testes or adrenals, and in the same way castration or adrenalectomy should result in a decreased concentration of oestrogens in urine. Therefore, no conclusion about the origin of oestrogens in men can be drawn from these qualitative experiments.

In this chapter, it will be attempted to review the available data on the source and production of oestrogens in the male. The first part of the chapter concentrates on the testis as a source of circulating oestradiol. The

Table 3.1. Concentrations of oestradiol (E_2) in testicular venous plasma (means ± S.D. (n))

Species/reference	E_2 in testicular venous plasma (pg/ml)	E_2 in peripheral venous plasma (pg/ml)	Testicular contribution to production rate of E_2 (%)*
Human			
Leonard et al. (1971)	948 ± 200 (5)	32 ± 21 (5)	"major part"
Kelch et al. (1972)	1049 ± 161 (8)	20 ± 4.5 (8)	14**
Longcope et al. (1972)	342 ± 275 (8)	32 ± 18 (8)	67**
Scholler et al. (1973)	1880 ± 1240 (9)	50 ± 28 (9)	16**
Baird et al. (1973)	2081 ± 825 (9)	32 ± 15 (9)	-
Dog			
Siegel et al. (1967)	456 ± 130 (5)	450 ± 240 (4)	-
Kelch et al. (1972)	100 ± 138 (4)	8.2 ± 2.1 (9)	15
Monkey			
Kelch et al. (1972)	50 ± 20 (4)	17 ± 10.4 (4)	24
Rat			
Appendix paper 1	17.5 ± 8.4 (43)	2.0 ± 0.9 (12)	25**

* Calculated as $\frac{(E_{2_{t.v.}} - E_{2_P}) / E_{2_P}}{(T_{t.v.} - T_P) / T_P}$ (t.v. = testicular venous concentration, P = peripheral concentration, T = testosterone)

** Corrected for different MCR for E_2 and T (Baird et al., 1969a)

second part of this chapter deals with the extra testicular production of oestradiol.

3.2. The testis as a source of oestrogens

3.2.1. Oestradiol in testicular venous blood

Final proof of secretion of a hormone from an endocrine gland can only be obtained by comparing the concentration of the hormone in the affluent (arterial) and effluent (venous) blood of the gland. In practice, it is generally assumed that the hormone concentration in the arterial blood or plasma is equal to that in the peripheral circulation. However, Free & Tillson (1973) showed that peripheral and glandular arterial concentrations of a hormone are not necessarily equal.

French et al. (1965) showed oestradiol secretion from the testes of a human pseudohermaphrodite by measuring a difference between the concentrations of the steroid in testicular venous and peripheral plasma. Results on the secretion of oestradiol from normal testes from different species are summarized in Table 3.1. In this table the contribution of testicular secretion of oestradiol to the blood production rate of the steroid is calculated from the ratios of testicular venous and peripheral concentrations of oestradiol and testosterone; the peripheral concentration of the latter steroid is assumed to result completely from testicular secretion. According to this calculation, the testis secretes 15-25% of the circulating oestradiol. This may lead to the conclusion that the oestrogens directly secreted by the testis play a quantitatively less important role in possible peripheral processes than the oestrogens formed from prehormones, which may be secreted by the adrenal or the testis also. A similar conclusion can be drawn from the calculations of blood pro-

duction rates and from peripheral production rates of oestradiol in men. These data will be discussed in section 3.3.

3.2.2. Oestradiol in testicular tissue

The presence of oestrogenic activity in testis tissue was first shown by Fellner (1921), who described the effects of extracts of bovine testes on uterus and mammary tissue of female rabbits, and concluded that the histological results obtained with testis extracts were comparable with those obtained after injections of ovarian or placental extracts. Many authors described the detection and measurement of oestrogenic hormones in testes from different species (see: Diczfalusy & Lauritzen, 1961, Parkes, 1966, Table 3.2.). It is difficult to predict the concentrations of the physiologically active, unconjugated oestradiol in the testis from stallion or man from the data in Table 3.2., since no correction for recovery was performed in the estimations in these species. Furthermore, hydrolytic procedures were used in these estimations, while Raeside (1969) showed that at least in stallion testes the larger part of testicular oestrogen is present as the sulphate conjugate.

Table 3.2. Oestradiol in testis tissue from different species

Species	Concentration (ng/g)	Reference
Horse	210	Beall (1940b)
Human (adult)	5.7	Goldzieher & Roberts (1952)
	8	Anliker et al. (1957)
(foetal)	1.9	Reyes et al. (1973)
Sheep (adult)	0.035, 0.065	Attal et al. (1972)
(foetal)	20	Attal (1969)
Rat	0.038	Appendix paper 2

3.2.3. Oestrogens in semen

Apart from the production of hormones, which are secreted in the testicular venous blood, the testis produces spermatozoa. These spermatozoa are ejaculated in the semen, together with excretions from prostate and seminal vesicles. Therefore, substances present in semen might be of testicular origin, and probably originate from the seminiferous tubules. The presence of oestrogenic material in human semen was first suggested by Green-Armytage (1943), and then confirmed by Green-Armytage et al. (1947), Riisfeldt (1948) and McCullagh & Schaffenburg (1951). These authors used a bioassay system for the assessment of oestrogen concentrations in semen. Diczfalusy (1952) was the first to assay seminal oestrogens by a physico-chemical technique, i.e. fluorimetry, using counter-current distribution as a means of separating and identifying oestrone, oestradiol and oestriol.

Schaffenburg & McCullagh (1954) showed the presence of oestrogenic material in bull spermatozoa, and suggested the germinal epithelium as a possible source of seminal oestrogens, whereas Raboch & Rezábek (1963) defined the Leydig cell as the place of oestrogen production after bioassay of seminal oestrogens from patients with different testicular disorders. Another possible explanation for the presence of oestrogens in semen is a transfer of oestrogens from blood to semen via prostate or seminal vesicles. This possibility is supported by the observations of Eriksson & Baker (1966), who observed that oestrogens were transferred to female rats after copulation with male rats, that were treated by peripheral oestradiol-benzoate injections. Furthermore, Stanek & Dostal (1972) showed the presence of plasma proteins in seminal vesicle fluid, and Steeno et al. (1969) and Tea et al. (1972) found a close correlation between peripheral plasma levels and seminal plasma levels of dehydroepiandrosterone sulphate and oestrone/oestradiol

respectively. The observation of the latter authors, that seminal oestrogen levels are higher than those in peripheral plasma, can be explained by the observation of Varkarakis et al. (1972), that oestrogens which were taken up from the blood are excreted in the prostatic fluid.

3.2.4. Regulation of the testicular production and secretion of oestrogens

Maddock & Nelson (1952) observed after prolonged administration of HCG to men an increased urinary excretion of oestrogens, concomitant with testicular Leydig cell hyperplasia. This increase in oestrogen excretion was larger than that of 17-ketosteroids. These observations were confirmed by Diczfalusy (1957), Givner et al. (1960), Jayle et al. (1962), Morse et al. (1962) and Smith (1971). Conti et al. (1955) and Frøland et al. (1973) excluded adrenal influences through administration of dexamethasone together with the trophic hormone. The same conclusion was reached by Fishman et al. (1967), who observed that the relative increase of the oestradiol production rate always superseded that of testosterone after administration of HCG to five men. Similar studies were performed in boars by Busch & Ittrich (1970) and Liptrap & Raeside (1971). It was concluded from these observations that the secretion of oestradiol is under the influence of HCG, and that the production of testicular oestradiol takes place in the interstitial compartment of the testis.

It is not possible, however, to discuss an increase of the testicular secretion of oestradiol on basis of data obtained by measuring urinary concentrations of the steroid, since the metabolism of its main peripheral precursor, testosterone (see section 3.3.), changes when the concentration is increased above physiological levels (Southren et al., 1968). This is certainly the case after stimulation

with HCG. Furthermore, the urinary method for the measurement of the production rate of oestradiol is not satisfactory (section 3.3.). Therefore, the method of choice for the study of the regulation of production and secretion of testicular oestradiol has to be the experiment at the level of the testis itself, and not the study of peripheral parameters. Such experiments have been described by Kelch et al. (1972), who observed that the concentrations of oestradiol in canine testicular venous blood were unchanged after administration of HCG. Weinstein et al. (1974) showed, however, that prolonged administration of HCG to men caused an increased secretion of oestradiol from the testis: this (eightfold) increase in the secretion of oestradiol was higher than that for testosterone (fivefold). The experiments described in the appendix papers 1 and 2 lead to the conclusion that the testicular secretion of oestradiol in the rat is not influenced by prolonged administration of HCG. The reason for this species difference is not clear.

In addition to the experiments described in the appendix papers 1 and 2, a more detailed study was performed in order to investigate the effects of hypophysectomy, prolactin administration and increased amounts of interstitial tissue on the concentration of oestradiol and testosterone in testis tissue and in testicular venous plasma. The experimental procedures used were similar to those described in appendix papers 1 and 2. The effect of hypophysectomy on steroid concentrations in testicular tissue and testicular venous plasma is shown in Fig. 3.1. and 3.2. Testosterone levels in both testicular venous blood and in testis tissue decrease with increasing length of hypophysectomy. The decrease in the tissue concentration of oestradiol shows the same trend. However, there was no significant change in the testicular venous concentration of this steroid after hypophysectomy, in the same way as there was no change after prolonged HCG administration (appendix paper 1). These results indicate that the net production of oestradiol in the testis is not influenced by trophic stimulation.

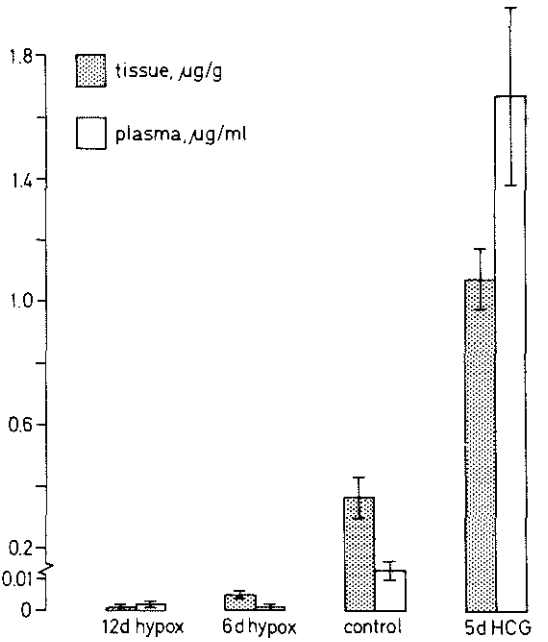

Fig. 3.1 Testosterone concentrations (means ± S.E.M.) in testicular venous plasma and testis tissue from hypophysectomized (HYPOX), intact (CONTROL) and HCG-treated (5 d HCG) intact rats.

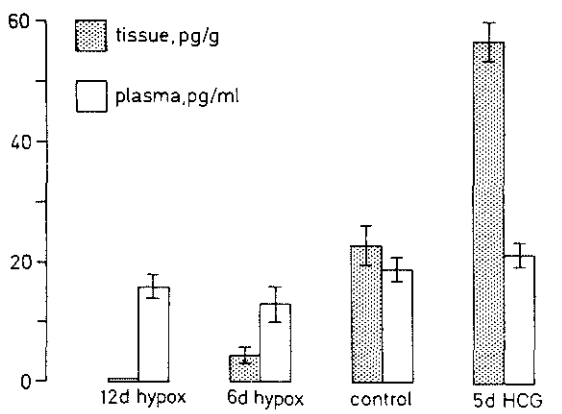

Fig. 3.2. Oestradiol concentrations (means ± S.E.M.) in testicular venous plasma and testis tissue from hypophysectomized (HYPOX), intact (CONTROL) and HCG-treated (5 d HCG) intact rats.

The amount of oestradiol present within the testis is influenced by hypophyseal factors, probably LH, as indicated by the effect of HCG. The mechanism regulating the testicular oestradiol concentrations is not clear. It is tempting to suggest that the concentration of oestradiol binding protein, which is present in interstitial tissue (Brinkmann et al., 1972) plays a role. A lack of specificity of the method used to estimate oestradiol in testicular venous plasma might be an alternative explanation for the data shown in Fig. 3.2. The changing testicular tissue/venous plasma ratios and the results presented in section 2.3.3.2. render this possibility less likely, however.

The intravenous injection of prolactin (200 µg, NIH-P-59) does not enhance the secretion or testicular tissue level of testosterone and oestradiol in intact rats (Tables 3.3. and 3.4.). This might be expected, since Hafiez et al. (1972) showed that prolactin has a permissive action on testicular testosterone production; no direct effects of this hormone could be shown. Finally, some experiments were performed in rats, which were fed with a diet, deficient in essential fatty acids (EFA-deficient rats). In these rats, an increase in the relative amount of interstitial tissue takes place as a result of the involution of the seminiferous tubules. Spermatogenesis is arrested at the secondary spermatocyte stage, and histology of the hypophysis suggests impaired androgen secretion from the testis (Panos & Finerty, 1954). On the other hand, Ahluwalia et al. (1968) suggested that the androgen production might be increased during incubations with testes from EFA-deficient rats. The concentrations of oestradiol and testosterone were measured in total testis tissue (Fig. 3.3.) and in testicular venous plasma (Fig. 3.4.) from EFA-deficient rats and control animals. It is concluded that the net production of testosterone and oestradiol in EFA-deficient rats does not differ from that in controls, assuming that the testicular blood flow is not altered. This observation is at variance with the results of Ahluwalia et al. (1968),

Table 3.3. Concentrations of oestradiol (E_2) and testosterone (T) in testicular venous plasma of intact male rats after administration of prolactin (means ± S.D.)

Treatment	E_2 (pg/ml)	T (ng/ml)	E_2:T (pg:ng)
none	11.3 ± 3.4 (6)	49.9 ± 27.5 (6)	0.28 ± 0.13 (6)
200 µg prolactin i.v.*	4.9 ± 2.5 (5)	79.8 ± 18.2 (5)	0.06 ± 0.03 (5)

The number of estimations is given in parentheses.

* 200 µg prolactin (NIH-P-59) were administered immediately before collection of testicular venous blood.

Table 3.4. Concentrations of oestradiol (E_2) and testosterone (T) in testis tissue of intact male rats after administration of prolactin (means ± S.D.)

Treatment	E_2 (pg/g)	T (ng/g)	E_2:T (pg:ng)
none	19.6 ± 11.0 (12)	186 ± 67 (6)	0.12 ± 0.05 (6)
200 µg prolactin i.v.*	16.4 ± 5.7 (10)	137 ± 92 (5)	0.16 ± 0.12 (5)

The number of estimations is given in parentheses.

* 200 µg prolactin (NIH-P-59) were administered immediately before collection of testicular venous blood.

probably because these authors did not correct their results for the altered ratio between interstitial tissue and seminiferous tubules in testes from EFA-deficient animals.

The high tissue concentrations for testosterone and oestradiol when compared with those in control animals might reflect that both steroids are localized in the interstitial tissue.

3.3. Extratesticular sources of oestradiol

From the data in Table 3.1. it appears that the testis secretes 15-25% of the total amount of oestradiol in the peripheral circulation in different species. These calculations were based on the ratios of the concentrations of oestradiol and testosterone in testicular venous and peripheral plasma, and on the assumption that peripheral testosterone is completely secreted by the testis. In this section, the testicular contribution to the total blood production will be calculated by comparison of the production rate of oestradiol with the rate of conversion of prehormones to this steroid. The difference between these quantities represents the amount of oestradiol which is secreted by endocrine glands: the testes, as discussed previously, or the adrenals; Corey & Britton (1934) were the first to describe oestrogenic activity in adrenal tissue. It will be concluded that the testis is the main glandular source of oestradiol, while about 80% of the circulating oestradiol is formed peripherally from testosterone or oestrone.

