THE ROLE OF MITOCHONDRIA IN TESTICULAR STEROID PRODUCTION

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM,
OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. B. LEIJNSE
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 8 OKTOBER 1975 DES NAMIDDAGS TE 4.15 UUR.

DOOR

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GEBOREN TE AMSTERDAM

1975
BRONDER-OFFSET B.V. – ROTTERDAM

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Dit proefschrift werd bewerkt in het instituut Biochemie II (Chemische Endocrinologie) van de Faculteit der Geneeskunde, Erasmus Universiteit te Rotterdam.
Het onderzoek werd mede mogelijk gemaakt door steun van de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO.

Voor Annalies

Errata

p. 37, line 10: for (57,62,63) read (92,135,167)
p. 40, line 20: for (102,210,202) read (102, 201,202)
p. 65, line 2: for (64) read (65)
p. 89, regel 36: lees "bijdrage" in plaats van "bijdragen"
p. 90, regel 11: lees "produceren" in plaats van "te produceren"

CONTENTS

2. G.J. van der Vusse, M.L. Kalkman and
H.J. van der Molen.
3β-Hydroxysteroid dehydrogenase in rat testis tissue. Inter- and subcellular localization and inhibition by cyanoketone and nagarse.
Biochim. Biophys. Acta 348 (1974) 404-414.

3. G.J. van der Vusse, M.L. Kalkman and
H.J. van der Molen.
Endogenous steroid production in cellular and subcellular fractions of rat testis after prolonged treatment with gonadotropins.
Biochim. Biophys. Acta 380 (1975) 473-485.

4. G.J. van der Vusse, M.L. Kalkman,
M.P.I. van Winsen and H.J. van der Molen.
On the regulation of testicular steroidogenesis. Short term effect of luteinizing hormone and cycloheximide in vivo and Ca^{2+} in vitro on steroid production by cell free systems.
Biochim. Biophys. Acta 398 (1975) 28-38.

LIST OF TRIVIAL NAMES AND ENZYMES

Trivial names used in this work	Systematic names
5α-androstanediol	- 5α-androstane-3α/3β,17β-diol
androstenediol	- 5-androstene-3β,17β-diol
androstenedione	- 4-androstene-3,17-dione
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
5α-dihydrotestosterone	- 17β-hydroxy-5α-androstan-3-one
17α,20α-dihydroxy-progesterone	- 17α,20α-dihydroxy-4-pregnen-3-one
estradiol-17β	- 1,3,5(10)-oestratriene-3,17β-diol
17α-hydroxypregnenolone	- 3β,17α-dihydroxy-5-pregnen-20-one
11β-hydroxyprogesterone	- 11β-hydroxy-4-pregnene-3,20-dione
17α-hydroxyprogesterone	- 17α-hydroxy-4-pregnene-3,20-dione
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-monochloro acetate

Trivial names used in this work	Systematic names and numbers
adenylate kinase	- ATP:AMP phosphotransferase (EC 2.7.4.3)
adenylate cyclase	- ATP pyrophosphate-lyase (cyclizing) (EC 4.6.1.1)
carboxyl esterase	- carboxylic-ester hydrolase (EC 3.1.1.1)
cholesterol esterase	- sterol-ester hydrolase (EC 3.1.1.13)
cytochrome c oxidase	- ferrocytochrome c:oxygen oxidoreductase (EC 1.9.3.1)
glucose-6-phosphatase	- D-glucose-6-phosphate phospho-hydrolase (EC 3.1.3.9)
glucose-6-phosphate dehydrogenase	- D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase (EC 1.1.1.49)
17α-hydroxylase	- steroid, hydrogen-donor: oxygen oxidoreductase (17α-hydroxylating) (EC 1.14.99.9)
3α-hydroxysteroid dehydrogenase	- 3α-hydroxysteroid:$NAD(P)^+$ oxidoreductase (EC 1.1.1.50)
3β-hydroxysteroid dehydrogenase	- 3(or 17)β-hydroxysteroid: $NAD(P)^+$ oxidoreductase (EC 1.1.1.51) or 3β-hydroxy-Δ^5-steroid:NAD^+ 3-oxidoreductase (EC 1.1.1.145)
17β-hydroxysteroid dehydrogenase	- 3(or 17)β-hydroxysteroid: $NAD(P)^+$ oxidoreductase (EC 1.1.1.51) or 17β-hydroxysteroid:$NADP^+$ 17-oxidoreductase (EC 1.1.1.64)
20α-hydroxysteroid dehydrogenase	- 20α-hydroxysteroid:$NAD(P)^+$ 20-oxidoreductase (EC 1.1.1.149)

lactate dehydrogenase	- L-lactate:NAP^+ oxidoreductase (EC 1.1.1.27)
"malic" enzyme	- L-malate:$NADP^+$ oxidoreductase (decarboxylating) (EC 1.1.1.40)
monoamine oxidase	- amine:oxygen oxidoreductase (deaminating) (EC 1.4.3.4.)
protein kinase	- ATP:protein phosphotransferase (EC 2.7.1.37)
NADPH-cytochrome c reductase	- NADPH:ferricytochrome oxidoreductase (EC 1.6.2.4)
5α-steroidreductase	- 5α-steroid:$NAD(P)^+$ Δ^4 oxidoreductase (EC 1.3.1)
steroidsulfatase	- sterol-sulphate sulphohydrolase (EC 3.1.6.2)
cholesterol side-chain cleaving enzyme complex	- cytochrome P-450 containing enzyme complex catalyzing the conversion of cholesterol to pregnenolone and isocaproic acid (NADPH dependent)
17α,20-lyase	- cytochrome P-450 containing enzyme complex catalyzing the conversion of 17α-hydroxyprogesterone to androstenedione and acetate (NADPH dependent)

LIST OF ABBREVIATIONS

ACTH	- adrenocorticotrophic hormone
BSA	- bovine serum albumin
Ci	- Curie
C.V.	- coefficient of variation $(\frac{\text{S.D. of estimated amount}}{\text{estimated amount}} \times 100\%)$
Cyclic AMP	- adenosine 3':5'-cyclic phosphate
DNA	- deoxyribonucleic acid
dpm	- disintegrations per minute
EDTA	- ethylenediaminetetra-acetate
g	- relative centrifugal force
h	- hour
HCG	- human chorionic gonadotrophin
ICSH	- interstitial-cell stimulating hormone
i.u.	- international unit
LH	- luteinizing hormone
min	- minute
n	- number of estimations
NAD^+	- nicotinamide-adenine dinucleotide (oxidized)
NADH	- nicotinamide-adenine dinucleotide (reduced)
$NADP^+$	- nicotinamide-adenine dinucleotide phosphate (oxidized)
NADPH	- nicotinamide-adenine dinucleotide phosphate (reduced)
p	- probability
rpm	- revolutions per minute
S.D.	- standard deviation
S.E.M.	- standard error of the mean
TLC	- thin-layer chromatography
Tris	- 2-amino-2-hydroxymethylpropane-1,3-diol

CHAPTER 1. INTRODUCTION AND STATEMENT OF PROBLEMS

The testicular gland is the main site of androgen production in the male. Androgens (e.g. testosterone, androstenedione and dihydrotestosterone) secreted by the testis and transported by the bloodstream to their target organs, influence several important physiological processes, such as maintenance of sex accessory glands, muscle development, hair growth, etc. (75). Inside the testis androgens are involved in the maintenance and regulation of spermatogenesis (195).

The testicular gland contains two main tissue compartments: the seminiferous tubules and the interstitial tissue. The interstitial tissue is composed of Leydig cells, macrophages, connective tissue, blood and lymph vessels and contributes approximately 10% to the total weight of the testis of the rat. Myoid cells, endothelial cells, Sertoli cells and cells in various stages of the spermatogenic cycle are the main constituents of the seminiferous tubules. It is generally accepted that the Leydig cell in the interstitial compartment is the main steroid producing cell type in the testis (58,84). However, studies with tracer amounts of radioactive precursors indicate that seminiferous tubules can metabolize steroid precursors to androgens (10,16,37, 214). In this regard it has been suggested that Sertoli cells might produce androgens in almost equal amounts as the Leydig cells (12-14,122). This notion might have important biological implications if steroids produced by the Sertoli cells would be involved in the maintenance of spermatogenesis, and if androgens produced by the Leydig cells would be secreted only into the bloodstream for transport to their extratesticular target organs.

The first aim of the present thesis was to investigate the steroidogenic capacities of isolated interstitial tissue and seminiferous tubules (chapter 3). The testis of the rat was chosen for these studies because the two tissue compartments are morphologically and histochemically easily discernable (21) and can be separated from the testis using simple dissection techniques (37,89,174). For species other than the rat the separation of the different types of tissue is difficult. The mass production of steroids from endogenous precursors in cell-free systems has been estimated, rather than measuring the conversion rate of exogenous labelled substrates. Therefore, the possible difficulty was prevented that added radioactive substrates may not completely mix with endogenous substrates and will not reflect true production rates. The present approach has permitted to compare the amounts of steroids produced in vivo with production rates in whole testis tissue, in isolated interstitial tissue and in seminiferous tubules.

Testicular steroid production is regulated and maintained by LH released from the pituitary gland (57,84,85). Surgical removal of this part of the brain (hypophysectomy) results in a rapid decrease of steroid production and ultimately the testes become atrophic.

To understand the hormonal regulation of steroidogenesis in molecular terms, knowledge of the cellular architecture and subcellular distribution of enzymes, substrates and cofactors, involved in steroid production, is indispensable. From incubation studies with radioactive precursors it was known that the enzymes required for one of the first steps in steroidogenesis, i.e. the conversion of cholesterol to pregnenolone occur in mitochondria (210), whereas the enzymes responsible for the conversion of pregnenolone to testosterone are localized in the endoplasmic reticulum (207). Incubation techniques with tracer amounts of radioactive cholesterol are complicated by the possibility that the results may not reflect true endogenous production. In addition, in previous studies the isolated subcellular

fractions have not always been characterized by proper marker enzymes. Consequently, the above mentioned localization of steroidogenic enzymes may be a simplification of the real cellular situation.

The second aim of this thesis was to study the subcellular production site of pregnenolone in rat testis tissue (chapter 3). The rates of pregnenolone formation from endogenous substrates have been estimated and the rates of production of pregnenolone in isolated subcellular fractions were compared with testosterone production from endogenous precursors in whole tissue homogenates.

The subcellular fractions were characterized with the use of specific marker enzymes in order to correct for mutual contamination. In addition, the subcellular localization of 3β-hydroxysteroid dehydrogenase, required for the conversion of pregnenolone to progesterone has been investigated.

The stimulating effect of LH on rat testicular steroid production has been clearly established both in vivo and in vitro (59,85). The conversion of cholesterol to pregnenolone appears to be rate-limiting in the metabolic steps resulting in androgen production (85). LH is thought to stimulate steroid production via cyclic AMP as intracellular messenger which ultimately has an indirect effect on the production rate of pregnenolone (59,85). Recently, the involvement of cyclic AMP dependent protein kinase activity and the synthesis or activation of a "labile" protein compound (41) in the regulation of testicular testosterone production has been proposed. However, the mechanism of action of cytoplasmic factors on pregnenolone formation in mitochondria is still unknown.

Therefore, the third aim of the work presented in this thesis was to investigate the effects of trophic hormones administered in vivo on testicular steroid production in isolated mitochondrial fractions (chapter 4 and 5). The mitochondrial production rates of pregnenolone have been compared with the amount of testosterone produced in vivo.

The results in section 4.2. and appendix paper III show the effect of long-term treatment with HCG on testicular steroid production and enzyme concentrations in rat testis preparations. In section 4.3. and 5.2. and appendix paper IV the results are given of the acute effect of LH on production rates of pregnenolone in isolated mitochondrial fractions compared with testosterone production rates in vitro and in vivo. The effect of variation of incubation conditions was studied on pregnenolone production rates in mitochondrial fractions from control and LH treated testes.

Finally, an attempt has been made to elucidate a possible role of Ca^{2+} ions in the mechanism of action of trophic hormones on the rate-limiting step in testicular steroid production (chapter 5).

CHAPTER 2. MATERIALS AND METHODS

2.1. Animals and preparation of biological material

2.1.1. Animals

Male Wistar rats (8-14 weeks old) were used for experiments described in appendix papers I and II and Wistar rats substrain R-Amsterdam (14-16 weeks old) for the experimental work, presented in appendix papers III and IV. The animals were kept under controlled light (14 h light and 10 h darkness) and temperature (20-22^{o}C) conditions. Animals were killed by decapitation.

2.1.2. Hormone treatment

In appendix paper III the effect of long-term treatment with HCG is described. Daily subcutaneous injections of 100 i.u. HCG dissolved in 0.2 ml 0.9% NaCl were given for 5 consecutive days. Control animals received 0.2 ml 0.9% NaCl only. The rats were killed by decapitation 24 h after the last injection and testis tissue preparations were analyzed for steroids.

Results of short-term effects of LH on testicular steroid production are described in appendix paper IV and in section 5.2. Rats were anaesthetized by intraperitoneal injection of 0.3 ml sodium pentobarbital (60 mg/ml). Either LH dissolved in 0.2 ml 0.9% NaCl, containing 0.2% bovine serum albumin (LH treated rats) or 0.2 ml 0.9% NaCl, containing 0.2% bovine serum albumin (control animals) was administered into the Vena Iliaca. Blood from the testicular vein was collected in heparinized tubes during 20 min

after hormone injection. Blood samples were centrifuged after collection and the plasma was stored at -20°C until analyzed. When the effect of LH treatment on steroid production rates in vitro was studied, one testis was removed and immediately chilled at 0°C in 0.25 M sucrose containing 1 mM EDTA (zero control testis). Subsequently the animals received via injection into the Vena Iliaca either a LH solution or a NaCl-albumin solution only. The second testis was removed 5-30 min after injection with LH (LH treated testis) or NaCl-albumin solution (control testis). No differences could be observed for in vitro production rates between preparations of "zero control testis" and "control testis". Therefore, for the experiments described in section 5.2. both testes were removed 20 min after injection.

2.1.3. Dissection and homogenization of testis tissue and isolation of subcellular fractions from testis tissue

After removal from the scrotum, testes were chilled in ice-cold 0.25 M sucrose, containing 1 mM EDTA. Then the tunica albuginea and the main blood vessels were removed and the remaining testis tissue was used for further preparations.

When testis tissue was dissected into interstitial tissue and seminiferous tubules, a modification of the technique of Christensen and Mason (37) was used. Whole testis tissue was covered in a petri disk with a small volume of ice-cold 0.25 M sucrose, containing 1 mM EDTA and was dissected with stainless steel forceps into interstitial tissue and seminiferous tubules. The isolated seminiferous tubules were washed 2 times with 10 ml of dissection medium. The purity of the isolated tissue fractions was checked by microscopic examination.

Tissues were homogenized at 0°C in 0.25 M sucrose, containing 1 mM EDTA, with an automatic Potter homogenizer

(Braun Melsungen, Germany) (3 times 15 sec at 1100 rpm). The volume of homogenization medium was adjusted, so that 10-15% homogenates (gram wet weight tissue/ml volume medium x 100%) were obtained.

In appendix paper I, tissue homogenates were separated at 4°C in a nuclear fraction N(500xg for 10 min pellet), a nuclei-free fraction E(500xg for 10 min supernatant), a heavy mitochondrial fraction M(3500xg for 10 min pellet), a light mitochondrial fraction L(10,000xg for 10 min pellet), a microsomal fraction P(105,000xg for 60 min pellet) and a particle-free supernatant S(105,000xg for 60 min supernatant). Fractions N, M and L were spun down in a SE-12 rotor using a Sorvall RC2-B superspeed centrifuge. The pellets obtained were washed twice with isolation medium. Fractions P and S were obtained using a Beckman 50 Ti rotor in a Beckman L2-65B ultracentrifuge. In appendix papers II-IV and section 5.2. a combined heavy and light mitochondrial fraction M(15,000xg for 10 min pellet) was isolated using a Sorvall HB-4 swing out rotor in a Sorvall RC-2B superspeed centrifuge. Fraction M was washed two times with isolation medium.

2.2. Incubation conditions

As a rule 6-8 mg protein of whole testis homogenates, 1-2 mg protein of interstitial tissue homogenates and 3-6 mg protein of seminiferous tubules homogenates were incubated at 33°C in an O_2/CO_2 (95:5, v/v) atmosphere in 2 ml medium (pH 7.3), containing sucrose (0.125 M), EDTA (0.5 mM), sodium succinate (20 mM), NAD^+ (1.0 mM), NADPH (1.0 mM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (0.3 unit), $CaCl_2$ (0.7 mM), $MgCl_2$ (5.0 mM), Tris-HCl (25 mM). Production of testosterone from endogenous precursors was determined as described in section 2.3.1.

Aliquots of mitochondrial fractions isolated from whole

testis tissue (1-2 mg protein/incubation), from interstitial tissue (0.3-0.6 mg/incubation) as well as from seminiferous tubules (2-6 mg/incubation) were rountinely incubated at 33°C in an O_2/CO_2 (95:5, v/v) atmosphere in 1-2 ml medium, pH 7.3, containing sucrose (0.125 M), EDTA (0.5 mM), sodium succinate (20 mM), NADPH (1.0 mM), glucose-6-phosphate dehydrogenase (0.3 unit), glucose-6-phosphate (10 mM), $CaCl_2$ (0.7 mM), $MgCl_2$ (5.0 mM), Tris-HCl (25 mM). Production rates of pregnenolone from endogenous substrates were estimated as described in section 2.3.2. Cyanoketone was present in the incubation medium to prevent further conversion of pregnenolone to progesterone and androgens (appendix paper II). In section 5.2. the effect of variations in the isolation medium and incubation conditions on mitochondrial pregnenolone production rates was investigated (for details see: Legends to figures of section 5.2.).

2.3. Estimation of steroids in biological samples

For the experiments presented in this thesis it was necessary to measure routinely small amounts of testosterone and of pregnenolone in rat testis tissue homogenates and subcellular fractions and, in addition, to determine changes in concentrations during in vitro incubation studies. Therefore, a technique appeared to be required which would permit the estimation of testosterone as well as of pregnenolone in the range of 1-100 ng. At the start of the experimental work in 1971 a variety of analytical methods for estimation of testosterone in biological samples had been described (see for review ref. 139). A gas-chromatographic technique with a satisfactory reliability (section 2.3.1.1.) was readily available for measurements of testosterone. For estimation of pregnenolone a modification of a gas-liquid chromatographic technique with electron capture detection of dehydroepiandrosterone was developed (section 2.3.2.1.). During recent years,

after the wide-scale introduction of radioimmunoassay techniques, a rapid development has taken place of analytical methods for estimation of steroids. Radioimmunoassay methods for estimating steroids are now present with high sensitivity and great practicability. During the progress of the experimental work described in this thesis, the more time-consuming and laborious gas-liquid chromatography was replaced by radioimmunoassay methods to determine testosterone and pregnenolone respectively (section 2.3.1.2. and 2.3.2.2.).

2.3.1. Estimation of testosterone

2.3.1.1. Gas-liquid chromatography

The estimation of testosterone in tissue samples was essentially as described by Brownie et al. (28). After isolation and purification of the testosterone containing samples gas-liquid chromatography was performed on a Pye Unicam 104, model 84, equipped with a ^{63}Ni electron capture detector or on a F and M gaschromatograph equipped with a ^{3}H electron capture detector. The 90 cm 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 monochloroacetate derivative of 20β-hydroxy-4-pregnen-3-one was used as internal standard for gas-liquid chromatography. The reliability of the method has been previously described (108,139). The sensitivity of detection of testosterone using gas-liquid chromatography with electron capture detection of the chloroacetate derivative of testosterone is 1-2 ng. The precision of the assay method in the range of 1-100 ng testosterone is 10-20%, when expressed as the coefficient of variation, calculated from the results of multiple estimations of the same sample (108,139).

2.3.1.2. Radioimmunoassay

Testosterone in plasma and testis tissue samples was estimated using the radioimmunoassay technique described by Verjans et al. (213), which was essentially a modification of the method first presented by Furuyama et al. (71).

Testosterone in plasma samples was extracted with hexane-ether (4:1, v/v) after addition of 25,000 dpm [1,2,5,6 ^{3}H]-testosterone as internal standard. Extracts were purified using alumina-columns. Testosterone in homogenates or subcellular fractions of rat testis tissue was extracted with ethyl acetate after addition of 20,000 dpm [1,2,5,6 ^{3}H]-testosterone as internal standard. Extracts were either purified using chromatography in a Bush A II system (150) or were used for radioimmunoassay directly after extraction. After incubation of aliquots of testosterone samples with antiserum, dextran-coated charcoal suspension was added to the incubation mixture for separation of free and bound testosterone (method b of Verjans et al. (213)). The precision of the radioimmunoassay technique when expressed as coefficient of variation was in the order of 5-20%, calculated from multiple estimation of amounts of testosterone in the range of 50-300 pg. The specificity of the radioimmunoassay of testosterone was investigated by comparing the ability of various steroids to displace [^{3}H]-testosterone from the antibody. Of the steroids investigated only 5α-dihydrotestosterone showed an appreciable cross-reactivity with the antiserum for testosterone. However, since the amounts of 5α-dihydrotestosterone present in adult rat testis tissue are low compared with testosterone levels, the specificity of the method described (213) is high enough for the type of study presented in this thesis. Estimates obtained by radioimmunoassay and by the gas-liquid chromatography technique of testosterone concentrations in homogenates and subcellular fractions from testis tissue showed a good correlation ($r=0.98$, $n=26$).

2.3.2. Estimation of pregnenolone

2.3.2.1. Gas-liquid chromatography

Pregnenolone in homogenates and subcellular fractions from testis tissue was initially estimated by gas-liquid chromatography. This technique was essentially similar to a method described by de Jong and van der Molen for estimating dehydroepiandrosterone in plasma (110). These authors suggested that the assay technique could be applied for other 3-hydroxy-5-ene steroids as well, since steroidal 3-hydroxy-5-ene can generally be oxidized to electron capturing 3,6-dioxo-4-ene compounds.

Pregnenolone was extracted from homogenates and subcellular fractions of rat testis tissue by ethyl acetate after addition of 100,000 dpm [16-^{3}H]-pregnenolone as internal standard to correct for losses during extraction and chromatography. After purification with paper chromatography, using a Bush A II system pregnenolone was oxidized to 4-pregnene-3,6,20-trione with CrO_3 (110). 4-Pregnene-3,6,20-trione was purified with thin-layer chromatography before estimation with electron capture detection after gas-liquid chromatography, essentially as described by de Jong and van der Molen (110). 4-Androstene-3,6,17-trione was used as internal standard for gas-liquid chromatography. The overall recovery of pregnenolone after oxidation and purification was 16 $\pm$ 4% (mean values $\pm$ S.D., n=68).

The accuracy and precision in the estimation of pregnenolone are summarized in table 2.I. In the order of 3-5 ng 4-pregnene-3,6,20-trione could still be estimated by this gas-liquid chromatographic method with acceptable accuracy and precision. However, smallest amounts of pregnenolone that could be detected in the biological samples were in the order of 20-30 ng because of the loss of pregnenolone during oxidation and purification.

Table 2.I Accuracy and precision in the estimation of pregnenolone added to water using gas-liquid chromatography with electron capture detection of 4-pregnene-3,6,20-trione

ng pregnenolone known	ng pregnenolone found ± S.D.	n	C.V. (%)
0	0.9 ± 0.6	15	66.6
10	13.9 ± 1.5	6	10.8
50	55.7 ± 11.2	21	20.1
100	104.5 ± 15.9	16	15.3

2.3.2.2. Radioimmunoassay

A radioimmunoassay technique for estimating pregnenolone was developed in collaboration with H.L. Verjans. New Zealand white rabbits were immunized against pregnenolone-3-hemisuccinate/bovine serum albumin and three months after the initial immunization an antiserum was obtained, which was suitable for use in the radioimmunoassay. Pretreatment of this antiserum before use was exactly as described for the antiserum against testosterone (213). Pregnenolone was extracted from homogenates and subcellular fractions with ethyl acetate after addition of 20,000 dpm [16-^{3}H]-pregnenolone as an internal standard. Extracts either were purified with paper chromatography using a Bush A II system or were directly used for radioimmunoassay. For the radioimmunoassay suitable aliquots of the pregnenolone containing extracts were added to 10 ml glass tubes (10-250 µl were added plus 20,000 dpm [16-^{3}H]-pregnenolone as a second internal standard) in addition to 250 µl of a buffer consisting of 8 mM sodium phosphate, pH 7.2, 140 mM NaCl, 0.05% human γ-globuline and 0.06% bovine serum albumin and containing 1 in 10,000 diluted antiserum. The contents of the tubes were incubated for 16 h at 4^{o}C. Four tubes con-

taining buffer and internal standard, but no antiserum, were assayed to determine non-specific binding. Bound and free pregnenolone were separated by a dextran-coated charcoal adsorption technique (213). Calibration curves were obtained with known amounts of pregnenolone in the range of 192 to 960 pg. Non-specific binding in the absence of antiserum was 0.62% ± 0.21 (mean value ± S.D., n=30). Data on the precision of estimation of known amounts of pregnenolone using radioimmunoassay without extraction and chromatography of the pregnenolone are summarized in table 2.II.

Table 2.II Precision of the calibration curve of radioimmunoassay obtained with known amounts of pregnenolone

pg pregnenolone added	[^{3}H]-pregnenolone bound in % (n=12) mean	S.D.	C.V. (%)
0	40.5	2.1	5.1
192	24.9	0.9	3.6
384	17.7	0.8	4.5
600	13.7	1.2	8.7
720	11.8	0.8	6.7
960	9.3	0.9	9.6

Accuracy of the method was assessed by replicate estimations of known amounts of pregnenolone extracted from a sucrose (0.25 M) - EDTA (1 mM) mixture. Table 2.III shows mean values and coefficients of variations. The data show a good correlation between the amounts of pregnenolone added and amounts estimated.

Table 2.III Accuracy and precision of radioimmunoassay of pregnenolone extracted from 0.25 M sucrose, containing 1 mM EDTA

ng pregnenolone added to sucrose - EDTA	n	ng pregnenolone found (mean)	S.D. (ng)	C.V. (%)
0	20	0.03	0.05	166
4.8	6	4.4	0.7	15
9.6	18	8.0	1.1	13
24	18	22.3	2.2	10
48	17	45.2	3.6	8
96	17	89.6	7.0	8

Recovery of pregnenolone after extraction from biological samples was 102% ± 2% (mean values ± S.D., n=30). The precision of the radioimmunoassay was estimated by measuring pregnenolone in the same biological samples at 5-8 different days (see table 2.IV). The interassay coefficient of variation was in the order of 5 to 11%.

Table 2.IV Precision of the radioimmunoassay of pregnenolone in biological samples. Each sample was analyzed at 5-8 different days. Interassay mean ± S.D. and interassay coefficient of variation are given

sample number	amount of pregnenolone estimated (ng)	number of assays	S.D. (ng)	C.V. (%)
1	47.0	5	3.7	8
2	47.2	5	3.6	8
3	71.9	7	3.8	5
4	144.3	8	15.2	11
5	149.9	8	16.2	11
6	156.1	8	8.3	5

Table 2.V shows data on the intra-assay precision. The amount of pregnenolone in each biological sample was estimated in sixfold. The coefficient of variation of the mean values was 4 to 17%.

Table 2.V Precision of the radioimmunoassay for estimation of pregnenolone in biological samples. Replicate assays of each sample were performed in the same series of assays on the same day. Intra-assay mean ± S.D. and intra-assay coefficient of variation are given

sample number	amount of pregnenolone estimated (ng)	number of replicate assays	S.D. (ng)	intra-assay c.v. (%)
I	30.3	6	5.1	16.8
II	46.8	6	5.3	11.4
III	78.3	6	5.6	7.1
IV	81.6	6	12.3	15.1
V	155.8	6	6.6	4.2
VI	355.7	6	18.3	5.1

The specificity of the antiserum was tested by comparing the ability of various steroids to displace [^{3}H]-pregnenolone from the antibody and was expressed as percentage cross-reaction i.e. the amount of a certain steroid that displaces 50% of [^{3}H]-pregnenolone relative to the amount of pregnenolone that displaces 50% of the [^{3}H]-pregnenolone.

The following data were obtained: pregnenolone, 100%; cortisol, 29%; estradiol-17β, 21%; progesterone, 12%; dehydroepiandrosterone, testosterone, cholesterol, 5α-dihydrotestosterone, 17α-hydroxyprogesterone, 11β-hydroxyprogesterone, corticosterone and cyanoketone, <1%.

For actual samples, specificity was also evaluated by comparison of the estimates of pregnenolone concentrations measured by the radioimmunoassay technique with results obtained with gas-liquid chromatography for estimation of

pregnenolone. The results of radioimmunoassay and gas-liquid chromatographic estimations showed a good correlation ($r=0.96$, $n=29$).

In studies with isolated mitochondrial fractions, cyanoketone was added to the incubation medium to prevent further conversion of pregnenolone. Under such conditions the main steroid produced is pregnenolone and interfering steroids are almost completely absent.

The use of a radioimmunoassay technique for estimation of pregnenolone has two important advantages, when compared with a gas-liquid chromatographic technique:

1. almost no loss of pregnenolone occurs during preparation of the samples for radioimmunoassay, whereas at least 85% of pregnenolone is lost during purification and oxidation to 4-pregnene-3,6,20-trione for gas-liquid chromatography. Therefore,the overall sensitivity of detection of pregnenolone using radioimmunoassay technique is 10-20 times higher than the sensitivity of gas-liquid chromatography.
2. the radioimmunoassay is much less laborious and time-consuming than the gas-liquid chromatographic technique.

Previously DiPietro and coworkers (47) and Abraham et al. (1) have described radioimmunoassays for estimation of pregnenolone. Sensitivity and precision of the described assays are comparable with the assay employed in this thesis. The specificity of the antisera used by these authors appeared, however, higher.