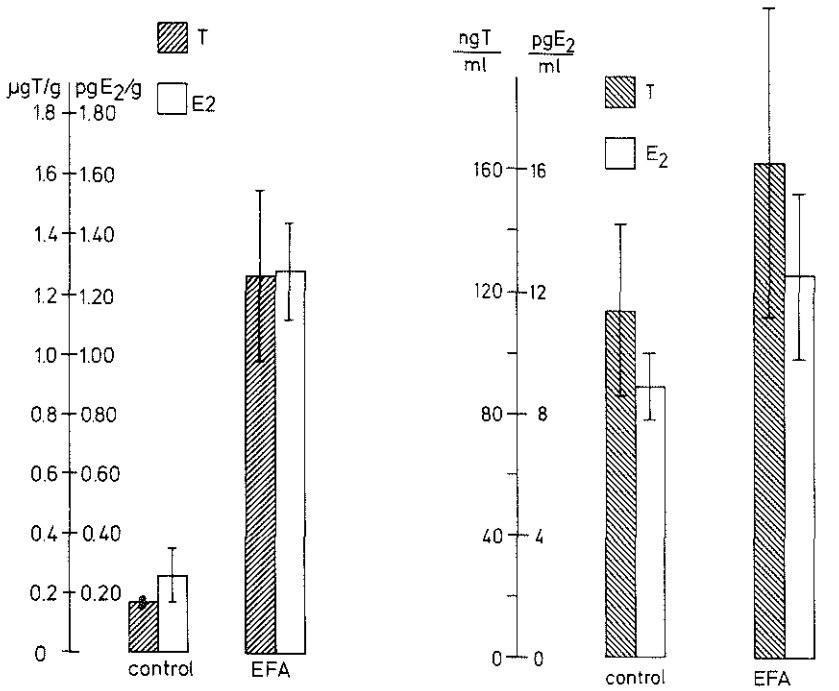

Fig. 3.3. Concentrations of oestradiol (E_2) and testosterone (T) in testicular tissue from normal rats and from rats fed with a diet, deficient in essential fatty acids (EFA) (means ± S.E.M.).

Fig. 3.4. Concentrations of oestradiol (E_2) and testosterone (T) in testicular venous plasma from normal rats and rats fed with a diet, deficient in essential fatty acids (EFA) (means ± S.E.M.).

3.3.1. Production rates of oestrogens in male mammals

The production rate of a steroid is defined as the amount of steroid entering the circulation per unit time. It is equal to the algebraic sum of the secretion rate of the steroid (the amount of steroid entering the circulation from an endocrine gland per unit time, S in Fig. 3.5.) and the peripheral conversion from prehormones to the steroid (P_1 + P_2, Fig. 3.5.). The theory and methods used for the estimation of steroid production rates have been reviewed by Tait & Burstein (1964). Two different types of methods can be discerned: procedures for measurements of the "urinary" and the "blood production rate" (PR_U and PR_B).

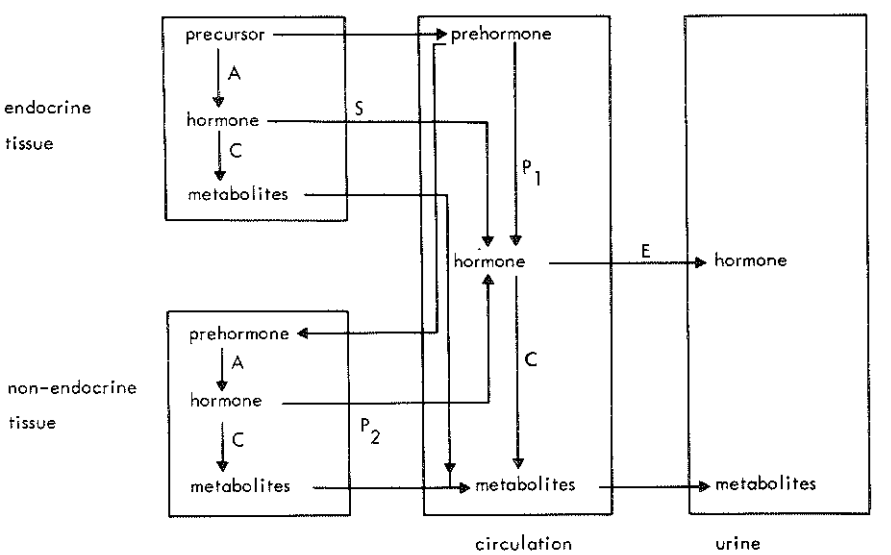

Fig. 3.5. Relationship between secretion (S), biosynthesis (A), degradation (C), net production (A-C=S), blood production rate ($S+P_1+P_2$) and excretion (E) of a hormone.

Table 3.5. Urinary production rates (μg/24 h) of oestradiol in normal men (means ± S.D. (n))

Radioactivity injected as	Production rate calculated from specific activity of urinary			Reference
	oestrone	oestradiol	oestriol	
Oestradiol	49, 90	47, 89	65, 169	Morse et al. (1962)
Oestradiol	67 ± 10 (5)	70 ± 13 (5)	234 ± 342 (5)	Lipsett et al. (1966)
Oestrone	46 ± 13 (12)			Crowell et al. (1967)
Oestradiol		29 ± 14 (9)		Eren et al. (1967)
Oestradiol		32 ± 13 (6)		Gabrilove et al. (1970)
Oestradiol		40		MacDonald et al. (1971)
Oestrone/ oestradiol	40 ± 12 (7)	42 ± 12 (7)		Kirschner & Taylor (1972)

In order to estimate PR_U, a known dose of radioactive steroid (R^* dpm) is injected intravenously. Urine is collected for a period t, which is sufficiently long to recover the total amount of radioactivity, associated with the steroid or one of its metabolites in the urine. Mass and radioactivity in the steroid or its metabolite are measured, and PR_U is calculated according to the formula:

$$\frac{\text{dpm excreted as metabolite}}{\text{dpm injected}} = \frac{\text{mass excreted as metabolite}}{\text{mass produced}},$$

$$\text{or} \quad \frac{\text{mass produced}}{t} = \frac{\text{mass excreted as metabolite}}{\text{dpm excreted as metabolite}} \times \frac{R^*}{t},$$

$$\text{or} \quad PR_U = \frac{R^*}{\sigma . t}, \text{ where } \sigma = \text{specific activity of the isolated metabolite}$$

Values of PR_U for oestradiol, measured in normal men, are summarized in Table 3.5. Calculations of PR_U from the specific activities of urinary oestrone or oestradiol agree well, suggesting that both urinary steroids originate from the same blood precursor steroids, including oestrone and oestradiol themselves (Kirschner & Taylor, 1972).

Blood production rates are calculated from the product of the plasma or blood concentration of the hormone (X) and its metabolic clearance rate (MCR): $PR_B = X \times MCR$. The MCR is defined as the volume of plasma or blood, which is cleared completely and irreversibly of the hormone per unit time. This quantity can be estimated by the method of single injection of a known amount of the radioactive steroid (R^*), or by continuous infusion of the radioactively labelled hormone until the steady state is reached. In both methods blood samples are taken at timed intervals after the injection or after the start of the infusion of labelled steroid. The concentration of the radioactive hormone in these samples (X', expressed as dpm/volume) is estimated and the MCR is calculated according to the formula:

Table 3.6. Plasma concentrations, plasma metabolic clearance rates (MCR_P) and blood production rates (PR_B) of oestradiol (E_2) in normal men (means ± S.D. (n))

Method for MCR_P	Method for plasma E_2*	E_2 (ng/l)	MCR_P (l/24 h)	PR_B (µg/24 h)	Reference
continuous inf.			1890 ± 330 (11)		Longcope et al. (1968)
continuous inf.			1640 ± 313 (5)		Hembree et al. (1969)
continuous inf.			1597 ± 234 (9)		Longcope & Tait (1971)
single injection	CPB	26 (3)	2430 (3)	62 (3)	Concolino et al. (1970)
	DID	26 ± 6 (7)			Baird & Guevara (1969)
	CPB	24 ± 6.5 (6)			Korenman et al. (1969)
	CPB	30 ± 9 (15)			Mayes & Nugent (1970)

* CPB = competitive protein binding method
DID = double isotope dilution method

$$MCR = \frac{R^{*}}{\int_{t=0}^{\infty} X' dt}$$

in which the denominator of the quotient can be explained graphically as the area under the t = f(X') curve (Fig. 3.6.). When continuous infusion is used, this formula can be transformed to:

$$MCR = \frac{R^{*}}{X'T}$$

where T = infusion time. Here too, the denominator in the quotient represents the area under the t = f(X') curve, which is a straight line parallel to the x-axis. Plasma concentrations and MCR-values of oestradiol for normal men are summarized in Table 3.6. The only study in which MCR

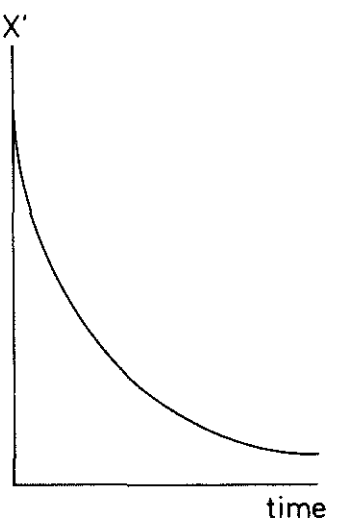

Fig. 3.6. Curve obtained after intravenous injection of a radioactive steroid. The area under the curve can be used in the estimation of the metabolic clearance rate of the steroid. X' = plasma concentration of the radioactive hormone.

and plasma concentration for oestradiol have been measured in the same subjects is that of Concolino et al. (1970). Calculation of the product of mean values for plasma concentration and MCR results in a PR_B of 45 µg/24 h.

It might be concluded from the agreement between the values for the production rates of oestradiol obtained by urinary and blood methods, that a reliable estimate of this production rate in men can be obtained using either of the two types of measurement. However, both the urinary method (Lipsett et al., 1966) and the blood method (Hembree et al., 1969) have been criticized. The influence of the enterohepatic circulation of oestrogens (Sandberg & Slaunwhite, 1957, Howard et al., 1969) and the existence of an oestrone sulphate pool, which is completely formed from circulating unconjugated oestrogens (Ruder et al., 1972, Longcope, 1972), make it unlikely that oestradiol is irreversibly metabolized, as is assumed in calculating the MCR. Finally, a diurnal rhythm in the plasma concentration of oestrogens in normal men has been described (Baird & Guevara, 1969). This phenomenon makes it more difficult to calculate PR_B from plasma concentrations and MCR.

Production rates of oestradiol in male subjects of other species than the human, have not been reported in the literature. For male rats the blood production rate of oestradiol was calculated in appendix paper 1 from the estimated concentration of the steroid in peripheral plasma and the MCR in normal men (Longcope et al., 1968), which was corrected for body weight according to the formula:

$$\frac{MCR}{(weight)^{\frac{3}{4}}} = \text{constant}$$

This approximation was found to be valid in a comparison between the MCR for oestradiol in female humans and rats by de Hertogh et al. (1970), even though sex steroid binding globulin is not present in rat plasma. The calculated MCR of 25.6 l/24 h in male rats with a body weight of 225 g,

combined with a peripheral plasma concentration of 2 pg/ml (appendix paper 1) resulted in a production rate of 51 ng/24 h.

3.3.2. Peripheral conversion of prehormones to oestradiol

Diczfalusy & Lauritzen (1961) concluded from studies on conversion of peripherally administered androgens to urinary oestrogens that this conversion could occur in the absence of steroid-secreting glands, thus excluding the possibility of androgen-stimulated oestrogen secretion. The same conclusion was reached by Braun-Cantilo et al. (1962), who administered tritiated testosterone to normal and ovariectomized women and isolated labelled oestrogens from urine: these authors showed the direct conversion from an androgen to oestrogens. In a similar way, the direct conversion of testosterone to urinary oestrogens was also shown by Ahmad & Morse (1965) and Epstein et al. (1966). Peripheral androstenedione is also a good precursor for urinary oestrogens (MacDonald et al., 1967) while the conversion of dehydroepiandrosterone and its sulphate to urinary oestrogens is low in men and non-pregnant women (Mancuso et al., 1965, Oertel & Menzel, 1970, Adams & Brown, 1971).

Quantitative data on the contribution of peripheral conversion of prehormones to the production rate of oestradiol, which was assumed to be 45 µg/24 h (Tables 3.5. and 3.6.), are summarized in Table 3.7. The theoretical aspects of this type of calculations have been reviewed extensively by Baird et al. (1968). Blood production rates of the prehormones are calculated from MCR and peripheral concentrations. The conversion ratio for the peripheral conversion of the prehormone to oestradiol, represents the ratio of ^{3}H- and ^{14}C-label in plasma oestradiol during simultaneous infusion of prehormone and oestradiol each marked with a different

Table 3.7. Data on the quantitative contribution of circulating androgens to oestradiol (E_2) production in men. Symbols are explained in the text

	Prehormone		
	testosterone	androstenedione	oestrone
Blood production rate of prehormone (mg/day)	7.2[§]	2.7[§]	0.164[§]
Data for calculation of PR_B			
Conversion ratio (prehormone → E_2)	0.0018[*]		0.05[§]
$MCR_{prehormone}$ (l/day)	1080[§]		2570[§]
MCR_{E_2} (l/day)	1890[§]		1890[§]
$\mathcal{D}^{prehormone \rightarrow E_2}_{BB}$	0.38%[*]		5%[§]
E_2 formed from prehormone (mg/day)	0.027		0.0082
% of total production of E_2	60		18
Data for calculation of PR_U			
Urinary conversion rate to E_2	0.3%[**]	0.82%[**]	
E_2 formed from prehormone (mg/day)	0.022	0.022	
% of total production of E_2	49	49	

[§] Baird et al. (1969a)

[*] Longcope et al. (1969)

[**] MacDonald et al. (1971)

radioactive label until steady state conditions are reached. Correction of the conversion ratio for the difference in MCR for prehormone and product (oestradiol) results in the transfer constant ρ. The transfer constant represents the fraction of circulating prehormone which is converted to oestradiol. The mass of oestradiol, arising from peripheral production from prehormones, is then calculated as the product of ρ and the production rate of the prehormone. On basis of such calculations, the total contribution of peripheral conversion of prehormones to the production rate of oestradiol in men is calculated as 78%.

Using urinary conversion ratios in these calculations, it appears from the data in Table 3.3. that the total production rate of oestradiol can be accounted for by the peripheral production of the steroid from testosterone and androstenedione. The urinary conversion rate is calculated after simultaneous injection of prehormone and product, each marked with a different radioactive label, collection of urine, and estimation of the isotopic ratio in the isolated product steroid or one of its metabolites. However, Kelly & Rizkallah (1973) showed that this type of calculation may be fallacious in case of the conversion of androgens to oestrogens, because the isotopic ratio was not constant in the oestrogens and their metabolites.

The site of the peripheral conversion of androgens to oestrogens is unknown, but it has been shown during incubation studies that this conversion can occur in brain tissue (Naftolin et al., 1971), in the liver (Wortmann et al., 1973), in fat cells (Schindler et al., 1972) and in intestinal bacteria (Goddard & Hill, 1972).

Quantitative studies on the peripheral conversion of androgens to oestrogens in male experimental animals have not yet been described.

3.3.3. The adrenal as a source of oestrogens

Baird et al. (1969b) and Saez et al. (1972) estimated concentration gradients of oestrone over the adrenal gland in human subjects but did not detect a secretion of oestradiol. Levy et al. (1965) and Meyer (1955) isolated 19-hydroxyandrostenedione after perfusion of human and bovine adrenals: this steroid is a possible intermediate in the biosynthesis of oestrogens from androstenedione. Beall (1940a) isolated oestrone from bovine adrenal tissue, and in vitro biosynthesis of oestrogens during incubation of normal adrenal tissue has been described, although the conversions were always very small (Engel & Dimoline, 1963, Chang et al., 1963, Vinson & Jones 1964). Feminizing adrenal tumours are a well-known clinical disorder (reviewed by Gabrilove et al., 1965 and Vague et al., 1970). Radioactive aromatic steroids have been isolated after incubation of tumour tissue with pregnenolone (Rose et al., 1969, Axelrod et al., 1969). The urinary excretion of oestrogens is increased after administration of ACTH and decreased after adrenalectomy (Brown et al., 1959, Engel, 1962, Schweppe et al., 1967); peripheral oestrone levels follow the same pattern (Saez et al., 1972). Such data, however, do not yield information on the question of direct secretion of oestrogens by the adrenal.

Summarizing, there is evidence that the adrenal secretes oestrone. There are no indications for the secretion of oestradiol from the normal adrenal gland, and information on the regulation of the adrenal oestrogen secretion is not available.