CHAPTER 3. LOCALIZATION OF STEROID PRODUCTION IN RAT TESTIS

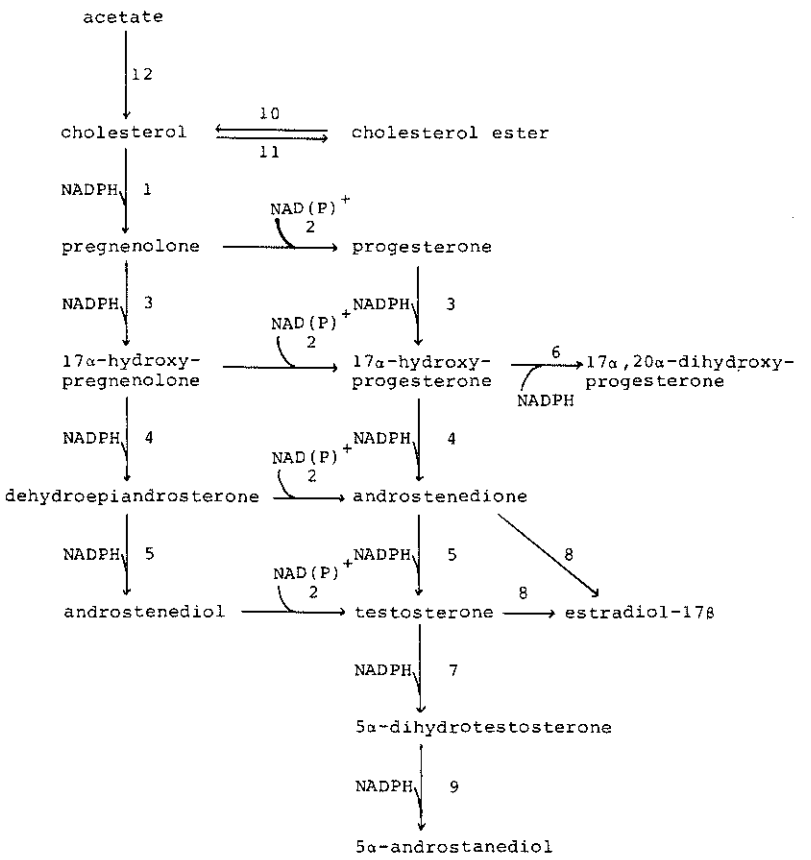
3.1. Introduction

The early observations of Berthold in 1849 (19) and Bouin and Ancel in 1903 (24) indicated the production of a compound with "androgenic activity" in the testicular gland. However, it was not until 1935 before David and co-workers (46) isolated this hormonal "principle" from testis tissue and identified the structure of the compound as testosterone. It is now generally accepted that testicular testosterone production is controlled by LH secreted by the pituitary gland (chapter 4).

In order to restrict the discussion of the site of steroid formation in the testis, steroidogenesis in testicular cells might be defined as the net production of testosterone from sterol precursors. This implies the occurrence of cholesterol, of the cholesterol side-chain cleaving enzyme system and of steroid metabolizing enzymes related to the conversion of pregnenolone to testosterone in that particular cell type. In figure 1 a scheme for testosterone production from cholesterol precursors is depicted. In rat testis pregnenolone is converted to testosterone via progesterone, 17α-hydroxyprogesterone and androstenedione, the so-called Δ^4-route (14,187,188,193,208,209). In contrast, the Δ^5-pathway via 17α-hydroxypregnenolone and dehydroepiandrosterone appears to be an important route besides the Δ^4-pathway for androgen formation in testis of rabbit, dog and man (13,30,60,141,176,223).

Initial observations on the localization of testosterone and its production site in the testis were restricted to the study of the correlation between hormone levels and

Figure 3.1.



Legends:
1 cholesterol side-chain cleaving enzyme
2 3β-hydroxysteroid dehydrogenase
3 17α-hydroxylase
4 17α,20-lyase
5 17β-hydroxysteroid dehydrogenase
6 20α-hydroxysteroid dehydrogenase
7 5α-steroid reductase
8 aromatizing enzyme system
9 3α-hydroxysteroid dehydrogenase
10 cholesterol esterase
11 cholesterol ester synthetase
12 cholesterol synthetizing system

biological effects with microscopic examination of testis tissues obtained under different physiological conditions. From such studies it was suggested that Leydig cells in the interstitial compartment of the testis are the main source of testosterone. On the other hand, no conclusive data were available to definitely exclude the possibility that cell types in the seminiferous tubules are involved in steroidogenesis. In this regard, Lacy and coworkers (123) have suggested that the androgens required for spermatogenesis are not supplied by the Leydig cells.

The first aim of this thesis was to investigate the cellular distribution of testicular steroid production. Therefore, production rates from endogenous precursors by preparations of both testicular tissue compartments were determined (appendix papers I and III). In addition, the distribution of 3β-hydroxysteroid dehydrogenase was measured between interstitial tissue and seminiferous tubules (appendix papers II and III).

For a proper understanding in molecular terms of the hormonal regulation of testosterone formation in the testicular gland, knowledge of the cellular architecture in relation to subcellular localization of substrate pools, enzyme systems and cofactors is indispensable. Therefore, the second aim of the present work was to investigate the subcellular localization of steroid production from endogenous precursors. In addition, the subcellular localization of 3β-hydroxysteroid dehydrogenase was investigated (appendix papers I and II).

In sections 3.2. and 3.3. the available data on cellular and subcellular localization of steroid production in rat testis have been reviewed.

3.2. Cellular localization of testicular steroid production

Several authors have shown that intact interstitial tissue will readily convert radioactive cholesterol to andro-

gens (10,89). However, the conversion rate of radioactive cholesterol by seminiferous tubules is very low or undetectable (10,89,220). The selectively restricted uptake of cholesterol by seminiferous tubules can explain this virtual lack of conversion of exogenous cholesterol (155,156). Irusta and Wasserman (100) have reported that a mitochondrial fraction from seminiferous tubules converts [^{3}H]-cholesterol to [^{3}H]-pregnenolone, but no quantitative comparison with an interstitial mitochondrial fraction was made. Bass et al. (10) and Wisner and Gomes (76,220) measured the cleavage of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol, as an estimate for side-chain cleavage activity in mitochondrial fractions obtained from interstitial tissue and seminiferous tubules. Since mitochondria isolated from interstitial tissue are 100-1000 times more active (on protein basis) in cleaving the side-chain of [26-^{14}C]-cholesterol than the corresponding tubular fraction, Wisner and Gomes (220) concluded that mitochondria in seminiferous tubules are lacking in cholesterol side-chain cleaving enzyme complex and that the small conversion rate in vitro can readily be caused by contaminating material from the interstitial tissue.

Incubation studies with tracer amounts of radioactive cholesterol are complicated by the possibility that the radioactive precursor is not taken up or does not completely mix with the endogenous cholesterol pool and therefore the results obtained might not reflect true production rates. This objection does not apply if androgen production from endogenous substrate pools is estimated. Endogenous testosterone production by intact interstitial tissue (42, 44), isolated Leydig cells (103,144) and homogenates of isolated interstitial tissue (108,218 and appendix paper III) has been clearly established. In contrast, data on testosterone production by analogous preparations of seminiferous tubules are contradictory. Cooke et al. (42,44) found no androgen production by isolated intact seminiferous tubules, whereas de Jong (108) estimated a small, but

significant production of testosterone. Lacy and coworkers (123) have claimed that seminiferous tubules produce testosterone from endogenous substrate in at least equal amounts as the interstitial compartments. However, these authors only estimated testosterone concentrations after 2 hours incubation and did not correct for zero time concentrations of this steroid, so that conclusions about the actual production of androgens by isolated seminiferous tubules are not possible. The production rate of testosterone in homogenates of seminiferous tubules in the presence of cofactors is about 500 times lower than the production rate in homogenates of interstitial tissue (appendix paper III). Seminiferous tubules would contribute only 2% to the testosterone production of total testis tissue in vitro, if it is assumed that the interstitial compartment contains 10% of the total testis protein (37,174).

The first step in androgen formation from sterol precursors is the conversion of cholesterol to pregnenolone. This step is catalyzed by the cholesterol side-chain cleaving enzyme complex, which, in rat testis, is localized in the mitochondrial fraction (see section 3.3.). The production of pregnenolone by isolated mitochondrial fractions obtained from interstitial tissue can be easily shown. The production rate of pregnenolone by mitochondrial fractions isolated from seminiferous tubules was 100-500 times lower (on protein basis) when compared with similar mitochondrial fractions from interstitial tissue (appendix papers I, II and III). Table 3.I summarizes data from appendix paper III. In vitro production rates in homogenates and mitochondrial fractions from whole testis tissue, interstitial tissue and seminiferous tubules are compared with the in vivo production rate of testosterone in rat testis as calculated by de Jong and coworkers (109). These data permit the conclusion, that the amount of steroids produced in vitro in preparations of rat interstitial tissue is quantitatively comparable with the amount of testosterone produced and secreted into the blood by the testicular gland in vivo.

Table 3.I Steroid production rates in vitro and in vivo

	production rate (nmol steroid/2 testes, hr)[1]
Whole testis tissue	
homogenate	23.7 ± 9.6
mitochondrial fraction	16.0 ± 4.5
Interstitial tissue[2]	
homogenate	13.6 ± 3.1
mitochondrial fraction	6.1 ± 3.5
Seminiferous tubules[2]	
homogenate	0.20 ± 0.18
mitochondrial fraction	0.16 ± 0.13
Testosterone production in vivo[3]	10.7 ± 7.3

1 In homogenates production rate of testosterone was measured. In isolated mitochondrial fractions the amount of pregnenolone produced has been estimated.

2 Data not corrected for losses of tissue during dissection.

3 de Jong, F.H., Hey, A.H. and van der Molen, H.J. (1973) (ref. 109).

Indirect evidence for the occurrence of enzymes catalyzing the conversion of pregnenolone to testosterone (3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17α,20-lyase and 17β-hydroxysteroid dehydrogenase) in the interstitial compartment can be derived from the patterns of radioactive products in incubation studies with tracer amounts of steroid precursors (14,15,37,61,72,89,98,167,197). From results obtained with the same incubation technique applied to seminiferous tubules, several authors have concluded that 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17α,20-lyase and 17β-hydroxysteroid dehydrogenase are also present in the spermatogenic compartment of rat testis (15,16,37,55,89,214). The poor kinetic conditions of those studies permit only a qualitative interpretation of the

results obtained and do not permit the calculation of the quantitative distribution of the enzymes related to androgen formation in the different tissue compartments.

In appendix paper II the quantitative distribution of 3β-hydroxysteroid dehydrogenase was calculated, by estimating the specific activity of this steroid dehydrogenase in both tissue compartments. At least 95-98% of the enzyme activity could be isolated in the interstitial tissue fraction. Recently, Menard and coworkers (135) reported that 70-80% of the cytochrome P_{450} dependent enzymes 17α-hydroxylase and 17α,20-lyase are present in the interstitial tissue compartment. Richards and Neville (167) and Henry and Gomes (92) found a relatively high 17β-hydroxysteroid dehydrogenase activity in isolated seminiferous tubules.

Conclusions on the capability of isolated seminiferous tubules to produce biological active steroids depend on the degree of purity of the preparations of seminiferous tubules or cell types of this tissue compartment used in studies in vitro. In most studies the degree of purity or lack of contaminating material from interstitial tissue has been evaluated by histological or electron-microscopic examinations.

Early histochemical studies revealed that 3β-hydroxysteroid dehydrogenase was predominantly localized in the interstitial cells of rat testis (22). In a recent publication Hovatta and coworkers (95) established that a certain number of cells, showing positive histochemical reaction for 3β-hydroxysteroid dehydrogenase can always be found at the peritubular tissue of seminiferous tubules, separated from total testis by the wet dissection technique as described by Christensen and Mason (37). Those cells were always located outside the basement membrane and seemed to be identical with Leydig cells. The number of 3β-hydroxysteroid dehydrogenase positive cells attached to the isolated seminiferous tubules was large enough to account for a significant steroid production by preparations of seminiferous tubules in vitro. From this observa-

tion, the very low activities in isolated seminiferous tubules of both the cholesterol side-chain cleaving enzyme complex and the 3β-hydroxysteroid dehydrogenase can easily be explained by interstitial contamination. Therefore, androgen formation from sterol precursors and from pregnenolone in the spermatogenic compartment is not very likely. On the other hand, the occurrence of 17α-hydroxylase, 17α,20-lyase and 17β-hydroxysteroid dehydrogenase in some cell types in the seminiferous tubules cannot be ruled out (57,62,63).

On basis of our present knowledge two different possibilities for the sites of androgen formation in rat testis can be proposed. Cholesterol is converted to pregnenolone and progesterone in the Leydig cells of the interstitial tissue. Most of the progesterone is probably metabolized to testosterone in the same cell type. Testosterone can then be secreted into the blood stream and via the testicular lymph into the seminiferous tubules. Uptake of radioactive progesterone and other steroids by seminiferous tubules in vivo has been shown by Parvinen et al. (155,156) and van Doorn et al. (49). Therefore, part of the progesterone and/or 17α-hydroxyprogesterone and androstenedione may be directly secreted into the seminiferous tubules and there metabolized to testosterone. Both alternatives imply that the concentration of testosterone in the seminiferous tubules is dependent on the steroidogenic activity of the interstitial tissue. According to the first theory tubular testosterone is directly derived from interstitial testosterone, whereas the second theory implies an indirect supply of androgens via conversion of steroid precursors. In order to prove the validity of the second theory it will be necessary to show that the production rate of androgens inside the tubules is high enough to meet the tubular requirements. Maximal production rates of testosterone in preparations of seminiferous tubules, free from contaminating interstitial material, have to be measured and compared with the level of testosterone required for maintaining

spermatogenesis, taking into consideration the turnover of testosterone by catabolizing enzymes and an efflux via the testicular efferent duct to the epididymis.

In conclusion, data at hand support the concept that the interstitial tissue compartment is the main site of androgen production in rat testis. It cannot definitively be excluded that the small production rate of testosterone and low level of 3β-hydroxysteroid dehydrogenase related to the conversion of pregnenolone to progesterone in preparations of seminiferous tubules in vitro reflect contaminating material from the interstitial tissue. In order to solve this problem iso-enzyme patterns of steroidogenic enzymes and the presence of specific marker enzymes for both interstitial tissue and seminiferous tubules have to be studied.

3.3. Subcellular localization of steroidogenesis in rat testis

Toren and coworkers (210) concluded that the cholesterol side-chain cleaving enzyme complex in whole testis could be isolated in the mitochondrial fraction. The enzyme activity was assayed by measuring the rate of cleavage of [^{14}C]-isocaproic acid from [26-^{14}C]-cholesterol. This enzyme system has an absolute requirement for NADPH and molecular oxygen (52,136,137,210). The mitochondrial localization of cholesterol side-chain cleaving enzyme complex is compatable with the results presented in appendix paper I. After isolation of subcellular fractions from whole testis tissue or interstitial tissue, it appears that endogenous steroid production occurs only in the isolated mitochondrial fractions. Therefore both the steroidogenic cholesterol pool (or at least an essential part of this pool) and the cholesterol side-chain cleaving enzyme complex must be localized in this subcellular fraction.

The mitochondrial localization of this enzyme complex has also been reported for the adrenal gland (82) and human

placental tissue (131). In contrast, the cholesterol side-chain cleaving enzyme complex in rat ovarian tissue appears to be localized in mitochondrial, microsomal as well as particle-free supernatant fractions (39,66,67,97). Yago and coworkers (221) have reported that this enzyme complex in bovine corpora lutea is predominantly localized in the mitochondria.

Submitochondrial localization of the testicular cholesterol side-chain cleavage enzyme has been studied in mitochondria obtained from a mouse Leydig cell tumour. Moyle et al. (145) have reported that the enzyme complex was located in the mitochondrial inner membrane fraction, as characterized by cytochrome c oxidase. Although complete separation of mitochondrial inner and outer membranes was not obtained, the same authors (145) concluded from the distribution pattern of monoamine oxidase, a specific marker for the outer membrane (181), that the bulk of mitochondrial cholesterol was present in the outer membrane. Parsons and Yamo (154) obtained similar results for cholesterol localization in guinea-pig liver mitochondria.

Evidence derived from studies using distribution patterns of marker enzymes supports the conclusion that cholesterol side-chain cleaving enzyme complex in the adrenal gland is localized in the mitochondrial inner membrane (222). However, it was observed by Billiar et al. (20) by controlled digestion of adrenal mitochondria by phospholipase A that a difference between the distribution of cholesterol side-chain cleaving enzyme complex and specific marker enzymes for mitochondrial inner membranes and the intermembrane space could be demonstrated. Therefore, these authors questioned the generally accepted submitochondrial distribution of this enzyme complex and proposed a localization in the outer membrane.

Tamaoki (207) states in a recent review that the conversion of pregnenolone to testosterone involving 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17α,20-lyase and 17β-hydroxysteroid dehydrogenase occurs in the endoplasmic

reticulum of the testis Leydig cells. His conclusion is based on results with subcellular fractions not characterized by specific marker enzymes in order to correct for mutual contamination. Since routinely 20-35% of each of these steroid metabolizing enzymes has been found in the isolated mitochondrial fraction and 65-80% in the microsomal fraction (188,207,209,217), it seems inaccurate to conclude that these enzymes are solely present in or attached to the endoplasmic reticulum. Results on the subcellular localization of 3β-hydroxysteroid dehydrogenase are described in more detail in appendix paper II. From differences between the distribution patterns of 3β-hydroxysteroid dehydrogenase and of carboxyl esterase, and from a difference in susceptibility of mitochondrial and microsomal 3β-hydroxysteroid dehydrogenase activity to the proteinase nagarse it was concluded that at least 7-15% of this steroid dehydrogenase is present in mitochondria.

A bimodal localization of this enzyme has also been proposed for the adrenal gland (9,45,117,133), human term placenta (114) and for ovarian tissue (102,210,202).

Sulimovici and coworkers (199) have further fractionated mitochondria obtained from whole testis tissue according the method described by Parsons et al. (153). The inner membrane fraction was identified with the aid of succinate dehydrogenase and cytochrome c oxidase and the corresponding outer membrane fraction with monoamine oxidase. At least 85% of mitochondrial 3β-hydroxysteroid dehydrogenase appeared to be localized in the inner membrane fraction. A point of criticism may be that most marker enzymes used in this study are 70-80% localized in the seminiferous tubules (appendix paper III), whereas 95-98% of the steroid dehydrogenase investigated is present in the interstitial compartment. Therefore results obtained with mitochondrial preparations from whole testis tissue are difficult to interpret.

Indirect evidence for the occurrence of 3β-hydroxysteroid dehydrogenase in the inner mitochondrial membrane

of rat adrenal cortex was obtained by Kream and Sauer (117). Microsomal 3β-hydroxysteroid dehydrogenase showed an absolute dependency for exogenous NAD^+ to convert pregnenolone into progesterone. Mitochondria, however, converted pregnenolone into progesterone in the absence of added NAD^+. Since the mitochondrial matrix space contains endogenous NAD^+ the authors concluded that this steroid dehydrogenase was localized in the inner membrane and had access to the matrix space. Moreover, progesterone formation from pregnenolone in mitochondria was almost completely inhibited by the addition of citric acid cycle substrates, most likely by reducing the intramitochondrial NAD^+, since these substrates had no effect on microsomal 3β-hydroxysteroid dehydrogenase. Depending on the intramitochondrial adenine-dinucleotide redox potential, the end product of the mitochondrial cholesterol side-chain cleaving that leaves the mitochondrion could be either pregnenolone or progesterone. Considering that the formation of pregnenolone by mitochondrial side-chain cleaving of cholesterol depends on the level of reduced NADPH in the matrix space and is stimulated by various citric acid cycle intermediates (203), it seems likely therefore that under conditions when mitochondrial pregnenolone synthesis is stimulated, most of this steroid is converted to progesterone outside the mitochondria.

A possible bimodal localization of 17α-hydroxylase, 17α,20-lyase and 17β-steroid dehydrogenase remains to be elucidated.

The submicrosomal localization of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17α,20-lyase and 17β-hydroxysteroid dehydrogenase has been extensively investigated by Inano and coworkers (207). Electron-microscopic examination of submicrosomal fractions obtained after sucrose-CsCl gradient centrifugation revealed the presence of a fraction with a smooth-surface membrane structure and a second fraction with membrane structures with ribosomal particles on the outer surface. Biochemical analysis showed that the

bulk of RNA was localized in the rough-surfaced microsomal subfraction whereas cytochrome P_{450} enzymes related to the conversion of pregnenolone to testosterone were predominantly present in the smooth-surfaced microsomal subfraction (99,207).

Shikita and colleagues (99,187,188,207,209) have reported the presence of an unknown factor in the particle-free supernatant that stimulated the activities of the enzymes related to the conversion of pregnenolone to testosterone. This factor is heat-stable (99) and is not involved in regeneration of respectively NAD^+ and NADPH (187,188), cofactors for respectively 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase, 17α,20-lyase and 17β-hydroxysteroid dehydrogenase. The chemical nature and the physiological significance of this factor remains unknown. In appendix paper II no evidence could be obtained for the existence of such a cytoplasmic factor, neither from the total recovery of this steroid dehydrogenase activity estimated in subcellular fractionation studies, nor from recombination of a particle-free fraction with aliquots of a microsomal fraction.

In summary it appears well established that the rate-limiting step in androgen formation, that is the conversion of cholesterol to pregnenolone, is mitochondrially localized. Data in appendix papers I-IV show that both the metabolic active precursor pool and the cholesterol side-chain cleaving enzyme complex are present in this subcellular fraction. Although isolated mitochondrial fractions in vitro produce in addition to pregnenolone significant amounts of testosterone, it is not possible to conclude that mitochondria in vivo synthetize androgens without cooperation of enzymes localized in the endoplasmic reticulum required for the conversion of pregnenolone to testosterone. For example, data in appendix paper II show that a small part (7-15%) of 3β-hydroxysteroid dehydrogenase in rat testis is present in mitochondria. Therefore, statements (207) that the endoplasmic reticulum is the only site for the

conversion of pregnenolone to testosterone must be considered with caution.

CHAPTER 4. HORMONAL REGULATION OF RAT TESTICULAR STEROIDOGENESIS

4.1. Introduction

Specific peptide hormones from the pituitary gland initiate, maintain and stimulate testicular steroidogenesis (58,59,84,85). LH (or ICSH) stimulates the production of androgens in the testis in vivo as well as in vitro. In addition, a supplementary role of follicle stimulating hormone and prolactin in the control of testicular steroid production and secretion has been suggested (7,62,78-80, 104,136).

Investigations concerning the hormonal regulation of testicular steroidogenesis have dealt with two main aspects of trophic stimulation of the testicular gland: short-term and long-term effects of trophic hormones. According to Hall (85) "short-term" effects are limited to effects on steroid production within 1 hour after addition of the trophic hormone to the animal or to the incubation medium. Effects observed after 1 hour are considered "long-term" effects.

In general, the acute effect of gonadotrophins may be restricted to intracellular changes in enzyme activities, in supply of cofactors and of substrates for the enzyme system responsible for the production of testosterone. Long-term effects of trophic hormones are a significant increase in testicular protein and in activities of enzymes related to steroidogenesis, and in tissue growth.

Investigation of the effect of long-term treatment with trophic hormones on testicular steroidogenesis was one of the aims of the present thesis. Adult rats with intact pituitaries were injected with HCG daily for 5 days. In

this way, the effects of superimposing exogenous trophic hormones over a long time on endogenous circulating LH were investigated. Most data presented in the literature on long-term administration of trophic hormones on testicular growth and on steroid production have been derived from studies with immature or hypophysectomized animals. Chronic treatment of immature rats with trophic hormones results in an increased androgen production and in increased levels of cholesterol side-chain cleaving enzyme complex and of enzymes related to the conversion of pregnenolone to testosterone (50,70,136,182,186). In essence, the acceleration of the development of testicular steroidogenesis during maturation has been compared with the experimental results obtained after long-term treatment of immature rats with gonadotrophins.

Administration of LH or HCG to hypophysectomized adult rats stimulates testosterone secretion in vivo, as well as testosterone production in vitro and elevates the amount of enzymes related to steroidogenesis (2,50,62,78-80,87,136, 164,165,177,196). This type of study has clearly established that the maintenance of testicular steroid production depends on the presence of a specific pituitary hormone (i.e. LH) in the blood and that exogenous gonadotrophins are able to replace endogenous hormones in their trophic action on the testicular gland.

The effect of chronic administration of trophic hormones on steroidogenesis in testis from adult rats with intact pituitaries is discussed in section 4.2.

The scheme for the molecular mechanism of the acute action of LH on testosterone production in the testis is mainly based on data obtained in studies on the adrenal gland. It is generally accepted that receptor sites for trophic hormones are present at the plasma membrane of target cells. Binding of the hormone by the receptor results in an activation of adenylate cyclase activity, localized in the cell membrane, which in turn will increase intracellular cyclic AMP concentrations. This cyclic nucleo-

tide can activate protein kinase activity, after binding to a specific receptor. It has been suggested that enhanced protein kinase activity may cause an increased phosphorylation of a ribosomal compound resulting in the formation of a labile protein. On the other hand activation of a newly synthetized protein cannot be excluded. This hypothetical labile protein factor may stimulate the transfer of cholesterol accumulated in the cytoplasma to the enzyme system, localized in mitochondria catalizing the conversion of cholesterol to pregnenolone (see ref. 183 for an extensive review). The significance of this concept for the hormonal stimulation of testicular steroidogenesis is discussed in section 4.3.

In the present thesis the acute effect of LH on testicular androgen formation has been investigated. Special attention has been given to the role of mitochondrial pregnenolone formation as the rate-limiting step in testicular testosterone production. The estimation of endogenous steroid production rates in homogenates and in subcellular fractions offered the possibility to compare steroid production rates from endogenous substrates in vitro and in vivo after acute trophic hormone stimulation. In addition, the rapidity of the increase of production rates in vitro after hormonal stimulation in vivo was compared with the increase observed in vivo (see appendix paper IV). Finally, a possible role of Ca^{2+} in the regulatory mechanism of the acute effect of trophic hormones on steroid formation has been investigated (chapter 5).

4.2. Long-term effects of trophic stimulation

It has been reported that the conversion of [^{14}C]-acetate to [^{14}C]-testosterone in slices of rabbit testis was stimulated after administration of ICSH for 3 days (86), but no effect on the conversion of labelled cholesterol to testosterone in rabbit testis homogenates was observed (87).

Long-term administration of HCG had only a stimulatory effect on the enzymic activity of 3β-hydroxysteroid dehydrogenase, 17α,20-lyase and 5α-steroid reductase, estimated in testicular microsomal fractions of rats younger than 22 days (186). Shikita and Hall (186) therefore proposed that testes of rats older than 22 days are under the influence of high levels of endogenous gonadotrophins, which maintain a maximally stimulated enzyme activity. De Jong and coworkers (108,109) have, however, shown that treatment with HCG for 5 consecutive days increased the testosterone concentration in rat testicular venous plasma, although it has also been reported that testosterone levels in peripheral plasma were not elevated after 3.5 days of LH administration (62). These results might have been caused by differences in the turnover or clearance of circulating testosterone and/or injected trophic hormone. Long-term HCG treatment in vivo caused a decreased testosterone production by decapsulated rat testis in vitro (108). In contrast, it was shown in appendix paper III that the same prolonged treatment with HCG stimulated testosterone production in homogenates of whole testis tissue, incubated in the presence of cofactors. From the increased pregnenolone production in isolated mitochondrial fractions, it was concluded that HCG stimulates testicular androgen formation by activating the mitochondrial processes involved in steroid production. Purvis et al. (165) have reported that prolonged HCG treatment of intact adult rats will increase the amount of mitochondrial cytochrome P_{450}, an essential component of the cholesterol side-chain cleaving enzyme complex. Long-term HCG administration will specifically stimulate steroid production in the interstitial compartment of the testis (appendix paper III). Although the total amount of interstitial tissue protein was not elevated after hormone treatment, a 40% increase in mitochondrial protein was observed.

Nussdorfer and colleagues (152) investigated with sterologic techniques the effect of chronic administration of

ACTH on the mitochondria of the zona fasciculata of the rat adrenal cortex. Up to the 9th day of hormone treatment the volume of the mitochondria significantly increased, whereas the number of mitochondria per cell was only slightly elevated. After 12 days of ACTH treatment they observed a large increase in the number of mitochondria per cell, with a concomittant decrease in their average volume. Unfortunately, no data are available that allow extrapolation of these phenomena observed in the adrenal gland to the testis. For that reason, it cannot be concluded that the increase of interstitial mitochondrial protein,cytochrome P_{450} and pregnenolone synthesis reflects the effect of HCG treatment either on the size of the mitochondria in the interstitial compartment or on the total number of mitochondria involved in steroidogenesis.

In contrast with the earlier mentioned results of Shikita and Hall (186), prolonged HCG treatment for 5 days caused an increase in specific activity of 3β-hydroxysteroid dehydrogenase (appendix paper III). It has been shown that microsomal cytochrome P_{450} decreased to one third of the level in control animals after 2 days of HCG administration (164). After 4 days of HCG injections cytochrome P_{450} levels were restored to control values, and further HCG treatment caused a rapid increase in microsomal cytochrome P_{450} content. A similar pattern was observed when cytochrome P_{450} dependent 17α-hydroxylase and 17α,20-lyase activities were determined. In contrast, the specific activity of 17β-hydroxysteroid dehydrogenase decreased slowly but consistent during chronic HCG treatment of intact adult rats (164).

Limited information is available on the effect of long-term trophic hormone treatment on cholesterol metabolism in rat testis. The incorporation of radioactive acetate into cholesterol has been found to be increased after chronic HCG administration (224). Prolonged HCG treatment in combination with aminoglutethimide, a specific inhibitor of the cholesterol side-chain cleaving enzyme complex, resulted in histochemical evidence for the accumulation of lipid

material in the Leydig cell of puberal rat testis (151). The chemical nature of the accumulated material remains to be established, although it has been suggested that these lipid droplets might consist of cholesterol (esters). However, van der Molen et al. (140) could not observe any effect of HCG treatment on concentrations of free and of esterified cholesterol levels in homogenates of whole testis and isolated interstitial tissue. This may indicate that the accelerated cholesterol biosynthesis (224) keeps pace with the stimulated conversion of this compound to androgens.