3.4. Conclusions

From the results presented in the appendix papers 1 and 2 and in this chapter, the following conclusions are drawn:

1. The testis of the normal adult rat secretes oestradiol.
2. In the rat and in man, the amount of oestradiol secreted by the testis constitutes about 20% of the total blood production rate for this steroid.
3. The testicular secretion of oestradiol is not affected by the prolonged administration of HCG or by hypophysectomy. However, the testicular tissue levels of oestradiol are clearly changed by these treatments.
4. No effect of FSH or prolactin on the testicular secretion or production of oestradiol or testosterone could be shown.
5. Oestradiol is mainly localized in the interstitial tissue of the testis.
6. The occurrence of oestrogens in seminal fluid does not reflect that oestrogens are produced in either the seminiferous tubules or interstitial tissue.
7. The direct adrenal contribution to the circulating concentration of oestradiol is negligible.

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CHAPTER 4. EXPERIMENTS IN VITRO

4.1. Introduction

A large number of reports describe the formation of oestrogens from radioactive or non-radioactive precursors in several tissues in vitro. The conversion of exogenous androgens to oestrogens takes place readily in placental tissue (see: Ryan, 1958, 1959 and Baggett et al., 1959). The mechanism of the conversion in placental tissue has been studied extensively, and since the observation that 19-hydroxyandrostenedione is a good precursor for oestrogen formation (Meyer, 1955), the biosynthesis of steroids, which contain an aromatic A-ring is thought to proceed via 19-oxygenated androstenedione or -testosterone as shown in Fig. 4.1. (see: Dorfman & Ungar, 1965 and Schwarzel et al., 1973).

Apart from the in vitro biosynthesis in placental tissue, the production of oestrogens during incubation studies has also been shown during incubation of the different compartments of the ovary (see: Savard, 1973), of adrenal tissue (section 3.3.3.) and of several non-endocrine tissues (section 3.3.2.). Furthermore, all precursors shown in Fig. 4.1., with the exception of 19-oxygenated androgens, were converted to oestrone or oestradiol during in vitro studies with testicular tissue from different species (Table 4.1.). The literature until 1967 on oestrogen biosynthesis in pathological human testes has been reviewed by Grant et al. (1967). With the exception of conversions in stallion testes, the amount of radioactivity recovered after incubation in the oestrogenic fractions was usually

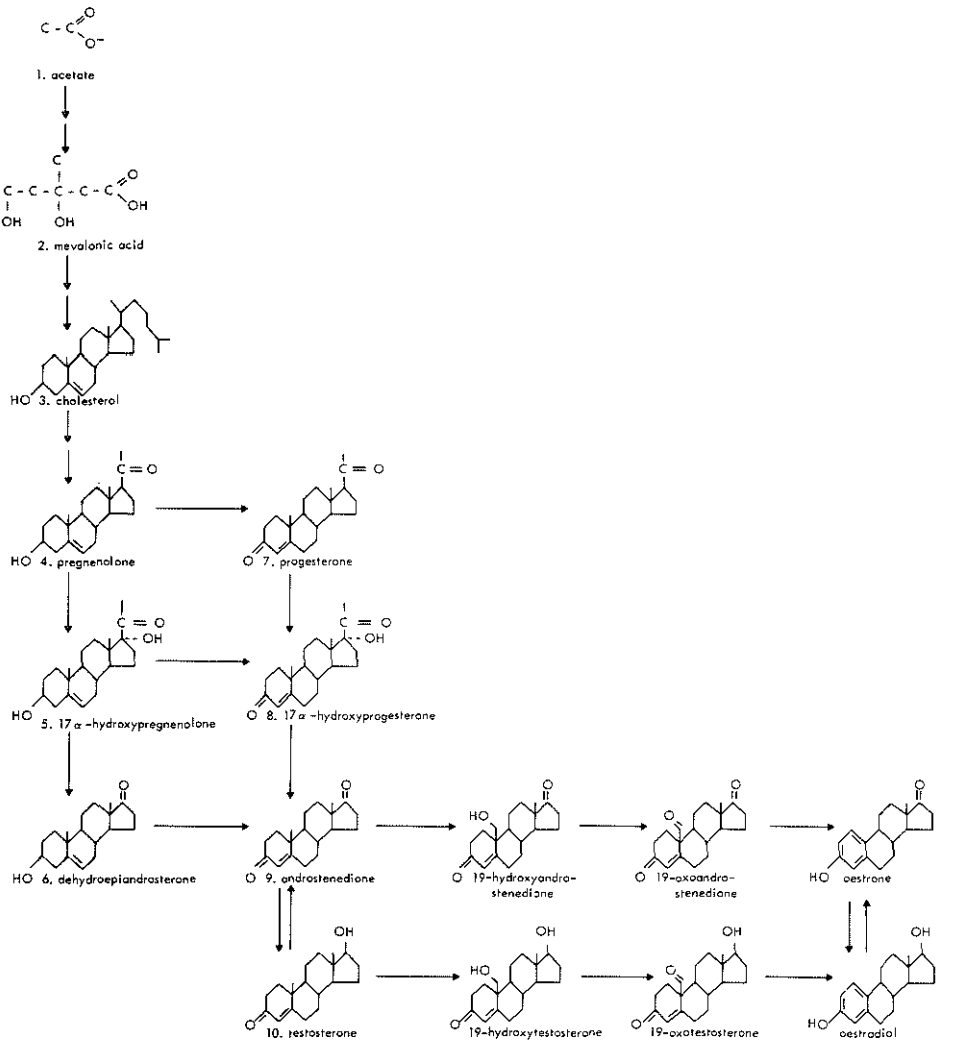

Fig. 4.1. Possible biosynthetic pathways leading to the formation of oestrone and oestradiol.

Table 4.1. Precursors converted to oestrone or oestradiol during incubation of testicular tissue preparations (numbers of precursors refer to Fig. 4.1.)

Precursor of oestrogens	Species				
	human	equine	canine	rat	chicken
1. acetate	1,2*				3
2. mevalonic acid	4				
3. cholesterol	1				
4. pregnenolone	5,6§,7+,8§,9+	10	11		
5. 17α-hydroxy-pregnenolone		10			
6. dehydroepian-drosterone	6§,7+,9+	10,12	11o,13		
7. progesterone		10,12		14	
8. 17α-hydroxy-progesterone		10,12			
9. androstenedione		10,12			
10. testosterone		10,12,15			

* pseudohermaphrodite
§ Leydig cell tumour
\+ testicular feminization
o Sertoli cell tumour

1. Rabinowitz (1958)
2. Rice et al. (1967)
3. Guichard et al. (1973)
4. Rabinowitz & Ragland (1958)
5. Axelrod (1965)
6. Pierrepoint et al. (1966)
7. Pérez-Palacios et al. (1971)
8. Ances et al. (1971)
9. Cardiff et al. (1972)
10. Bedrak & Samuels (1969)
11. Pierrepoint et al. (1967)
12. Oh & Tamaoki (1970)
13. Pierrepoint (1968)
14. Ficher & Steinberger (1971)
15. Baggett et al. (1959)

lower than 1%, while some authors (Wade et al., 1968, Rice et al., 1966, Bardin et al., 1969) were not able to identify any radioactive oestrogens after incubation of human testicular material with androgen or ^{14}C-acetate.

The data obtained in the studies mentioned are all qualitative in nature: although conversion of precursors to oestrogens was observed, the degree of dilution of the exogenous radioactive or non-radioactive precursor with endogenous precursors was not taken into account in any of these experiments. Therefore, in the experiments described in this chapter the endogenous production of oestradiol and testosterone was measured during incubation of rat testicular tissue. It was also attempted to measure the conversion of radioactive androgens to oestradiol under the same conditions in order to assess the precursor role of the androgens quantitatively. The results obtained during these in vitro studies will be compared with those from the in vivo experiments discussed in chapter 3. Finally, it was attempted to resolve the question (see 1.4.) about the intratesticular localization of the biosynthesis of oestrogens. Therefore, in addition to the incubations with total testis tissue, separated seminiferous tubules and interstitial tissue were also incubated.

4.2. Materials and methods

4.2.1. Animal techniques

Wistar rats with a body weight of 200-250 g were used throughout this study, with the exception of the rats that were fed with a diet deficient in essential fatty acids.

Testicular tissue was obtained immediately after decapitation of the animals and was kept in ice cold buffer solution until it was incubated (usually not longer than 30 min, when total testis was incubated; dissection

generally took 60 min more). Dissection of testicular tissue into seminiferous tubules and interstitial tissue was performed as described by Christensen & Mason (1965) in a dissection medium containing sucrose (0.25M) and EDTA (1 mM).

4.2.2. Steroids and steroid estimations

Non-radioactive steroids were obtained from Steraloids (Pawling, U.S.A.) and used without further purification. Cyanoketone is a product of Stirling-Winthrop, New York, U.S.A. Radioactive androgens and oestrogens were obtained from New England Nuclear (Boston, U.S.A.) or from the Radiochemical Centre (Amersham, England). [1,2,6,7-^{3}H]-Androstenedione was prepared from [1,2,6,7-^{3}H]-testosterone by chromium trioxide oxidation. Specific activities of these steroids, as quoted by the manufacturer and place of attachment of the radioactive atoms to the steroid nucleus are shown in Table 4.2. Radioactive steroids were purified before use by paper- and thin-layer chromatography, using

Table 4.2. Specific activities of steroids used in incubation studies

Steroid	Specific activity
[4-^{14}C]-androstenedione	60 mCi/mMol
[4-^{14}C]-testosterone	58.8 mCi/mMol
[4-^{14}C]-dehydroepiandrosterone	58.8 mCi/mMol
[4-^{14}C]-oestradiol	58 mCi/mMol
[1,2,6,7-^{3}H]-androstenedione	85 Ci/mMol
[1,2,6,7-^{3}H]-testosterone	85 Ci/mMol
[2,4,6,7-^{3}H]-oestradiol	105 Ci/mMol
[6,7-^{3}H]-oestrone	48 Ci/mMol
[6,7-^{3}H]-oestradiol	40 Ci/mMol

purity criteria as described earlier (de Jong & van der Molen, 1972). Endogenous oestradiol and testosterone were estimated by radioimmunoassay, as described in chapter 2. Protein was estimated according to the method of Lowry et al. (1951).

4.2.3. Incubation conditions

When testes were incubated in Krebs-Ringer buffer, the tissue was homogenized in this buffer, pH 7.4, containing 0.2% glucose (w/v), using a Potter-type homogenizer. Incubations were performed after addition of NADPH and NADH (final concentration 1 mM), glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (0.3 Kornberg Units). The homogenates were incubated for 3 h in a shaking water bath at 34°C under an atmosphere of 95% O_2 and 5% CO_2.

In a second series of incubations, 800xg supernatant of homogenized total testis tissue, seminiferous tubules or interstitial tissue was used. Testes were homogenized in a solution containing sucrose (0.25M) and EDTA (1 mM), using an automatic Potter homogenizer. After centrifugation at 800xg for 10 min, the supernatant was collected and incubated in a medium and under conditions as described by van der Vusse et al. (1973). Dissection of testis tissue before these incubations took place under the homogenization medium. When radioactive or non-radioactive steroids were incubated with tissue or tissue homogenates, these steroids were added to the incubation vessel dissolved in ethanol. The solvent was evaporated under nitrogen at 45°C and the residue was taken up in 50 µl ethanol. Then buffer solutions and tissue or tissue homogenates were added.

4.2.4. Techniques used for the separation and identification of steroids

After incubation of testis homogenates with radioactive androgens non-radioactive oestrone and oestradiol (25 µg each) were added to the incubation vessels as carrier steroids. Steroids were extracted from the incubation medium with ethyl acetate and the extracts were purified according to the scheme in Fig. 4.2. ^{3}H-Labelled steroid was only added after incubation with ^{14}C-labelled precursors. In one experiment, radioactive oestrogens were purified after incubation by chromatography on Sephadex LH-20 columns.

After incubation of ^{3}H-labelled precursors with testi-

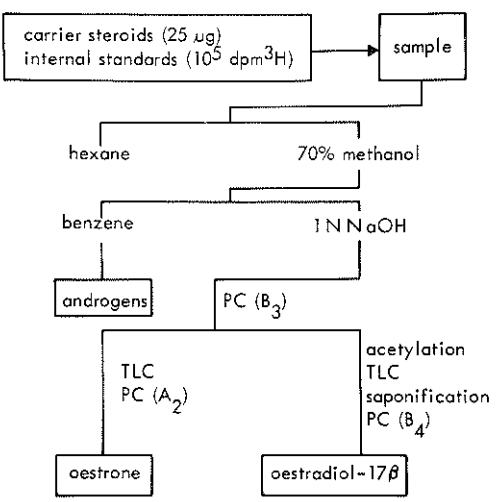

Fig. 4.2. Outline of extraction and purification of radioactive oestradiol and oestrone after incubation of labelled precursors (10^6 dpm ^{14}C- or ^{3}H-steroid) with testicular tissue. PC = paper chromatography in the Bush systems indicated. TLC = thin-layer chromatography: toluene-ethyl acetate 4-1 (v/v) for oestrone and toluene-ethyl acetate 9-1 (v/v) for oestradiol.

Table 4.3. Production of oestradiol and testosterone by rat testicular tissue under different conditions

	Incubation conditions	Oestradiol (pg/testis/3 h)	Testosterone (ng/testis/3 h)
In vivo[1)]		690	4625
In vitro			
Whole testis	intact cells in Krebs-Ringer buffer[2)]	30	116
	homogenates in sucrose-EDTA[3)]	40	4800
Interstitial tissue	intact cells in Krebs-Ringer buffer[2)]	--	82
	homogenates in sucrose-EDTA[3)]	--	5250
Seminiferous tubules	intact cells in Krebs-Ringer buffer[2)]	12	--
	homogenates in sucrose-EDTA[3)]	--	1200

1) appendix paper 1

2) appendix paper 2

3) Figure 4.3.

cular 800xg supernatants, the purification scheme as discussed earlier (Fig. 4.2.) was followed. After the second paper chromatographic step, part of the sample was counted for ^{3}H, and ^{14}C-oestradiol (720 dpm) was added to the samples. After enzymatic oxidation (Rosenfield, 1971) followed by paper chromatography (Bush A_2, 24 h) the oestrone fraction was isolated. Part of the sample was counted, and the remainder was acetylated and chromatographed (thin-layer chromatography, toluene-ethyl acetate 4-1, v/v). Subsequently, the oestrone acetate fraction was counted.

4.3. Results

4.3.1. Measurement of endogenous hormones

Results of a first series of experiments in which non-homogenized testis tissues were incubated in Krebs-Ringer buffer were reported in Table 5 of appendix paper 2. Since the production of endogenous steroids in total testis under these conditions was low in comparison with in vivo steroid production (Table 4.3.) a second series of experiments using the conditions as described by van der Vusse et al. (1973) was performed (see: Fig. 4.3. and Table 4.3.). The experiments on the production of endogenous steroids in isolated seminiferous tubules and interstitial tissue, as described in appendix paper 2, were also repeated under the conditions of van der Vusse et al. (1973). Results of these incubations are summarized in Table 4.4. With the exception of the 800xg pellet of the interstitial tissue and the dissection medium, all fractions contained amounts of oestradiol, which were not significantly different from the blank value of 3.1 $\pm$ 3.3 (S.D.) pg (n=12), obtained in the same series of estimations.