To summarize, chronic treatment of adult rats for several days with HCG resulted in an increased testosterone production in vivo (108,109) as well as in vitro (appendix paper III). The increase in androgen formation may be explained by an elevated pregnenolone production in mitochondrial fractions from the interstitial compartment. In addition, levels of 3β-hydroxysteroid dehydrogenase (appendix paper III), 17α-hydroxylase and 17α,20-lyase were increased after prolonged hormone treatment (164). In contrast a small decrease of 17β-hydroxysteroid dehydrogenase activity was observed (164). It is shown in appendix paper III that the same prolonged HCG treatment did not elevate the specific activities of interstitial mitochondrial and interstitial microsomal enzymes which are not directly related to androgen formation. It is concluded that chronic treatment with HCG results in a specific increase of enzyme levels responsible for the formation of androgens in the testicular gland.

4.3. Short-term effects of trophic stimulation

4.3.1. General aspects

Infusion of HCG via the spermatic artery of the dog testis resulted in an almost instantaneous increase of tes-

tosterone secretion (57,58). Intravenous administration of HCG or LH to normal adult rats increased the testosterone concentration both in testis tissue and in testicular venous plasma within 20 min after injection (108,109 and appendix paper IV). Trophic hormone in vitro stimulates the conversion of labelled cholesterol to testosterone by slices of rabbit testis (83,88) as well as the production of androgens from endogenous precursors by intact testis tissue and isolated cells within 30 to 60 min after addition of the hormone (41,54,103,120,146,147,171,172). Rapid responses of steroid production to trophic hormones have also been observed in studies with preparations of the adrenal gland (11,147,168,183) and of ovarian tissue (3,4, 170).

In recent years more experimental evidence has been obtained about the intracellular mechanism of action of LH in the testicular gland. Therefore, an attempt can now be made to discuss the validity of the general working hypothesis given in section 4.2. for the action of trophic hormones on steroidogenesis in the testicular gland.

4.3.1.1. Receptors for gonadotrophic hormones

The occurrence of receptor sites for HCG and LH in rat testis have been described (34,35,53). Binding of labelled LH and HCG occurs mainly in the interstitial compartment of the testis (68,118,126). According to the working hypothesis trophic hormone receptors should be present at the plasma membrane and functionally related to the adenylate cyclase, localized at the inner side of the Leydig cell membrane. However, Pulsinelli and Eik-Nes (163), Hollinger (94) and Sulimovici and coworkers (200,206) have reported the presence of adenylate cyclase activity in isolated mitochondrial fractions obtained from dog (94,163) and rat testes (200,206).

4.3.1.2. Cyclic AMP as second messenger

HCG and LH can increase intracellular cyclic AMP concentration in whole testis, in dissected interstitial tissue and in isolated partially purified Leydig cells (40,103, 172,173,174,178). Since the increase of intracellular cyclic AMP concentration precedes the elevation of steroid production (171,173) and since cyclic AMP and dibutyryl cyclic AMP have shown to stimulate testosterone production in vitro (38,178), it has been suggested that this cyclic nucleotide is the intracellular ("second") messenger in trophic hormone stimulation of testosterone production (see for review ref. 173).

Recent publications have cast a doubt on the obligatory and unique role of cyclic AMP as the second messenger in the sequence of intracellular events provoked by trophic hormones. A large discrepancy was observed between the amount of LH required to stimulate testosterone production and to elevate the intracellular concentration of cyclic AMP (44,146,161). Low amounts of LH stimulated testosterone production whereas no significant effect on cyclic AMP concentration was observed. This may indicate that hormonal control of steroidogenesis under certain conditions is not mediated by cyclic AMP, but by other unknown cellular factor(s). Beall and Sayers (11) have reported a similar observation during stimulation of corticosteroid production in isolated adrenal cells by low doses of ACTH. However, failure to demonstrate an increase in the concentration of cyclic AMP may simply reflect the inadequacy of the assay techniques used for the estimation of the cyclic nucleotide. Moreover, a multiple intracellular compartmentation of cyclic AMP may exist, whereas only one of those compartments, comprising a small fraction of total cellular cyclic AMP, may be involved in the activation of steroid production. Recently, Schulster and coworkers (130,184) and Nakamura et al. (149) have shown that stimulation of corticosteroid production by low amounts of ACTH is always pre-

ceded by a small, but significant increase in cyclic AMP concentration. In their opinion, there is no strong evidence that cyclic AMP does not act as second messenger in hormonal stimulation of steroidogenesis.

A direct in vitro effect of cyclic AMP on 3β- and 17β-hydroxysteroid dehydrogenases in rat testis has been reported (27,204). The concentration of cyclic AMP used in these studies were in the millimolar range, whereas cyclic AMP in interstitial tissue after LH stimulation is present in micromolar concentrations (172). Although an accumulation of cyclic AMP at the enzymatic site cannot be excluded it does not appear very likely that these inhibitory effects have a physiological meaning. Data of Catt and coworkers (36) do not favour the possibility that cyclic AMP will inhibit steroid metabolizing enzymes because most of cyclic AMP produced by trophic stimulation is extruded from the cell and can be recovered in the incubation medium.

4.3.1.3. Protein kinase activity

The presence of cyclic AMP dependent protein kinase activity in rat testicular interstitial tissue has been established (18,43,161). Exogenous histone is generally used as a substrate for estimation of protein kinase activity. In order to elucidate an obligatory role of protein kinase in the stimulating action of LH on androgen production, phosphorylation of specific endogenous protein(s) in the Leydig cell has to be shown. Murakami and Ichii (148) have reported that ACTH treatment in vivo activates the incorporation of radioactively labelled phosphate into mitochondrial, microsomal as well as cytosol protein in the adrenal gland. The nature of the phosphorylated compounds has not been further investigated. However, no data are available which demonstrate that LH activates the phosphorylation of endogenous proteins in rat testis, resulting in a drastic change in either biological or enzymatic activity of the

phosphorylated protein factor.

4.3.1.4. Synthesis of "labile" proteins

Studies carried out with inhibitors of protein synthesis have provided indirect evidence that newly synthetized proteins play an important role in trophic stimulation of steroid production in rat testis (41,86 and appendix paper IV). It was found that cycloheximide and puromicine, specific inhibitors of ribosomal protein synthesis in the endoplasmic reticulum (158), prevented the trophic stimulation of androgen production. Chloramphenicol, an inhibitor of mitochondrial protein synthesis, seemed to be without effect on testosterone production in isolated Leydig cells (103), although Hall and Eik-Nes (86) have reported that chloramphenicol inhibited the incorporation of labelled acetate into testosterone in slices of rabbit testis.

Until now, attempts to show a stimulating effect of LH on protein synthesis in various preparations of intact adult rats have been unsuccessful. This experimental result may support the theory that LH acts by activating newly synthetized protein(s) although it cannot be ruled out that the present assay methods are not adequate enough to detect the increased synthesis of a particular protein.

Cooke et al. (41) have calculated that the decay half-life of the newly synthetized protein involved in stimulated testosterone formation is less than 13 minutes. A protein factor with a rapid turnover rate involved in hormone stimulated steroidogenesis has also been postulated for ovarian tissue (5) and adrenal cells (183).

4.3.2. Role of mitochondria in testicular steroidogenesis

It is generally accepted that the conversion of cholesterol to pregnenolone, which is restricted to mitochondria,

is rate-limiting in overall testosterone production (85). The ultimate locus of hormonal stimulation of androgen production may therefore be present in this particular subcellular fraction. The results reported in appendix paper IV have clearly established that stimulated testosterone production after LH treatment is caused by increased pregnenolone formation from endogenous precursors in the mitochondria. Experimental data concerning the essential link between hormonal stimulated events in the cytosol of the Leydig cell and the increased conversion of cholesterol into pregnenolone are still limited.

The production of pregnenolone in the mitochondrion is a result of the activity of a highly integrated system, involving an enzyme complex in the inner mitochondrial membrane, a steroidogenic cholesterol pool, molecular oxygen and NADPH. The enzyme system, (the so-called cholesterol side-chain cleaving enzyme complex) has been shown to contain cytochrome P_{450} and an iron-sulfur protein: "testodoxin" (analogous to adrenodoxin in adrenal mitochondria (96,203)) as well as an FAD-containing protein: NADPH-"testodoxin" reductase (132).

No experimental evidence exists to conclude that short-term treatment with trophic hormones increases the concentration of one of those components of the cholesterol side-chain cleaving enzyme complex. In contrast, prolonged hormonal treatment for 5 consecutive days was necessary to show an elevated content of mitochondrial cytochrome P_{450} (132,165).

The biochemical mechanism of cleaving the side-chain of cholesterol, resulting in the formation of pregnenolone and isocaproic acid, is not completely elucidated (31,183,204). Hydroxylation of the carbon atoms at 20- and at 22-positions (32,33,48,169,189), formation of 20α-peroxide (128) or a direct desaturation of 20-22 carbon bond, resulting in a Δ^{20-22} cholesterol (116) has been proposed as the first step in splitting the side-chain in adrenal mitochondria. Independent of the exact mechanism involved, investigations

with adrenal mitochondria have revealed that the rate of association of cholesterol with cytochrome P_{450} may be rate-limiting in overall pregnenolone formation (29,111, 191). Since all these factors are required for the biosynthesis of pregnenolone, LH can theoretically exert its stimulating effect via one or a combination of the following factors:

1. enhancement of the supply of molecular oxygen for the enzyme system.
2. net change in intramitochondrial NADPH/$NADP^+$ ratio.
3. release of pregnenolone from the mitochondria to prevent feed back inhibition by this steroid of cholesterol side-chain cleaving enzyme activity (115).
4. an increase of rate of association of cholesterol with cytochrome P_{450}, through a stimulation of the intramitochondrial transfer of cholesterol from a metabolically inert pool to a steroidogenic pool as well as by an increase of the supply of cholesterol from extramitochondrial sources.

ad 1: The increased blood flow through the testicular gland under influence of gonadotrophins resulting in an elevated O_2 supply to the hydroxylation chain has been proposed as a possible explanation of hormonal stimulated androgen formation (85). Hamberger and Steward (90) reported that LH stimulated oxygen consumption by interstitial cells. Since the increase in oxygen consumption has not been stoechiometrically correlated with an increase in testosterone formation, the observed phenomenon may reflect more a positive effect of trophic hormones on the total metabolic state of the interstitial cells than a driving force for the conversion of cholesterol into pregnenolone.

ad 2: The concept that trophic hormone action is mediated by changes in NADPH/$NADP^+$ in cytosol and matrix space of the mitochondria of the steroid producing cell has been considered for a long time. According to the original

scheme of Haynes and Berthet (91) the metabolism of glucose-6-phosphate via the pentose phosphate pathway enhanced the availability of NADPH for steroidogenesis. Since cyclic AMP is involved in the activation of phosphorylase activity (91) it was suggested that trophic hormones can stimulate steroid production by elevating the amount of glucose-6-phosphate originating from the breakdown of glycogen. The inner mitochondrial membrane is impermeable for NAD(P)H (77). However, it is now known, that reducing equivalents of cytoplasmic NAD(P)H can be transported across the inner mitochondrial membrane via shuttle mechanisms (see review 138). Moreover, the presence of "malic" enzyme in mitochondria of various steroidogenic cell types (143,159,160,190, 198) provides a second mechanism to raise intramitochondrial NADPH/$NADP^+$ ratios. Although data concerning the involvement of NADPH in trophic stimulation of testicular steroid production are limited compared with the adrenal gland, some experimental evidence has been provided that glycolysis and Krebs cycle activity may be related to gonadotrophic stimulated androgen production. The increase in testosterone production from endogenous substrates by interstitial tissue in the presence of LH was greater when glucose was added to the incubation medium (172). Isolated mitochondrial fractions convert more labelled cholesterol to pregnenolone and produce more pregnenolone from endogenous substrates in the presence of Krebs cycle intermediates (23, and section 5.2.), most probably through an elevation of the intramitochondrial NADPH/$NADP^+$ ratio. For testis no data are available which allow the conclusion that the stimulating effect of trophic hormones on steroid production can be explained solely by an increase of reduced NADPH at the rate-limiting step in androgen formation.

ad 3: Pregnenolone can inhibit the conversion of radioactive cholesterol to pregnenolone by mitochondrial preparations from bovine adrenal cortex (115), bovine corpus luteum (97) and rat testis (51). Koritz and Hall (115) have

suggested that trophic hormones might stimulate the conversion of cholesterol into pregnenolone, by increasing the rate of removal of pregnenolone from mitochondria. Trophic hormone action may induce a modification in mitochondrial membrane structure, causing mitochondrial swelling and concomittant increase of permeability for pregnenolone. There is, however, little experimental evidence to support this hypothesis. Incubation of quartered adrenal glands (64) as well as isolated mitochondrial fractions from the adrenal gland (191), from ovarian tissue (5) and from testicular interstitial tissue (appendix paper II) in the presence of cyanoketone (a specific inhibitor of 3β-hydroxysteroid dehydrogenase activity (74)), resulted in an accumulation of pregnenolone. Under these conditions no inhibition of pregnenolone formation was observed. Johnson and Nelson (105) have reported that addition of albumin to the incubation medium caused a transfer of endogenous pregnenolone from adrenal mitochondria to the medium, without affecting the production rate of pregnenolone. In appendix paper IV it was shown that LH treatment in vivo of normal adult rats caused a five times increase in pregnenolone concentrations in isolated testicular mitochondrial fractions. Despite the mitochondrial accumulation of pregnenolone, in vitro production rates of pregnenolone by mitochondrial fractions isolated from hormone stimulated testis were increased when compared with relevant control samples. Therefore, it is not very likely that release of pregnenolone from the mitochondria is an important factor in control mechanism of steroidogenesis.

ad 4: Metabolism of cholesterol as related to steroid production has been extensively explored in the adrenal gland and ovarian tissue. The generally accepted hypothesis concerning the role of cholesterol dynamics in regulating steroid production has been formulated in great detail by Garren and coworkers (73) and by Boyd and Trzeciak (26). ACTH stimulates the hydrolysis of esterified cholesterol,

accumulated in lipid storage particles in the cytosol (26, 73). Activation of cholesterol esterase activity by ACTH treatment has been established (26,211). Trzeciak and Boyd (211) have presented strong evidence that cholesterol esterase activity can be regulated by cyclic AMP. Hormonal activation of the hydrolysis of esterified cholesterol in the lipid droplets appears to be a cycloheximide-insensitive process (73). This may indicate that the hypothetical protein compound is not involved in this particular step of cholesterol metabolism. The accumulation of free cholesterol in the lipid droplets which results after cycloheximide administration (73) indicates that the transport of free cholesterol from the storage particles to the mitochondria requires a newly synthetized protein factor. Farese (63) was able to isolate from supernatant fractions of ACTH stimulated adrenal quarters a protein moiety that showed a small, but significant stimulation of steroid production by mitochondrial fractions isolated from control adrenal quarters. However, there is no information about possible cholesterol binding properties of this protein factor. Kan et al. (112,113,212) have reported about a cholesterol binding protein in preparations of adrenal mitochondria, which was capable to enhance the conversion of cholesterol to pregnenolone in incubations of acetone powders of adrenal mitochondrial fractions. However, no change in the level of this protein compound after ACTH treatment of intact or hypophysectomized rats was observed (113). Electron microscopic studies (73,119,185) have revealed that mitochondria and the abundant lipid droplets in adrenal cells are in close contact with each other and ACTH treatment resulted in a fusion of these subcellular particles (119,185). A possible role of a labile newly synthetized protein factor in this fusion process remained unclear.

On the other hand, an intramitochondrial shift of cholesterol from a metabolically inert cholesterol pool to cytochrome P_{450}, an essential component of cholesterol side-chain cleaving enzyme system, may occur by trophic stimula-

tion (29,111,191). Since no change in total mitochondrial cholesterol concentration after in vivo ACTH treatment was observed (191), cholesterol supply from extramitochondrial sources must be in equilibrium with the trophic hormone induced intramitochondrial substrate shift and subsequent increased conversion to pregnenolone. It is doubtful whether this scheme for activation of adrenal steroid production can be applied integrally to the mechanism of action of LH on androgen formation in the testis of the rat.

In striking contrast with the adrenal gland and ovarian tissue, very few lipid droplets and esterified cholesterol are present in the steroidogenic compartment of rat testis (see table 4.I). The concentration of free cholesterol, however, is comparable (73,140,157). Free cholesterol is predominantly localized in the mitochondrial and microsomal fractions (140,215). No acute effect of LH administered in vivo on free cholesterol as well as on esterified cholesterol in isolated interstitial tissue could be observed (140), although under such conditions testosterone levels in testicular venous plasma (109 and appendix paper IV) and in testicular tissue are elevated and rates of mitochondrial pregnenolone production are increased (appendix paper IV). This discrepancy can be explained by the fact that the theoretical amount of cholesterol required for steroid production is rather small (table 4.I). Therefore, in contrast with the adrenal gland dramatic changes in cholesterol levels in rat testis tissue cannot be expected. No experimental data are available, which allow the definitive conclusion that LH stimulates testicular steroid production either by an increased transport or supply from extramitochondrial sources to the mitochondrion, or by an intramitochondrial shift of a large inert cholesterol pool to the cholesterol side-chain cleaving enzyme complex.

With respect to the cholesterol ester content of testis tissue, it is of interest that differences exist between rats and mice which are normally used for laboratory work. In mouse testes the cholesterol esters account for 10-40%

Table 4.I Concentrations of free and esterified cholesterol in rat adrenal cortex and rat testis interstitial tissue and pregnenolone production rates from endogenous substrate by isolated mitochondrial fractions

	Tissue			
	Adrenal cortex		Interstitial tissue	
Homogenate				
free cholesterol (nmol/mg protein)	50	(73)	20	(140)
esterified cholesterol (nmol/mg protein)	400 - 450	(26,73)	0.3	(140)
% esterified cholesterol	90		1.5	
Mitochondrial fraction				
free cholesterol (nmol/mg mitochondrial protein)	10 - 15	(26,191)	25 - 100	(215)
pregnenolone production (nmol/mg mitochondrial protein, 10 min)	2 - 5	(26,191)	0.3 - 0.5*	(215)

incubation was performed at 27°C in a medium containing 0.250 M sucrose, 20 mM KCl, 10 mM potassium phosphate, 5.0 mM $MgCl_2$, 15 mM Tris HCl (pH 7.3), 6.0 mM isocitrate and 4 µM cyanoketone.

* incubation was performed at 33°C in a medium containing 0.125 M sucrose, 35 mM Tris HCl (pH 7.3), 5.0 mM $MgCl_2$, 20 mM sodium succinate and 0.1 µM cyanoketone.

Numbers between brackets are references.

of the total cholesterol (6,8,162). LH treatment in vivo caused a significant decrease in total esterified cholesterol content of the mouse testis (6,8). In addition, LH may stimulate steroid production in isolated Leydig cell tumours in part by increasing the concentration of free cholesterol within the cell, released from esterified cholesterol (162). This indicates that hormonal control of mouse testis steroid production in this respect is consistent with the model of Garren et al. (73) postulated for the adrenal gland.

4.3.3. Conclusions

An increase in the blood concentration of LH of adult male rats with intact pituitaries results in a rapid increase of testicular testosterone secretion (108,109 and appendix paper IV). A concomittant increase in testicular testosterone concentration takes place, indicating an elevated production of androgens in vivo by trophic hormone action. Testosterone production rates in whole testis tissue homogenates are markedly increased when testes are removed from the animal 20 min after intravenous injection of LH (appendix paper IV). The increase in pregnenolone production estimated in isolated mitochondrial fractions is most probably the cause of increased testosterone formation. Increased pregnenolone production rates support the generally accepted theory that the mitochondrial conversion of cholesterol to pregnenolone is rate-limiting in steroid biosynthesis and that this step is the locus of acute trophic action.

Table 4.II summarizes in quantitative terms data from appendix paper IV, reflecting the effect of intravenous injection of LH on testicular testosterone production in vivo as well as on steroid production in vitro. Table 4.II clearly shows that a good correlation exists between the increase in amounts of testosterone synthetized in vivo

Table 4.II Acute effect of LH in vivo on testosterone production in vivo and steroid production in vitro

	control	LH treated
in vivo		
testosterone secretion rate[1] (nmol testosterone/testis, 20 min)	1.3	3.1
testosterone concentration[2] (nmol testosterone/testis)	0.35	3.5
in vitro		
testosterone production rate[3] (nmol testosterone/testis equivalent, 20 min)	1.6	6.6
pregnenolone production rate[4] (nmol pregnenolone/testis equivalent, 20 min)	1.3	4.5

1 Calculated from data in Table I, appendix paper IV, assuming that 10% of testosterone is bound to erythrocytes and that the haematocrit value is 45% (109). Values of 0.25 ml/gram testis, min for testicular blood flow were obtained from Jones (107) and Free and Tillson (69).

2 Data from Table II, appendix paper IV, assuming that one rat testis contained 110 mg protein (appendix paper III).

3 Data from Fig. 3A, appendix paper IV. Testes were removed 20 min after hormone injection. Testosterone production rates estimated in whole testis tissue homogenates.

4 Data from Fig. 3B, appendix paper IV. Testes were removed 20 min after hormone injection. Pregnenolone production rates were estimated in isolated mitochondrial fractions.

during the first 20 min after LH injection, and steroid production rates in vitro estimated in preparations of testis, removed 20 min after injection of the trophic hormone.

Injection of cycloheximide prior to LH prevents the stimulating effect of LH treatment on steroid production estimated in vivo as well as in vitro (appendix paper IV). This observation may offer indirect evidence that synthesis

of a protein compound is involved in the hormonal regulation of testicular steroidogenesis. Cooke et al. (41) calculated from data obtained from superfusion experiments that this hypothetical protein will have a half-life of 13 min or shorter.

Data presented in appendix paper IV and section 5.2. may suggest that stimulation of mitochondrial pregnenolone production by trophic hormones is caused by an increase in availability of sterol substrate for the conversion to pregnenolone by cholesterol side-chain cleaving activity. Information on the molecular mechanism of hormone stimulation of mitochondrial pregnenolone synthesis in rat testis is still limited.

CHAPTER 5. THE ROLE OF Ca^{2+} IN HORMONAL REGULATION OF TESTICULAR STEROIDOGENESIS

5.1. Introduction

Recent experimental evidence, mainly from adrenal preparations, indicates that Ca^{2+} ions may play a key role in the hormonal regulation of production and secretion of steroids. A possible interaction between cyclic AMP and Ca^{2+} ions in hormonal control mechanisms of a variety of tissues has been reviewed by Rasmussen (166).

Extracellular Ca^{2+} appears to be required for the increase in steroid production by rat adrenal gland in response to ACTH (21,81). Sayers and coworkers (180) have suggested that the magnitude of the signal generated by binding of ACTH to the membrane receptor site and the transmission of this signal through the membrane to the adenylate cyclase compartment is related to the extracellular Ca^{2+} concentration. Since the stimulation of corticosteroid synthesis by dibutyryl cyclic AMP was hardly affected by extracellular Ca^{2+}, Haksar and Peron (81) concluded that Ca^{2+} has a larger effect on the events prior to the formation of the second messenger cyclic AMP, than on the events resulting from increased cyclic AMP production. In contrast, Bowyer and Kitabchi (25) concluded that the action of Ca^{2+} may occur both before and after the stimulation of cyclic AMP. Dufau et al. (56) have reported that binding of gonadotrophic hormones to isolated Leydig cells is not influenced by Ca^{2+} ions. The production of cyclic AMP and testosterone in response to gonadotrophic stimulation was, however, reduced in the absence of extracellular Ca^{2+} ions.

Several important observations have been reported about

intracellular effects of Ca^{2+} in steroidogenic tissue. Farese (64) observed that low concentrations of Ca^{2+} ions can stimulate the transfer of amino acid from the amino acyl transfer-RNA complex to protein in cell-free preparations of adrenal glands. With soluble fractions from ACTH stimulated adrenal cells (which contain an increased transfer enzyme activity) no effect of Ca^{2+} was observed. Moreover, Ca^{2+} can activate malic enzyme in adrenal mitochondria (160), but it is not clear whether Ca^{2+} increases the availability of cytosol $NADP^{+}$ for the intramitochondrially localized cholesterol side-chain cleaving enzyme complex via this mechanism.

Mitochondria can readily concentrate Ca^{2+} from the surrounding medium (125). Therefore, mitochondria can play a role in modifying the cellular ionic environment. The intramitochondrial Ca^{2+} concentration is not necessarily constant, but can be influenced by changes in the rate of Ca^{2+} uptake and in extramitochondrial Ca^{2+} concentrations. It has been found that high extramitochondrial concentrations of Ca^{2+} can cause a swelling of adrenal mitochondria in vitro with a concomittant increase in pregnenolone production rate when NADPH is present in the incubation medium (93,192). Studies with isolated mitochondria from adrenal cortex suggest that the association of cholesterol with cytochrome P_{450} is rate-limiting in overall pregnenolone production rate and that ACTH increases the association rate of substrate and enzyme complex (29,111,192). In this respect Simpson et al. (192) have reported that Ca^{2+} ions in vitro induce changes in cytochrome P_{450} difference spectra similar to the changes that occur after ACTH stimulation in vivo. These authors suggest that Ca^{2+} is involved in a shift of intramitochondrial cholesterol from a metabolic inert pool to the cholesterol side-chain cleaving enzyme complex. The low concentrations of cytochrome P_{450} in testicular mitochondrial fractions (132), however, do not permit a similar type of study with testicular mitochondria.

ACTH can influence the distribution of Ca^{2+} in the adrenal gland (101,127). It has been reported, that ACTH and dibutyryl cyclic AMP cause a significant stimulation of $^{45}Ca^{2+}$ uptake by rat adrenal glands in vitro (127). Within 5 minutes after hormone treatment a marked accumulation of $^{45}Ca^{2+}$ was observed in mitochondrial and microsomal fractions (127). Jaanus and Rubin (101) have suggested that increased Ca^{2+} levels in subcellular fractions are the result of a translocation and redistribution of Ca^{2+} between the intracellular components, without significant uptake of extracellular Ca^{2+}.

Addition of Ca^{2+} ions will also stimulate the conversion of labelled cholesterol to steroids by isolated mitochondrial fractions of rat testis (52). In appendix paper IV it was concluded that LH administration to intact, adult rats caused a difference in mitochondrial sensitivity to Ca^{2+}. These preliminary observations have been extended in section 5.2.

5.2. Experimental results

Adult intact rats were anaesthetized with nembutal and were then intravenously injected with 25 µg LH (LH treated testes) or with 0.2 ml 0.9% NaCl - 0.2% serum bovine albumin solution only (control testes). Testes were removed from the animal 20 min after injection. Mitochondrial fractions were isolated as previously described (appendix paper III).

Influence of incubation time on pregnenolone production rates.

Figures 1 and 2 summarize the influence of incubation time on pregnenolone production rates in mitochondrial fractions from control and LH treated testes. Figure 1 shows a biphasic pattern of pregnenolone formation by mitochondrial fractions isolated in sucrose-EDTA from both con-

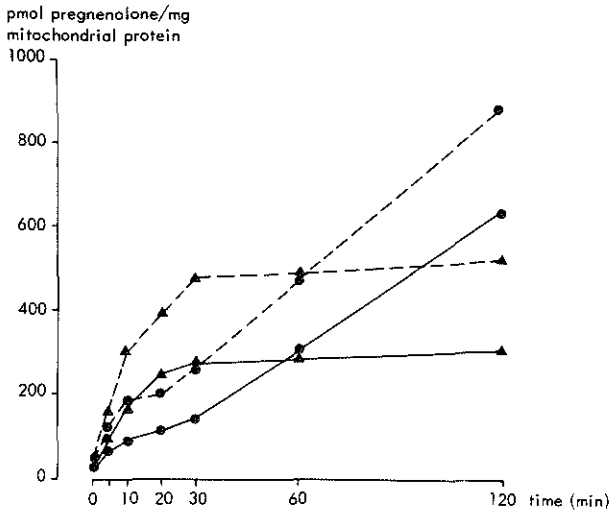


Figure 1 Time course of the concentrations of pregnenolone in mitochondrial fractions isolated in 0.25 M sucrose, containing 1 mM EDTA, from luteinizing hormone treated and control testes.

The incubation medium contained 0.125 M sucrose, 0.5 mM EDTA, 20 mM sodium succinate, 5.0 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.3), 0.1 µM cyanoketone and 0.7 mM $CaCl_2$. ▲, aliquots of mitochondrial protein incubated in 2 ml incubation medium only. ●, aliquots of mitochondrial protein in incubation medium plus 1.0 mM NADPH, 10 mM glucose 6-phosphate and 0.3 unit glucose 6-phosphate dehydrogenase. (——) solid line, 0.95 mg mitochondrial protein from control testes. (---) broken line, 1.0 mg mitochondrial protein from luteinizing hormone treated testes.

trol and LH treated testes, when incubated in the presence of 20 mM succinate and 0.7 mM Ca^{2+} ions. An initial phase (10-20 minutes) of high pregnenolone production was followed by a very slow rate of production up to 120 min incubation time. In mitochondrial fractions obtained from LH treated testes production rates during the first 10 min were increased when compared to relevant control samples. Production rates after 30 min incubation were almost zero. Addition of NADPH to the incubation mixture containing succinate and Ca^{2+} ions resulted in a triphasic pattern. An ini-

tial phase lasting 10 min of high pregnenolone production was followed by a slower production rate during about 20 minutes. Thereafter a rapid increase in production rates by mitochondrial fractions isolated from control testes as well as from LH treated testes took place. The stimulating effect of NADPH on pregnenolone production after longer incubation times may result from an increased permeability of mitochondrial membranes for NADPH. During incubations lasting 10-30 min an unexpected phenomenon has been observed when NADPH was present in the incubation medium. The presence of NADPH resulted in lower succinate supported production rates of pregnenolone during short incubation times (figure 1). This might reflect a lower production of pregnenolone as well as an increased catabolism. Therefore, studies were performed with tracer amounts of [16-^{3}H]-pregnenolone added to the incubation mixture at time zero. After incubation the steroid products were analyzed using silicagel thin-layer chromatography with trichloromethane: diethyl ether (85:15, v/v) as eluent fluid, and it appeared that during incubation in the presence of added NADPH a part of the radioactive steroid was metabolized to a more polar compound. In the absence of added NADPH little or no conversion of labelled pregnenolone took place (data not shown).