Finally, the concentrations of oestradiol and testoste-

Table 4.4. Oestradiol (E_2) and testosterone (T) in various fractions of rat testis tissue before and after incubation of isolated tissue fractions homogenized in sucrose-EDTA solution (means ± S.D. (n))

Tissue fraction	protein content (mg)	incubation time (h)	E_2 (pg/mg protein)	T (pg/mg protein)	E_2/T (pg/ng)
Interstitial tissue					
800 x g pellet	2.5 ± 1.0(6)	0	2.2 ± 1.3 (6)	1.9 ± 0.8 (6)	2.2 ± 3.1 (6)
800 x g pellet	3.3 ± 1.0(6)	0	<2.5 (5)	134 ± 75 (6)	<0.02
	3.1 ± 0.9(6)	3	<2.5 (6)	1180 ± 254 (6)	<0.002
Seminiferous tubules					
800 x g pellet	33.7 ± 11.0(6)	0	<0.25(6)	0.18 ± 0.04(6)	<1.40
800 x g pellet	33.2 ± 5.2(6)	0	<0.25(6)	0.82 ± 0.57(6)	<0.30
	31.8 ± 4.8(6)	3	<0.25(6)	18.1 ± 9.3 (6)	<0.02
Dissection medium					
	17.3 ± 7.0(4)	0	0.74 ± 0.35(4)	2.9 ± 0.5 (4)	0.26 ± 0.10(4)

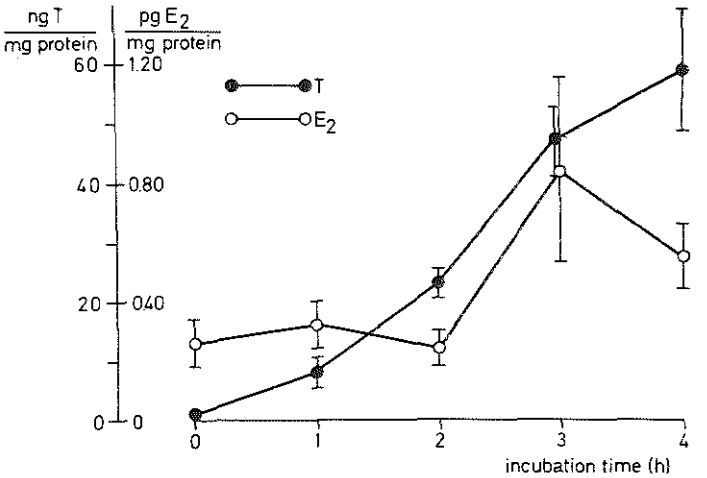

Fig. 4.3. In vitro production (means ± S.E.M., n=6) of oestradiol and testosterone by 800xg supernatant of homogenized rat testes. Incubation conditions were as described by van der Vusse et al. (1973).

rone were estimated in isolated seminiferous tubules and interstitial tissue from EFA-deficient rats. The results of these estimations are summarized in Table 4.5. The concentrations of oestradiol and testosterone in interstitial tissue from normal rats (Table 4 in appendix paper 2) and in EFA-deficient rats are not significantly different. However, the concentrations of both steroids are significantly higher in tubular material from EFA-deficient rats when compared with those in normal rats ($P < 0.02$). This difference may be explained by a larger interstitial contamination of the tubular fraction.

Table 4.5. Oestradiol (E_2) and testosterone (T) in testicular tissue from essential fatty acid deficient rats (means ± S.D. (n))

Tissue	E_2 (pg/g)	T (ng/g)
Total testis	128 ± 54 (11)	1265 ± 685 (6)
Interstitial tissue	180 ± 115 (6)	291 ± 123 (7)
Tubules	106 ± 70 (6)	57 ± 27 (7)

4.3.2. Conversion of radioactive precursors

4.3.2.1. Interconversion and degradation of oestrogens

After incubation of [4-^{14}C]-oestrone or [4-^{14}C]-oestradiol (10^5 dpm) with testis tissue homogenates (equivalent with 100 mg tissue) in Krebs-Ringer buffer, non-radioactive carriers and tritium-labelled oestrone and oestradiol (10^5 dpm) were added. Steroids were extracted and purified using the method outlined in Fig. 4.2. Radioactivity present in appropriate aliquots was estimated after each purification step: $^3H/^{14}C$ ratios did not change after thin-layer chromatography indicating that purity of the oestrogens was reached at this stage of purification. The percentages conversion of oestrone and oestradiol, corrected for losses during extraction and purification, are summarized in Table 4.6.

Table 4.6. Catabolism of oestrogens during incubations of rat testis homogenates

Substrate	% of substrate isolated as		
	E_1	E_2	E_1+E_2
Oestrone (E_1)	36.2	65.1	101.3
Oestradiol (E_2)	11.8	90.6	102.4

In order to get an impression of the catabolism of an amount of oestradiol, comparable to that endogenously present in testicular tissue (Table 1 in appendix paper 2), [6,7-^{3}H]-oestrone ($2x10^{4}$ dpm) and [2,4,6,7-^{3}H]-oestradiol ($2x10^{4}$ dpm) were incubated with testicular homogenates (equivalent with 1 g of tissue) under the conditions described by van der Vusse et al. (1973). After incubation the steroids were extracted and chromatographed on Sephadex LH-20 microcolumns as described in appendix paper 2. The radioactivity in the oestrone and oestradiol fractions from the Sephadex LH-20 columns was counted. The results of these estimations are summarized in Fig. 4.4.

4.3.2.2. Biosynthesis of oestrogens

Results of incubations of [4-^{14}C]-dehydroepiandrosterone ($1.1x10^{6}$ dpm), [4-^{14}C]-androstenedione ($1.5x10^{6}$ dpm) or [4-^{14}C]-testosterone (10^{6} dpm) with homogenates of rat and horse testis tissue (equivalent to 100 mg wet weight) in Krebs-Ringer buffer are summarized in Table 4.7. The oestrogens were extracted and purified as depicted in Fig. 4.2. The amount of ^{14}C-label found in the oestrogen frac-

Table 4.7. Conversion of androgens to oestrogens during incubations with (+) or without (-) testis homogenates*

Substrate		rat testis		equine testis	
		oestrone	oestradiol	oestrone	oestradiol
Testosterone	-	0.036	0.005	0.013	0.003
	+	0.037	0.012	1.26	40.9
Androstenedione	-	0.036	0.014	0.004	0.005
	+	0.024	0.007	28.0	37.4
Dehydroepian-drosterone	-	0.038	0.004	0.017	0.020
	+	0.022	0.021	7.6	19.7

* % of incubated substrate

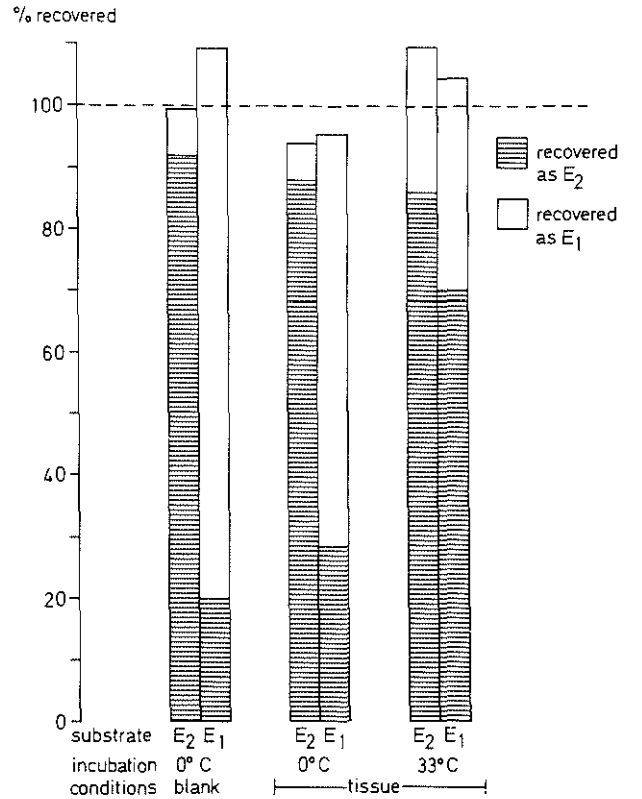

Fig. 4.4. Radioactivity eluted in the oestrone and oestradiol fractions from Sephadex LH-20 microcolumns after 3 hr incubation of rat testicular tissue 800xg supernatant with ^{3}H-oestrone (E_1, 40 pg) or ^{3}H-oestradiol (E_2, 20 pg). Incubation conditions were as described by van der Vusse et al. (1973). Results are expressed as percentage of the incubated substrate recovered in the different fractions. Mean values of 5-6 incubations are given.

tions isolated from rat testis incubations was not different from that in tissue-free incubations, while all three androgens were converted to oestrone and oestradiol during incubation with equine testis tissue. $^{3}H/^{14}C$ ratios for both steroids did not change after the thin-layer chromatographic step.

The results of incubations of 10^{6} dpm of [1,2,6,7-^{3}H]-androstenedione or [1,2,6,7-^{3}H]-testosterone with 800xg supernatants of rat testis homogenates (equivalent to 1 g of tissue), suggested conversion rates to oestradiol of approximately 1 $^{o}/oo$ (Fig. 4.5.), when corrected for the results of blank incubations, for mean recovery (43.2 $\pm$ 4.5 (S.D.) %, n=27) after paper- and thin-layer chromatography, and for ^{3}H-loss from the 1β- and 2β-position (Fishman et al., 1969, Osawa & Spaeth, 1971). No radioactivity associated with oestrone, was detected at this stage of purification. Addition of the Bush B_3 paper chromatographic step to the purification of ^{3}H-oestradiol, and subsequent addition of [4-^{14}C]-oestradiol (720 dpm) and further purification of the steroid by the method described in section 4.2.4. resulted in a complete dissociation of ^{3}H- and ^{14}C-radioactivity in all oestradiol fractions. The results from incubations where testosterone (10 µg/incubation) or cyanoketone (0.5 µg/incubation) were added, showed the same dissociation of radioactive labels. Testosterone always accounted for more than 80% of the radioactivity in the androgenic fractions isolated after incubation. Similar experiments with isolated seminiferous tubules and interstitial tissue yielded comparable results: the ^{3}H-radioactivity which was isolated after paper- and thin-layer chromatography, was completely dissociated from the ^{14}C-label after subsequent purification.

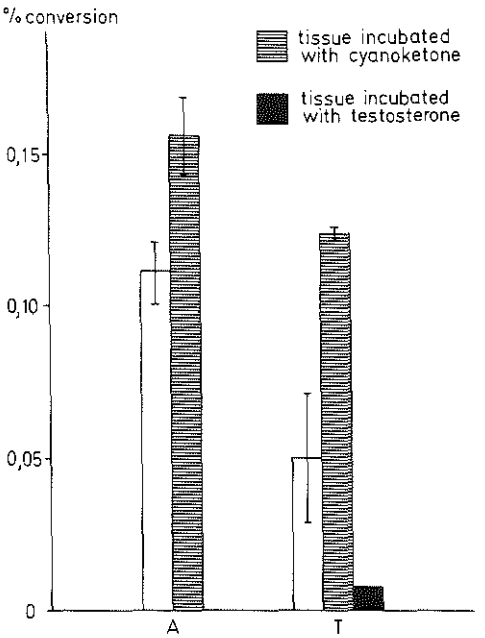

Fig. 4.5. Apparent percentage conversion of tritiated androstenedione (A) or testosterone (T) to labelled oestradiol after 3 h incubation with 800xg supernatant of rat testis homogenates. Incubation and dissection procedures were as described by van der Vusse et al. (1973).
After incubation oestradiol was extracted and purified according to the procedure in Fig. 4.2., with the exception of the last paper chromatographic step.

4.4. Discussion

4.4.1. Endogenous production of oestradiol

The testicular production of endogenous oestradiol in vitro was much lower than that observed under in vivo conditions (see Table 4.3.). On the other hand, by changing the incubation conditions, it was possible to increase the production of testosterone during incubation to a level comparable to that observed during in vivo experiments (Table 4.3.). This difference between the relative productions of oestradiol and testosterone in vivo and in vitro may reflect that conditions which favour the production of testosterone are not favourable for production of oestradiol. A similar conclusion was drawn from the vivo experiments described in appendix paper 1: a prolonged trophic stimulation of the secretion of testosterone did not increase the secretion of oestradiol. It is not likely that the low production of oestradiol is caused by absence of cofactors: NADPH and molecular oxygen, which are necessary for oestrogen formation in placental homogenates (see: Talalay, 1965), and in the microsomal fraction from equine testes (Oh & Tamaoki, 1971), were present. A second explanation for the low production rate of oestradiol during incubations of testicular homogenates is the possible inhibition of the aromatization reaction by testosterone catabolites, which may accumulate in the incubation medium. Such an inhibition was shown to occur during incubation of placental microsomes (Schwarzel et al., 1973).

The conclusion of appendix paper 2, that under in vitro conditions oestradiol may be synthetized in tubular tissue could not be confirmed by the results shown in Table 4.4., because of the extremely low levels of oestradiol in the samples. The localization of measurable levels of oestradiol in the 800xg pellet of the interstitial tissue suggests that this steroid is mainly localized in the

nuclei of the interstitial tissue, as might be expected on basis of the results of Mulder et al. (1973). This observation on the interstitial localization of oestradiol is also in agreement with the results described in Table 4 of appendix paper 2 and with the results obtained in EFA-deficient rats (Fig. 3.3. and Table 4.5.). It cannot be decided, however, whether the high concentration of oestradiol in isolated interstitial tissue reflects the real situation in vivo, because redistribution of the steroid might have occurred during the dissection procedure. The relatively high concentration of oestradiol in the dissection medium, which contrasts with the level of testosterone (Table 4.4.), supports the latter possibility.

Finally, the apparent, albeit small production of testosterone in isolated seminiferous tubules during the incubations of 800xg supernatants (Table 4.4.) is at variance with the data reported by Cooke et al. (1972). It is not clear if this discrepancy is due to the difference in incubation conditions or to an interstitial inpurity in the tubular fraction.

4.4.2. Production of radioactive oestradiol

The quantitative extent of oestrogen catabolism in rat testicular tissue is small (Table 4.6. and Fig. 4.4.). The relative amounts of oestrone and oestradiol, found after incubation, depends largely on the cofactors added: in the presence of a NADPH-generating system in the incubation medium, most of the steroid will be reduced. This occurs with large as well as with small amounts of oestrone or oestradiol: the results of the incubation studies with ^{14}C- or ^{3}H-labelled steroids do not differ. The catabolism to other steroids is quantitatively not important, since almost 100% of the incubated radioactivity was isolated as oestrone and oestradiol after incubation. These results with rat testis tissue are in agreement with those of Lucis & Lucis (1969), who found only very small conversions

Table 4.8. Theoretical production rates of radioactive oestradiol (E_2) from ^{14}C- or ^{3}H-testosterone (T) during incubation of testis homogenates

Incubation conditions	Amount of tissue	Endogenous T (ng)	Radioactive T			S.A. of T ($\frac{dpm}{pg}$)	E_2 produced		
			label	dpm	mass		mass (pg)	dpm	% of T
Total homogenate in Krebs-Ringer buffer	100 mg	5.5[1)]	^{14}C	10^6	2.2 µg	0.45	2[1)]	0.9	0.0001
800xg supernatant sucrose-EDTA	50 mg protein	50[2)]	^{3}H	$2x10^6$	3.1 ng	38	25[2)]	475[3)]	0.05

1) values from Table 5, appendix paper 2

2) values from Fig. 4.3.

3) corrected for loss of 1β- and 2β-^{3}H atoms (see section 4.3.2.)

S.A. = specific activity

to 6-oxygenated products after incubation of rat and human testes with ^{14}C-oestrone. However, Axelrod & Goldzieher (1962) and Wade et al. (1968) found significant amounts of 2-hydroxy- and 2-methoxy-derivatives after incubation of human testis tissue from a normal man and from a patient with testicular feminization.

Table 4.8. shows the radioactivity which should theoretically be found in the oestradiol fractions after incubation of testis tissue homogenates with ^{14}C- or ^{3}H-labelled testosterone. It is assumed that production of endogenous testosterone does not take place, and that testosterone is the only precursor of oestradiol. It is evidently impossible to detect conversion from ^{14}C-testosterone to oestradiol using the conditions shown: the calculated conversion (0.0001%) is lower than the corresponding values for blank incubations (Table 4.7.). A similar conclusion applies to the incubations with ^{3}H-testosterone when a 60-fold increase in endogenous testosterone production takes place (Fig. 4.3.): this results in a comparable decrease in specific activity of the labelled steroid. Finally, a 0.05% conversion of testosterone to oestradiol might be expected in incubations where the endogenous production of testosterone is blocked by the addition of cyanoketone (van der Vusse et al., 1974). However, no production of labelled oestradiol could be detected in the latter incubates (see 4.3.2.2.). This may be due to the possibility that testosterone is not the obligatory precursor for oestrogen biosynthesis, or that cyanoketone influences the aromatizing system in the testis at lower concentrations than those used to inhibit placental aromatization (Schwarzel et al., 1973).

The observation that no production of radioactively labelled oestradiol could be observed during incubation of 800xg supernatants of homogenates of seminiferous tubules and interstitial tissue is in agreement with the results shown in Table 4.4.