Figure 2 shows the effect of duration of incubation on pregnenolone concentrations in control and LH stimulated testicular mitochondrial fractions isolated in sucrose without EDTA. In the absence of extramitochondrial succinate and Ca^{2+} ions control mitochondrial fractions produced pregnenolone only during the first 10 min of incubation. Thereafter production rates were almost negligible. Addition of 0.5 mM Ca^{2+} to the incubation medium at time zero increased the initial production rate but had no effect on the production rates observed after 10 min incubation. Addition of 20 mM succinate hardly influenced production rates during the first 10 min, but prevented the decrease of pregnenolone production rates, probably by preventing

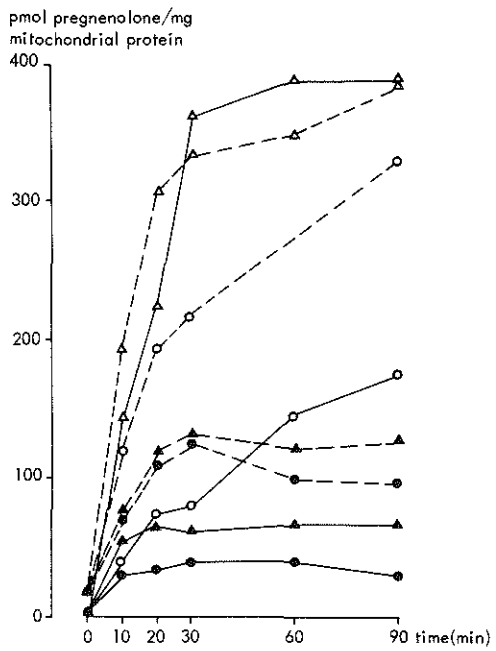


Figure 2 Time course of the pregnenolone concentrations in mitochondrial fractions isolated in 0.25 M sucrose in the absence of EDTA, from control and luteinizing hormone treated testes.

The composition of the incubation medium was 0.125 M sucrose, 5.0 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.3) and 0.1 mM cyanoketone. Aliquots of mitochondrial fractions were incubated in: ●, 2 ml incubation medium only. ▲, incubation medium containing 0.5 mM $CaCl_2$. ○, incubation medium containing 20 mM sodium succinate. △, incubation medium containing 20 mM sodium succinate as well as 0.5 mM $CaCl_2$. (——) solid lines, mitochondrial protein from control testes. (---) broken lines, mitochondrial protein from luteinizing hormone treated testes. Each point reflects mean values of 2-3 incubations.

the depletion of intramitochondrial donors of reducing equivalents. Addition of both succinate and Ca^{2+} ions resulted in an increase in the production of pregnenolone during the first 30 min. This initial phase was followed by a slower rate of production for 60 min. Pregnenolone

production in mitochondrial fractions from LH treated testes was maintained during the first 20 min. Thereafter production rates were almost negligible. Addition of 0.5 mM Ca^{2+} had no stimulating effect on the initial production of pregnenolone. However, during 90 min incubation 20 mM succinate maintained production of pregnenolone in mitochondrial fractions from LH treated testes. The presence of 0.5 mM Ca^{2+} in addition to succinate caused a small stimulation of initial production rates. Mitochondrial fractions from control and LH stimulated testes gave almost similar production rates when Ca^{2+} and succinate were present in the incubation mixture. These data strongly suggest that Ca^{2+} in vitro can mimic the stimulatory effect of LH in vivo on succinate supported pregnenolone production.

The results in figure 2 may indicate that after LH treatment in vivo a metabolically active cholesterol pool has been built up and that Ca^{2+} in vitro does not stimulate further accumulation of cholesterol into this active pool. Information on the mechanism of the intramitochondrial cholesterol shift is still limited. Studies on fractionation of mitochondria obtained from Leydig cell tumors (145) have shown that cholesterol side-chain cleaving enzyme is predominantly located in the inner mitochondrial membrane, whereas the bulk of cholesterol was present in the outer membrane fractions. Ca^{2+} might activate cholesterol transport from an inactive pool to the enzyme complex; at the other hand changes in the conformation of mitochondrial membrane structures, provoked by Ca^{2+}, may facilitate the contact between enzyme complex and the substrate. Experiments with aged mitochondria (figures 3A and B) may support this theoretical explanation.

Effect of ageing of mitochondrial protein on pregnenolone production rates.

Figures 3A and 3B summarize the effect of ageing of mitochondrial preparations, isolated in sucrose without EDTA. Pregnenolone production rates in the presence of either

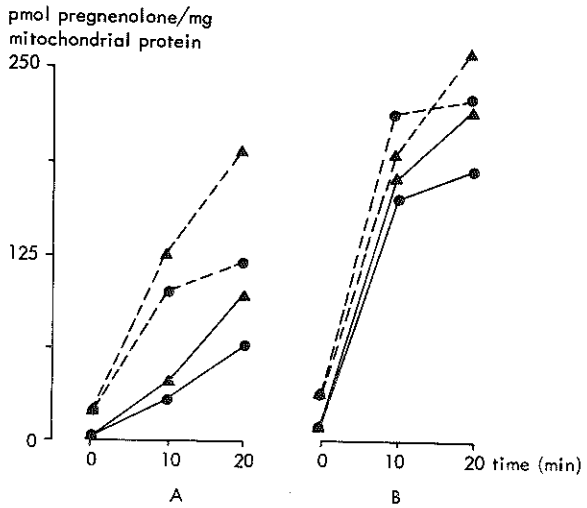


Figure 3 Influence of ageing of mitochondrial fractions isolated in 0.25 M sucrose in the absence of EDTA from luteinizing hormone treated and control testes on the time course of the pregnenolone concentrations.

The incubation medium contained 0.125 mM sucrose, 5.0 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.3), 20 mM sodium succinate and 0.1 uM cyanoketone. Figure 3A: freshly prepared mitochondrial fractions incubated in: ▲, 2 ml incubation medium only. ●, incubation medium containing 1.0 mM NADPH, 10 mM glucose 6-phosphate dehydrogenase. (——) solid line, 0.91 mg mitochondrial protein from control testes. (---) broken line, 0.93 mg mitochondrial protein from luteinizing hormone treated testes. Figure 3B: aliquots of mitochondrial fractions from control and luteinizing hormone treated testes were aged by pre-incubation for 60 min at 33°C in 0.25 M sucrose. Incubation conditions were identical to those as described in the legend to Figure 3A.

succinate or succinate plus NADPH were higher in freshly prepared mitochondrial fractions from LH treated rats than in control preparations (figure 3A). After ageing for 60 min at 33°C in 0.25 M sucrose the rates of pregnenolone production by control mitochondrial fractions in the presence of succinate or succinate plus NADPH increased and equalled production rates by relevant LH treated mitochondrial fractions (figure 3B). It is well known that ageing

of mitochondria involves changes in the ultrastructure. The effect of ageing of steroidogenic mitochondria on pregnenolone production rates may reflect, therefore, the loss of a distinct compartmentalized localization of enzyme complex and substrate pool in freshly prepared mitochondria from control testes. In this regard Kahnt and coworkers (111) and Johnson et al. (106) observed that pregnenolone production rates of rat adrenal cortex mitochondria increased after disruption (either by swelling in distilled water or by sonication) of the mitochondria whereas production rates in sonicated mitochondria obtained from control and ACTH treated adrenal glands became similar (106).

Effect of ruthenium red on Ca^{2+} stimulated pregnenolone production rates.

The involvement of binding of Ca^{2+} by testicular mitochondria in Ca^{2+} stimulated pregnenolone production was investigated with ruthenium red. Ruthenium red is a specific inhibitor of mitochondrial Ca^{2+} binding and prevents the accumulation of Ca^{2+} inside the mitochondria (175,194). Figure 4 shows that ruthenium red inhibited Ca^{2+} stimulated pregnenolone production in isolated mitochondrial fractions by almost 70%. Production rates in the absence of Ca^{2+} were not affected by ruthenium red. These results may indicate that testicular mitochondria can accumulate Ca^{2+} from the surrounding medium by a specific Ca^{2+} binding and uptake mechanism. It is not known if the 30% stimulation of pregnenolone production in the presence of Ca^{2+} and ruthenium red was caused by Ca^{2+} accumulation in the mitochondria by a ruthenium red insensitive transport mechanism, or is merely reflecting improper incubation conditions.

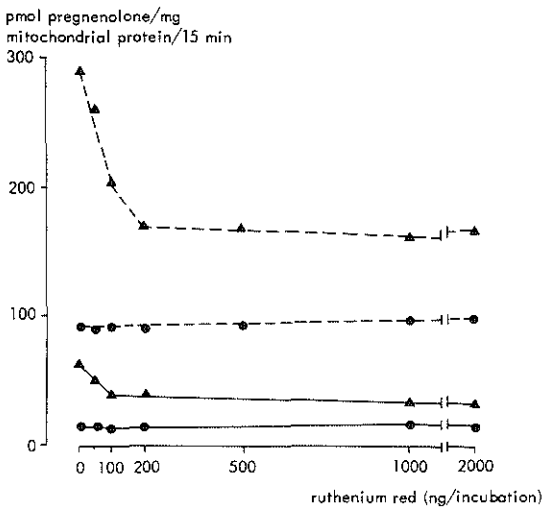


Figure 4 Effect of ruthenium red on Ca^{2+} stimulated pregnenolone production rates by mitochondrial fractions isolated in 0.25 M sucrose, containing 1 mM EDTA, from luteinizing hormone treated and control testes.

Aliquots of mitochondrial fractions were incubated in 1 ml medium, containing 0.125 M sucrose, 0.5 mM EDTA, 20 mM sodium succinate, 5.0 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.3), 0.1 µM cyanoketone, 10 mM NADPH, 10 mM glucose 6-phosphate and 0.3 units glucose 6-phosphate dehydrogenase for 15 min at 33°C. ●, incubation medium alone. ▲, incubation medium containing 0.7 mM $CaCl_2$. (——), solid line, 1.25 mg mitochondrial protein from control testes. (---), broken line, 1.3 mg mitochondrial protein from luteinizing hormone treated testes.

5.3. Conclusions

From experimental data presented in appendix paper IV and section 5.2. it is concluded that Ca^{2+} can stimulate testosterone production in vitro, via a stimulation of the mitochondrial pregnenolone production. Ca^{2+} fulfills the criterium for a potential obligatory factor involved in the trophic stimulation of steroid production, since Ca^{2+} had a greater effect on succinate supported pregnenolone production rates in control mitochondrial fractions than on production rates in mitochondrial fractions isolated from LH

treated testes (section 5.2.). Ca^{2+} may stimulate the formation of pregnenolone via an effect on the rate of association of cholesterol and cytochrome P_{450} through an intramitochondrial cholesterol shift or changes in mitochondrial membrane conformations. At the other hand, it cannot be excluded that Ca^{2+} also increases the availability of reducing equivalents for cholesterol side-chain cleaving enzyme activity.

The experiments with ruthenium red, a specific inhibitor of Ca^{2+} uptake by mitochondria (175,194), indicate that specific mitochondrial Ca^{2+} binding and uptake were involved in stimulation of pregnenolone production rates by Ca^{2+} ions added to the incubation mixture (section 5.2.).

Information on the possible role of Ca^{2+} in testicular steroidogenesis in vivo or in intact cells in vitro is limited at this moment. Therefore, it is not possible to postulate a detailed mechanism of action of LH on androgen formation involving Ca^{2+} ions.

REFERENCES

1. Abraham, G.E., Buster, J.E., Kyle, F.W., Corrales, P.C. and Teller, R.C. (1973) J. Clin. Endocrinol. Metab. 37, 140-144.
2. Appell, R.A. (1973) Steroids 22, 351-364.
3. Armstrong, D.T. (1968) Recent Progr. Horm. Res. 3, 255-320.
4. Armstrong, D.T., O'Brien, J. and Greep, R.O. (1964) Endocrinology 57, 488-500.
5. Arthur, J.R. and Boyd, G.S. (1974) Eur. J. Biochem. 49, 117-127.
6. Bartke, A. (1971) J. Reprod. Fertil. 25, 153-156.
7. Bartke, A. (1973) Acta Endocrinol. 177, 348.
8. Bartke, A. and Shire, J.G.M. (1972) J. Endocrinol. 55, 173-184.
9. Basch, R.S. and Finegold, M.J. (1971) Biochem. J. 125, 983-989.
10. Bass, J.J., Bell, J.B.G. and Lacy, D. (1973) J. Endocrinol. 56, 331-332.
11. Beall, R.J. and Sayers, G. (1972) Arch. Biochem. Biophys. 148, 70-76.
12. Bell, J.B.G. (1972) Steroids Lipid Res. 3, 315-320.
13. Bell, J.B.G. and Lacy, D. (1974) Proc. Roy. Soc. London B. 186, 99-120.
14. Bell, J.B.G., Vinson, G.P., Hopkin, D.J. and Lacy, D. (1968) Biochim. Biophys. Acta 164, 412-420.
15. Bell, J.B.G., Vinson, G.P. and Lacy, D. (1968) Biochem. J. 110, 58-59.
16. Bell, J.B.G., Vinson, G.P. and Lacy, D. (1971) Proc. Roy. Soc. London B 176, 433-443.
17. Bell, J.J., Cheng, S.C. and Harding, B.W. (1973) Ann. N.Y. Acad. Sci. 212, 290-306.
18. Bernard, E.A. and Wasserman, G.F. (1974) Canad. J. Biochem. 52, 563-569.
19. Berthold, A.A. (1849) Arch. Anat. Physiol. Wiss. 16, p. 42.
20. Billiar, R.B., Alousi, M.A., Knappenberger, M.H. and Little, B. (1971) Arch. Biochem. Biophys. 144, 30-50.
21. Birmingham, M.K., Elliot, F.H. and Valere, P.H.I. (1953) Endocrinology 53, 687-689.
22. Blackshaw, A.W. (1970) in The Testis (Johnson, A.D., Gomes, W.R. and VanDemark, N.L., eds.) Vol. II, p. 73-123, Academic Press, New York and London.
23. Boguslawski, W., Klimek, J. and Zelewski, L. (1972) FEBS Lett. 27, 181-183.
24. Bouin, P. and Ancel, P. (1903) Arch. Zool. Exptl. Gen. 1, 437-523.
25. Bowyer, F. and Kitabchi, A.E. (1974) Biochem. Biophys. Res. Commun. 57, 100-105.
26. Boyd, G.S. and Trzeciak, W.H. (1973) Ann. N.Y. Acad. Sci. 212, 361-377.
27. Brinkmann, A.O. (1973) unpublished results.
28. Brownie, A.C., van der Molen, H.J., Nishiwaza, E.E. and Eik-Nes, K.B. (1964) J. Clin. Endocrinol. Metab. 24, 1091-1102.
29. Brownie, A.C., Simpson, E.R., Jefcoate, C.R., Boyd, G.S., Orme-Johnson, W.H. and Beinert, H. (1972) Biochem. Biophys. Res. Commun. 46, 489-490.

30. de Bruijn, H.W.A. (1973) On testicular steroid biosynthesis, thesis Erasmus University, Rotterdam.
31. Burstein, S. and Gut, M. (1971) Rec. Progr. Horm. Res. 27, 303-344.
32. Burstein, S., Kimball, H.L. and Gut, M. (1970) Steroids 15, 809-857.
33. Burstein, S., Middleditch, B.S. and Gut, M. (1974) Biochem. Biophys. Res. Commun. 61, 692-697.
34. Catt, K.J., Dufau, M.L. and Tsuruhara, T. (1972) J. Clin. Endocrinol. Metab. 34, 123-132.
35. Catt, K.J., Tsuruhara, T. and Dufau, M.L. (1972) Biochim. Biophys. Acta 279, 194-201.
36. Catt, K.J., Watanabe, K. and Dufau, M.L. (1972) Nature New Biol. 239, 280-281.
37. Christensen, A.K. and Mason, N.R. (1965) Endocrinology 76, 646-656.
38. Connel, G.M. and Eik-Nes, K.B. (1968) Steroids 12, 507-512.
39. Cook, B., Struthers, A.D., Denholm, R.B. and Taylor, P.D. (1974) J. Steroid Biochem. 5, 309.
40. Cooke, B.A., van Beurden, W.M.O., Rommerts, F.F.G. and van der Molen, H.J. (1972) FEBS Lett. 25, 83-86.
41. Cooke, B.A., Clotcher, W.F., de Jong, C.M.M., Renniers, A.C.H.M. and van der Molen, H.J. (1974) J. Endocrinol. 63, 17-18.
42. Cooke, B.A., de Jong, F.H., van der Molen, H.J. and Rommerts, F.F.G. (1972) Nature New Biol. 237, 255-256.
43. Cooke, B.A., van der Kemp, J.W.C.M. and van der Molen, H.J. (1975) J. Endocrinol. 64, 69P-70P.
44. Cooke, B.A., Rommerts, F.F.G., van der Kemp, J.W.C.M. and van der Molen, H.J. (1974) Mol. Cell. Endocrinol. 1, 99-111.
45. Cowan, R.A., Grant, J.K., Giles, C.A. and Biddlecombe, W. (1972) Biochem. J. 126, 12p.
46. David, K., Dingemanse, E., Freud, J. and Laqueur, E. (1935) Z. Physiol. Chem. 233, 281-282.
47. DiPietro, D.L., Brown, R.D. and Strott, C.A. (1972) J. Clin. Endocrinol. Metab. 35, 729-735.
48. Dixon, R., Furutachi, T. and Lieberman, S. (1970) Biochem. Biophys. Res. Commun. 40, 161-165.
49. van Doorn, L.G., de Bruijn, H.W.A., Galjaard, H. and van der Molen, H.J. (1974) Biol. Reprod. 10, 47-53.
50. Dorfman, R.I., Menon, K.M.J. and Forchielli, E. (1968) Research on steroids, vol. III, 15-31.
51. Dorfman, R.I., Sharman, D., Menon, K.M.J. and Forchielli, E. (1967) Proc. 3th Asia and Oceania Congr. Endocrinol. pp. 171-177, Manilla.
52. Drosdowski, M., Menon, K.M.J., Forchielli, E. and Dorfman, R.I. (1965) Biochim. Biophys. Acta 104, 229-236.
53. Dufau, M.L. and Catt, K.J. (1973) Nature New Biol. 242, 246-248.
54. Dufau, M.L., Catt, K.J. and Tsuruhara, T. (1972) Endocrinology 90, 1032-1040.
55. Dufau, M.L., de Kretser, D.M. and Hudson, B. (1971) Endocrinology 88, 825-832.

56. Dufau, M.L., Tsuruhara, T., Watanabe, K. and Catt, K.J. (1973) Abstract book of 55th Annual meeting of Amer. Endocr. Soc. Chicago, abstr. 157.
57. Eik-Nes, K.B. (1964) Physiol. Rev. 44, 609-630.
58. Eik-Nes, K.B. (1970) in The Androgens of the Testis (Eik-Nes, K.B., ed.) p. 1-48, Marcel Dekker, New York.
59. Eik-Nes, K.B. (1971) Rec. Progr. Horm. Res. 27, 517-535.
60. Eik-Nes, K.B. and Kekre, M. (1963) Biochim. Biophys. Acta 78, 449-456.
61. Ellis, L.C. and Van Kampen, K.R. (1971) Rad. Res. 48, 146-163.
62. El Safoury, S. and Bartke, A. (1974) J. Endocrinol. 61, 193-198.
63. Farese, R.V. (1967) Biochemistry 6, 2052-2065.
64. Farese, R.V. (1971) Endocrinology 89, 958-962.
65. Farese, R.V. (1971) Endocrinology 89, 1064-1074.
66. Flint, A.P.F. and Armstrong, D.T. (1971) Nature New Biol. 231, 60-61.
67. Flint, A.P.F. and Armstrong, D.T. (1971) Biochem. J. 123, 143-152.
68. Frederik, P.M. (1975) In the press.
69. Free, M.J. and Tillson, S.A. (1973) Endocrinology 93, 874-879.
70. Frowein, J. (1973) J. Endocrinol. 57, 437-449.
71. Furuyama, S., Mayes, D.M. and Nugent, C.A. (1970) Steroids 16, 415-428.
72. Galena, H.J. and Terner, C. (1974) J. Endocrinol. 60, 269-276.
73. Garren, L.D., Gill, G.N., Masui, H. and Walton, G.M. (1971) Rec. Progr. Horm. Res. 27, 433-474.
74. Goldman, A.S., Yakovac, W.C. and Bongiovanni, A.M. (1965) Endocrinology 77, 1105-1118.
75. Gomes, W.R. (1970) in The Testis (Johnson, A.D., Gomes, W.R. and VanDemark, N.L. eds.) pp. 67-138, Academic Press, New York.
76. Gomes, W.R. and VanDemark, N.L. (1974) in Annual Rev. Physiol. (Comroe, J.H., Sonnenschein, R.R. and Zierler, K.L. eds.) Vol. 36, pp. 307-330, Annual Review Inc. Palo Alto.
77. Greville, G.D. (1969) in Citric Acid Cycle (Lowenstein, J.M. ed.) pp. 1-36, Marcel Dekker, New York.
78. Hafiez, A.A., Bartke, A. and Lloyd, C.W. (1972) J. Endocrinol. 53, 223-230.
79. Hafiez, A.A., Lloyd, C.W. and Bartke, A. (1971) J. Endocrinol. 52, 327-332.
80. Hafiez, A.A., Philpott, J.E. and Bartke, A. (1971) J. Endocrinol. 50, 619-623.
81. Haksar, A. and Péron, F.G. (1972) Biochem. Biophys. Res. Commun. 47, 445-450.
82. Halkerston, I.D.K., Eichhorn, J. and Hechter, O. (1961) J. Biol. Chem. 236, 374-380.
83. Hall, P.F. (1966) Endocrinology 78, 690-698.
84. Hall, P.F. (1970) in The Testis (Johnson, A.D., Gomes, W.R. and VanDemark, N.L., eds.) Vol. II, p. 1-72, Academic Press, New York.
85. Hall, P.F. (1970) in The Androgens of the Testis (Eik-Nes, K.B. ed.) p. 73-116, Marcel Dekker, New York.
86. Hall, P.F. and Eik-Nes, K.B. (1962) Biochim. Biophys. Acta 63, 411-422.
87. Hall, P.F. and Eik-Nes, K.B. (1963) Biochim. Biophys. Acta 71, 438-447.
88. Hall, P.F. and Eik-Nes, K.B. (1964) Biochim. Biophys. Acta 86, 604-609.
89. Hall, P.F., Irby, D.C. and de Kretser, D.M. (1969) Endocrinology 84, 488-496.

90. Hamberger, L.A. and Stewart, V.W. (1968) Endocrinology 83, 855-860.
91. Haynes, R.C. and Berthet, L. (1957) J. Biol. Chem. 255, 115-124.
92. Henry, H. and Gomes, W.R. (1972) Fed. Proc. 31, 813.
93. Hirshfield, I.N. and Koritz, S.B. (1964) Biochemistry 3, 1994-1998.
94. Hollinger, M.A. (1970) Life Sci. 9, 533-540.
95. Hovatta, O., Nykänen, M. and Kormano, M. (1974) Histochemistry 40, 25-30.
96. Huang, J.J. and Kimura, T. (1971) Biochem. Biophys. Res. Commun. 44, 1065-1070.
97. Ichii, S., Forchielli, E. and Dorfman, R.I. (1963) Steroids 2, 631-656.
98. Inano, H. (1974) J. Steroid Biochem. 5, 145-149.
99. Inano, H., Inano, A. and Tamaoki, B. (1970) J. Steroid Biochem. 1, 83-99.
100. Irusta, O. and Wasserman, G.F. (1971) Acta Physiol. Lat. Amer. 21, 260-261.
101. Jaanus, S.D. and Rubin, R.P. (1971) J. Physiol. 213, 581-598.
102. Jackanicz, T.M. and Armstrong, D.T. (1968) Endocrinology 83, 769-776.
103. Janszen, F.H.A. (1975) J. Endocrinol., submitted for publication.
104. Johnson, B.H. and Ewing, L.L. (1971) Science 173, 635-637.
105. Johnson, L.R. and Nelson, D.H. (1972) Proc. 57th meeting Biochem. Soc. Aberdeen, pp. 9-10.
106. Johnson, L.R., Ruhman-Wennhold, A. and Nelson, D.H. (1973) Ann. N.Y. Acad. Sci. 212, 307-318.
107. Jones, P. (1971) Br. J. Radiol. 44, 841-849.
108. de Jong, F.H. (1974) Testicular oestradiol-17 , thesis Erasmus University, Rotterdam.
109. de Jong, F.H., Hey, A.H. and van der Molen, H.J. (1973) J. Endocrinol. 57, 277-284.
110. de Jong, F.H. and van der Molen, H.J. (1972) J. Endocrinol. 53, 461-474.
111. Kahnt, F.W., Milani, A., Steffen, H. and Neher, R. (1974) Eur. J. Biochem. 44, 243-250.
112. Kan, K.W., Ritter, M.C., Ungar, F. and Dempsey, M.E. (1972) Biochem. Biophys. Res. Commun. 48, 423-429.
113. Kan, K.W. and Ungar, F. (1973) J. Biol. Chem. 248, 2868-2875.
114. Koide, S.S. and Torres, M.T. (1965) Biochim. Biophys. Acta 105, 115-120.
115. Koritz, S.B. and Hall, P.F. (1964) Biochemistry 3, 1298-1304.
116. Kraaipoel, R.J., Degenhart, H.J., Leferink, J.G., van Beek, V., de Leeuw-Boon, H. and Visser, H.K.A. (1975) FEBS Lett. 50, 204-209.
117. Kream, B. and Sauer, L.A. (1975) Abstract book 57th Annual meeting Amer. Endocr. Soc. New York, abstr. 434.
118. de Kretser, D.M., Catt, K.J. and Paulsen, C.A. (1971) Endocrinology 80, 332-337.
119. Kuo, T.H. and Tchen, T.T. (1973) J. Biol. Chem. 248, 6679-6683.
120. Lacroix, E., Eechautee, W. and Leusen, I. (1973) Ann. d'Endocrinol. 34, 611-615.
121. Lacy, D. (1967) Endeavour 26, 101-108.
122. Lacy, D. (1974) in Male Fertility and Sterility (Mancini, R.E. and Martini, L. eds.) pp. 175-205, Academic Press, London.
123. Lacy, D., Vinson, G.P., Collins, P., Bell, J., Fyson, P., Pudney, J. and Pettitt, A.J. (1968) Excerp. Med. Int. Congr. 184, 1019-1029.

124. Lefkowitz, R.J., Roth, J. and Pastan, I. (1970) Nature 228, 864-866.
125. Lehninger, A.L. (1970) Biochem. J. 119, 129-138.
126. Leidenberger, F. and Reichert, L.E. (1972) Endocrinology 91, 135-143.
127. Leier, D.J. and Jungmann, R.A. (1973) Biochim. Biophys. Acta 329, 196-210.
128. van Lier, J.E. and Smith, L.L. (1970) Biochim. Biophys. Acta 210, 153-163.
129. Loir, M. and Lanneau, M. (1974) Exp. Cell Res. 83, 319-327.
130. Mackie, C., Richardson, M.C. and Schulster, D. (1972) FEBS Lett. 23, 345-348.
131. Mason, J.I. and Boyd, G.S. (1971) Eur. J. Biochem. 21, 308-321.
132. Mason, J.I., Estabrook, R.W. and Purvis, J.L. (1973) Ann. N.Y. Acad. Sci. 212, 406-419.
133. McCune, R.W., Roberts, S. and Young, P.L. (1970) J. Biol. Chem. 245, 3859-3867.
134. Meistrich, M.L., Bruce, W.R. and Clermont, Y. (1973) Exp. Cell Res. 79, 213-227.
135. Menard, R.H., Latif, S.A. and Purvis, J.L. (1975) Abstract book 57th Annual meeting of Amer. Endocr. Soc. New York, abstr. 479.
136. Menon, K.M.J., Drosdowsky, M., Dorfman, R.I. and Forchielli, E. (1965) Steroids suppl. I, 95.
137. Menon, K.M.J., Dorfman, R.I. and Forchielli, E. (1967) Biochim. Biophys. Acta 148, 486-494.
138. Meyer, A.J. and van Dam, K. (1974) Biochim. Biophys. Acta 346, 213-244.
139. van der Molen, H.J. (1970) in The Androgens of the Testis (Eik-Nes, K.B. ed.) pp. 145-215, Marcel Dekker, New York.
140. van der Molen, H.J., Bijleveld, M.J., van der Vusse, G.J. and Cooke, B.A. (1972) Endocrinol. 57, vi-vii.
141. van der Molen, H.J. and Eik-Nes, K.B. (1971) Biochim. Biophys. Acta 248, 343-362.
142. Morrison, G., Meigs, R.A. and Ryan, K.J. (1965) Steroids suppl. II, 177-188.
143. Mounib, M.S. and Eisan, J.S. (1972) Endocrinology 91, 1375-1379.
144. Moyle, W.R., Jungas, R.L. and Greep, R.O. (1973) Biochem. J. 134, 407-414.
145. Moyle, W.R., Jungas, R.L. and Greep, R.O. (1973) Biochem. J. 134, 415-424.
146. Moyle, W.R. and Ramachandran, J. (1973) Endocrinology 93, 127-134.
147. Moyle, W.R., Kong, Y.C. and Ramachandran, J. (1973) J. Biol. Chem. 248, 2409-2417.
148. Murakami, N. and Ichii, S. (1973) Endocrinol. Japon. 20, 421-424.
149. Nakamura, M., Ide, M., Okabayashi, T. and Tanaka, A. (1972) Endocrinol. Japon. 19, 443-448.
150. Neher, R. (1964) Steroid Chromatography, pp. 96-97, Elsevier, Amsterdam.
151. Nüesch, H. and Siebenmann, R. (1973) Virchows Arch. Abt. A Path. Anat. 358, 149-162.
152. Nussdorfer, G.G., Rebuffat, P., Mazzochi, G., Belloni, A.S. and Meneghelli, V. (1974) Cell Tiss. Res. 150, 79-94.
153. Parsons, D.F., Williams, G.R. and Chance, B. (1966) Ann. N.Y. Acad. Sci. 137, 643-666.
154. Parsons, D.F. and Yano, Y. (1967) Biochim. Biophys. Acta 135, 362-364.

155. Parvinen, M., Hurme, P. and Niemi, M. (1970) Endocrinology 87, 1082-1084.
156. Parvinen, M. and Niemi, M. (1971) Steroidologica 2, 129-137.
157. Perlman, P.L. (1950) Endocrinology 46, 341-346.
158. Pestka, S. (1971) Ann. Rev. Micr. Biol. 25, 487-562.
159. Pfeiffer, D.R., Kimura, T. and Tchen, T.T. (1972) FEBS Lett. 22, 165-168.
160. Pfeiffer, D.R. and Tchen, T.T. (1973) Biochem. Biophys. Res. Commun. 50, 807-813.
161. Podesta, E. and Dufau, M.L. (1975) Abstract book 57th Annual meeting Amer. Endocr. Soc. New York, abstr. 215.
162. Pokel, J.D., Moyle, W.R. and Greep, R.O. (1972) Endocrinology 91, 323-325.
163. Pulsinelli, W.A. and Eik-Nes, K.B. (1969) Fed. Proc. 29, 918.
164. Purvis, J.L., Canick, J.A., Latif, S.A., Rosenbaum, J.H., Hologgitas, J. and Menard, R.H. (1973) Arch. Biochem. Biophys. 159, 39-49.
165. Purvis, J.L., Canick, J.A., Rosenbaum, J.H., Hologgitas, J. and Latif, S.A. (1973) Arch. Biochem. Biophys. 159, 32-38.
166. Rasmussen, H. (1970) Science 170, 404-412.
167. Richards, G. and Neville, A.M. (1973) Nature 244, 359-361.
168. Richardson, M.C. and Schulster, D. (1972) J. Endocrinol. 55, 127-139.
169. Roberts, K.D., Bandy, L. and Lieberman, S. (1969) Biochemistry 8, 1259-1270.
170. Robinson, J., Stevenson, P.M., Boyd, G.S. and Armstrong, D.T. (1975) Moll. Cell. Endocrinol. 2, 149-155.
171. Rommerts, F.F.G., Cooke, B.A., van der Kemp, J.W.C.M. and van der Molen, H.J. (1972) FEBS Lett. 24, 251-254.
172. Rommerts, F.F.G., Cooke, B.A., van der Kemp, J.W.C.M. and van der Molen, H.J. (1973) FEBS Lett. 33, 114-118.
173. Rommerts, F.F.G., Cooke, B.A. and van der Molen, H.J. (1974) J. Steroid Biochem. 5, 279-285.
174. Rommerts, F.F.G., van Doorn, L.G., Galjaard, H., Cooke, B.A. and van der Molen, H.J. (1973) J. Histochem. Cytochem. 21, 572-579.
175. Rossi, C.W., Vasington, F.D. and Carafoli, E. (1973) Biochem. Biophys. Res. Commun. 50, 846-852.
176. Samuels, L.T. and Eik-Nes, K.B. (1968) Metabolism of steroid hormones, in Metabolic Pathways (Greenberg, D.M. ed.) Vol. II, pp. 169-220, Academic Press, New York.
177. Samuels, L.T. and Helmreich, M.L. (1956) Endocrinology 58, 435-442.
178. Sandler, R. and Hall, P.F. (1966) Endocrinology 79, 647-649.
179. Saswitha (1974) in Dood gaan, Dood zijn. Wetenschappelijke Uitgeverij, Amsterdam.
180. Sayers, G., Beall, R.J. and Seelig, S. (1972) Science 175, 1131-1133.
181. Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967) J. Cell Biol. 32, 719-735.
182. Schoen, E.J. and Samuels, L.T. (1965) Acta Endocrinol. 50, 365-378.
183. Schulster, D. (1974) in Adv. in Steroid Biochem. Pharmacol. (Briggs, M.H. and Christie, G.A. eds.) pp. 233-295, Academic Press, New York.

184. Schulster, D., Richardson, M.C. and Mackie, C. (1972) Biochem. J. 129, 8p-9p.
185. Sharma, R.K., Hashimoto, K. and Kitabchi, A.E. (1972) Endocrinology 91, 994-1003.
186. Shikita, M. and Hall, P.F. (1967) Biochim. Biophys. Acta 136, 484-497.
187. Shikita, M., Kakizaki, H. and Tamaoki, B. (1964) Steroids 4, 521-531.
188. Shikita, M. and Tamaoki, B. (1965) Endocrinology 76, 563-569.
189. Shimizu, K., Gut, M. and Dorfman, R.I. (1962) J. Biol. Chem. 237, 699-702.
190. Simpson, E.R., Cooper, D.Y. and Estabrook, R.W. (1968) Rec. Progr. Horm. Res. 24, 523-562.
191. Simpson, E.R., Jefcoate, C.R., Brownie, A.C. and Boyd, G.S. (1972) Eur. J. Biochem. 28, 442-450.
192. Simpson, E.R., Jefcoate, C.R., McCarthy, J.L. and Boyd, G.S. (1974) Eur. J. Biochem. 45, 181-184.
193. Slaunwhite, W.R. and Burgett, M.J. (1965) Steroids 6, 721-735.
194. Sordahl, L.A. (1975) Arch. Biochem. Biophys. 167, 104-115.
195. Steinberger, E. (1971) Physiol. Rev. 51, 1-22.
196. Steinberger, E. and Ficher, M. (1973) Steroids 22, 425-443.
197. Steinberger, E., Steinberger, A., Vilar, O., Salamon, I.I. and Sud, B.N. (1967) in Endocrinology of the Testis, Ciba Found. Coll. on Endocrinol. (Wolstenholme, G.E.W. and O'Connor, M. eds.) pp. 56-81, Churchill Ltd., London.
198. Stevenson, P.M. and Taylor, P.L. (1971) FEBS Lett. 19, 251-254.
199. Sulimovici, S., Bartoov, B. and Lunenfeld, B. (1973) Biochim. Biophys. Acta 321, 27-40.
200. Sulimovici, S., Bartoov, B. and Lunenfeld, B. (1975) Biochim. Biophys. Acta 377, 454-462.
201. Sulimovici, S. and Boyd, G.S. (1968) Eur. J. Biochem. 3, 332-345.
202. Sulimovici, S. and Boyd, G.S. (1969) Eur. J. Biochem. 7, 549-558.
203. Sulimovici, S.I. and Boyd, G.S. (1969) in Vitamins and Hormones (Harris, R.S., Wool, I.G., Lorraine, J.A. and Munson, P.L. eds.) pp. 199-234, Academic Press, New York.
204. Sulimovici, S. and Lunenfeld, B. (1971) in Gonadotropins (Saxen, B.B., Beling, C.B. and Gandy, H.M. eds.) pp. 287-300, Wiley-Interscience, New York.
205. Sulimovici, S. and Lunenfeld, B. (1972) J. Steroid Biochem. 3, 781-790.
206. Sulimovici, S. and Lunenfeld, B. (1973) Biochem. Biophys. Res. Commun. 55, 673-679.
207. Tamaoki, B. (1973) J. Steroid Biochem. 4, 89-118.
208. Tamaoki, B. and Shikita, M. (1966) Eur. J. Steroids 1, 351-359.
209. Tamaoki, B. and Shikita, M. (1966) in Steroid Dynamics (Pincus, G., Nakao, T. and Tait, J.F. eds.) pp. 493-530, Academic Press, New York.
210. Toren, D., Menon, K.M.J., Forchielli, E. and Dorfman, R.I. (1964) Steroids 3, 381-390.
211. Trzeciak, W.H. and Boyd, G.S. (1973) Eur. J. Biochem. 37, 327-333.
212. Ungar, F., Kan, K.W. and McCoy, K.E. (1973) Ann. N.Y. Acad. Sci. 212, 276-289.

213. Verjans, H.L., Cooke, B.A., de Jong, F.H., de Jong, C.M.M. and van der Molen, H.J. (1973) J. Steroid Biochem. 4, 665-676.
214. Vinson, G.P. and Whitehouse, B.J. (1973) J. Endocrinol. 58, iii-iv.
215. van der Vusse, G.J. (1975) Unpublished results.
216. van der Vusse, G.J., Kalkman, M.L. and van der Molen, H.J. (1973) Biochim. Biophys. Acta 297, 179-185.
217. van der Vusse, G.J., Kalkman, M.L. and van der Molen, H.J. (1974) Biochim. Biophys. Acta 348, 404-414.
218. van der Vusse, G.J., Kalkman, M.L. and van der Molen, H.J. (1975) Biochim. Biophys. Acta 380, 473-485.
219. Welsh, M.J. and Wiebe, J.P. (1975) Endocrinology 96, 618-624.
220. Wisner, J.R. and Gomes, W.R. (1975) Abstract book 57th Annual meeting of the Amer. Endocr. Soc. New York, abstr. 192.
221. Yago, N., Dorfman, R.I. and Forchielli, E. (1967) J. Biochem. 62, 345-352.
222. Yago, N. and Ichii, S. (1969) J. Biochem. 65, 215-224.
223. Yanaihara, T. and Troen, P. (1972) J. Clin. Endocr. Metab. 34, 783-792.
224. Ying, B.P., Chang, Y.-J. and Gaylor, J.L. (1965) Biochim. Biophys. Acta 100, 256-262.

SUMMARY

The testicular gland is the main androgen producing organ in male animals. The production of androgens is regulated by LH, secreted by the pituitary. The experimental work described in this thesis concerns the role of the rate-limiting step in overall testosterone formation in rat testis, that is the mitochondrially localized production of pregnenolone.

In order to properly characterize cellular and subcellular fractions used in this study, the presence and distribution of specific marker enzymes had to be established in these fractions. Carboxyl esterase has been used to characterize testicular interstitial tissue. Cytochrome c oxidase and monoamine oxidase have been applied to identify mitochondrial fractions, whereas carboxyl esterase, rotenone-insensitive NADPH-cytochrome c reductase and steroid sulfatase have been used as marker enzymes for microsomal fractions. Glucose-6-phosphate dehydrogenase and lactate dehydrogenase were assayed as reference enzymes for the particle-free supernatant fraction. In appendix paper I, however, it has been suggested that a small part of lactate dehydrogenase activity is localized in mitochondria of specific cell types in the seminiferous tubules.

As a measure for steroid production, the production rates of endogenous testosterone in tissue homogenates and of endogenous pregnenolone in isolated mitochondrial fractions have been determined rather than the formation of steroids from exogenous labelled precursors. In this way the possibility was avoided that exogenous substrates may not completely mix with endogenous substrate pools and that the conversion rates may not reflect true production rates of endogenous steroids. In addition, estimation of the rate of steroid production from endogenous precursors offered the possibility to compare endogenous production rates in

vitro with the production of steroids in vivo.

The mass of testosterone and of pregnenolone was initially estimated using gas-liquid chromatography with electron capture detection technique (chapter 2 and appendix papers I, II and III). This technique was in the course of these investigations replaced by more sensitive and less time-consuming radioimmunoassay techniques (chapter 2 and appendix papers III and IV).

Five main aspects has been studied:

1. The cellular localization of the endogenous production of androgens and of enzyme systems related to androgen formation (chapter 3, appendix papers I, II and III).

From the experiments on the cellular localization of steroidogenesis in rat testis, in interstitial tissue and in seminiferous tubules, it was concluded that interstitial tissue contributes at least 97% to total testicular steroid production in vitro. Similar conclusion can be drawn from a comparison of pregnenolone production rates in mitochondrial fractions isolated from those tissue compartments. It was also concluded that at least 95% of the 3β-hydroxysteroid dehydrogenase activity was located in the interstitial compartment of the testicular gland. The results presented (chapter 3 and appendix papers I, II and III) do not provide sufficient evidence to exclude the possibility that seminiferous tubules are producing pregnenolone and progesterone from endogenous precursors. However, the quantitative contribution of these steroids produced in cell types in the seminiferous tubules to the production in total testis would be very small, and the low production rates measured in isolated seminiferous tubules may well reflect contamination of material from the interstitial tissue.

2. The subcellular localization of the production of androgens (chapter 3 and appendix papers I, II and III).

From the experiments on the localization of steroid production in isolated subcellular fractions from rat testis it was concluded that only isolated mitochondrial fractions synthetize steroids from endogenous substrates. Both the cholesterol side-chain cleaving enzyme system and a cholesterol substrate pool are present in this particular fraction. Testosterone and pregnenolone were the main steroids produced in the absence of cyanoketone. The production of testosterone in isolated mitochondrial fractions may have been caused by contamination with microsomal enzymes required for the conversion of pregnenolone to testosterone. In this regard the difference in distribution pattern of 3β-hydroxysteroid dehydrogenase indicated that 7-15% of this steroid dehydrogenase is located in the mitochondria.

3. The effect of long-term treatment with HCG in vivo on endogenous steroid production and on enzyme activities in testis of adult male rats (chapter 4 and appendix paper III).

Intact adult rats who had received daily subcutaneous injections of 100 i.u. HCG for 5 days showed increased testosterone concentrations in total homogenates of all testis tissues investigated. The production of testosterone from endogenous precursors was markedly elevated in homogenates of whole testis tissue and this increase in steroid formation after long-term HCG treatment could be explained by an increased production in the interstitial compartment. Long-term treatment with HCG increased pregnenolone production in mitochondrial fractions isolated from interstitial tissue. This observation confirms the generally accepted theory that enhanced androgen production is caused by an increase in mitochondrial pregnenolone formation.

The specific activity of 3β-hydroxysteroid dehydrogenase was increased in homogenates of whole testis tissue, isolated interstitial tissue and seminiferous tubules.

Long-term HCG treatment had no effect on the specific activities of marker enzymes such as cytochrome c oxidase, monoamine oxidase, steroid sulfatase and lactate dehydrogenase. In contrast, the specific activity of carboxyl esterase was decreased.

4. The acute effect of LH on testicular steroid production (section 4.3. and appendix paper IV).

Within 20 min after LH injection concentrations of testosterone in testicular venous plasma and in testicular tissue were 3, respectively 10 times higher than in relevant samples of control rats. Pregnenolone concentrations in isolated mitochondrial fractions from testes removed from the animal 20 min after LH treatment were 5 times increased.

The production rates of testosterone in homogenates of whole testis tissue and of pregnenolone in isolated mitochondrial fractions were significantly elevated within 5 to 10 min after intravenous LH injection. The stimulating effect of LH on testosterone production in vivo as well as on testosterone production in testicular homogenates and on pregnenolone production in isolated mitochondrial fractions was abolished, when cycloheximide was administered intravenously 10 min prior to LH.

5. A possible role of Ca^{2+} ions in the cellular mechanism of hormonal regulation of steroid production (chapter 5 and appendix paper IV).

Addition of Ca^{2+} ions to the incubation medium stimulated both testosterone production rates in whole testis tissue homogenates and the rates of pregnenolone production in mitochondrial fractions. Addition of Ca^{2+} had a greater effect on pregnenolone production rates in control mitochondrial fractions than in LH stimulated fractions (section 5.2.). In this respect, Ca^{2+} in vitro can mimic the effect of LH in vivo on mitochondrial steroid production.

After ageing of mitochondrial protein, rates of pregnenolone formation in control mitochondrial fractions were increased and were almost similar to production rates in comparable fractions from LH stimulated testes.

The presence of ruthenium red, a specific inhibitor of binding and transport of mitochondrial Ca^{2+}, resulted in a 70% reduction of the Ca^{2+} stimulated pregnenolone production rates in mitochondrial fractions from control and LH treated testes. Production rates in the absence of Ca^{2+} were not affected by ruthenium red.

In chapter 5 a possible role of Ca^{2+} ions in the hormonal regulation of steroid production has been discussed.

SAMENVATTING

Bij manlijke dieren vindt de produktie van androgene steroiden voornamelijk plaats in de testikel. Luteiniserend hormoon (LH), dat uitgescheiden wordt door de hypofyse, reguleert de testikulaire steroidproduktie. In dit proefschrift zijn de resultaten beschreven van onderzoekingen over de snelheidsbeperkende stap bij de produktie van testosteron in de testikel van de rat: de produktie van pregnenolon, gelokaliseerd in de mitochondria.

Voor de karakterisering van de cellulaire en subcellulaire frakties die gebruikt zijn, is de aanwezigheid en verdeling van specifieke "marker"enzymen in deze frakties bepaald. Carboxyl esterase werd gebruikt om het interstitiële weefsel van de testikel te karakteriseren. Met behulp van cytochroom c oxidase en monoamine oxidase werden mitochondriale frakties geïdentificeerd. Carboxyl esterase, rotenon-ongevoelige NADPH-cytochroom c reduktase en steroid sulfatase werden gebruikt als "marker"enzymen voor de mikrosomale frakties. Glucose-6-fosfaat dehydrogenase en lactaat dehydrogenase werden bepaald als enzymen die karakteristiek zijn voor de oplosbare fraktie van de testikel. Uit appendix publikatie I blijkt tevens dat een klein gedeelte van de testikulaire lactaat dehydrogenase aktiviteit is gelokaliseerd in mitochondria van specifieke celtypen in de seminifere tubuli.

De produktiesnelheden van endogeen testosteron in weefselhomogenaten en van endogeen pregnenolon in geïsoleerde mitochondriale frakties werden bepaald als maat voor de produktie van steroidhormonen in vitro. Deze methode biedt twee voordelen boven de techniek waarbij de omzettingssnelheid van toegevoegd radioaktief substraat bestudeerd wordt. De mogelijkheid wordt vermeden dat exogeen substraat niet volledig mengt met het reeds aanwezige endogene substraat zodat de omzettingssnelheid van radioaktieve

prehormonen niet de ware produktiesnelheden van de endogene steroiden reflekteert. Ten tweede kan men met behulp van de hoeveelheden steroiden, die geproduceerd worden uit endogeen substraat, een reëlere vergelijking maken tussen de produktiesnelheden in vitro en in vivo.

De massa van testosteron en pregnenolon in biologische monsters werd aanvankelijk bepaald met behulp van gaschromatografie (hoofdstuk 2 en appendix publikaties I, II en III). Deze techniek werd in de loop van het onderzoek vervangen door gevoeliger en minder tijdrovende radioimmunologische bepalingsmethoden (hoofdstuk 2 en appendix publikaties III en IV). Met behulp van deze bepalingstechniek was het mogelijk om de invloed en het effekt van veranderingen in de inkubatiekondities op de initiële produktiesnelheden van pregnenolon na kortdurende inkubaties van mitochondriale frakties te onderzoeken.

Vijf aspekten werden bestudeerd:

1. De cellulaire lokalisatie van de produktie van androgenen uit endogeen aanwezig substraat en van enzymen, die betrokken zijn bij de synthese van androgenen (hoofdstuk 3, appendix publikaties I, II en III).

Uit de resultaten van de experimenten betreffende de cellulaire lokalisatie van de steroidproduktie in de testikel van de rat werd gekonkludeerd, dat interstitieel weefsel minstens 97% bijdraagt aan de totale produktie van testosteron in vitro. Inkubatiestudies met mitochondriale frakties, geïsoleerd uit interstitieel weefsel en seminifere tubuli, leidden tot dezelfde konklusie.

Van de totale aktiviteit van het enzym 3β-hydroxysteroid dehydrogenase, dat de omzetting van pregnenolon in progesteron katalyseert, is tenminste 95% aanwezig in het interstitiële kompartiment. Op grond van de resultaten, vermeld in hoofdstuk 3 en appendix publikaties I, II en III, kan niet worden uitgesloten dat de seminifere tubuli pregnenolon en progesteron uit endogeen aanwezig substraat kunnen produceren. De kwantitatieve bijdragen van de steroidpro-

duktie in de seminifere tubuli tot de totale steroidproduktie in de testikel is evenwel gering. De geringe produktiesnelheid van steroiden in geïsoleerde seminifere tubuli in vitro zou het gevolg kunnen zijn van verontreiniging met kleine hoeveelheden materiaal afkomstig uit interstitieel weefsel.

2. De subcellulaire lokalisatie van de steroidproduktie (hoofdstuk 3 en appendix publikaties I, II en III).

Uit experimenten met geïsoleerde subcellulaire frakties uit testisweefsel bleek dat alleen mitochondriale frakties signifikante hoeveelheden endogene steroiden te produceren. Uit deze resultaten kan worden gekonkludeerd dat in de mitochondriale fraktie zowel het enzymkomplex noodzakelijk voor de afsplitsing van een gedeelte van de zijketen van cholesterol aanwezig is, als ook het substraat voor dit enzymkomplex. In afwezigheid van cyanoketon, een remmer van 3ß-hydroxysteroid dehydrogenase aktiviteit, werd voornamelijk pregnenolon en testosteron gesynthetiseerd. De produktie van testosteron in geïsoleerde mitochondriale frakties kan mogelijk veroorzaakt zijn door verontreiniging van deze fraktie met mikrosomaal gelokaliseerde enzymen, welke noodzakelijk zijn voor de omzetting van pregnenolon tot testosteron. In appendix publikatie II werd gekonkludeerd dat ongeveer 7-15% van 3ß-hydroxysteroid dehydrogenase mitochondriaal gelokaliseerd is.

3. Het effekt van langdurige behandeling van volwassen manlijke ratten met gonadotropinen in vivo op de testikulaire produktie van endogene steroiden (sektie 4.2. en appendix publikatie III).

Dagelijkse subcutane toediening van 100 i.u. HCG aan intakte volwassen ratten, resulteerde in een toename in de koncentratie van testosteron in homogenaten van zowel totaal testisweefsel als interstitieel weefsel en seminifere tubuli. Na de hormoonbehandeling was ook de produktie van testosteron in homogenaten van totaal testisweefsel ver-

hoogd. De gestimuleerde produktie van testosteron in homogenaten van interstitieel weefsel kan de verhoogde produktie van endogeen testosteron in totaal testisweefsel volledig verklaren. Tevens werd een verhoogde produktie van pregnenolon waargenomen in mitochondriale frakties geïsoleerd uit totaal testisweefsel en interstitieel weefsel. Deze resultaten bevestigden de theorie dat hormonaal gestimuleerde produktie van androgenen veroorzaakt wordt door een toename in de mitochondriale pregnenolonsynthese.

Na langdurige behandeling met HCG was de specifieke aktiviteit van 3β-hydroxysteroid dehydrogenase in homogenaten van totaal testisweefsel, interstitieel weefsel en seminifere tubuli verhoogd, maar er was geen effekt op de specifieke aktiviteiten van "marker"enzymen als cytochroom c oxidase, monoamine oxidase, steroid sulfatase en lactaat dehydrogenase aantoonbaar. De specifieke aktiviteit van carboxyl esterase nam af.

4. Het akute effekt van gonadotropinen op de testikulaire steroidproduktie (sektie 4.3. en appendix publikatie IV).

Binnen 20 min na intraveneuze toediening van LH aan intakte volwassen ratten steeg de koncentratie van testosteron in testikulair veneus plasma en in testikulair weefsel met een faktor 3, respektievelijk 10. De koncentratie van pregnenolon in mitochondriale frakties uit testikels welke 20 min na toediening van LH uit de rat verwijderd werden was 5 maal hoger dan in vergelijkbare kontrole mitochondriale frakties.

Binnen 5 min na intraveneuze injektie van LH was de produktiesnelheid van testosteron in weefselhomogenaten en van pregnenolon in mitochondriale frakties verhoogd. Het stimulerende effekt van LH op de testosteronproduktie in vivo en in vitro en op de produktie van pregnenolon in geïsoleerde mitochondriale frakties werd geblokkeerd, wanneer 10 min voor de toediening van LH in vivo cycloheximide intraveneus werd toegediend.

5. De mogelijke rol die Ca^{2+} ionen spelen in het cellulaire mechanisme van de hormonaal gereguleerde produktie van steroiden (hoofdstuk 5 en appendix publikatie IV).

Toevoeging van Ca^{2+} ionen stimuleerde de produktie van testosteron in weefselhomogenaten en van pregnenolon in mitochondriale frakties. Toevoeging van Ca^{2+} aan het inkubatiemedium kan het stimulerende effekt van LH in vivo op de mitochondriale pregnenolonproduktie nabootsen, aangezien Ca^{2+} een groter stimulerend effekt had op de produktie van pregnenolon in kontrole mitochondriale frakties dan in mitochondriale frakties geïsoleerd uit LH gestimuleerde testikels (sektie 5.2.).

Veroudering van mitochondriaal eiwit verhoogde de produktiesnelheid van pregnenolon in mitochondriale frakties van kontrole testikels. Na veroudering waren de mitochondriale produktiesnelheden van pregnenolon in kontrole en LH gestimuleerde testikels vrijwel gelijk.

Het stimulerend effekt van Ca^{2+} op de pregnenolonproduktie in mitochondriale frakties (zowel voor kontrole als LH gestimuleerde testikels) was ongeveer 70% lager in aanwezigheid van ruthenium red, een specifieke remmer van binding en transport van Ca^{2+} in mitochondria.

De mogelijke rol van Ca^{2+} ionen in de hormonale regulatie van de testikulaire steroidproduktie is uitvoeriger beschreven in hoofdstuk 5.

CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1945 te Amsterdam geboren. In 1964 werd het getuigschrift gymnasium B behaald aan het Cartesius Lyceum te Amsterdam. Aan de Gemeentelijke Universiteit van deze stad begon hij in hetzelfde jaar met de scheikundestudie. Hij behaalde het kandidaatsexamen in de wiskunde en natuurwetenschappen (kode S2) in 1968 en het doctoraalexamen met hoofdvak biochemie en bijvakken fysisch en synthetisch organische chemie in 1971. Sinds september 1971 is hij verbonden als wetenschappelijk medewerker aan de afdeling Chemische Endocrinologie (Biochemie II) van de Erasmus Universiteit te Rotterdam.

NAWOORD

In het voor U liggende proefschrift zijn de resultaten verwerkt van het experimentele onderzoek dat van september 1971 tot juni 1975 door mij verricht is op de afdeling Chemische Endocrinologie (Biochemie II) van de Erasmus Universiteit te Rotterdam.

Velen hebben op theoretische en praktische wijze bijgedragen tot het verschijnen van dit proefschrift:
Henk van der Molen als organisator en promotor,
Dr. G.S. Boyd en Jasper Scholte als coreferenten, Brian Cooke als kritisch lezer van de manuskripten en de overige medewerkers van de afdelingen Biochemie I en II als kommentatoren tijdens werkbesprekingen op dinsdagmiddagen en/of donderdagavonden.
Het praktische werk werd mede verricht door Ienke Kalkman, Mary van der Planck en, als stagiaires van de Delftse Analistenschool, Anneke Moespot en Hans Goedemans. Inge van Dop heeft als keuze-praktikante enige maanden aan het onderzoek meegewerkt. Pim Clotscher droeg zorg voor het goed funktioneren van de gebruikte apparatuur.

De manuskripten zijn getypt door Willie Bakhuizen en Marja Decae. Het grafische werk is grotendeels verzorgd door de Audiovisuele Dienst.

Voor al deze steun ben ik iedereen zeer erkentelijk.

De mensheid wroet, met een onvoorstelbaar geduld en vindingrijkheid, diep in de hem omringende natuur naar het ontstaan van het leven. Zelfs breekt men zich het hoofd over het probleem van het mogelijke bestaan van buitenaardse vormen van intelligentie, evenals men de invloed nagaat van de incubatiewarmte op de kunstmatige synthese van bepaalde steroïden in de zaadballen van ratten. Ogenschijnlijk ontsnapt er niets aan onze aandacht. Maar is dat werkelijk zo?

Saswitha (179)

APPENDIX PAPERS

Reprinted from

Biochimica et Biophysica Acta, 297 (1973) 179–185

BBA Report

BBA 21354

Endogenous production of steroids by subcellular fractions from total rat testis and from isolated interstitial tissue and seminiferous tubules

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(Received December 28th, 1972)

SUMMARY

The endogenous production of pregnenolone and testosterone in subcellular fractions of rat testis tissue was estimated. A medium containing cofactors was used and the incubations were carried out for 120 min at 33 °C. Only mitochondrial fractions produced the estimated steroids. Seminiferous tubules and interstitial tissue were isolated from rat testis tissue using a wet dissection technique. Mitochondrial fractions were isolated from the homogenized isolated tissue compartments and production of pregnenolone and testosterone after incubation of these fractions was estimated. The interstitial mitochondrial fraction produced 1200–2600 pmoles per mg mitochondrial protein per 2 h which was at least 60 times higher than the production in similar fractions obtained from seminiferous tubules. Production in the mitochondrial fraction of whole testis tissue was 300–600 pmoles per mg protein per 2 h.

Conversion of cholesterol to pregnenolone and isocaproic acid is an important rate-limiting step in steroid production[1]. The enzyme system (the so-called cholesterol side-chain cleaving enzyme) responsible for this conversion, consists of cytochrome P-450, a flavoprotein and a non-heme iron protein[2]. It is generally considered that this enzyme system is located in mitochondria and that further metabolism of pregnenolone, *e.g.* to testosterone, occurs in the endoplasmic reticulum and cytosol[2]. Information concerning the site and specificity of the metabolism of cholesterol to steroids in the testis has mainly been obtained from observations on the conversion of radioactive cholesterol to either radioactive steroids[3] or radioactive isocaproic acid[3,4]. Although metabolism of a radioactive substrate may give valuable information about the presence of enzymes it does not give quantitative information about the production of endogenous steroids. Further-

more, this type of study is complicated by the possibility that the radioactive precursor does not completely mix with the endogenous pool and therefore the results obtained may not reflect true endogenous conversions.