4.5. Conclusions

From the results presented in appendix paper 2 and in this chapter, the following conclusions are drawn:

1. Oestradiol and testosterone are mainly localized in the interstitial tissue.
2. In dissected interstitial tissue oestradiol is mainly localized in the 800xg pellet, while testosterone is found in the 800xg supernatant.
3. Conditions favouring the in vitro production of testosterone do not enhance the biosynthesis of oestradiol.
4. Biosynthesis of oestradiol could only be shown in whole tissue preparations of total testis tissue and seminiferous tubules and in total testis homogenates.
5. Biosynthesis of testosterone was observed during incubations of whole tissue preparations of total testes and interstitial tissue and in homogenates of total testes, interstitial tissue and seminiferous tubules.
6. Radioactively labelled androgens were not converted to oestrogens under conditions where endogenous production of oestradiol could be shown. This is probably a result of the dilution of the radioactive steroids with endogenous hormones.

4.6. References

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CHAPTER 5. THE SIGNIFICANCE OF THE TESTICULAR PRODUCTION OF OESTRADIOL

5.1. Introduction

The effects of exogenous oestrogens on different processes in the male animal have been extensively studied: oestrogens have been found to influence the prostate (Harper et al., 1971, Leav et al., 1971, Bonne & Raynaud, 1973), adrenal function (Sobrinho et al., 1971), the pineal gland (Nagle et al., 1972), the neurohypophysis (Legros & Grau, 1973), the metabolism of testosterone (Bird et al., 1971) and testicular and hypophyseal function.

Until recently the latter two effects of the administration of oestrogens to male animals were thought to be correlated via the negative feedback action of oestrogens on hypothalamic-hypophyseal processes which result in the secretion of trophic hormones; the administration of oestrogens leads to "physiological hypophysectomy" (Steinberger & Nelson, 1955). The role of testicular oestradiol can be only of limited significance in this respect, because the testis secretes only a minor part of the amount of circulating oestradiol (chapter 3). Recent studies (Mallampati & Johnson, 1973, Danutra et al., 1973) showed, however, that the administration of oestrogens to intact male rats did not influence levels of circulating trophic hormones, while testosterone concentrations in peripheral blood fell steeply. These results would suggest a direct effect of oestrogens on testicular processes. This latter possibility will be discussed in more detail in this chapter.

5.2. Influence of oestrogens on testicular function

5.2.1. Effects of longterm oestrogen treatment on testicular enzymes

As early as 1941 Huggins et al. suggested that oestrogenic steroids might influence the testicular secretion of androgens. These authors also indicated that this action of the oestrogenic steroids might reflect either a negative feedback action on the secretion of gonadotrophic hormones from the hypophysis, or a direct action on testicular processes. The inhibition of hypophyseal gonadotrophin release has been suggested in almost all experiments on the effect of oestrogens under simultaneous administration of trophic hormones: Meyer (1968) showed that injection of oestradiol-dipropionate inhibits rat testicular glucose-6-phosphate dehydrogenase, lactate dehydrogenase, NAD cytochrome-c reductase, cytochrome oxidase and 3β-hydroxysteroid dehydrogenase, but this inhibition could be reversed through administration of gonadotrophins. Samuels et al. (1967) showed a similar effect of HCG on 17α-hydroxylase and 17-20 lyase in rat testes after diethylstilboestrol treatment. The latter authors also indicate a possible direct effect of diethylstilboestrol on 17β-hydroxysteroid dehydrogenase in rat testes, and they showed a significant direct effect on the testicular concentration of enzymes involved in steroidogenesis in BALB/c mice. Effects of diethylstilboestrol on testicular RNA synthesis and on the growth of interstitial cell tumours in these animals have also been reported (Samuels et al., 1968).

The only study of this type which was performed in the human male is that of Slaunwhite et al. (1962), who found a decreased activity of 17β-hydroxysteroid dehydrogenase activity during in vitro studies of testis tissue, obtained

from men with prostatic cancer, who were treated with oestrogens. This decrease could, however, be reversed by the administration of HCG in vivo.

Finally, Danutra et al. (1973) described a discrepancy between LH and testosterone levels in rats after oestrogen administration: plasma LH levels remained normal while plasma testosterone concentrations were decreased. This might be the first evidence for a direct action of oestrogens on testicular processes in the rat. However, these observations were not confirmed by Verjans et al. (1974) who in a comparable study observed a close correlation between the decrease of circulating LH levels and the decrease of testicular and plasma testosterone concentrations.

5.2.2. Effects of oestrogens on spermatogenesis

The influence of exogenous oestrogens on spermatogenesis and fertility has recently been reviewed by Steinberger (1971). This author concludes from data, which were mainly obtained from experiments in rats, that the negative influence of oestrogens on spermatogenesis is mediated via the hypophysis. Steinbeck et al. (1971) postulated that the deleterious action of oestrogens on spermatogenesis is not only pituitary-, but primarily Leydig cell-dependent: after oestrogen treatment the interstitial tissue degenerates first, while after cessation of administration the Leydig cells recover firstly, followed by the recovery of spermatogenesis. A similar observation was recorded by Steinberger & Nelson (1955) who reported that the administration of testosterone propionate caused reversal of the oestrogen induced decrease in testicular hualuronidase, a marker enzyme for advanced stages of spermatogenesis. Elkington & Blackshaw (1970) and Lacy & Lofts (1965) restored spermatogenesis in oestrogen treated rats with in-

jections of PMS, testosterone and FSH. Similar experiments were performed in mice by Davies et al. (1974) who did not observe any effect of FSH alone on spermatogenesis. A direct effect of oestrogens on spermatogenesis has never been reported.

5.2.3. Effects observed during incubations of testicular tissue with oestrogens

Direct effects of oestrogens on testicular metabolism in vitro have been investigated particularly with respect to the enzymes involved in steroidogenesis. The oestrogen concentrations used in these studies are generally in the range of between 10^{-7} and 10^{-4}M, hence several orders of magnitude higher than in the physiological situation (appendix paper 2). It is likely, therefore, that non-physiologic effects of these high oestrogen concentrations (Samuels et al., 1967) might have caused the observed inhibition of 3β-hydroxysteroid dehydrogenase-Δ^5-Δ^4-isomerase in the rat (Kaartinen et al., 1971) and in man (Yanaihara & Troen, 1971), of 17α-hydroxylase in mouse (Samuels et al., 1967) and in man (Balatti et al., 1967), of 17-20 lyase in mouse (Samuels et al., 1967) and of 17β-hydroxysteroid dehydrogenase in rats (Kaartinen et al., 1971). Finally, Vera Cruz et al. (1970) observed an increased oxygen uptake and CO_2 production by testicular tissue in vitro after addition of oestradiol. The oestrogen concentrations used were again aphysiologic.

5.3. Summary and conclusions

In their 1947 review, Emmens & Parkes conclude that "in the absence of any conclusive evidence in either direction, one may conclude, provisionally, that the presence of a certain amount of estrogen in the normal male is incidental rather than functionally important, and that its entire absence would have little or no deleterious effect on the animal". At present, this statement may require further specification, because there appears to be a significant role for circulating oestrogens in the male with respect to the stimulation of sexual behaviour (Larsson et al., 1973, Joshi & Raeside, 1973, Södersten, 1973, Baum & Vreeburg, 1973). The conclusion of Emmens & Parkes may, however, still apply to testicular oestrogens which contribute only a minor part of the total oestrogen production in the male. The presence of a receptor for oestradiol in testicular interstitial tissue, which in some respects seems to behave like the uterine receptor (Mulder et al., 1973), and the observation in the ovary that responsiveness to FSH depends on the local presence of oestradiol (Goldenberg et al., 1973) might indicate, however, that the validity of this conclusion is only of passing significance, pending further investigations.

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SUMMARY

The presence of oestrogens in urine and blood from male animals is well established. The origin of oestrogenic steroids in the male is not clear, however. The study described in this thesis concerns the role of the testis in the production of oestrogens; the following aspects of the testicular production and secretion of oestradiol were investigated:

I. The regulation of testicular production and secretion of oestradiol in vivo (chapter 3, appendix papers 1 and 2).

II. The localization of oestradiol in the two main testicular tissue compartments, the seminiferous tubules and the interstitial tissue (chapter 4, appendix paper 2).

III. The localization of the production of oestradiol in the testicular compartments using incubation studies (chapter 4, appendix paper 2).

In order to measure the low endogenous levels of oestradiol in male rats, it was necessary to evaluate the sensitivity and specificity of methods for the estimation of this steroid (chapter 2). In this respect three different methods used for the estimation of oestradiol were compared. Competitive protein binding assay, using rabbit uterine cytosol as the source of binding protein, lacked specificity. A double competitive protein binding technique appeared more specific, but the sensitivity of the method was not satisfactory. Adequate specificity and sensitivity were obtained only by using radioimmunoassay for the estimation of oestradiol.

Testosterone, which was measured in each sample as a reference, was estimated either by gas-liquid chromatography or radioimmunoassay. Both techniques were shown to

provide reliable results (chapter 2).

I. On basis of the data from the literature (see chapter 3), and of the experiments described in the appendix papers 1 and 2, it has been concluded that:
   1. the testis secretes oestradiol in all species studied thusfar.
   2. in the rat, as in the human male, the contribution of the testicular secretion of oestradiol to the total production rate of the steroid is in the order of 20%.
   3. the intravenous administration of HCG causes an increase in the testicular production of oestradiol and testosterone in the rat.
   4. prolonged administration of HCG to rats causes an increased concentration of oestradiol and testosterone in testicular tissue. The secretion of oestradiol remains unchanged, while the secretion of testosterone increases sharply.
   5. hypophysectomy does not influence the concentration of oestradiol in testicular venous plasma, while the testis tissue concentrations of oestradiol and testosterone and the testicular venous plasma concentration of testosterone are decreased.
   6. the administration of FSH to intact or hypophysectomized rats does not influence the concentration of either testosterone or oestradiol in testis tissue or testicular venous plasma.
   7. the intravenous administration of prolactin to intact rats has no stimulatory effect on the concentration of oestradiol or testosterone in testicular venous plasma.

II. The distribution of oestradiol and testosterone between seminiferous tubules and interstitial tissue was investigated by measuring the endogenous concentrations of the steroids in the isolated tissue compartments from normal rats and in testes from rats, fed with a diet which was deficient in essential fatty acids (chapter 4 and appendix paper 2). It has been concluded that:

1. oestradiol and testosterone are mainly localized in the interstitial tissue.
2. in dissected interstitial tissue oestradiol is mainly localized in the 800xg pellet, while testosterone is found in the 800xg supernatant.

III. The production of oestradiol and testosterone during in vitro studies of total testis tissue or separated interstitial tissue and seminiferous tubules has been discussed in chapter 4 and appendix paper 2. The following conclusions have been drawn from the experiments performed:

1. conditions favouring the in vitro production of testosterone do not enhance the biosynthesis of oestradiol.
2. biosynthesis of oestradiol could only be shown in whole tissue preparations of total testis tissue and seminiferous tubules and in total testis homogenates.
3. biosynthesis of testosterone was observed during incubations of whole tissue preparations of total testes and interstitial tissue and in homogenates of total testes, interstitial tissue and seminiferous tubules.
4. radioactively labelled androgens were not converted to oestrogens under conditions where endogenous production of oestradiol could be shown. This is probably a result of the dilution of the radioactive steroids with endogenous hormones.

Finally, the significance of the testicular production of oestradiol was discussed (chapter 5). It is concluded that so far no evidence for a direct effect of testicular oestradiol on any process has been shown, although some results in the literature might indicate an intra testicular function for the steroid.

SAMENVATTING

Oestrogene steroiden zijn aanwezig in urine en bloed van mannelijke dieren. De herkomst van deze hormonen is echter niet eenduidig bepaald. Het in dit proefschrift beschreven onderzoek heeft betrekking op de mogelijke rol van de testis bij de produktie van oestradiol. In dit verband werden de volgende aspekten van de testikulaire produktie en sekretie van oestradiol onderzocht:

I. De regulatie van de produktie en sekretie van oestradiol door de testis in vivo (hoofdstuk 3 en de artikelen 1 en 2 van de appendix).
II. De koncentraties van oestradiol in de twee belangrijkste kompartimenten van de testis: de seminifere tubuli en het interstitiële weefsel (hoofdstuk 4 en artikel 2 van de appendix).
III. De biosynthese van oestradiol in deze kompartimenten (hoofdstuk 4 en artikel 2 van de appendix).

Voor de bepaling van de lage endogene koncentraties van oestradiol in mannelijke ratten was het noodzakelijk de gevoeligheid en specificiteit van methoden voor de bepaling van dit steroid te evalueren (hoofdstuk 2). Drie methoden voor de bepaling van oestradiol werden vergeleken. Een kompetitieve eiwitbindingstechniek, waarin de cytosolreceptor van de konijne-uterus als bindend eiwit wordt gebruikt, bleek niet voldoende specifiek te zijn. Een dubbele kompetitieve eiwitbindingsmethode was meer specifiek, maar de gevoeligheid van deze methode was niet bevredigend. Gevoeligheid en specificiteit van een radioimmunologische techniek bleken toereikend voor de bepaling van oestradiol in plasma en weefsel van de mannelijke rat.

Testosteron, het steroid dat als referentie werd bepaald in ieder plasma- of testisweefselmonster, werd bepaald met behulp van een gas chromatografische of een radioimmunolo-

gische methode. Met beide technieken werden betrouwbare resultaten bereikt (hoofdstuk 2).

I. Op basis van gegevens uit de literatuur (zie hoofdstuk 3) en van de resultaten van de experimenten die in de artikelen 1 en 2 van de appendix werden beschreven, werd het volgende gekonkludeerd:
   1. de testis secerneert oestradiol in alle species die tot dusver bestudeerd zijn.
   2. de testikulaire sekretie van oestradiol vormt in de rat, evenals in de mens, ongeveer 20% van de totale produktiesnelheid van het steroid.
   3. na intraveneuze toediening van HCG wordt in de rat een toename van de testikulaire produktie van oestradiol en testosteron waargenomen.
   4. na langdurige toediening van HCG aan ratten wordt een verhoogde koncentratie van testosteron en oestradiol in testisweefsel waargenomen. De sekretie van oestradiol verandert niet, terwijl een sterke stijging van de testosteronsekretie optreedt.
   5. hypofysektomie beïnvloedt de koncentratie van oestradiol in testikulair veneus plasma niet. De koncentraties van oestradiol en testosteron in testisweefsel en de koncentratie van testosteron in testikulair veneus plasma zijn verlaagd na hypofysektomie.
   6. na toediening van FSH aan intakte of gehypofysektomeerde ratten treedt geen verandering op in de koncentraties van oestradiol en testosteron in testisweefsel of testikulair veneus plasma.
   7. na intraveneuze toediening van prolactine aan intakte ratten worden geen verhoogde koncentraties van oestradiol en testosteron in testisweefsel of testikulair veneus plasma gevonden.

II. De verdeling van testosteron en oestradiol tussen seminifere tubuli en interstitieel weefsel werd onderzocht door de endogene steroidkoncentraties in de geisoleerde weefselkompartimenten te meten. Uit de resultaten van experimenten met testes van normale ratten en van ratten, die gevoed waren met een dieet, waarin geen essentiële vetzuren aanwezig waren,werden de volgende konklusies getrokken (hoofdstuk 4 en artikel 2 van de appendix):

1. oestradiol en testosteron zijn voornamelijk in het interstitiële weefsel gelokaliseerd.
2. in gedissekteerd interstitieel weefsel is oestradiol voornamelijk gelokaliseerd in het 800xg sediment, terwijl testosteron zich voornamelijk in de 800xg supernatant bevindt.

III. Experimenten over de produktie van oestradiol en testosteron gedurende inkubaties van totaal testisweefsel of gescheiden interstitieel weefsel en seminifere tubuli van de rat worden besproken in hoofdstuk 4 en in artikel 2 van de appendix. De resultaten van deze experimenten leidden tot de volgende konklusies:

1. omstandigheden die de in vitro produktie van testosteron bevorderen, hebben geen stimulerende invloed op de biosynthese van oestradiol.
2. biosynthese van oestradiol kan alleen worden aangetoond in inkubaties van totaal testisweefsel en seminifere tubuli en in homogenaten van totaal testisweefsel.
3. biosynthese van testosteron wordt waargenomen in inkubaties van totaal testisweefsel en interstitieel weefsel, terwijl de homogenaten van totaal testisweefsel, interstitieel weefsel en seminifere tubuli testosteron produceerden tijdens inkubaties.
4. radioaktieve androgenen worden niet omgezet naar gemerkte oestrogenen onder omstandigheden, waaronder wel endogene produktie van oestradiol plaats

kan hebben. Deze diskrepantie wordt mogelijk veroorzaakt door verdunning van de radioaktieve steroiden met endogeen hormoon.