We have therefore estimated the endogenous production of steroids by subcellular fractions from rat testis tissue.

The results in Fig. 1 show the subcellular distribution of endogenous steroid production after fractionation of a homogenate of total rat testis tissue.

Testes from 3 Wistar rats of 8–10 weeks old were pooled and were homogenized with an automatic Potter homogenizer (B. Braun Melsungen) for 3 × 15 s at 1100 rev./min. Nuclear fraction N; 500 × *g* for 10 min), nuclei-free fraction (E; 500 × *g* for 10 min, supernatant), heavy mitochondrial fraction (M; 3500 × *g* for 10 min), light mitochondrial fraction (L; 10 000 × *g* for 10 min), microsomal fraction (P; 105 000 × *g* for 60 min) and particle-free supernatant (S; 105 000 × *g* for 60 min, supernatant) were obtained by differential centrifugation. For determination of steroid production in a typical experiment 7.9 mg protein of N fraction, 5.2 mg of E fraction, 5.6 mg of M fraction, 3.8 mg of L fraction, 7.3 mg of P fraction and 2.5 mg of S fraction were incubated for 120 min at 33 °C in 95% O_2 + 5% CO_2 atmosphere in a fortified medium (see legend Fig. 1).

The results in Fig. 1 show the relative specific activity or concentration of DNA as a marker for the nuclear fraction, cytochrome *c* oxidase for mitochondrial fractions, carboxylesterase for microsomal fraction and lactate dehydrogenase for particle-free supernatant. It was found that part of the lactate dehydrogenase activity remained in the mitochondrial fractions, even after repeated washing. The rate of production of steroids was measured as the increase in endogenous concentrations of testosterone and pregnenolone. Routinely pregnenolone and testosterone accounted for 80–90% of the steroids produced during 120 min incubation at 33 °C. Other steroids measured were progesterone and androstenedione (van der Vuss, G.J. and Kalkman, M.L., unpublished results). Protein was measured by the method of Lowry *et al.*[5] using bovine serum albumin as a standard. The sum of production of pregnenolone and testosterone in N and E fraction is 101 nmoles/2 h. The relative contribution of the different fractions to the sum of production is 3.1% for N fraction, 27.4% for M fraction, 64.5% for L fraction, 3.4% for P fraction and 0.4% for S fraction. The recovery is 98.8%. It is apparent that only mitochondrial fractions produced steroids in appreciable amounts. The production of testosterone (from pregnenolone) in the mitochondrial fractions may be due to microsomal contamination. These results are in agreement with experiments of Toren *et al.*[3], who located the cholesterol side-chain cleaving enzyme in the 6500 × *g* for 25 min pellet, using radioactive cholesterol as a substrate.

Considering the different cell types in the testis it is still not clear whether only the interstitial tissue, which is supposed to produce the bulk of testicular steroids, is active in steroid biosynthesis, or whether the seminiferous tubules can also produce small amounts of steroids. Lacy and co-workers[6] have suggested that Sertoli cells in the seminiferous tubules might convert cholesterol to steroids which are required for maintenance of spermatogenesis. Christensen and Mason[7], Hall *et al.*[8] and Bell *et al.*[9] have reported the

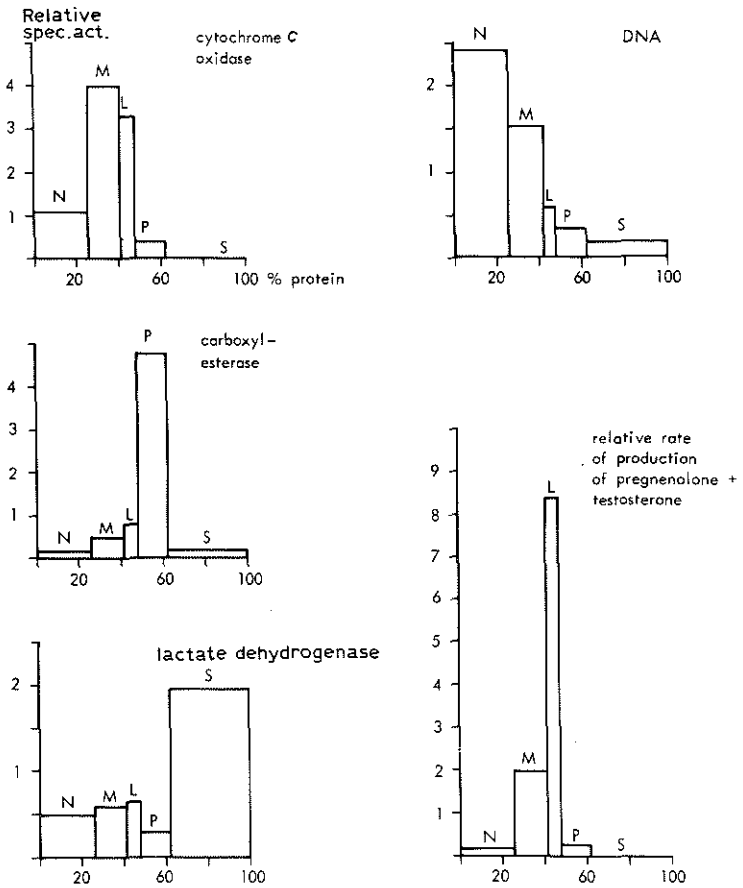

Fig. 1. Subcellular distribution of steroid production in rat testis tissue from endogenous precursors. Graphs show relative specific enzyme activities of marker enzymes and the relative specific rate of production of pregnenolone and testosterone in subcellular fractions of rat testis tissue. Subcellular fractions were isolated according to the method introduced by De Duve *et al.*[12]; cytochrome *c* oxidase activity (EC 1.9.3.1) was measured according to the method of Schnaitman *et al.*[13] and used as a marker for mitochondria; DNA according to the method of Burton[14] and was used as a marker for nuclei; lactate dehydrogenase (EC 1.1.1.27) according to the method of Kornberg[15]; carboxylesterase activity (EC 3.1.1.1) was measured with *p*-nitrophenyl acetates as a substrate and was used as a marker for microsomes; aliquots of the fractions were incubated for 120 min at 33 °C in a 95% O_2 + 5% CO_2 atmosphere in a medium containing sucrose (0.125 M), EDTA (0.5 mM), succinate (20.0 mM), NADPH (1.0 mM), glucose 6-phosphate (10.0 mM), glucose-6-phosphatase (3 K.U.), phosphate (25.0 mM), Ca^{2+} (0.7 mM), Mg^{2+} (5.0 mM) at pH 7.2 in 2.0 ml; pregnenolone and testosterone were extracted from the incubation medium with 3 × 5 ml ethyl acetate containing [16-^{3}H]pregnenolone and [1,2-^{3}H]testosterone as internal standards and were estimated by gas–liquid chromatography using electron capture detection. Pregnenolone was measured by a modification of the method of De Jong *et al.*[16] and testosterone by the method of Brownie *et al.*[17].

presence of enzyme activities in seminiferous tubules for the conversion of pregnenolone and progesterone to testosterone. The results of such experiments may greatly depend upon the purity of the isolated tissues and contamination of seminiferous tubules with only a small amount of interstitial tissue and may easily result in an apparent steroid biosynthetic activity. Hall *et al.*[8] could not demonstrate a significant conversion of radioactive cholesterol to steroids by isolated intact seminiferous tubules. It has also been reported that isolated tubules incubated *in vitro* did not produce testosterone (Cooke *et al.*[10]).

In order to estimate the difference in biosynthetic activity between the different testis tissues, we have measured the production of endogenous pregnenolone and testosterone rather than the conversion of radioactive precursors in mitochondrial fractions from whole testis tissue, seminiferous tubules and interstitial tissue.

For each experiment the testis of one Wistar rat, 10 weeks old, were decapsulated. 2.7 g (Expt 1, Table I and Fig. 2) and 3.1 g (Expt 2, Table I) testis tissue was divided in two parts 1.9 g (Expt 1) and 2.2 g (Expt 2) was used for dissection into interstitial tissue and seminiferous tubules by a method introduced by Christensen and Mason[7]. The rest of the testis tissue remained undissected. From the loss of esterase activity and protein it was estimated that the loss of tissue during dissection was approximately 40–50%. Fig. 2 shows the relative contribution of mitochondrial fractions from seminiferous tubules and interstitial tissue to endogenous steroid production in testis. The distribution of carboxylesterase activity in the top panel supports the good separation between tubular and interstitial tissue (Rommerts, F.F.G., Van Doorn, L.G., Galjaard, H., Cooke, B.A. and Van der Molen, H.J., unpublished). Combined heavy and light mitochondrial fractions were obtained from these isolated tissues. Although we have used the presence of lactate dehydrogenase as a marker for the particle-free supernatant, it has been reported by Machado de Domenech *et al.*[11] that in the testis a different lactate dehydrogenase isoenzyme is located inside the mitochondria from primary spermatocytes. Therefore, the presence of lactate dehydrogenase activity in the mitochondrial fractions was considered specific for the presence of mitochondria from tubular origin and was used as a marker for contamination of mitochondria from interstitial tissue by mitochondria from tubules. The lower panel of Fig. 2 shows the distribution of the relative specific rate of production of pregnenolone and testosterone. The sensitivity of the techniques used for detection of the steroids permitted estimation of 1–3 pmoles testosterone[18] and 6–10 pmoles pregnenolone[17]. The endogenous amounts of steroids produced by mitochondrial fractions from interstitial tissue could easily be measured. Routinely, 0.25–0.40 mg of mitochondrial protein from interstitial tissue contained, after incubation, amounts in the order of 20–25 pmoles pregnenolone and 500–700 pmoles testosterone, while 0.80–1.50 mg mitochondrial protein from the tubular compartment contained after incubation 15–20 pmoles pregnenolone and 4–12 pmoles testosterone (Table I).

When expressed per mg mitochondrial protein per 2 h the production of pregnenolone and testosterone in the mitochondrial fraction of seminiferous tubules was at least 60 times lower, when compared with the production in the interstitial mitochondrial

TABLE I

ENDOGENOUS PRODUCTION OF PREGNENOLONE AND TESTOSTERONE BY MITOCHONDRIAL FRACTIONS FROM RAT TESTIS TISSUE. INTERSTITIAL TISSUE AND SEMINIFEROUS TUBULES

Combined heavy and light mitochondrial fractions from total testis tissue, interstitial tissue and seminiferous tubules were incubated and steroids were estimated as described in the legend to Fig. 1. All results are mean values of duplicate estimations.

Expt	*Source of mitochondrial fraction*	*Mitochondrial protein*		*Pregnenolone (pmoles)*		*Testosterone (pmoles)*		*Total production of pregnenolone and testosterone (pmoles/2 h/mg protein)*
		Total amount (mg)	*Protein per incubation (mg)*	*Time of incubation (min)*		*Time of incubation (min)*		
				0	*120*	*0*	*120*	
1	Total testis	10.3	1.47	11	25	3	410	286
	Interstitial tissue	2.5	0.40	6	20	0	483	1242
	Seminiferous tubules	11.9	1.28	2	21	2	12	23
2	Total testis	5.7	0.86	12	29	2	481	576
	Interstitial tissue	1.8	0.26	8	24	2	663	2604
	Seminiferous tubules	5.8	0.68	2	14	3	4	18

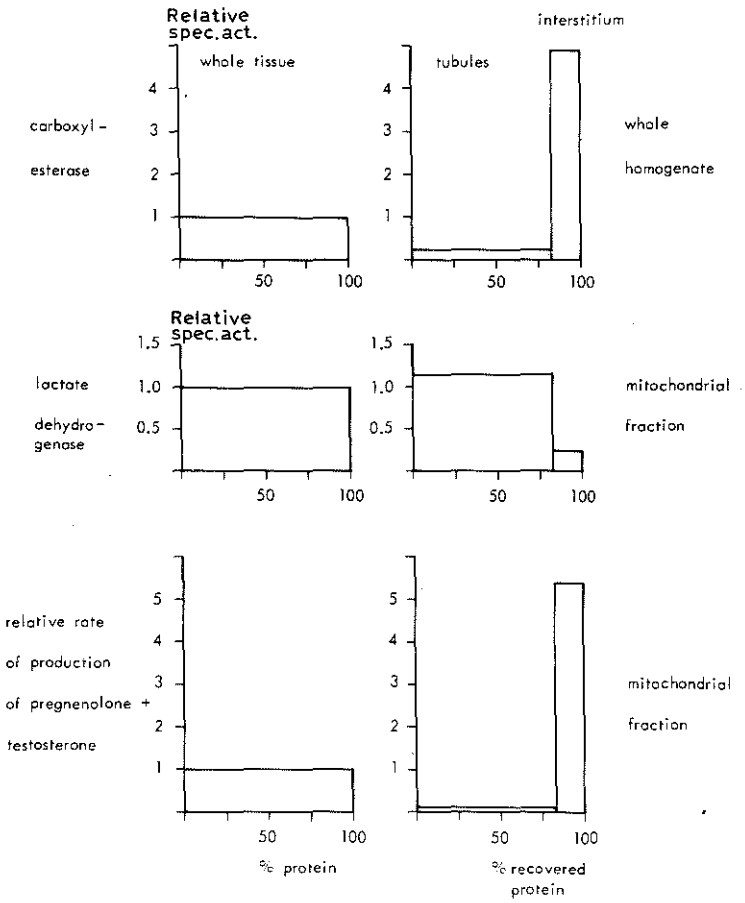

Fig. 2. Intercellular distribution of endogenous production of pregnenolone and testosterone in rat testis tissue (Expt 1 from Table I). Top panel: relative specific activity of carboxylesterase in homogenates of whole testis and in homogenates of isolated seminiferous tubules and interstitial tissue; middle panel: relative specific activity of lactate dehydrogenase (EC 1.1.1.27) in isolated mitochondrial fractions from whole testis and from isolated seminiferous tubules and interstitial tissue; lower panel: relative specific rate of production of pregnenolone and testosterone, measured in mitochondrial fractions from whole testis tissue and from isolated seminiferous tubules and interstitial tissue. In this experiment heavy and light mitochondrial fractions were combined. See legend to Fig. 1 for incubation conditions for steroid production and estimations of activity of lactate dehydrogenase and carboxylesterase.

fraction. From the data in Table I it may appear that the production in the mitochondrial fractions isolated from interstitial tissue contributes 92–97% to the production of mitochondrial fractions isolated from total testis tissue, if losses of activity during the isolation procedure are comparable. In the light of these findings we conclude that the mitochondrial fraction of interstitial tissue is the main source of steroids from endogenous precursors in rat testicular tissue.

Further investigation is required to establish if the small production of steroids in the tubular mitochondrial fraction is due to contamination by interstitial mitochondria; this contamination cannot be detected with the marker enzymes used in the present investigation. It cannot be excluded that the low rate of steroid production, if any, in the mitochondrial fraction from seminiferous tubules under our experimental conditions is caused by deprivation of extramitochondrial precursors, although Cooke *et al.*[10] could not show testosterone production in intact tubules. The observation of Hall *et al.*[8] that the tubular compartment does not convert exogenous radioactive cholesterol to steroids might reflect either the absence of enzyme activities or the restricted penetration of cholesterol into the seminiferous tubules, as shown by Parvinen *et al.*[18].

REFERENCES

1 Hall, P.F. (1970) in *The Androgens of the Testis* (Eik-Nes, K.B., ed.), pp. 73–116, Marcel Dekker, New York
2 Samuels, L.T. and Eik-Nes, K.B. (1968) in *Metabolic Pathways* (Greenberg, D.M., ed.), Vol. II, pp. 169–220, Academic Press, London and New York
3 Toren, D., Menon, K.M.J., Forchielli, E. and Dorfman, R.I. (1964) *Steroids* 3, 381–390
4 Menon, K.M.J., Dorfman, R.I. and Forchielli, E. (1967) *Biochim. Biophys. Acta* 148, 486–494
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275
6 Lacy, D. and Pettit, A.J. (1970) *Br. Med. Bull.* 26, 87–91
7 Christensen, A.K. and Mason, N.R. (1965) *Endocrinology* 76, 646–656
8 Hall, P.F., Irby, D.C. and De Kretser, D.M. (1969) *Endocrinology* 84, 488–496
9 Bell, J.B.G., Vinson, G.P. and Lacy, D. (1971) *Proc. R. Soc. London Ser. B* 176, 433–443
10 Cooke, B.A., De Jong, F.H., Van der Molen, H.J. and Rommerts, F.F.G. (1972) *Nature New Biol.* 237, 255–256
11 Machado de Domenech, E., Domenech, C.E., Aoki, A. and Blanco, A. (1972) *Biol. Reprod.* 6, 136–147
12 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) *Biochem. J.* 60, 604–617
13 Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967) *J. Cell Biol.* 32, 719–735
14 Burton, K. (1956) *Biochem. J.* 62, 315–323
15 Kornberg, A. (1955) in *Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O., eds), Vol. 1, pp. 441–443, Academic Press, London and New York
16 De Jong, F.H. and Van der Molen, H.J. (1972) *J. Endocrinol.* 53, 461–474
17 Brownie, A.C., Van der Molen, H.J., Nishiwaza, E.E. and Eik-Nes, K.B. (1964) *J. Clin. Endocrinol. Metab.* 24, 1091–1102
18 Parvinen, M., Hurme, P. and Niemi, M. (1970) *Endocrinology* 87, 1082–1084

Reprinted from

Biochimica et Biophysica Acta, 348 (1974) 404–414

BBA 56446

3β-HYDROXYSTEROID DEHYDROGENASE IN RAT TESTIS TISSUE

INTER- AND SUBCELLULAR LOCALIZATION AND INHIBITION BY CYANOKETONE AND NAGARSE

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(Received January 3rd, 1974)

SUMMARY

The quantitative inter- and subcellular distribution of 3β-hydroxysteroid dehydrogenase (3β-hydroxysteroid:NAD (P)$^+$ oxidoreductase, EC 1.1.1.51) has been studied in testis tissue of the rat. The specific activity in homogenates of isolated interstitial tissue was 150–500 times higher than the specific activity in homogenates of seminiferous tubules. It was concluded that 95–98% of 3β-hydroxysteroid dehydrogenase is located in the isolated interstitial tissue.

Subcellular fractionation of testis homogenates and the distribution of marker enzymes indicated that mitochondria contribute 7–15% to total 3β-hydroxysteroid dehydrogenase in rat testis tissue.

Nagarse (subtilipeptidase, EC 3.4.21.14) treatment or cyanoketone (2α-cyano-4,4',17α-trimethyl-17β-hydroxy-5-androsten-3-one) inhibited 3β-hydroxysteroid dehydrogenase activity in rat testis tissue.

Measurements of steroid production from endogenous substrates in isolated mitochondrial fractions showed that addition of cyanoketone to incubation mixtures or pretreatment of homogenates of interstitial tissue with nagarse caused a acncumulation of pregnenolone at the expense of testosterone.

INTRODUCTION

Testis tissue contains two different compartments: interstitial tissue, containing Leydig cells, connective tissue and blood vessels, and seminiferous tubules with Sertoli cells and cells in various stages of spermatogenesis. The steroidogenic properties of interstitial tissue have been clearly established [1–6]. Steroid production from endogenous substrates by seminiferous tubules either in vivo or in vitro remains uncertain [2, 5, 6]. It has been suggested, however, that mitochondrial fractions of seminiferous tubules may convert [26-^{14}C]cholesterol to isocaproic acid [7] and could therefore produce pregnenolone and testosterone [2]. Nevertheless, this enzyme activity is very low when compared with the same activity in analogous incubation studies with mitochondrial fractions from interstitial tissue.

The conversion of radioactive pregnenolone to progesterone and androgens by

preparations of seminiferous tubules [8–11] indicate the presence of 3β-hydroxysteroid dehydrogenase in this tissue compartment. However, no data are available for the quantitative intercellular distribution of this steroid dehydrogenase in testis tissues.

With respect to subcellular distribution of 3β-hydroxysteroid dehydrogenase in testis Shikita et al. [12] have shown that this enzyme activity is particle-bound, whereas the occurrence of this enzyme in the inner membrane fraction of mitochondria, isolated from total testis tissue was recently reported [13].

The aim of the present study was to determine the quantitative distribution of 3β-hydroxysteroid dehydrogenase between interstitial tissue and seminiferous tubules. It was also attempted to measure the contribution of the mitochondrial 3β-hydroxysteroid dehydrogenase to the total enzyme activity.

Finally the effect of inhibition of 3β-hydroxysteroid dehydrogenase by cyanoketone or nagarse on mitochondrial steroid production from endogenous substrates was studied.

MATERIALS AND METHODS

Pregnenolone, progesterone and testosterone were obtained from Steraloids, Pawling, U.S.A. and were used without further purification. Cofactors were purchased from Boehringer, Mannheim, Germany. Cyanoketone is a product of Stirling-Winthrop, New York, U.S.A. Nagarse was obtained from Serva, Heidelberg, Germany. *p*-Nitrophenyl acetate is a product of Koch-Light Lab., Colnbrook, England. [16-^{3}H]Pregnenolone was obtained from CEN, Mol, Belgium. Other radioactive steroids and [1-^{14}C]tyramine were obtained from Radiochemical Centre, Amersham, England. Radioactive steroids were purified until less than 1% radioactive contaminants could be detected with paper chromatography.

Male Wistar rats of 12–14 weeks old were killed by decapitation. Testes were removed and immediately chilled in ice-cold 0.25 M sucrose, containing 1 mM EDTA. After removing the tunica albuginea the weight of the testes was determined.

In some experiments testis tissue was dissected into interstitial tissue and seminiferous tubules according to the method described by Christensen and Mason [4]. During the dissection procedure the tissue was covered with ice-cold 0.25 M sucrose, containing 1 mM EDTA. The dissected tissues were washed 2 times with the dissection medium. Tissues were homogenized at 4 °C in 0.25 M sucrose, containing 1 mM EDTA with an automatic Potter homogenizer (Braun Melsungen, Germany) for 3 times 15 s at 1100 rev./min. Nuclear fractions (N, $500 \times g_{av}$ for 10-min pellet), nuclei-free fractions (E, $500 \times g_{av}$ for 10-min supernatant), mitochondrial fractions (M, $15000 \times g_{av}$ for 10-min pellet), microsomal fractions (P, 1 h, $105000 \times g_{av}$ pellet) and particle-free supernatant (S, 1 h, $105000 \times g_{av}$ supernatant) were obtained by differential centrifugation. N, E. and M fractions were isolated using a Sorvall HB-4 swing-out rotor in a Sorvall RC 2-B centrifuge. N and M fractions were washed twice with 0.25 M sucrose, containing 1 mM EDTA. P and S fractions were obtained with a Beckmann 50 Ti rotor in a Beckmann L2-65B ultracentrifuge.

Assay of enzyme activities

Glucose-6-phosphate dehydrogenase activity (EC 1.1.1.4) was determined according to Glock and McLean [14] at 25 °C.

3β-Hydroxysteroid dehydrogenase (EC 1.1.1.51) activity was determined routinely by following the conversion of 0.1 mM [16-^{3}H]pregnenolone to [16-^{3}H]progesterone at 33 °C in the presence of 1 mM NAD^+ in 25 mM potassium phosphate buffer pH 7.35 or 9.0, 0.125 M sucrose, 0.5 mM EDTA. Incubation volume was 1.0 ml. Pregnenolone was added in 20 μl ethanol to the incubation mixture. The reaction was stopped with 3 ml ethyl acetate, containing 12000 dpm [4-^{14}C]pregnenolone and 2000 dpm [4-^{14}C]progesterone as internal standards. The incubation mixture was extracted 3 times with 3 ml ethyl acetate. The collected extracts were evaporated under N_2 and subjected to paper chromatography on Whatman No. 20 paper strips in a Bush A II system (light petroleum (b.p. 60–90 °C)–methanol–water (10: 7: 3, by vol.).

Detection of radioactive steroids on paper chromatograms was carried out with a Packard radiogramscanner (Model 7200). The spots corresponding with pregnenolone and progesterone were eluted separately with 5 ml ethanol. After evaporation of the ethanol the residue was acetylated with an acetic anhydride–pyridine mixture (1:1, v/v) for 30 min at 45 °C, followed by evaporation of the reagents under N_2. After chromatography of the residue on paper in a Bush AII system the spots corresponding with progesterone and pregnenolone acetate were eluted with ethanol in counting vials. The ethanol was evaporated and radioactivity was counted in a modified Bray's solution [15]. Incubation times and amounts of protein were chosen so that not more than 15% of the substrate was converted. No other metabolites than progesterone could be detected in this assay. In this assay the rate-limiting dehydrogenase activity was estimated since the isomerization is very rapid [16, 17].

Monoamine oxidase (EC 1.4.3.4.) activity was determined by the method of McCaman et al. [18], using [1-^{14}C]tyramine as the substrate at 32 °C.

Rotenone-insensitive NADPH–cytochrome *c* reductase (EC 1.6.2.3) activity was measured in the presence of 0.15 μM rotenone by following the reduction of cytochrome *c* at 550 nm at 25 °C [19].

Cytochrome *c* oxidase (EC 1.9.3.1) activity was assayed according to the method of Schnaitman et al. [20] at 32 °C.

Adenylate kinase (EC 2.7.4.3) was assayed by the method of Sottocasa et al. [21] at 25 °C.

Carboxyl esterase (EC 3.1.1.1) was measured with *p*-nitrophenyl acetate as substrate, by following the increase of extinction at 400 nm at 20 °C [22].

Glucose 6-phosphatase (EC 3.1.3.9) was assayed as described by De Duve et al. [23].

Steroid sulfatase (EC 3.1.6.2) was assayed essentially as described by Payne et al. [24] following the hydrolysis of [4-^{14}C]dehydroepiandrosterone sulfate (17-oxo-5-androsten-3β-yl sulfate) at 32 °C and pH 6.5.

The endogenous production of steroids by mitochondrial fractions was estimated by incubating aliquots of mitochondrial fractions, at 33 °C in a O_2–CO_2 (95:5, v/v) atmosphere in a medium containing sucrose (0.125 M), EDTA (0.5 mM), succinate (20 mM), NADPH (1.0 mM), glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (0.3 units), $CaCl_2$ (0.7 mM), $MgCl_2$ (5.0 mM), Tris–HCl buffer (25.0 mM) at pH 7.35 in 1 ml. The reaction was stopped by adding 4 ml ethyl acetate containing 100000 dpm [16-^{3}H]pregnenolone and 120000 dpm [1,2-^{3}H]testosterone as internal standards. The reaction mixture was extracted 3 times with 4 ml

ethyl acetate. Pregnenolone was purified by paper and thin-layer chromatography and after oxidation to 4-pregnen-3,6,20-trione this compound was estimated using gas–liquid chromatography with electron capture detection. This method is essentially a modification of the method of De Jong and Van der Molen [25] for estimation of dehydroepiandrosterone (3β-hydroxy-4-androsten-17-one). Testosterone was determined by the method of Brownie et al. [26] The smallest amount of pregnenolone and testosterone that could be measured was respectively 6–10 and 1–3 pmoles. Endogenous steroid production was calculted from the difference in the amount of pregnenolone or testosterone present in the incubations after 60 min incubation and at zero time.

Cyanoketone was added to the incubation mixture in 20 μl ethanol. Protein was measured by the method of Lowry et al. [27] using bovine serum albumine as a standard.

For gaschromatography either a Pye Unicam 104, Model 84 or an F and M gaschromatograph equipped with a ^{63}Ni or ^{3}H electron capture detector was used.

Counting of radioactivity was done in either a Nuclear Chicago Mark I or a Packard 3375 liquid-scintillation counter to precision of 2% or less.

RESULTS

Assay of 3β-hydroxysteroid dehydrogenase activity

Insolubility of pregnenolone at concentrations higher than 0.1 mM prevents the determination of the Michaelis-Menten constants of 3β-hydroxysteroid dehydrogenase in homogenates of rat testis tissue since the K_m is higher than the saturation concentration. For the comparison of specific activities in various tissue fractions and in subcellular fractions 0.1 mM pregnenolone was used in all experiments communicated in this paper.

The conversion of pregnenolone to progesterone reached a maximum at pH 9.0. The ratio of the enzyme activity, assayed in the range pH 9.0–10.0 and at pH 7.35 in homogenates of isolated tissues as well as subcellular fractions, is in the order of 1.6. Sonication of aliquots of tissue homogenates before enzyme assay had no influence on enzyme activity (data not shown).

Intercellular distribution of 3β-hydroxysteroid dehydrogenase

In Table I the results of a typical experiment are summarized. The specific activities of 3β-hydroxysteroid dehydrogenase are compared with those of other particle-bound enzymes such as steroid sulfatase, carboxyl esterase and rotenone-insensitive NADPH–cytochrome *c* reductase.

Combined results of four experiments gave the following specific activities of 3β-hydroxysteroid dehydrogenase at pH 7.35 in homogenates: of undissected tissue 0.20 $\pm$ 0.06 nmoles/mg protein per min (mean value $\pm$ S.D.), of interstitial tissue 1.59 $\pm$ 0.40 and of seminiferous tubules 0.007 $\pm$ 0.004. The ratio of specific activity of the enzyme in homogenates of interstitial tissue and seminiferous tubules was 260 $\pm$ 162. The ratio of specific activities measured in homogenates of interstitial tissue and of seminiferous tubules exceeded the value of the ratio of 25.3 for carboxyl esterase, which is a useful biochemical marker for interstitial tissue as recently shown by Rommerts et al. [22]. The ratio of specific activities of other microsomal enzymes

TABLE I

INTERCELLULAR DISTRIBUTION OF 3β-HYDROXYSTEROID DEHYDROGENASE IN RAT TESTIS TISSUE

Enzyme assays were performed as described in Materials and Methods.
Two rat testes (2.83 g wet wt) were divided in two parts. 1.36 g remained undissected and 1.47 g was dissected into interstitial tissue and seminiferous tubules. Enzyme activities were determined in homogenates of the undissected testis tissue, of interstitial tissue and of seminiferous tubules. All results are mean values of triplicate determinations

Enzyme	Enzyme activity (nmoles/mg protein per min)			Ratio b/c
	Total testis tissue (a)	Interstitial tissue (b)	Seminiferous tubules (c)	
3β-Hydroxysteroid dehydrogenase (pH 7.35)	0.120	1.180	0.007	168
3β-Hydroxysteroid dehydrogenase (pH 9.0)	0.170	1.580	0.010	158
Steroid sulfatase	0.016	0.081	0.013	6.2
Carboxylesterase	240	1520	60	25.3
NADPH–cytochrome *c* reductase	3.0	6.9	3.3	2.1
Protein (mg/fraction)	117.4	7.1	71.2	

as steroid sulfatase and rotenone-insensitive NADPH–cytochrome *c* reductase showed a less distinct difference between interstitial tissue and seminiferous tubules.