Tenslotte is in hoofdstuk 5 de mogelijke betekenis van de testikulaire oestradiol produktie besproken (hoofdstuk 5). Tot nu toe zijn geen direkte effekten van testikulaire oestrogenen op intra- of extratestikulaire processen aangetoond, hoewel in de literatuur enkele indikaties voor een mogelijke intratestikulaire funktie voor oestradiol vermeld zijn.

CURRICULUM VITAE

Schrijver dezes werd in 1945 te Oudenrijn geboren. Hij behaalde in 1963 het getuigschrift gymnasium B aan het Utrechts Stedelijk Gymnasium. In hetzelfde jaar begon hij met de scheikundestudie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen in de wiskunde en natuurwetenschappen (letter g) werd afgelegd in 1967, terwijl het doctoraalexamen (hoofdvak: analytische chemie, bijvak: endocrinologie) in 1969 behaald werd. In 1967 was hij enige tijd als student-assistent verbonden aan het Analytisch-Chemisch Laboratorium van de Rijksuniversiteit te Utrecht. In juni 1969 trad hij als wetenschappelijk medewerker in dienst van de afdeling Biochemie II van de Medische Faculteit te Rotterdam, waar het in dit proefschrift beschreven onderzoek verricht werd. Sedert april 1971 is hij verbonden aan de afdeling Interne Geneeskunde III. In oktober 1970 werd het diploma Brandwacht 2e klasse (beschikking Ministerie van Binnenlandse Zaken 15-1-1970 nr. EB 70/U23) behaald.

APPENDIX PAPERS

J. Endocr. (1973), **57**, 277–284
Printed in Great Britain

EFFECT OF GONADOTROPHINS ON THE SECRETION OF OESTRADIOL-17β AND TESTOSTERONE BY THE RAT TESTIS

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(*Received* 14 *September* 1972)

SUMMARY

Concentrations of oestradiol-17β and testosterone were estimated in peripheral venous plasma and testicular venous plasma of adult male rats before and after administration of human chorionic gonadotrophin (HCG) or follicle-stimulating hormone (FSH). The concentration of oestradiol-17β in peripheral plasma, as measured with a radioimmunological technique, was $2{\cdot}0 \pm 0{\cdot}9$ (S.D.) pg/ml ($n = 12$). Peripheral testosterone concentrations were $2{\cdot}4 \pm 1{\cdot}8$ (S.D.) ng/ml ($n = 21$). Concentrations of oestradiol-17β and testosterone in testicular venous plasma were significantly higher than those in peripheral plasma. After intravenous administration of HCG (100 i.u.), oestradiol-17β and testosterone concentrations in testicular venous plasma increased significantly. After prolonged s.c. administration of HCG (5 days) the concentration of oestradiol-17β in testicular venous plasma did not change significantly, although the concentration of testosterone increased more than ten times. Intravenous administration of HCG after 5 days of pretreatment with HCG caused a significant increase in oestradiol-17β concentrations in testicular venous plasma. The increase in testosterone concentration was not significant under these conditions.

Intravenous administration of FSH did not change oestradiol-17β or testosterone concentrations in testicular venous plasma.

INTRODUCTION

Until very recently only indirect and conflicting evidence existed for the secretion of steroids with a phenolic A-ring by the mammalian testis. Oestrogens have been detected and their concentrations measured in testicular tissue of foetal sheep (Attal, 1969), of the horse (Beall, 1940) and of the human being (Goldzieher & Roberts, 1952; Anliker, Perelman, Rohr & Ruzicka, 1957), and synthesis of oestrogens by equine testicular tissue *in vitro* is well recorded (Bedrak & Samuels, 1969; Oh & Tamaoki, 1970, 1971). Eik-Nes (1967) showed conversion of radioactive androstenedione to oestrone and oestradiol during perfusion of the dog testis *in vivo*.

Conversions of radioactive precursors to oestrogens *in vitro* have been reported by Axelrod (1965) and Sharma & Gabrilove (1971) for man and by Inaba, Nakao & Kamata (1967) and Ficher & Steinberger (1971) for the rat. Pierrepoint, Galley, Griffiths & Grant (1967), however, could not show a significant conversion of pregnenolone or dehydroepiandrosterone to oestrogens in the testis of the normal dog and F. H. de Jong, A. H. Hey & H. J. van der Molen (unpublished observations) did not obtain radioactive oestrogens after incubation of rat testicular tissue with radioactively labelled testosterone, androstenedione or dehydroepiandrosterone. Peripheral conversion of androgens to oestrogens in men could account for at least part of the blood production rate of oestradiol-17β (Baird, Horton, Longcope & Tait, 1968, 1969). However, calculations by MacDonald, Rombaut & Siiteri (1967) and MacDonald, Grodin & Siiteri (1971) suggest that all the oestradiol-17β produced in the male human being is derived from peripheral conversion of androstenedione and testosterone.

Direct proof for the secretion of oestrogens by the testis can only be obtained by measuring concentrations of steroids in blood from the testicular vein, and comparing them with the concentrations in the testicular artery or in peripheral blood. Eik-Nes (1967) postulated that the testicular secretion of oestrogens in the dog is less than 4 ng/h. Siegel, Forchielli, Dorfman, Brodey & Prier (1967) could not show that concentrations of oestrogens in dog testicular venous plasma were higher than in peripheral plasma of the same animal. Recently, Kelch, Jenner, Weinstein, Kaplan & Grumbach (1972) and Leonard, Flocks & Korenman (1971) estimated plasma oestradiol-17β gradients across human, monkey and dog testes. The present study was undertaken to assess the testicular secretion of oestradiol-17β *in vivo* in the rat. Testicular venous and peripheral blood levels of this steroid were measured before and after administration of human chorionic gonadotrophin (HCG) or follicle-stimulating hormone (FSH). Testosterone levels were measured in the same plasma samples to compare oestradiol secretion with the secretion of this testicular androgen.

MATERIALS AND METHODS

Animal techniques

Adult male Wistar rats with a body weight of 200–250 g were used. Peripheral blood was usually collected in heparinized beakers after decapitation of the animals. Some rats were anaesthetized with sodium pentobarbitone (18 mg/rat, administered intraperitoneally) and then injected with heparin (500 i.u./rat, injected into one of the tail veins) before decapitation in order to investigate the influence of these conditions on the concentrations of oestradiol-17β and testosterone in peripheral blood. Blood from the testicular vein was obtained under sodium pentobarbitone anaesthesia by a modification of the technique described by Bardin & Peterson (1967). After opening the scrotum and underlying tissue, taking care not to sever any blood vessels, heparin was injected into a tail vein. In some experiments the heparin injection was followed immediately or after 10 min by intravenous administration of either saline (0·9 % NaCl in water), saline containing HCG (Pregnyl, Organon, Oss, 100 i.u.) or a solution of 0·05 % human γ-globulin in saline containing FSH (NIH-FSH-S9, 5 μg). Epididymal and fat tissue were separated from the testis.

The rat was placed on its back on a test tube rack and each testis was placed in a small funnel. One of the veins just under the capsule was cut in both testes, and blood was collected for 30 min in small centrifuge tubes which were placed under the funnels. Blood was collected in one or two portions, depending on the time and duration of gonadotrophin injection. Blood was centrifuged immediately after collection and plasma was stored at -15 °C until analysed. Plasma samples from two to six rats were pooled when necessary. In some experiments rats were injected subcutaneously with 100 i.u. HCG daily for a period of 5 days.

Steroid estimations

Radioimmunoassay of oestradiol-17β. Purification of solvents and radioactive steroids, cleaning of glassware and counting of radioactivity were carried out as described by de Jong & van der Molen (1972), with the exception of methanol and toluene used for column chromatography (BHD, Analar). These solvents were used without prior purification. Buffer solutions, antibody solution and dextran-coated charcoal suspension were as described by Hotchkiss, Atkinson & Knobil (1971). The antibody, raised against an oestradiol-17β-6-(*O*-carboxymethyl)oxime–bovine serum albumin (BSA) complex was a gift from Dr D. Exley. The properties of this antibody with regard to specificity have been described by Exley, Johnson & Dean (1971). All samples were assayed at least in duplicate with different volumes of plasma. The method consisted of the following steps. After addition of [2,4,6,7-^{3}H]oestradiol-17β (1×10^4 d.p.m., sp.act. 100 Ci/mmol, New England Nuclear Corporation, Boston) to the plasma sample (0·5–2·0 ml for testicular venous plasma, 5·0 ml for peripheral venous plasma), the plasma was extracted three times with two volumes of ether. The combined extracts were taken to dryness under nitrogen at 45 °C and applied to Sephadex LH-20 microchromatography columns which were packed in Pasteur pipettes as described by Wu & Lundy (1971). The columns were prewashed with 5 ml toluene:methanol (50:50, v/v) followed by 7 ml toluene:methanol (90:10, v/v). After application of the plasma extract to the column, the fraction containing oestrone was eluted with 2·6 ml toluene:methanol (9:1, v/v). The oestradiol-17β fraction was eluted subsequently with 3·0 ml of the same solvent mixture and taken to dryness. The antiserum (200 μl of a 1:21 000 dilution) was then added to the dry residue. After mixing on a vortex mixer the solution was left overnight at 4 °C and 50 μl were then taken for estimation of recovery. An aliquot of 100 μl was transferred to another tube and 500 μl of a suspension of dextran-coated charcoal were added. After mixing and standing at 4 °C for 10 min the tube was centrifuged at 4 °C (1200*g* for 15 min). An aliquot (500 μl) of the supernatant was counted to estimate the amount of radioactivity bound to antibody. The mass of oestradiol-17β was calculated from the percentage of bound radioactivity by comparison with a standard curve, prepared in the same way. The amounts of oestradiol-17β in the original samples were calculated after correction for recovery and mass of added radioactive oestradiol. The accuracy and precision of the method are given in Table 1. The recovery of tritiated oestradiol-17β from plasma samples was 71·0 ± 5·0 (S.D.) % (n = 107); the recovery for samples in the standard curve was 73·0 ± 4·0 % (n = 96). Method blanks were virtually zero. When only radioactive oestradiol-17β was added to water and assayed, no significant difference could be

detected between the amount of oestradiol-17β calculated from the standard curve ($5{\cdot}4 \pm 4{\cdot}1$ (S.D.) pg) and the amount added ($6{\cdot}6 \pm 0{\cdot}4$ pg) ($P > 0{\cdot}20$, $n = 10$). For routine use standard curves were constructed for amounts of 5–100 pg oestradiol-17β. Plasma volumes were always chosen in such a way that the amount of oestradiol-17β in the unknown samples was between 10 and 70 pg.

Estimation of testosterone. Testosterone was measured using the method of Brownie, van der Molen, Nishizawa & Eik-Nes (1964), which consists of estimation of testosterone chloroacetate using electron capture detection after gas–liquid chromatography. Single estimations were performed.

The significance of differences between results for different groups of animals was calculated using a two-tailed Student's *t*-test (Snedecor & Cochran, 1967).

Table 1. *Accuracy and precision of the estimation of oestradiol-17β (E_2) by radioimmunoassay (known amounts of oestradiol-17β were added to water and the solution was processed by the method described in the text)*

Amount of E_2 added (pg)	Mean amount of E_2 found $\pm$S.D. (pg)	Coefficient of variation*
12·6	$12{\cdot}9 \pm 6{\cdot}4$ (8)	49·6
30	$33{\cdot}2 \pm 9{\cdot}4$ (10)	28·3
50	$55{\cdot}5 \pm 12{\cdot}7$ (10)	22·9
100	$106{\cdot}0 \pm 18{\cdot}4$ (8)	17·8
Plasma pool I	$24{\cdot}9 \pm 7{\cdot}7$ (8)	30·9
Plasma pool II	$63{\cdot}6 \pm 7{\cdot}5$ (7)	11·8

* Coefficient of variation (%) = 100 × S.D. (in pg)/pg E_2 found.
Number of estimations in parentheses.

Table 2. *Concentrations of oestradiol-17β (E_2) and testosterone (T) in peripheral and testicular venous plasma of intact male rats (means $\pm$ S.D.)*

Plasma	E_2 (pg/ml)	T (ng/ml)	E_2:T (pg:ng)
Peripheral	$2{\cdot}0 \pm 0{\cdot}9$ (12)	$2{\cdot}4 \pm 1{\cdot}8$ (21)	$1{\cdot}13 \pm 0{\cdot}29$ (5)
Peripheral during anaesthesia	$3{\cdot}4 \pm 2{\cdot}4$ (8)	2·8 (pool)	1·2
Testicular venous	$17{\cdot}5 \pm 8{\cdot}4$ (43)	$110{\cdot}0 \pm 80{\cdot}5$ (19)	$0{\cdot}18 \pm 0{\cdot}16$ (18)

The number of estimations is given in parentheses.

RESULTS

The results of the estimations of oestradiol-17β and testosterone in peripheral and in testicular venous plasma of the rat are summarized in Table 2. Under the conditions used, no significant differences could be detected between the concentrations of oestradiol-17β and testosterone in peripheral plasma during anaesthesia and in peripheral plasma which was obtained after decapitation without prior anaesthesia ($P > 0{\cdot}1$ and $P > 0{\cdot}1$). The concentrations of oestradiol-17β and testosterone in testicular venous plasma were significantly higher than concentrations of these steroids in peripheral plasma ($P < 0{\cdot}001$ and $P < 0{\cdot}001$, respectively). The ratio of the concentrations of oestradiol-17β and testosterone in testicular venous plasma was significantly lower than the ratio for peripheral plasma ($P < 0{\cdot}001$).

Table 3 shows the concentrations of oestradiol-17β and testosterone in testicular

venous plasma after administration of HCG. The concentrations of both steroids were increased significantly after acute HCG administration ($P < 0{\cdot}002$ and $P < 0{\cdot}002$, respectively). The ratio of the concentrations of oestradiol-17β and testosterone was decreased ($P < 0{\cdot}05$). Continuous administration of HCG for 5 days did not change the concentration of oestradiol in testicular venous plasma significantly when compared with levels in untreated rats ($0{\cdot}1 > P > 0{\cdot}05$). Testosterone concentrations, however, were increased ($P < 0{\cdot}001$) under these conditions. Additional i.v. administration of 100 i.u. HCG after 5 days pretreatment with HCG resulted in an increase in the testicular venous oestradiol-17β concentration ($P < 0{\cdot}002$), while the increase in testosterone concentration was not significant ($P > 0{\cdot}1$).

Table 3. *Concentrations of oestradiol-17β (E_2) and testosterone (T) in testicular venous plasma of intact male rats after administration of human chorionic gonadotrophin (HCG)* (*means* ± S.D.)

Treatment*	E_2 (pg/ml)	T (ng/ml)	E_2:T (pg:ng)
None	19·0± 8·5 (18)	129± 91 (9)	0·178±0·106 (8)
100 i.u., i.v.	41·3±24·1 (16)	666± 397 (8)	0·070±0·055 (8)
5×100 i.u., s.c. (5 d)	21·7± 7·6 (16)	1675± 707 (6)	0·013±0·007 (6)
5×100 i.u., s.c. (5 d) +100 i.u., i.v.	37·5±15·1 (13)	2592±1219 (5)	0·021±0·013 (5)

The number of estimations is given in parentheses.
* 100 i.u., i.v. = 100 i.u. of HCG were administered intravenously immediately before collection of testicular venous blood. 5×100 i.u., s.c. (5 d) = 100 i.u. of HCG were administered subcutaneously each day during 5 days.

Table 4. *Concentrations of oestradiol-17β (E_2) and testosterone (T) in testicular venous plasma of intact male rats after administration of follicle-stimulating hormone (FSH)* (*means* ± S.D.)

Treatment	E_2 (pg/ml)	T (ng/ml)	E_2:T (pg:ng)
None	19·5± 7·0 (17)	81±31 (4)	0·27 (2)
5 μg FSH i.v.*	23·1±11·4 (29)	111±73 (7)	0·28±0·31 (5)

The number of estimations is given in parentheses.
* 5 μg of FSH (NIH-FSH-S9) were administered intravenously immediately before collection of testicular venous blood.

Finally, the effect of acute administration of FSH on testicular venous plasma concentrations of oestradiol-17β and testosterone is shown in Table 4. The levels of both oestradiol and testosterone did not increase as a result of the FSH administration ($P > 0{\cdot}1$ and $P > 0{\cdot}1$, respectively).

DISCUSSION

The concentrations of testosterone in peripheral and in testicular venous plasma estimated during the present study compare well with reported values (Bardin & Peterson, 1967; Rivarola, Snipes & Migeon, 1968). The peripheral plasma concentrations of oestradiol-17β that were estimated during the present study are ten times lower than those reported by Labhsetwar (1972). The difference may be caused by a greater specificity of the antibody to oestradiol-17β used in our study. However, that

non-oestrogenic material, possibly originating from the adrenal, might have been bound by the antiserum in the present study cannot be disregarded. The theoretically possible presence of oestradiol-17β oxygenated at carbon atom 6 in the sample is not likely after column chromatography, but cannot be excluded with certainty. On the other hand, the finding of comparable levels of oestradiol-17β in peripheral plasma from male rats by using a double competitive protein-binding technique, as described by Robertson, Mester & Kellie (1971) (F. H. de Jong, A. H. Hey & H. J. van der Molen, unpublished results), makes these possibilities less probable.

A possible influence of anaesthesia or surgical stress on testosterone concentration can almost be ruled out, when blood production rates for testosterone are compared with the testicular production rate of testosterone. The latter may be calculated from testicular blood flow (Jones, 1971) and the testosterone concentration in testicular venous plasma. The blood production rate for testosterone is 80 μg/day, assuming that 10 % of the steroid is bound to erythrocytes and that the haematocrit value is 45 %. The testicular production rate, based on a testicular weight of 3 g per rat, is 74 μg/day, or 92 % of the blood production rate. This indicates that anaesthesia and collection of testicular venous blood did not influence the testicular testosterone secretion to a large extent. Similarly, no significant effect of anaesthesia on peripheral concentrations of oestradiol-17β or testosterone could be shown (Table 2).

The observation of a difference between oestradiol-17β concentrations in testicular venous plasma and in peripheral plasma reflects that the rat testis secretes oestradiol-17β. A testicular oestradiol-17β secretion of 11 ng/day can be calculated from testicular blood flow and the concentration of oestradiol-17β in testicular venous plasma, assuming that the concentration of oestradiol-17β in plasma from the testicular artery is equal to the peripheral venous level. Since the metabolic clearance rate (MCR) for oestradiol-17β in the male rat has not yet been reported, it is not possible to calculate the blood production rate for this steroid in the male rat. However, de Hertogh, Ekka, VanderHeyden & Hoet (1970) measured the MCR for oestradiol-17β in the female rat and observed a close agreement with the MCR for oestradiol-17β estimated by Longcope, Layne & Tait (1968) for women, after correction for body weight. Applying the same correction for body weight differences to the MCR for oestradiol-17β in the male human being as estimated by Longcope *et al.* (1968), the MCR for oestradiol-17β in the male rat is calculated to be 25·6 l/day. This would reflect a blood production rate of oestradiol-17β of 51 ng/day. Hence, the testicular secretion of 11 ng/day in the rat reflects that only 21 % of the total circulating oestradiol-17β is secreted by the testis. This percentage agrees well with the observation of Kelch *et al.* (1972), who calculated that in men approximately 25 % of circulating oestradiol is secreted by the testis. The larger part of circulating oestradiol-17β may be derived from peripheral conversion of testicular and adrenal androgens. The presence of a binding principle for oestradiol-17β in interstitial tissue (Brinkmann, Mulder & van der Molen, 1972) might, however, be an indication for a physiological significance of oestradiol-17β in the testis itself.

Concentrations of both oestradiol-17β and testosterone in testicular venous plasma increased acutely after intravenous administration of HCG. After 5 days of subcutaneous HCG stimulation, testicular venous oestradiol-17β levels were equal to those in non-stimulated rats, although additional i.v. administration of HCG gave

an increase in oestradiol-17β levels (Table 3). Testosterone concentrations in the same samples were increased ten times after 5 days of HCG treatment and increased only slightly after additional i.v. HCG. This discrepancy between the effects of HCG on oestradiol-17β and testosterone levels, which is reflected by their varying ratios, can be explained if the two steroids are synthesized in different compartments of the testis or by assuming a constant production of oestradiol-17β by an enzyme system with a very limited capacity, whilst HCG administration stimulates release of oestradiol-17β from some storage compartment. Follicle-stimulating hormone does not stimulate the testicular secretion of testosterone or oestradiol-17β under the conditions used in the present investigation. If the action of this trophic hormone is mediated through cyclic AMP, this observation is in agreement with the conclusions of Dorrington, Vernon & Fritz (1972) and of Cooke, van Beurden, Rommerts & van der Molen (1972), who did not observe an increase in cyclic AMP levels during incubation of testicular tissue or seminiferous tubules from intact adult rats with FSH. The suggestion of Eik-Nes (1962) that his observation of increased testosterone secretion by the perfused dog testis after administration of FSH would be caused by LH impurities in the FSH preparation and a synergistic effect of FSH, is in agreement with the present results.

The authors gratefully acknowledge the gift of antibodies raised against the oestradiol-17β-6-(*O*-carboxymethyl)oxime–BSA complex from Dr D. Exley (London), and the gift of FSH from the NIH Endocrine Study Section.

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J. Endocr. (1974), **60**, 409–419
Printed in Great Britain

OESTRADIOL-17β AND TESTOSTERONE IN RAT TESTIS TISSUE: EFFECT OF GONADOTROPHINS, LOCALIZATION AND PRODUCTION *IN VITRO*

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(*Received* 14 *June* 1973)

SUMMARY

Concentrations of oestradiol-17β and testosterone were estimated in testicular tissue from intact and hypophysectomized rats. Within 30 min after intravenous injection of human chorionic gonadotrophin (HCG) or follicle-stimulating hormone (FSH) to intact animals the tissue concentrations of both steroids were not significantly changed. Prolonged s.c. administration of HCG (5 days) caused an increase in the tissue levels of both steroids, which was further increased when the prolonged treatment was followed by an intravenous injection with this trophic hormone. FSH had no influence on tissue concentrations of oestradiol-17β or testosterone in hypophysectomized rats.

Assay of separated seminiferous tubules and interstitial tissue indicated that oestradiol-17β and testosterone were mainly localized in the interstitial tissue. Incubations of these constituents showed that oestradiol-17β was produced in the seminiferous tubules, while testosterone was produced in the interstitial compartment.

INTRODUCTION

Secretion of oestradiol-17β by the testis of a number of mammalian species has been demonstrated by measuring differences between the concentrations of this steroid in peripheral and testicular venous plasma (Leonard, Flocks & Korenman, 1971; Kelch, Jenner, Weinstein, Kaplan & Grumbach, 1972; Scholler, Grenier, Castanier, Di Maria, Niaudet, Millet & Netter, 1973; Baird, Galbraith, Fraser & Newsam, 1973; de Jong, Hey & van der Molen, 1973*a*). Furthermore, the concentration of oestradiol-17β has been estimated in testicular tissue from the horse (Beall, 1940), from sheep during the foetal and postnatal period (Attal, 1969; Attal, Andre & Engels, 1972) and from the human male (Goldzieher & Roberts, 1952; Anliker, Perelman, Rohr & Ruzicka, 1957). The influence of trophic hormones on testicular venous plasma concentrations of oestradiol-17β in the rat was studied by de Jong

et al. (1973*a*), but it was not clear whether the rise in oestradiol-17β secretion after the intravenous administration of human chorionic gonadotrophin (HCG) reflected an increased release or an increased synthesis of steroid. Therefore, the testicular tissue concentrations of oestradiol-17β before and after treatment with HCG were studied in the present investigation.

Follicle-stimulating hormone (FSH) did not influence testicular venous plasma concentrations of oestradiol-17β or testosterone in the rat under the conditions used by de Jong *et al.* (1973*a*). However, it has been suggested that testicular processes in intact animals are maximally stimulated by endogenous FSH, thus preventing a further stimulation by exogenously added FSH (Means & Hall, 1968). In this respect it has been shown that FSH has also no influence on the concentration of cyclic AMP in testicular tissue from intact rats during incubation, although this trophic hormone causes an increase in the cyclic AMP concentration in testis tissue from hypophysectomized rats (Dorrington, Vernon & Fritz, 1972). Therefore, the effect of FSH on the testicular tissue concentration of oestradiol-17β and testosterone in hypophysectomized rats has been studied. Finally, it has been suggested by Scholler, Grenier & Castanier (1972) and by de Jong *et al.* (1973*a*) that oestradiol-17β and testosterone might be synthesized in different testicular compartments. The distribution of these two steroids between interstitial tissue and seminiferous tubules and their production *in vitro* by total and dissected testis tissues have therefore been studied.

A preliminary account of some of the present results was given at the ninth Acta Endocrinologica Congress (de Jong, Hey & van der Molen, 1973*b*).

MATERIALS AND METHODS

Animal techniques

Adult male Wistar rats with a body weight of 200–250 g were used in the experiments with intact animals. Hypophysectomized rats were from the R-Amsterdam strain, which is an inbred Wistar substrain. These animals also weighed 200–250 g. Hypophysectomies were performed by the transauricular approach (Dr R. Welschen and Mrs M. Loonen-Rutte, Department of Anatomy, Erasmus University, Rotterdam). The concentration of testosterone in the testicular tissue was used as a criterion for the completeness of hypophysectomy, since a visual inspection of the sellae turcicae proved unreliable. Hypophysectomized rats were discarded if their testicular testosterone concentration exceeded 10 ng/g.

When the effect of injected gonadotrophins was studied, rats were anaesthetized with sodium pentobarbitone. Heparin was injected into one of the tail veins, followed by saline (0·9 % NaCl in water), saline containing HCG (Pregnyl, Organon, Oss, 100 i.u.) or a solution of 0·05 % human γ-globulin in saline containing FSH (NIH-FSH-S9, 5 or 10 μg). Testicular venous blood was collected during a 30 min period (de Jong *et al.* 1973*a*). The testes were then removed from the animal, immediately placed on ice and kept at -15 °C until analysed. In some experiments rats were injected subcutaneously with 100 i.u. HCG or 10 μg FSH daily for a period of 5 days. FSH treatment started on the day after hypophysectomy. Total testis tissue was dissected using the technique of Christensen & Mason (1965). The dissections were performed

in a Krebs–Ringer buffer, pH 7·4, containing 0·2 % glucose. Isolated seminiferous tubules were washed three times as described by Rommerts, van Doorn, Galjaard, Cooke & van der Molen (1973).

Incubation procedures

Testes were removed immediately after decapitation. The testes were placed in a dish on ice and decapsulated. To both testes from one animal, 1·5 ml of an icecold Krebs–Ringer buffer, pH 7·4, containing 0·2 % glucose, was added. One of the testes was then kept at −15 °C and served as an unincubated control. The other testis was incubated for 180 min at 34 °C in an atmosphere of 95 % O_2 and 5 % CO_2 and was then placed at −15 °C until analysed. The same procedure was followed for dissected interstitial tissue and seminiferous tubules.

Steroid estimations

Radioimmunoassay of oestradiol-17β

Materials used for the radioimmunoassay of oestradiol-17β were described by de Jong *et al.* (1973*a*). After addition of tritiated testosterone and oestradiol-17β, testicular tissue was homogenized by sonification in 1·5 ml distilled water or buffer solution. Proteins were precipitated with acetone (5 ml, Union Chimique Belge, p.a. quality). After centrifugation the acetone layer was removed and the precipitate washed with 2 ml of acetone. The combined acetone layers were evaporated under nitrogen at 45 °C until only water remained. This water layer was extracted twice with ether (3 ml each time) and the combined ether layers taken to dryness. The residue was partitioned between 2 ml methanol:water (70:30, v/v) and 2 ml *n*-hexane. After a second extraction with 2 ml *n*-hexane the methanolic layer was dried down under nitrogen at 45 °C. The residue was applied to a Sephadex LH-20 microcolumn. Testosterone was eluted from the column with 2·6 ml toluene:methanol (9:1, v/v) and the oestradiol-17β fraction was collected in the next 3 ml of eluate. After evaporating the solvent of this oestradiol-17β fraction under nitrogen at 45 °C, radioimmunoassay was performed, using the antibody as described by Exley, Johnson & Dean (1971). Details of the procedure are given by de Jong *et al.* (1973*a*). The effectiveness of the extraction procedure for oestradiol-17β from rat testis tissue was investigated by extracting tritiated oestradiol-17β from part of a testis, into which the steroid was infused via the spermatic artery (Frederik & van Doorn, 1973). The amount of radioactivity remaining in the precipitated protein was estimated after treatment with soluene (Packard) and amounted to 7 % of the number of d.p.m. recovered (mean of duplicate determinations).

Accuracy and precision, as well as blank values for the estimation, were reported previously (de Jong *et al.* 1973*a*). The overall recovery for the estimation of oestradiol-17β in testis tissues was 52·9 ± 6·6 (S.D.) % (n = 296). Data on the specificity of the method, with regard to oestradiol-17β concentrations in peripheral plasma from ovariectomized and adrenalectomized prepubertal female rats, are given by Meijs-Roelofs, Uilenbroek, de Jong & Welschen (1973).

Estimation of testosterone

Testosterone was measured using the method of Brownie, van der Molen, Nishizawa & Eik-Nes (1964), which consists of estimation of testosterone chloroacetate using electron capture detection after gas–liquid chromatography.

The significance of differences between results for different groups was calculated using a two-tailed Student's *t*-test (Snedecor & Cochran, 1967).

RESULTS

The results of the estimations of oestradiol-17β and testosterone in testis tissue from untreated and HCG-treated rats are summarized in Table 1. The intravenous administration of HCG did not significantly increase the concentration of oestradiol-17β ($P > 0{\cdot}5$) or testosterone ($P > 0{\cdot}2$) above that found in untreated rats.

Table 1. *Concentrations of oestradiol-17β and testosterone in testicular tissue of intact male rats after administration of human chorionic gonadotrophin (HCG) (means* ± S.D.)

Treatment*	No. of estimations	Oestradiol-17β (pg/g)	Testosterone (ng/g)	Oestradiol-17β: testosterone (pg:ng)
None	6	44·6 ± 18·5	372 ± 287	0·16 ± 0·08
100 i.u., i.v.	8	48·8 ± 18·8	700 ± 764	0·12 ± 0·06
5 × 100 i.u., s.c. (5 days)	10	66·0 ± 14·0	1351 ± 374	0·05 ± 0·02
5 × 100 i.u., s.c. (5 days) + 100 i.u., i.v.	9	90·2 ± 23·4	1491 ± 579	0·07 ± 0·02

* 100 i.u., i.v. = 100 i.u. HCG were administered intravenously immediately before collection of testicular venous blood. 5 × 100 i.u., s.c. (5 days) = 100 i.u. HCG were administered subcutaneously each day for 5 days.

Table 2. *Concentrations of oestradiol-17β and testosterone in testicular tissue of intact male rats after administration of follicle-stimulating hormone (FSH) (means* ± S.D.)

Treatment*	No. of estimations	Oestradiol-17β (pg/g)	Testosterone (ng/g)	Oestradiol-17β: testosterone (pg:ng)
None	18	30·4 ± 17·0	373 ± 239	0·11 ± 0·07
5 μg, i.v.	10	38·8 ± 26·6	258 ± 145	0·22 ± 0·28
10 μg, i.v.	6	43·5 ± 24·9	350 ± 288	0·16 ± 0·08

* FSH was injected intravenously immediately before collection of testicular venous blood.

Repeated subcutaneous administration of HCG caused a significant increase in the testicular concentrations of both oestradiol-17β and testosterone ($P < 0{\cdot}05$ and $P < 0{\cdot}001$, respectively). The relative increase in testosterone concentration was larger than the increase in oestradiol-17β concentration as indicated by a significant decrease of the oestradiol-17β:testosterone ratio ($P < 0{\cdot}01$). When the prolonged treatment with HCG was combined with an acute intravenous dose of the gonadotrophin, a further increase in testicular concentration of oestradiol-17β was observed ($P < 0{\cdot}02$), while the concentration of testosterone did not increase significantly ($P > 0{\cdot}50$). As a consequence, the oestradiol-17β:testosterone ratio was significantly

increased ($P < 0{\cdot}05$), when compared with that of the animals receiving only subcutaneous injections of HCG.