Subcellular distribution of 3β-hydroxysteroid dehydrogenase in homogenates of rat testis tissue

The results in Table II and Fig. 1 show the distribution of 3β-hydroxysteroid dehydrogenase activity in subcellular fractions isolated from homogenates of total

TABLE II

SUBCELLULAR DISTRIBUTION OF 3β-HYDROXYSTEROID DEHYDROGENASE AND SOME MARKER ENZYMES FOR SUBCELLULAR PARTICLES IN FRACTIONS ISOLATED FROM TESTIS TISSUE

Enzyme assays were performed on subcellular fractions isolated from four pooled testes (6.35 g wet wt) as described in Materials and Methods. N, nuclear fraction; E, nuclei-free supernatant; M, mitochondrial fraction; P, microsomal fraction and S, 1 h, $105000 \times g_{av}$ supernatant. The enzyme activities present in the N+E fraction isolated from 4 testes are expressed as μmoles/min. All results are mean values of triplicate determinations.

Enzyme	Enzyme activity (μmoles/min) in (N+E) fraction	Relative distribution of enzyme activity in percent of the activity in N+E fraction (%)					Recovery (%)
		N+E	N	M	P	S	
3β-Hydroxysteroid dehydrogenase (pH 7.35)	0.066	100	19.9	20.1	62.4	3.0	105.4
3β-Hydroxysteroid dehydrogenase (pH 9.0)	0.095	100	18.2	16.5	55.1	4.7	94.5
Cytochrome *c* oxidase	69.5	100	29.8	66.6	11.2	0.0	107.6
Monoamine oxidase	0.41	100	35.4	48.2	11.5	1.0	96.1
Carboxyl esterase	145.7	100	15.2	13.9	59.4	8.9	97.4
Steroid sulfatase	0.010	100	30.2	21.3	57.4	0.0	108.9
NADPH–cytochrome *c* reductase	0.82	100	23.4	22.2	51.4	8.7	105.7
Glucose-6-phosphate dehydrogenase	6.18	100	12.7	4.8	4.4	72.8	94.7
Protein (mg/4 testes)	435.4	100	34.0	15.0	13.7	33.1	95.8

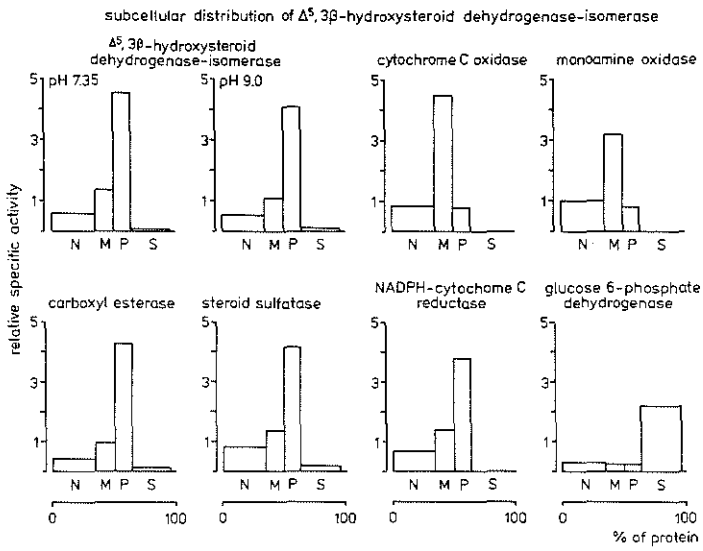

Fig. 1. Subcellular distribution of 3β-hydroxysteroid dehydrogenase in total testis tissue of the rat in relation to some marker enzymes. Abscissae: the percentage of the total protein content in each fraction is presented as cummulative values. Ordinates: relative specific activity (percentage of total enzyme activity/percentage of total protein content) of various fractions. N, nuclear fraction; E, nuclei-free supernatant; M, mitochondrial fraction; P, microsomal fraction and S, 1 h, $105000 \times g_{av}$ supernatant.

testis tissue in relation to known marker enzymes for subcellular particles and cytosol. The conversion of pregnenolone to progesterone occurred in all five fractions obtained by differential centrifugation. However, 3β-hydroxysteroid dehydrogenase activity assayed at both pH 7.35 and 9.0 showed a distribution comparable with that of microsomal enzymes carboxyl esterase, steroid sulfatase and rotenone-insensitive NADPH–cytochrome *c* reductase. Glucose-6-phosphatase activity measured was extremely low in homogenates of rat testis tissue and could not be used as a marker for microsomes of rat testis, in contrast with a gluconeogenic organ like liver [23]. Because appreciable amounts of steroid sulfatase and NADPH-cytochrome *c* reductase are present in the seminiferous tubules (Table I), the subcellular distribution patterns of these two latter enzymes cannot be used as reliable markers to establish the subcellular localization of 3β-hydroxysteroid dehydrogenase in interstitial tissue. Carboxyl esterase appears to be the most reliable marker.

The ratio of enzyme activities of 3β-hydroxysteroid dehydrogenase in microsomal and mitochondrial fractions was 2.7 ± 0.6 (mean value ± S.D. from three fractionation studies), of carboxyl esterase 3.9 ± 0.6 and of cytochrome *c* oxidase 0.11 ± 0.06. When corrected for mutual contamination of the mitochondrial and microsomal fractions, it can be concluded that the contribution of mitochondria to total 3β-hydroxysteroid dehydrogenase activity in rat testis tissue is 7–15%.

The recovery of the activity of 3β-hydroxysteroid dehydrogenase assayed at pH 7.35 and 9.0 is in the order of 100% (Table II) whereas addition of an aliquot of the particle-free supernatant to the particulate fractions did not influence the specific activity of 3β-hydroxysteroid dehydrogenase (data not shown).

This observation strongly suggests that the enzyme activity measured in a separate fraction is not dependent on the presence of any of the other fractions and does not support the conclusion of Shikita et al. [12] that a stimulating factor for the particle-bound 3β-hydroxysteroid dehydrogenase is present in the particle-free supernatant.

Effect of cyanoketone and nagarse on 3β-hydroxysteroid dehydrogenase activity in homogenate of rat testis tissue

Concentrations of 0.1 μM cyanoketone, a potent inhibitor of 3β-hydroxysteroid dehydrogenase in other steroidogenic tissues [28, 29] almost completely inhibited the conversion of pregnenolone to progesterone in incubation studies with 0.5–1.0 mg protein of homogenates of total testis tissue and 0.05–0.1 mg protein of interstitial tissue. On the other hand, the addition of the proteinase nagarse to the testis tissue homogenate (up to 0.20 mg/mg total tissue protein) inactivated only 80–85 % of 3β-hydroxysteroid dehydrogenase activity (data not shown).

In Table III the effect of nagarse pretreatment on the activity of 3β-hydroxysteroid dehydrogenase and some other enzymes present in the mitochondrial and microsomal fraction of isolated interstitial tissue is summarized. Preincubation of homogenates of isolated interstitial tissue with 0.22 mg nagarse/mg protein at 4 °C for 30 min according to De Jong and Hülsmann [30] caused an inactivation of 3β-hydroxysteroid dehydrogenase measured in the mitochondrial fraction of approx. 88 % and in the microsomal fraction of 98 %. At least 6 % of total activity of both mitochondrial and microsomal fraction was unaffected by the nagarse pretreatment.

The activity of cytochrome *c* oxidase, monoamine oxidase, carboxyl esterase and adenylate kinase was essentially not influenced by the pretreatment with nagarse.

TABLE III

INFLUENCE OF NAGARSE TREATMENT OF RAT INTERSTITIAL TISSUE HOMOGENATE ON THE ACTIVITY OF 3β-HYDROXYSTEROID DEHYDROGENASE

Four rat testes (5.71 g wet wt) were dissected into interstitial tissue and seminiferous tubules under 0.25 M sucrose – 1 mM EDTA at 0 °C. The homogenate of interstitial tissue contained 4.6 mg protein/ml. Nagarse (2.5 mg) was added to an aliquot (2.4 ml) of this homogenate and subsequently stirred for 30 min at 4 °C before differential centrifugation to obtain mitochondrial and microsomal fractions as described under Materials and Methods.

Fraction	Enzyme	Enzyme Activity (nmoles/min per fraction)	
		Control	Nagarse
Mitochondrial	3β-Hydroxysteroid dehydrogenase	3.25	0.38
	Cytochrome *c* oxidase	806	890
	Monoamine oxidase	14.6	14.1
	Adenylate kinase	286	243
	Carboxyl esterase	4108	5230
	Protein (mg/fraction)	2.6	2.7
Microsomal	3β-Hydroxysteroid dehydrogenase	4.85	0.10
	Cytochrome *c* oxidase	238	323
	Monoamine oxidase	4.76	4.93
	Carboxyl esterase	8290	9760
	Protein (mg/fraction)	1.7	1.7

Effect of cyanoketone and nagarse on mitochondrial steroid production from endogenous precursors

As previously shown by our laboratory [2] relatively crude mitochondrial fractions of rat testis tissue and of isolated interstitial tissue produce pregnenolone and testosterone. In order to obtain a simple parameter for the total endogenous steroid production by mitochondrial fractions, we have studied the effect of various concentrations of cyanoketone on the amount of pregnenolone and testosterone pro-

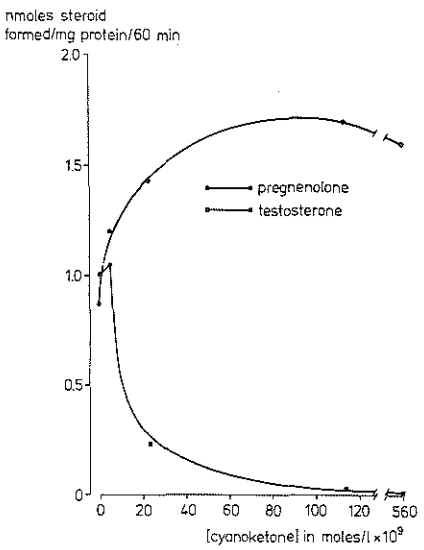

Fig. 2. Influence of cyanoketone on steroid production from endogenous substrate by the mitochondrial fraction from isolated interstitial tissue. Four testes (6.10 g wet wt) were dissected into interstitial tissue and seminiferous tubules in 0.25 M sucrose – 1 mM EDTA at 0 °C. Mitochondrial fraction was isolated from the interstitial tissue as described under Materials and Methods. Yield of mitochondrial protein: 6.3 mg. Mitochondrial protein (0.46 mg/ml) was incubated for 60 min at 33 °C with various amounts of cyanoketone. Results are mean value of duplicate incubations. See Materials and Methods for incubation conditions and assay of pregnenolone and testosterone.

TABLE IV

EFFECT OF NAGARSE TREATMENT OF HOMOGENATE OF INTERSTITIAL TISSUE ON STEROID PRODUCTION FROM ENDOGENOUS SUBSTRATE BY THE ISOLATED MITOCHONDRIAL FRACTION

See Table III for details of nagarse treatment. 0.26 mg mitochondrial protein isolated from nagarse treated interstitial tissue homogenates and 0.26 mg of a control sample were incubated for 60 min at 33 °C. The results of duplicate incubations are given.

Mitochondrial fraction from	Steroid production (pmoles/mg protein per 60 min)	
	Pregnenolone	Testosterone
Control homogenate	960	1025
	940	945
Nagarse-treated homogenate	2250	18
	3340	20

duced by mitochondrial fractions from isolated interstitial tissue during 60 min at 33 °C. At concentrations of cyanoketone higher than 0.1 μM only pregnenolone was produced and testosterone levels during incubation did not change (Fig. 2). Simpson et al. [31] have previously shown that addition of cyanoketone to mitochondrial fractions from rat adrenal tissue will cause accumulation of pregnenolone.

After preincubation of homogenates of isolated interstitial tissue with 0.22 mg nagarse/mg protein at 4 °C for 30 min, mitochondrial fractions were obtained that produced large amounts of pregnenolone at the expense of testosterone (Table IV). In control experiments (homogenates of interstitial tissue incubated at 4 °C for 30 min without nagarse) pregnenolone and testosterone were produced in almost equal amounts.

DISCUSSION

The specific activity of 3β-hydroxysteroid dehydrogenase in homogenates of isolated interstitial tissue is 150–500 times higher than the specific activity in homogenates of isolated seminiferous tubules. Assuming that 10% of rat testis protein is present in the interstitial compartment [4, 22], the conclusion can be derived that approx. 97% of the total enzyme activity in testis is in the interstitial compartment and only 3% in the seminiferous tubules. The present finding of 3β-hydroxysteroid dehydrogenase activity in interstitial tissue is in agreement with the observations of Bell et al. [1] and histochemical studies of Woods and Domm [32].

The occurrence of enzyme activities for conversion of radioactive pregnenolone by preparations of isolated seminiferous tubules of immature [11] and adult [8–10] rats has been communicated. The present results do not exclude the possibility that pregnenolone can be converted to progesterone by preparations of isolated seminiferous tubules. The quantitative contribution of the seminiferous tubules to this enzyme activity would be small, however, and the very low specific activity measured in homogenates of seminiferous tubules may well reflect contamination of material from the interstitial tissue.

When corrected for mutual contamination of the mitochondrial and microsomal fraction on basis of carboxyl esterase and cytochrome *c* oxidase (Tables II and III), it can be concluded that mitochondria are able to convert pregnenolone to progesterone. Their contribution to the total 3β-hydroxysteroid dehydrogenase activity is 7–15%.

In homogenates of testis tissue cyanoketone completely inhibits 3β-hydroxysteroid dehydrogenase, whereas the proteinase nagarse reduces the activity of this steroid dehydrogenase to approx. 15% of the control value. This difference can be explained by the chemical nature of the inhibitor. Nagarse, because of its protein structure, cannot easily pass membranes [33–35] in contrast to cyanoketone, a lipophilic steroid. After pretreatment of homogenates of interstitial tissue with nagarse, followed by isolation of washed subcellular fractions, 3β-hydroxysteroid dehydrogenase activity in the microsomal fraction was almost completely abolished whereas approx. 6% of the total activity in mitochondrial and microsomal fraction remained active (Table IV).

Nagarse can only destroy dehydrogenase activity which is not shielded by intact membranes. Therefore it may be expected that inactivation of mitochondrial ac-

tivity must reflect either the presence of microsomal activity on the outside of intact mitochondria or the mitochondrial membranes might be disrupted, so that nagarse can invade the mitochondria.

At present no evidence can be given for the intactness of the mitochondrial outer membrane. Therefore it can be concluded that at least 6% of testicular 3β-hydroxysteroid dehydrogenase, which is not inactivated after nagarse pretreatment, is inside the mitochondrial outer membrane. Localization of 3β-hydroxysteroid dehydrogenase in the mitochondrial innermembrane fraction has been reported by Sulimovici et al. [13]. They used submitochondrial fractions from mitochondria isolated from whole testis tissue. Assming that only 10% of testis mitotondria are deived from the interstitial tissue, their conclusions for total testis based on marker enzymes present in both interstitial tissue and seminiferous tubules compartment must be carefully interpreted.

Either pretreatment of testis tissue fractions with nagarse or cyanoketone does inactivate 3β-hydroxysteroid dehydrogenase and inhibits metabolism of endogenous pregnenolone. Under such conditions the endogenous pregnenolone production in isolated mitochondrial fractions can be used as paramter for total steroid production.

ACKNOWLEDGMENT

The authors thank Miss J. Moespot for expert technical assistance.

REFERENCES

1 Bell, J. B. G., Vinson, G. P., Hopkin, D. J. and Lacy, D. (1968) Biochim. Biophys. Acta 164, 412–420
2 Van der Vusse, G. J., Kalkman, M. L. and Van der Molen, H. J. (1973) Biochim. Biophys. Acta 297, 179–185
3 Rommerts, F. F. G., Cooke, B. A., Van der Kemp, J. W. C. M. and Van der Molen, H. J. (1973) FEBS Lett. 33, 114–118
4 Christensen, A. K. and Mason, N. R. (1965) Endocrinology 76, 646–656
5 Hall, P. F., Irby, D. C. and De Kretser, D. M. (1969) Endocrinology 84, 488–496
6 Cooke, B. A., De Jong, F. H., Van der Molen, H. J. and Rommerts, F. F. G. (1972) Nature New Biol. 237, 255–256
7 Bass, J. J., Bell, J. B. G. and Lacy, D. (1973) J. Endocrinol. 56, 321–322
8 Bell, J. B. G., Vinson, G. P. and Lacy, D. (1971) Proc. Roy. Soc. London B. 176, 433–443
9 Dufau, M. L., De Kretser, D. M. and Hudson, B. (1971) Endocrinology 88, 825–832
10 Ellis, L. C. and Van Kampen, K. R. (1971) Radiat. Res. 48, 146–163
11 Yamada, M., Yasue, S. and Matsumoto, K. (1973) Endocrinology 93, 81–87
12 Shikita, M., Kakizaki, H. and Tamaoki, B. (1964) Steroids 4, 521–531
13 Sulimovici, S., Bartoov, B. and Lunenfeld, B. (1973) Biochim. Biophys. Acta 321, 27–40
14 Glock, G. E. and McLean, D. (1953) Biochem. J. 55, 400–408
15 Bray, G. A. (1960) Anal. Biochem. 1, 279–285
16 Basch, R. S. and Finegold, M. J. (1971) Biochem. J. 125, 983–989
17 Neville, A. H. and Engel, L. L. (1968) Endocrinology 83, 864–872
18 McCaman, R. E., McCaman, M. W., Hunt, J. M. and Smith, M. S. (1965) J. Neurochem. 12, 15–23
19 Sottocasa, G. L., Kuylensterna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438
20 Schnaitman, C., Erwin, V. G. and Greenawalt, J. W. (1967) J. Cell Biol. 32, 719–735
21 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) In Methods in Enzymology (Colowick, S. P. and Kaplan, N.O., eds) Vol. 10, p. 448. Academic Press, New York and London

22 Rommerts, F. F. G., Van Doorn, L. G., Galjaard, H., Cooke, B. A. and Van der Molen, H. J. (1973) J. Histochem. Cytochem. 21, 572–579
23 De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 6, 604–617
24 Payne, A. H. (1972) Biochim. Biophys. Acta 258, 473–483
25 De Jong, F. H. and Van der Molen, H. J. (1972) J. Endocrinol. 53, 461–474
26 Brownie, A. C., Van der Molen, H. J., Nishizawa, E. E. and Eik-Nes, K. B. (1964) J. Clin. Endocrinol. Metab. 24, 1091–1102
27 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
28 Goldman, A. S. (1968) J. Clin. Endocrinol. Metab. 28, 1539–1546
29 Goldman, A. S., Yakovac, W. C. and Bongiovanni, A. M. (1965) Endocrinology 77, 1105–1118
30 De Jong, J. W. and Hülsmann, W. C. (1970) Biochim. Biophys. Acta 197, 127–135
31 Simpson, E. R., Jefcoate, C. R., Brownie, A. C. and Boyd, G. S. (1972) Eur. J. Biochem. 28, 442–450
32 Woods, J. E. and Domm, L. V. (1968) Gen. Comp. Endocrinol. 559–570
33 De Jong, J. W. (1971) Biochim. Biophys. Acta 245, 288–298
34 Pande, S. V. and Blanchaer, M. L. (1970) Biochim. Biophys. Acta 202, 43–48
35 Van Tol, A. and Hülsmann, W. C. (1970) Biochim. Biophys. Acta 223, 416–428

Reprinted from

Biochimica et Biophysica Acta, 380 (1975) 473—485

BBA 56565

ENDOGENOUS STEROID PRODUCTION IN CELLULAR AND SUBCELLULAR FRACTIONS OF RAT TESTIS AFTER PROLONGED TREATMENT WITH GONADOTROPINS

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(Received August 28th, 1974)

Summary

Steroid production and enzyme activities were examined in preparations of whole testis tissue, isolated interstitial tissue and seminiferous tubules obtained from adult rats with intact pituitaries receiving daily subcutaneous injections of 100 I.U. human chorionic gonadotropin for 5 days and from control animals.

After human chorionic gonadotropin administration testosterone concentrations were increased in total homogenates of whole testis tissue, interstitial tissue and seminiferous tubules. The testosterone production from endogenous precursors was enhanced only in total homogenates of whole testis tissue and interstitial tissue obtained from testes of human chorionic gonadotropin-treated rats. The production of testosterone in the corresponding homogenates of isolated seminiferous tubules was very low.

The specific activity of 3β-hydroxysteroid dehydrogenase was increased in total homogenates of whole testis tissue, isolated interstitial tissue and seminiferous tubules. No effect was observed on the specific activities of marker enzymes such as cytochrome *c* oxidase, monoamine oxidase, steroid sulfatase and lactate dehydrogenase, whereas the specific activities of carboxyl esterase were decreased in homogenates of whole testis tissue and interstitial tissue. Total activity of monoamine oxidase was increased in homogenates of interstitial tissue of testes from human chorionic gonadotropin treated rats.

After the same prolonged human chorionic gonadotropin treatment the concentration of pregnenolone was increased in mitochondrial fractions of whole testis tissue, interstitial tissue and seminiferous tubules, and the amount of protein isolated in the mitochondrial fraction of interstitial tissue increased by 40%.

Steroid production (estimated as pregnenolone) from endogenous precursors by mitochondrial fractions of whole testis tissue and interstitial tissue were

increased after human chorionic gonadotropin treatment, for whole testis from 580 pmol/mg mitochondrial protein per h to 1420 pmol/mg per h; and for interstitial tissue from 2665 pmol/mg per h to 7050 pmol/mg per h. The production of pregnenolone in mitochondrial fractions obtained from isolated seminiferous tubules was very low and contributed hardly at all to the total pregnenolone production in mitochondrial fractions of whole testis tissue from normal rats as well as from human chorionic gonadotropin-treated rats.

Introduction

Luteinizing hormone plays an important role in the maintenance and stimulation of steroid biosynthesis in rat testis. Hypophysectomy of adult rats causes a decrease of testicular weight, a decreased concentration of testosterone in testis tissue and plasma [1,2], and a marked decrease of cytochrome P_{450} [3] and of enzyme activities related to steroid biosynthesis [3—5]. Administration of human chorionic gonadotropin to hypophysectomized rats prevents these effects of removal of the pituitary on testicular steroid biosynthesis [3,6,7].

It is also clearly established that prolonged human chorionic gonadotropin treatment of immature rats results in increased testicular weight and increased activity of enzymes involved in the biosynthesis of androgens [5,8]. For adult rats with intact pituitaries it has been reported that prolonged administration of human chorionic gonadotropin results in an increased testicular testosterone production in vivo [1], in an increased mitochondrial and microsomal cytochrome P_{450} concentration [3,9] and in higher activities of 17α-hydroxylase (EC 1.14.1.7), steroid 17α,20-lyase and 5α-steroid reductase (EC 1.3.1.99) [3,9].

Mitochondrial conversion of cholesterol to pregnenolone appears to be a rate-limiting step in steroid biosynthesis in the ovary, adrenal and testis [10] and trophic stimulation of steroid production by adrenocorticotropin hormone or gonadotropins is thought to result in a stimulation of the mitochondrial activity for pregnenolone formation. Little information is available, however, on the effect of prolonged treatment with human chorionic gonadotropin of intact adult rats on the mitochondrial pregnenolone production.

We have previously reported that production of steroids from endogenous precursors is a good parameter for steroid biosynthesis in cell-free preparations of rat testis [11,12]. Therefore, in the present experiments we have studied the effect of long-term human chorionic gonadotropin treatment on testicular steroid biosynthesis from endogenous precursors in tissue homogenates and in isolated mitochondrial fractions of whole testis tissue, interstitial tissue and seminiferous tubules. In addition, the effects of human chorionic gonadotropin treatment on testis 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) and on the activities of cytochrome *c* oxidase (EC 1.9.3.1), monoamine oxidase (EC 1.4.3.4), steroid sulfatase (EC 3.1.6.2), carboxyl esterase (EC 3.1.1.1) and lactate dehydrogenase (EC 1.1.1.27) and on the protein concentrations in different tissue compartments have been investigated.

Materials and Methods

Human chorionic gonadotropin was obtained from Organon, Oss, Holland (Pregnyl, 500 I.U./flask).

Pregnenolone, testosterone and progesterone were purchased from Steraloids, Pawling, U.S.A. and were used without further purification.

Cyanoketone (2α-cyano-4,4′,17α-trimethyl-17β-hydroxy-5-androsten-3-one), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, is a product of Stirling-Winthrop, New York, U.S.A. [16-^{3}H] pregnenolone was obtained from CEN, Mol, Belgium. Other radioactive steroids and [1-^{14}C] tyramine were purchased from The Radiochemical Centre, Amersham, England. Radioactive steroids were purified until less than 1% radioactive impurities could be detected with paper chromatography.

p-Nitrophenyl acetate is a product of Koch-Light Lab., Colnbrook, England. Permablend II was obtained from Packard S.A., Zurich, Switzerland. Co-factors were obtained from Boehringer, Mannheim, Germany.

Adult male Wistar rats substrain R-Amsterdam (14—16 weeks old, 200—250 g) were used in this study. The animals were kept under controlled light (14 h light and 10 h darkness) and temperature (20—22° C) conditions. Laboratory chow and tap water were provided ad libitum. Daily subcutaneous injections of 100 I.U. human chorionic gonadotropin in 0.9% NaCl were given for a period of 5 days. Control animals received 0.2 ml vehicle only. The animals were killed by decapitation. Testes were removed and immediately chilled in ice-cold 0.25 M sucrose, containing 1 mM EDTA. After removing the tunica albuginea the weight of the testes was determined. Testis tissue was dissected into interstitial tissue and seminiferous tubules, and homogenates of testis tissues and mitochondrial fractions of those tissues were prepared as described previously [12].

Assay of enzyme activities

Lactate dehydrogenase was assayed according to Kornberg [13] at 25° C.

Carboxyl esterase was assayed with *p*-nitrophenyl acetate as substrate, by following the increase of extinction at 400 nm at 20° C in 0.1 M Tris · HCl buffer, pH 8.0.

The activities of 3β-hydroxysteroid dehydrogenase, monoamine oxidase, cytochrome *c* oxidase and steroid sulfatase were assayed as described previously [12].

Estimation of steroid production

The production of testosterone from endogenous substrates in tissue homogenates was estimated by incubating aliquots of homogenates of whole testis tissue (6—8 mg protein), of interstitial tissue (0.8—1.8 mg protein) or of seminiferous tubules (2.5—6 mg protein) at 33° C in an O_2/CO_2 (95 : 5, v/v) atmosphere in 2 ml medium, at pH 7.35, containing sucrose (0.125 M), EDTA (0.5 mM), sodium succinate (20 mM), NAD^+ (1.0 mM), NADPH (1.0 mM), glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (0.3 unit) $CaCl_2$ (0.7 mM), $MgCl_2$ (5.0 mM), Tris · HCl (25 mM). The reaction was stopped by adding 3 ml ethyl acetate containing 120 000 dpm [1,2-^{3}H] testo-

sterone as internal standard. The reaction mixture was extracted three times with 3 ml ethyl acetate and testosterone was purified with paper chromatography with a Bush A II system (light petroleum (b.p. 60—90°C)/methanol/water (10 : 7 : 3, by vol.) [14]). Recovery of the added radioactive testosterone after extraction and purification was in the order of 80%. Corrections were applied for the losses to calculate the steroid contents of the original samples.

The production of pregnenolone by mitochondrial fractions was measured as described previously [12] by incubating aliquots of mitochondrial fractions from whole testis tissue (1.0—1.5 mg mitochondrial protein), from interstitial tissue (0.3—0.5 mg protein) or from seminiferous tubules (1.0—2.0 mg protein). A solution (20 μl) of cyanoketone in ethanol was added to the incubation mixture to inhibit further conversion of pregnenolone. Final cyanoketone concentrations were 0.1 μM in incubation studies with mitochondrial fractions from testes of control animals and 0.25 μM in incubation studies with mitochondrial fractions from testes of human chorionic gonadotropin-treated rats. The reaction was stopped by adding 3 ml ethyl acetate containing 100 000 dpm [16-^{3}H] pregnenolone as internal standard. The reaction mixture was extracted three times with 3 ml ethyl acetate. Pregnenolone was purified by paper chromatography with a Bush A II system. Recovery of the added radioactive pregnenolone after extraction and purification was in the order of 60%. Corrections were applied for the losses to calculate the pregnenolone contents of the original samples.

Those steroids that could theoretically interfere with the binding of testosterone or pregnenolone to the antisera (see below), were separated during paper chromatography using a Bush A II system. R_f values measured for various steroids are: 17α-hydroxypregnenolone, 0.0; 5-androstenediol, 0.0; 17α-hydroxyprogesterone, 0.11; testosterone, 0.14; dehydroepiandrosterone, 0.26; 4-andro-androstenedione, 0.40; 5α-dihydrotestosterone, 0.40; pregnenolone, 0.50; progesterone, 0.61; oestrone, 0.07; oestradiol, 0.0 and oestriol, 0.0.

Testosterone cannot be separated from 17α-hydroxyprogesterone in this system, but the last mentioned steroid does not interfere with the binding of testosterone [15]. It is unlikely that significant amounts of any of those steroids are present in the incubation mixture with mitochondrial fractions. The addition of cyanoketone prevents further conversion of pregnenolone by blocking 3β-hydroxysteroid dehydrogenase activity [12]. Endogenous steroid production was calculated from the difference in the amount of pregnenolone or testosterone present in the incubation mixtures after 1 h and at zero time.