The effect of the i.v. administration of a single dose of FSH on testicular levels of oestradiol-17β and testosterone is shown in Table 2. Neither the administration of 5 μg FSH, nor of 10 μg FSH had a significant influence on the testicular levels of oestradiol-17β or testosterone in intact rats (P was always $> 0{\cdot}10$). The results in

Table 3. *Concentrations of oestradiol-17β and testosterone in testicular tissue of hypophysectomized male rats after administration of follicle-stimulating hormone (FSH) (means* $\pm$ S.D.)

Treatment*	Oestradiol-17β (pg/g)	Testosterone (ng/g)
None	$4{\cdot}5 \pm 5{\cdot}6$ (18)	$5{\cdot}1 \pm 2{\cdot}7$ (9)
10 μg, i.v.	$3{\cdot}9 \pm 4{\cdot}4$ (16)	$4{\cdot}5 \pm 2{\cdot}7$ (8)
5×10 μg, s.c. (5 days)	$1{\cdot}4 \pm 2{\cdot}7$ (10)	$3{\cdot}7 \pm 1{\cdot}3$ (5)
5×10 μg, s.c. (5 days) + 10 μg, i.v.	$1{\cdot}0 \pm 2{\cdot}3$ (12)	$3{\cdot}0 \pm 1{\cdot}0$ (6)

The number of estimations is given in parentheses.

* 10 μg i.v. = 10 μg FSH (NIH-FSH-S9) were administered intravenously immediately before collection of testicular venous blood. 5×10 μg, s.c. (5 days) = 10 μg FSH (NIH-FSH-S9) were administered subcutaneously each day for 5 days.

Table 4. *Concentrations of oestradiol-17β and testosterone in dissected testis tissue from intact rats (means* $\pm$ S.D.)

Tissue*	Oestradiol-17β (pg/g)	Testosterone (ng/g)	Oestradiol-17β: testosterone (pg/ng)
Untreated rats:			
Seminiferous tubules	$20{\cdot}5 \pm 11{\cdot}7$ (4)	$21{\cdot}4 \pm 6{\cdot}9$ (6)	$0{\cdot}93 \pm 0{\cdot}41$ (4)
Interstitial tissue	179 ± 58 (3)	226 ± 113 (4)	0·73 (2)
HCG-treated rats:			
Seminiferous tubules	$12{\cdot}2 \pm 6{\cdot}0$ (4)	174 ± 53 (4)	$0{\cdot}07 \pm 0{\cdot}03$ (4)
Interstitial tissue	197 ± 89 (7)	3428 ± 410 (4)	$0{\cdot}06 \pm 0{\cdot}02$ (4)

The number of estimations is given in parentheses.

* Seminiferous tubules and interstitial tissue were obtained by dissection of testes from untreated rats or from rats which were injected subcutaneously with 100 i.u. chorionic gonadotrophin (HCG) each day for 5 days.

Table 3 show that also after the administration of FSH to hypophysectomized rats no significant increase in the concentrations of oestradiol-17β or testosterone occurred after intravenous, repeated subcutaneous or a combination of the subcutaneous and intravenous injections of FSH (P was always $> 0{\cdot}10$).

Table 4 summarizes the concentrations of oestradiol-17β and testosterone found in seminiferous tubules and interstitial tissue after dissection of testes from untreated rats and rats treated with HCG for 5 days. The concentrations of both oestradiol-17β and testosterone were higher in interstitial tissue than in seminiferous tubules for the normal ($P < 0{\cdot}01$ and $P < 0{\cdot}01$) as well as for the HCG-treated rats ($P < 0{\cdot}001$ and $P < 0{\cdot}001$).

The results from the estimations of oestradiol-17β and testosterone before and after incubation of total testis tissue, seminiferous tubules or interstitial tissue are

summarized in Table 5. Because the initial steroid levels in the tissue samples from individual animals showed a large variation, the ratios of the concentration after 180 min incubation over the concentration at the start of the incubation were calculated, and the significance of differences was calculated from these figures. The concentration of oestradiol-17β increased significantly during incubation of total tissue and of seminiferous tubules from the normal rat. The testosterone concentration increased significantly during incubation of total tissue and of interstitial tissue from the normal rat. A significant decrease in testosterone levels was observed after incubation of testis from rats, which were treated 5 days with HCG.

When steroid concentrations were calculated /mg protein rather than /g tissue, essentially similar relationships were obtained.

Table 5. *Concentrations of oestradiol-17β (E_2, pg/g) and testosterone (T, ng/g) in testicular tissue before and after 180 min incubation (means* ± S.D.)

Tissue*	Steroid	Incubation time (t) 0 min	Incubation time (t) 180 min	$\frac{t=180\text{ min}}{t=0\text{ min}}$
Untreated rats				
Total testis	E_2	11·7 ± 4·0 (7)	31·6 ± 15·1 (7)	2·42 ± 0·61 (6)
	T	55 ± 32·5 (4)	132 ± 28·3 (4)	3·12 ± 1·75 (4)
Seminiferous tubules	E_2	20·2 ± 11·7 (4)	39·2 ± 19·5 (4)	1·94 ± 0·33 (4)
	T	21·4 ± 6·9 (6)	30·3 ± 19·8 (6)	1·49 ± 0·96 (6)
Interstitial tissue	E_2	179 ± 58 (7)	181 ± 57 (4)	0·94 ± 0·11 (3)
	T	226 ± 113 (4)	663 ± 286 (4)	3·67 ± 1·86 (4)
HCG-treated rats:				
Total testis	E_2	64·7 ± 42·3 (14)	69·0 ± 44·8 (16)	1·02 ± 0·26 (14)
	T	1062 ± 231 (8)	594 ± 78 (8)	0·57 ± 0·09 (8)

The number of estimations is given in parentheses.

* Testis tissue from untreated rats or from rats which were injected subcutaneously with 100 i.u. human chorionic gonadotrophin (HCG) each day for 5 days was incubated for 180 min in a Krebs–Ringer buffer, pH 7·4, which contained 0·2% glucose.

Incubations were performed at 34 °C in an atmosphere of 95% O_2:5% CO_2.

DISCUSSION

The intravenous administration of HCG into intact rats causes an increase in the testicular venous plasma concentration of testosterone and oestradiol-17β (de Jong *et al.* 1973*a*). This increase in venous plasma levels reflects increased secretion as a result of an increased biosynthesis of both steroids, since the testis tissue concentrations of testosterone and oestradiol-17β were not decreased after the 30 min period of testicular venous blood collection (Table 1). The influence of intravenous administration of HCG on the biosynthesis of oestradiol-17β was even more pronounced after pretreatment with HCG for 5 days: a significant increase in the secretion rate of oestradiol-17β was accompanied by a significant rise of the testicular tissue concentration of the steroid. The rate of testosterone biosynthesis seems to be maximally stimulated after prolonged pretreatment with HCG: additional intravenous administration of HCG did not further increase the plasma or tissue concentrations.

After 5 days of HCG treatment the relative increase of the testicular venous testosterone concentration was larger than the increase of the testis tissue level

(Fig. 1). This indicates that the secretion of testosterone is faster in the rat treated for 5 days with HCG than in the untreated animal. On the other hand, the repeated administration of trophic hormone raised the tissue content of oestradiol-17β, although the testicular venous plasma level was not increased (Fig. 2). It is not clear if this apparent retention of oestradiol-17β by testicular tissue is caused by intratesticular

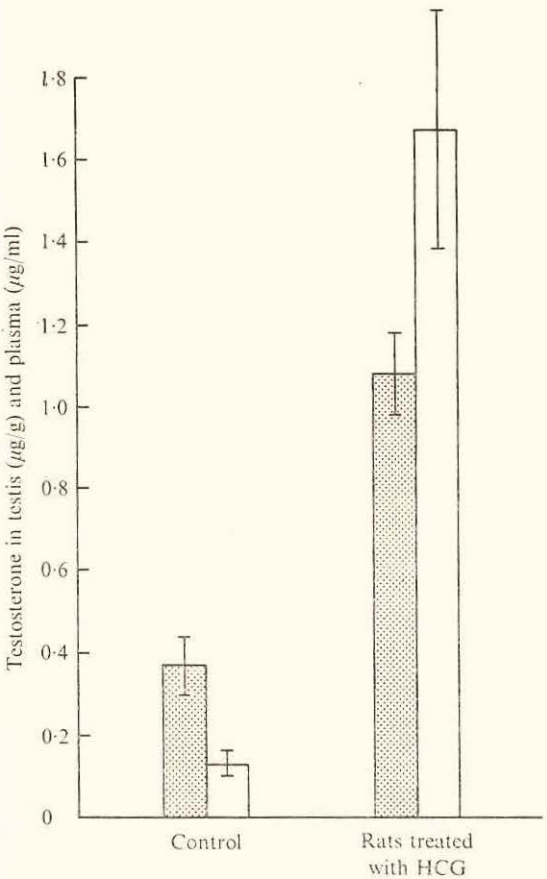

Fig. 1

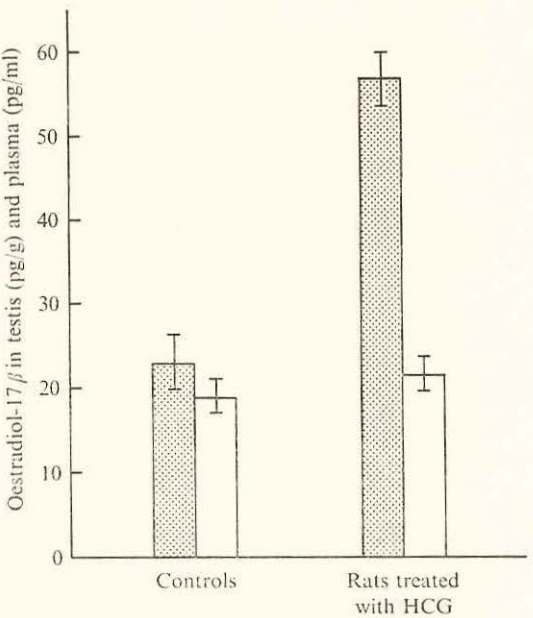

Fig. 2

Fig. 1. Concentrations of testosterone in testis tissue (stippled bars) and testicular venous plasma (open bars) from normal intact rats and rats which were injected subcutaneously with 100 i.u. of human chorionic gonadotrophin (HCG) per day for 5 days (means $\pm$ S.E.M.). (Data partly from de Jong *et al.* 1973*a*.)

Fig. 2. Concentrations of oestradiol-17β in testis tissue (stippled bars) and testicular venous plasma (open bars) from normal intact rats and rats which were injected subcutaneously with 100 i.u. human chorionic gonadotrophin (HCG) per day for 5 days (means $\pm$ S.E.M.). (Data partly from de Jong *et al.* 1973*a*.)

metabolism of the steroid, occurring also under conditions *in vitro* (Table 5), which should take place before oestradiol-17β reaches the blood stream. This possibility suggests a different localization of the enzymes which synthesize or metabolize testosterone and oestradiol-17β, since the secretion of testosterone is increased, although intratesticular metabolism of testosterone is greater than that of oestradiol-

17β under conditions *in vitro* (Table 5). On the other hand, the retention of oestradiol-17β in the tissue may be caused by changes in the concentration of testicular receptor proteins, which bind the steroid with a high affinity (Brinkmann, Mulder, Lamers-Stahlhofen, Mechielsen & van der Molen, 1972).

Since no data are available on the distribution of oestrogen precursors and the aromatizing enzyme system in the different intracellular or intratesticular compartments, it cannot be concluded if HCG influences the biosynthesis of oestradiol-17β by increasing the tissue concentrations of androgens, the most likely precursors for aromatic steroids, or by changing the activity of the aromatizing enzyme system.

Intravenous administration of FSH to intact or hypophysectomized rats had no detectable effects on the tissue concentrations of testosterone or oestradiol-17β (Tables 2 and 3) in agreement with earlier data on the concentration of these steroids in testicular venous plasma after FSH administration to intact rats (de Jong *et al.* 1973*a*).

After dissection of total testis tissue, oestradiol-17β, like testosterone, was mainly present in interstitial tissue (Table 4). However, the possibility of redistribution of oestradiol-17β during the dissection period cannot be excluded, since the steroid is quickly secreted and the interstitial tissue contains a receptor protein for oestradiol-17β (Brinkmann *et al.* 1972). The increased oestradiol-17β:testosterone ratios in both tubules and interstitial tissue when compared with total testis in the normal rat indicate that the dissection procedure results in an apparent loss of testosterone from the tissue.

The intratesticular localization of oestrogen production has been amply discussed and several investigators suggest that oestrogens may be produced in Sertoli cells. Huggins & Moulder (1945) isolated oestrogens from a Sertoli cell tumour, while Lacy, Lofts, Ryan & Hopkin (1966) isolated oestradiol-17β from the lipid droplets from Sertoli cells of heat-treated rat testes. Teilum (1949) described feminizing Sertoli cell tumours in men. Incubation studies with human Sertoli cell tumours showed conversion of androgens (Neher, Kahnt, Roversi & Bompiani, 1965; Sharma, Dorfman & Southren, 1965) of progesterone (French, Baggett, van Wijk, Talbert, Hubbard, Johnston, Weaver, Forchielli, Rao & Sarda, 1965) to oestrogens. Cameron, Markwald & Worthington (1972) observed ultrastructural changes in Sertoli cells after FSH administration to immature rats. These changes were interpreted as an augmentation of the steroidogenic potential of these cells. However, Griffiths, Grant & Whyte (1963) and Pierrepoint, Griffiths, Grant & Steward (1966) did not observe an increased production of oestrogens after incubation of human and canine Sertoli cell tumours with radioactive precursors.

Other observations indicate that oestrogen production may also occur in interstitial tissue. Maddock & Nelson (1952) and Jayle, Scholler, Sfikakis & Héron (1962) observed that the amount of urinary oestrogens excreted after prolonged HCG treatment of normal men increased relatively more than the 17-oxosteroid excretion did. These authors concluded from the hyperplasia of the Leydig cells that these cells secreted oestrogens. Moreover, Rice, Cleveland, Sandberg, Ahmad, Politano & Savard (1967) observed oestrogen formation in a Leydig cell preparation, while Pierrepoint *et al.* (1966) and F. H. de Jong, B. A. Cooke & H. J. van der Molen (unpublished results) observed oestrogen production by an interstitial cell tumour.

This evidence is contradictory, and many of the conclusions are based on indirect evidence (urinary steroids) or unphysiological situations (tumours). The present investigation shows that oestradiol-17β can be synthesized *in vitro* in the tubular compartment of the testis, while no significant increase in the testosterone content of the same samples could be demonstrated. The testosterone production takes place in the interstitial compartment, as observed earlier by Cooke, de Jong, van der Molen & Rommerts (1972). Testosterone concentrations decreased during incubations with testicular tissue from rats that were pretreated with HCG for 5 days. This may reflect steroid metabolism during incubation and is in agreement with the observations of Shikita & Hall (1967) in immature rats. The metabolism of oestrogens during incubations is not quantitatively important, except for the interconversion between oestradiol-17β and oestrone (Lucis & Lucis, 1969).

A possible physiological role of the testicular production of oestradiol-17β remains to be elucidated. The quantitative contribution of testicular oestradiol-17β to the amounts of oestrogen in the peripheral circulation is probably of minor importance (de Jong *et al.* 1973*a*). However, local effects of oestradiol-17β in the testis might be considered. Studies which report direct effects of oestrogens on testicular processes *in vivo* or *in vitro* have been carried out with amounts of oestrogen which are far in excess of the endogenous concentrations reported in the present investigation. The presence of a receptor protein for oestradiol-17β in interstitial tissue (Brinkmann *et al.* 1972), which is found also in the nuclei of the interstitial cells (Mulder, Brinkmann, Lamers-Stahlhofen & van der Molen, 1973), might suggest a role of the oestradiol-17β in the regulation of interstitial tissue function, thus providing a means of communication between tubular and interstitial cells.

We thank Dr Exley for the gift of antibodies raised against the oestradiol-17β-6-(*O*-carboxymethyl)oxime–BSA complex and the NIH Endocrine Study Section for the gift of FSH. We thank Dr. R. Welschen and Mrs M. Loonen-Rutte for performing the hypophysectomies and Mrs H. B. W. Clotscher-Rooseboom, Mrs M. J. L. Lamers-Wilbers and Mrs S. M. van Woerkom-van Bree for expert technical assistance.

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