Estimation of testosterone and pregnenolone

Testosterone was estimated by a radioimmunoassay technique described by Verjans et al. [15], which was essentially a modification of the method of Furuyama et al. [16].

Pregnenolone was determined by a radioimmunoassay technique using an antiserum obtained from New Zealand white rabbits immunized against pregnenolone-3-hemisuccinate/bovine serum albumin. Three months after the initial immunization an antiserum was obtained, which was suitable for use in the radioimmunoassay. Pretreatment of this antiserum before use was exactly as

described for the antiserum against testosterone [15]. Estimations of pregnenolone with radioimmunoassay were performed in essentially the same way as the determination of testosterone in tissue samples [15].

The specificity of the antiserum was tested by comparing the ability of various steroids to displace [^{3}H]pregnenolone from the antibody and was expressed as percentage cross reaction, i.e. the amount of a certain steroid that displaces 50% of [^{3}H]pregnenolone relative to the amount of pregnenolone that displaces 50% of the [^{3}H]pregnenolone. The following data were obtained: pregnenolone, 100%; cortisol, 29%; oestradiol-17β, 21%; progesterone 12%; dehydroepiandrosterone, testosterone, cholesterol, 5α-dihydrotestosterone, 17-hydroxyprogesterone, 11β-hydroxyprogesterone, corticosterone and cyanoketone, <1%. For actual samples, specificity was also evaluated by comparison of the pregnenolone concentrations measured by the radioimmunoassay technique with results obtained by a method employing gas-liquid chromatography for estimation of pregnenolone. The latter method was essentially a modification of the method of De Jong and Van der Molen [17] for estimation of dehydroepiandrosterone. Radioimmunological and gas-liquid chromatographic results showed a good correlation ($r = 0.961$, $n = 29$).

Sensitivity and precision of the present radioimmunological assay of pregnenolone were comparable with those of the assays described by DiPietro et al. [18] and Abraham and coworkers [19]. The smallest amounts of pregnenolone that could be detected in biological samples are in the order of 5—10 pmol. The precision of assays in this range was in the order of 20% (intra-assay variance).

Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as a standard.

Counting of radioactivity was done in a scintillation fluid prepared by dissolving 80 g naphthalene and 5 g Permablend II in a mixture of 500 ml toluene and 500 ml methoxy ethanol. Radioactivity was estimated using an automated liquid scintillation system (Nuclear Chicago model Isocap/300 or a Packard 3375). Samples were counted to an accuracy of 2% or better. The significance of differences between results for different groups was calculated using a two-tailed Student's *t*-test [21].

Results

The effect of prolonged human chorionic gonadotropin treatment on testis weight, protein and steroid concentration

Values for testicular weight, the amounts of total and mitochondrial protein, testosterone concentrations in homogenates of testis tissues and pregnenolone concentrations in mitochondrial fractions for normal rats and for rats after prolonged subcutaneous administration of human chorionic gonadotropin are given in Table I. No significant effect of human chorionic gonadotropin treatment was observed in testicular weight and in amounts of protein of whole testis, isolated interstitial tissue and seminiferous tubules. Whereas the amounts of protein measured in mitochondrial fractions obtained from whole testis tissue and seminiferous tubules showed no difference after human chorionic gonadotropin treatment, the amount of protein, isolated in the mitochondrial fraction of interstitial tissue showed a 40% ($P < 0.05$) increase.

TABLE I

Testicular weight, protein concentrations in testis tissues and in mitochondrial fractions and concentrations of testosterone in tissue homogenates and of pregnenolone in mitochondrial fractions of testis tissue from normal rats (control) and from rats treated with human chorionic gonadotropin (HCG) (*MEANS ± S.D.).

	Whole testis			Interstitial tissue**			Seminiferous tubules**		
	n	control	HCG	*n*	control	HCG	*n*	control	HCG
Wet weight (g/2 testes)	9	2.65 ± 0.13	2.58 ± 0.13						
Protein (mg/2 testes)	9	214 ± 16	218 ± 18	6	12.1 ± 3.7	14.9 ± 3.8	6	84.4 ± 17.4	76.4 ± 14.4
Testosterone (pmol/mg protein)	4	6.2 ± 2.8	38.4 ± 14.5	5	18.8 ± 11.3	68.8 ± 17.3	5	5.1 ± 2.9	16.4 ± 2.2
Mitochondrial protein (mg/2 testes)	6	28.8 ± 3.9	27.8 ± 5.3	6	2.1 ± 0.4	3.0 ± 0.6	6	9.4 ± 2.3	8.5 ± 1.1
Pregnenolone (pmol/mg mitochondrial protein)	6	29.3 ± 1.7	45.7 ± 11.0	6	37.0 ± 15.1	115 ± 77	6	1.3 ± 0.8	5.1 ± 2.5

* 100 I.U. human chorionic gonadotropin were administered subcutaneously each day for 5 days.

** Values are not corrected for losses of protein during dissection.

The concentrations of testosterone in homogenates of whole testis, interstitial tissue and seminiferous tubules were significantly increased with factors of 6.1, 3.7 and 3.2, respectively ($P < 0.05$, $P < 0.025$ and $P < 0.025$) after human chorionic gonadotropin treatment.

Pregnenolone concentrations in isolated mitochondrial fractions from whole testis tissue, interstitial tissue and seminiferous tubules were likewise increased with factors of 1.6, 3.1 and 3.9, respectively ($P < 0.05$, $P < 0.10$ and $P < 0.05$).

The influence of prolonged human chorionic gonadotropin treatment on testosterone production and enzyme activities in homogenates of rat testis tissues

Data in Table II show that continuous administration of human chorionic gonadotropin to intact adult rats resulted in a 2.4-fold increase in testosterone production from endogenous precursors in homogenates of whole testis tissue ($P < 0.05$) (production is expressed as pmol/mg protein per h or as nmol/2 testes per h).

Testosterone production in homogenates of interstitial tissue, expressed either per mg interstitial tissue or per amount of interstitial tissue obtained from two testes was significantly increased with factors of 2.0 and 2.4, respectively ($P < 0.05$ and $P < 0.05$), whereas no significant increase was observed in incubations with material from seminiferous tubules. No corrections were made for losses of material during the dissection procedure and therefore the sum of the testosterone production in homogenates of interstitial tissue and seminiferous tubules is lower than the production in preparations of whole, undissected tissue. In order to obtain an impression about the specificity of the trophic effect of human chorionic gonadotropin on testicular enzymes the activities of one of the enzymes involved in the formation of androgens from pregnenolone i.e. 3β-hydroxysteroid dehydrogenase and of some mitochondrial, microsomal and soluble enzymes not directly related to steroid biosynthesis in rat testis have been studied (Table II).

As a result of the human chorionic gonadotropin treatment a significant 2.1—2.5-fold increase of the specific activity of 3β-hydroxysteroid dehydrogenase in homogenates of whole testis tissue, interstitial tissue and seminiferous tubules was found ($P < 0.001$, $P < 0.05$ and $P < 0.05$, respectively). When expressed as total activities (in munits/2 testes) the increase of 3β-hydroxysteroid dehydrogenase in whole testis tissue and interstitial tissue was 2.3- and 2.6-fold, respectively ($P < 0.001$ and $P < 0.025$), whereas the total activity of this enzyme in isolated seminiferous tubules was very low, which is in agreement with previous observations [12]. In contrast, human chorionic gonadotropin administration had no effect on the specific activity of mitochondrial enzymes, such as cytochrome *c* oxidase and monoamine oxidase, on the microsomal steroid sulfatase and on the soluble lactate dehydrogenase measured in homogenates of whole testis tissue and of dissected interstitial tissue and seminiferous tubules. Only the activity of monoamine oxidase (when expressed as munits per interstitial tissue obtained from two testes) showed a tendency to increase ($P < 0.05$). However, in homogenates of whole testis tissue and of interstitial tissue the specific activity of carboxyl esterase decreased significantly after prolonged human chorionic gonadotropin treatment with a factor 0.6 and 0.7, respectively ($P < 0.05$ and $P < 0.05$).

TABLE II

TESTOSTERONE PRODUCTION IN VITRO AND ENZYME ACTIVITIES IN HOMOGENATES OF RAT (HCG)* (MEANS ± S.D.)

	Whole testis		
	n	control	HCG
Testosterone production pmol/mg per h	4	112 ± 47	267 ± 44
nmol/2 testes per h**		23.7 ± 9.6	60.2 ± 15.7
Cytochrome c oxidase munits/mg	4	155 ± 55	123 ± 41
units/2 testes**		31.6 ± 10.8	26.3 ± 8.1
Monoamine oxidase munits/mg	4	0.84 ± 0.15	1.05 ± 0.20
munits/2 testes**		174 ± 29	218 ± 36
Carboxyl esterase units /mg	7	0.28 ± 0.09	0.16 ± 0.075
units/2 testes**		59.1 ± 21.4	35.7 ± 17.8
Steroid sulfatase μunits/mg	4	19.4 ± 3.9	21.6 ± 4.4
munits/2 testes**		3.83 ± 0.77	4.38 ± 0.87
3β-Hydroxysteroid dehydrogenase μunits/mg	9	140 ± 25	322 ± 60
munits/2 testes**		30.2 ± 5.5	70.5 ± 13.1
Lactate dehydrogenase units/mg	5	0.55 ± 0.11	0.57 ± 0.11
units/2 testes**		113 ± 19	118 ± 20

* 100 I.U. human chorionic gonadotropin were administered subcutaneously each day for 5 days.
** Values are not corrected for losses of tissue during dissection.

Pregnenolone production and activities of cytochrome c oxidase and monoamine oxidase in isolated mitochondrial fractions

The results in Table III show that the amount of pregnenolone formed from endogenous precursors in mitochondrial fractions, obtained from whole testis tissue was increased 2.4-fold ($P < 0.025$) after prolonged treatment with human chorionic gonadotropin. The production of pregnenolone in the presence of cyanoketone in mitochondrial fractions obtained from testes of human chorionic gonadotropin-treated and control animals is linear with time of incubation (Fig. 1). Therefore, the production of pregnenolone measured in

TABLE III

PREGNENOLONE PRODUCTION AND ENZYME ACTIVITIES IN ISOLATED MITOCHONDRIAL GONADOTROPIN (HCG)* (MEANS ± S.D.)

	Whole testis		
	n	control	HCG
Pregnenolone production pmol/mg per h	5	580. ± 165	1420 ± 390
nmol/2 testes per h**		16.0 ± 4.5	38.3 ± 6.9
Cytochrome c oxidase munits/mg	4	680 ± 231	612 ± 170
units/2 testes**		19.1 ± 3.6	18.1 ± 1.0
Monoamine oxidase munits/mg		n.d.	n.d.
munits/2 testes**		n.d.	n.d.

* 100 I.U. human chorionic gonadotropin were administered subcuteaneously each day for 5 days.
** Values not corrected for losses of protein during dissection and fractionation.
n.d., Not determined.

TABLE II (Continued)

TESTIS TISSUES AFTER PROLONGED TREATMENT WITH HUMAN CHORIONIC GONADOTROPIN

Interstitial tissue			Seminiferous tubules		
n	control	HCG	*n*	control	HCG
5	1041 ± 228	2081 ± 628	5	2.1 ± 1.7	6.8 ± 4.5
	13.6 ± 3.1	33.1 ± 3.8		0.20 ± 0.18	0.57 ± 0.49
5	206 ± 71	235 ± 36	5	182 ± 97	159 ± 44
	2.51 ± 1.00	3.61 ± 1.42		15.4 ± 7.9	12.7 3.4
4	3.02 ± 1.15	4.15 ± 0.28	4	0.82 ± 0.15	0.84 ± 0.10
	35.8 ± 12.3	61.2 ± 11.5		71.7 ± 15.2	73.5 ± 5.4
4	1.25 ± 0.05	0.82 ± 0.16	4	0.061 ± 0.009	0.056 ± 0.005
	16.6 ± 1.0	13.5 ± 2.8		5.77 ± 1.81	4.75 ± 1.10
4	56.0 ± 11.2	60.8 ± 7.5	4	16.2 ± 3.4	17.4 ± 4.3
	0.69 ± 0.20	1.01 ± 0.18		1.48 ± 0.47	1.42 ± 0.38
4	1030 ± 140	2200 ± 420	4	8 ± 2	20 ± 6
	12.8 ± 1.8	33.9 ± 5.1		0.72 ± 0.19	1.78 ± 0.77
5	0.17 ± 0.08	0.18 ± 0.04	5	0.56 ± 0.10	0.55 ± 0.08
	2.13 ± 1.09	3.02 ± 0.84		50.2 ± 17.7	44.3 ± 13.4

vitro after 60 min of incubation may be expected to give a reliable reflection of the effect of human chorionic gonadotropin treatment on pregnenolone synthesis. Mitochondrial fractions isolated from interstitial tissue from testes of human chorionic gonadotropin-treated rats showed an increased production of pregnenolone with a factor 2.6 ($P < 0.025$). Production of pregnenolone by mitochondrial fractions from seminiferous tubules was slightly increased after prolonged human chorionic gonadotropin treatment in vivo ($0.10 > P > 0.05$) but the production was very low when compared with the in vitro production of pregnenolone by mitochondrial fractions from the interstitial compartment

FRACTIONS OF RAT TESTIS TISSUES AFTER PROLONGED TREATMENT WITH HUMAN CHORIONIC

Interstitial tissue			Seminiferous tubules		
n	control	HCG	*n*	control	HCG
5	2665 ± 1000	7050 ± 2850	6	17.5 ± 15.0	37.5 ± 21.2
	6.1 ± 3.5	20.7 ± 6.9		0.16 ± 0.13	0.32 ± 0.12
6	391 ± 117	342 ± 130	6	551 ± 133	520 ± 251
	0.83 ± 0.25	0.99 ± 0.34		5.42 ± 2.56	4.48 ± 2.39
6	4.72 ± 1.29	6.19 ± 0.92	6	2.86 ± 0.54	3.08 ± 0.54
	11.5 ± 3.6	18.5 ± 4.1		26.2 6.9	25.8 ± 5.5

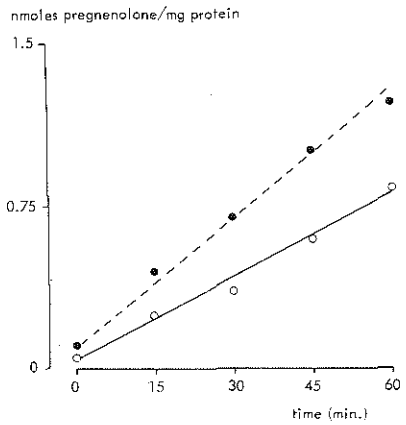

Fig. 1. Time course of the production of pregnenolone from endogenous precursors by mitochondrial fractions of whole testis tissue. ●———●, incubations with mitochondrial protein from testes of intact adult rats treated with 100 I.U. human chorionic gonadotropin subcutaneously each day for 5 days. ○———○, incubations with mitochondrial protein from testes of control rats. Results of duplicate incubations are given. See Materials and Methods for incubation conditions and assay of pregnenolone.

of the same testes. No significant effect of human chorionic gonadotropin was observed on the specific activities of cytochrome *c* oxidase and monoamine oxidase assayed in the mitochondrial fractions. In contrast, the total amount of monoamine oxidase in the mitochondrial fractions obtained from interstitial tissue was increased by 60% after human chorionic gonadotropin treatment ($P < 0.05$).

Discussion

The present results show that prolonged treatment with human chorionic gonadotropin of adult rats with intact pituitaries results in an increased testosterone production from endogenous substrates in homogenates of whole testis tissue. These in vitro results are compatible with in vivo data published by De Jong et al. [22], who observed after prolonged human chorionic gonadotropin treatment of intact adult rats an increase of the testicular production rate of testosterone from 10.7 ± 7.3 nmol/2 testes per h to 137 ± 57 nmol/2 testes per h. Although the production of testosterone in vitro is of the same order of magnitude (23.7 ± 9.6 nmol/2 testes per h for control rats and 60.2 ± 15.7 nmol/2 testes per h for human chorionic gonadotropin-treated animals) as the in vivo production, the lower percentage increase after human chorionic gonadotropin treatment in the in vitro studies may be caused by the difference in strain of animals used or a partial loss of stimulating factors(s) during homogenization of the testicular preparations.

Assuming that 10% of rat testis protein is present in the interstitial compartment [23,24], it can be concluded that for these in vitro experiments the contribution of interstitial testosterone production to the production in whole testis tissue is approx. 98%. With respect to the apparently very small testosterone production in homogenates and pregnenolone production in isolated mitochondrial fractions obtained from seminiferous tubules it cannot be ex-

cluded that cell type(s) in the spermatogenic compartment of the testis produce this small amount of steroids, but on the other hand this apparent tubular production may well reflect contamination with material from the interstitial tissue.

The in vitro results with isolated interstitial tissue preparations indicate that the increased testicular steroid production is caused by stimulation of the steroid production in the interstitial compartment. In that respect the present results are compatible with results concerning in vitro stimulation by luteinizing hormone of testosterone biosynthesis in isolated interstitial tissue and seminiferous tubules, obtained from hypophysectomized rats [25]. Hall and Eik-Nes [6] have reported that long-term treatment of intact rabbits with human chorionic gonadotropin had no effect on the conversion of radioactive cholesterol into testosterone by testis homogenates. However, such observations may be caused by poor mixing of the added radioactively labelled compound with the endogenous cholesterol pools. This difficulty does not apply if steroid production from endogenous precursors is measured as in the present study. Prolonged treatment with human chorionic gonadotropin does increase the total activity of 3β-hydroxysteroid dehydrogenase in rat testis tissue. This increase can be explained by the increase of enzyme activity present in the interstitial compartment. This increased specific activity of 3β-hydroxysteroid dehydrogenase and the lack of increase of the specific activities of other particle-bound enzymes, not directly related to steroid biosynthesis, indicate a specific effect of human chorionic gonadotropin. The present results do not support the suggestion of Shikita and Hall [26] that the activity of 3β-hydroxysteroid dehydrogenase in testes of rats older than 22 days is already maximally stimulated by the endogenous circulating gonadotropins and cannot be further increased by added human chorionic gonadotropin. Moreover, Purvis et al. [3] have recently reported an increase in activity of the microsomal cytochrome P_{450}-dependent enzymes 17α-hydroxylase and steroid $C_{17,20}$-lyase after long-term treatment with human chorionic gonadotropin.

Human chorionic gonadotropin treatment resulted in a 2—3 times higher pregnenolone production from endogenous precursors in testicular mitochondrial fractions in the presence of cyanoketone, in fractions obtained from whole testis tissue as well as from isolated interstitial tissue. This observation strongly suggests that the enhanced androgen biosynthesis may be caused by stimulation of the mitochondrial pregnenolone production.

The increased pregnenolone production in isolated mitochondrial fractions might reflect (1) an increased amount of Leydig cell mitochondria, assuming that Leydig cells are the steroid producing cells in the interstitial tissue; (2) an increased concentration of components of the cholesterol side-chain cleaving complex per mitochondrion; (3) an accumulation of precursors for pregnenolone synthesis (e.g. cholesterol) in the isolated mitochondrial fraction, caused by an activation of the supply of these precursors and (4) a combination of the above mentioned phenomena. The significant increase of the amount of protein measured in the mitochondrial fraction from the interstitial tissue and of the mitochondrial enzyme monoamine oxidase in total homogenates and in mitochondrial fractions from this tissue compartment may indicate that human chorionic gonadotropin increases the amount of interstitial tissue mitochon-

dria. An increase of some components of the cholesterol side-chain cleaving complex has also been reported [9,27].

Little is known about the effect of long-term human chorionic gonadotropin treatment on cholesterol pools in testes of intact adult rats. Ying et al. [28] have shown that the biosynthesis of cholesterol is accelerated after one week of human chorionic gonadotropin treatment, whereas Van der Molen et al. [29] have reported that for the strain of rats used in our studies no difference can be observed in cholesterol and cholesterol esters concentrations in homogenates of whole testis and isolated interstitial tissue before and after human chorionic gonadotropin treatment. In contrast with the adrenal gland, in which organ most of the cholesterol is esterified and accumulated in lipid droplets [30], in testicular interstitial tissue less than 5% of cholesterol is esterified [29] and the free cholesterol is mainly recovered in mitochondrial and microsomal fractions after tissue fractionation (Van der Vusse, unpublished results). Therefore the hypothesis of de-esterification of cholesterol esters in lipid droplets and a subsequent translocation of cholesterol from these droplets into the mitochondria appears less likely as mechanism of action of human chorionic gonadotropin on testicular steroid biosynthesis. A possible effect of gonadotropins on cholesterol uptake by Leydig cells and on an intracellular and intramitochondrial shift of cholesterol remains to be investigated.

From the present data it is evident that endogenous steroid production by isolated testis mitochondria can be employed as a useful parameter for trophic stimulation of testicular steroid production in vivo.

Acknowledgement

The authors thank J.H.J. Goedemans and Miss M.P.I. Van Winsen for their technical assistance in part of the work. The investigations were supported in part by the Foundation for Medical Research FUNGO which is subsidized by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

1 De Jong, F.H., Hey, A.H. and Van der Molen, H.J. (1974) J. Endocrinol. 60, 409—419
2 Hafiez, A.A., Lloyd, C.W. and Bartke, A. (1972) J. Endocrinol. 52, 327—332
3 Purvis, J.L., Canick, J.A., Latif, S.A., Rosenbaum, J.H., Hologgitas, J. and Menard, R.H. (1973) Arch. Biochem. Biophys. 159, 39—49
4 Samuels, L.T. and Helmreich, M.L. (1956) Endocrinology 58, 435—442
5 Menon, K.M.J., Drosdowsky, M., Dorfman, R.I. and Forchielli, E. (1965) Steroids, Suppl. I, 95—111
6 Hall, P.F. and Eik-Nes, K.B. (1963) Biochim. Biophys. Acta 71, 438—447
7 Menon, K.M.J., Dorfman, R.I. and Forchielli, E. (1967) Biochim. Biophys. Acta 148, 486—494
8 Schoen, E.J. and Samuels, L.T. (1965) Acta Endocrinol. 50, 365—378
9 Purvis, J.L., Canick, J.A., Rosenbaum, J.H., Hologgitas, J. and Latif, S.A. (1973) Arch. Biochem. Biophys. 159, 32—38
10 Hall, P.F. (1970) In The Androgens of the Testis (Eik-Nes, K.B., ed.), pp. 73—115, Marcel Dekker Inc., New York
11 Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1973) Biochim. Biophys. Acta 297, 179—185
12 Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1974) Biochim. Biophys. Acta 348, 404—414
13 Kornberg, A. (1955) In Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds), Vol. 1, pp. 441—443, Academic Press, New York

14 Neher, R. (1964) Steroid chromatography, pp. 96—97, Elseviers Publishing Co.
15 Verjans, H.L., Cooke, B.A., De Jong, F.H., De Jong, C.M.M. and Van der Molen, H.J. (1973) J. Steroid Biochem. 4, 665—676
16 Furuyama, S., Mayes, D.M. and Nugent, C.A. (1970) Steroids 16, 415—428
17 De Jong, F.H. and Van der Molen, H.J. (1972) J. Endocrinol. 53, 461—474
18 DiPietro, D.L., Brown, R.D. and Strott, C.A. (1972) J. Clin. Endocrinol. Metabol. 35, 729—735
19 Abraham, G.E., Buster, J.E., Kyle, F.W., Corrales, P.C. and Teller, R.C. (1973) J. Clin. Endrocrinol. Metabol. 37, 140—144
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, 6th edn, Ames: Iowa State University Press
22 De Jong, F.H., Hey, A.H. and Van der Molen, H.J. (1973) J. Endocrinol. 57, 277—284
23 Christensen, A.K. and Mason, N.R. (1965) Endocrinology 76, 646—656
24 Rommerts, F.F.G., Van Doorn, L.G., Galjaard, H., Cooke, B.A. and Van der Molen, H.J. (1973) J. Histochem. Cytochem. 21, 572—579
25 Cooke, B.A., Rommerts, F.F.G., Van der Kemp, J.W.C.M. and Van der Molen, H.J. (1974) MOl. Cell. Endocrinol. 1, 99—111
26 Shikita, M. and Hall, P.F. (1967) Biochim. Biophys. Acta 136, 484—497
27 Mason, J.I., Estabrook, R.W. and Purvis, J.L. (1973) Ann. N.Y. Acad. Sci. 212, 406—419
28 Ying, B.P., Chang, Y.-J. and Gaylor, J.L. (1965) Biochim. Biophys. Acta 100, 256—262
29 Van der Molen, H.J., Bijleveld, M.J., Van der Vusse, G.J. and Cooke, B.A. (1972) J. Endocrinol. 57, vi—vii
30 Garren, L.D., Gill, G.N., Masui, H. and Walton, G.H. (1971) Recent Progr. Horm. Res. 27, 433—474

Reprinted from

Biochimica et Biophysica Acta, 398 (1975) 28—38

BBA 56623

ON THE REGULATION OF RAT TESTICULAR STEROIDOGENESIS

SHORT TERM EFFECT OF LUTEINIZING HORMONE AND CYCLOHEXIMIDE IN VIVO AND Ca^{2+} IN VITRO ON STEROID PRODUCTION IN CELL-FREE SYSTEMS

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(Received January 31st, 1975)

Summary

An attempt has been made to correlate the rapid effect of luteinizing hormone on testicular steroid production in vivo with testicular steroid concentrations and in vitro steroid production rates in testis tissue preparations.

Within 20 min after intravenous administration of 25 μg luteinizing hormone, increases were observed in testosterone concentrations in testicular venous plasma and in whole testis tissue and in pregnenolone concentrations in isolated testis mitochondrial fractions. Testosterone production by whole testis homogenates and pregnenolone production by isolated mitochondrial fractions were significantly increased within 5 min after in vivo administration of luteinizing hormone.

Injection of cycloheximide 10 min prior to luteinizing hormone prevented the stimulating effect of luteinizing hormone on steroid levels in testicular venous plasma and testis tissue and on steroid production rates by preparations of rat testis tissue. Cycloheximide treatment of control animals did not significantly alter testosterone concentrations and testosterone production rates in vitro, although mitochondrial pregnenolone concentrations and production rates were decreased.

Testosterone production by whole testis homogenates as well as the pregnenolone production by isolated mitochondrial fractions obtained from luteinizing hormone treated testes and control glands showed a biphasic time curve. A period (5—10 min) of high steroid production was followed by a period of lower steroid production.

Addition of 25 μg luteinizing hormone or 10^{-8}—10^{-5} M adenosine 3′:5′-monophosphate (cyclic AMP) to the incubation medium had no effect on pregnenolone production by isolated mitochondrial fractions.

Administration of luteinizing hormone in vivo markedly enhanced the stimulating effect of Ca^{2+} on testosterone production by whole testis homogenates and on pregnenolone production by isolated mitochondrial fractions.

Introduction

Testicular steroid production can be stimulated by luteinizing hormone in vivo as well as in vitro [1—3]. The action of luteinizing hormone is thought to be mediated by intracellular cyclic AMP [4] and protein synthesis or activation of protein factor(s) seems to be involved. This has also been suggested for the adrenal gland [5], ovary [6] and the testis [7], but the precise molecular mechanism of the stimulation of the steroid biosynthetic pathway is poorly understood. Mitochondrial conversion of cholesterol to pregnenolone appears to be a rate-limiting step in steroid biosynthesis [2] and stimulation of steroid production by gonadotropins may be caused by a stimulation of the mitochondrial activity for pregnenolone synthesis.

Several authors [1,8] have shown, that rapid changes in gonadotropin levels in blood cause a rapid response in testicular steroid production in vivo. In this respect we report on the short-term effect of intravenously administered luteinizing hormone in intact adult male rats on testosterone production in vivo, as well as on testosterone concentrations in whole testis tissue and pregnenolone concentrations in isolated mitochondrial fractions. In order to study the molecular mechanism of hormone-activated steroid biosynthesis, we have tried to correlate the in vitro steroid production in cell-free preparations of rat testes with the in vivo biosynthesis. We have previously shown [9,10,11] that pregnenolone and testosterone production from endogenous precursors is a good parameter for rat testicular steroid biosynthesis. Therefore, we have studied the in vitro testosterone production by whole testis tissue homogenates and pregnenolone production by isolated mitochondrial fractions from rat testis after in vivo luteinizing hormone treatment. The possible involvement of protein synthesis was studied by investigating the effect of cycloheximide administered prior to luteinizing hormone on testosterone production in vivo and on testosterone and pregnenolone production in vitro. It has been reported that Ca^{2+} can activate the conversion of radioactive cholesterol by testis mitochondrial fractions [12] as well as pregnenolone synthesis by mitochondrial fractions isolated from adrenal glands [13,14]. However, no data are available on the possible role of Ca^{2+} in trophic hormone stimulated pregnenolone formation. In the present study the effect of luteinizing hormone treatment in vivo on Ca^{2+} stimulated mitochondrial pregnenolone production in vitro was investigated.

Materials and Methods

Luteinizing hormone (ovine NIH-LH-S18, 1.03 units/mg) was a gift from the National Institute of Health, Bethesda, Md., U.S.A. Cycloheximide was purchased from Boehringer Mannheim, Germany. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was obtained from Aldrich Chemic al Com-

pany Inc., Milwaukee, U.S.A. Steroids, cofactors and other chemicals were purchased as previously described [9]. Sodium pentobarbital is a product of Abbott S.A., Saint Remy sur Avry, France.

Adult male Wistar rats substrain R-Amsterdam (14—16 weeks old) were used in this study. Luteinizing hormone dissolved in 0.2 ml 0.9% NaCl, containing 0.2% bovine serum albumin, was administered into the Vena iliaca. Blood from the testicular vein was obtained under sodium pentobarbital anaesthesia as described by De Jong et al. [8]. Blood was collected in heparinized tubes for 20 min after intravenous injection of 25 μg luteinizing hormone or of 0.2 ml NaCl/albumin solution only. Blood samples were centrifuged after collection and the plasma was stored at —20°C until analysed.

When the short-term effect of gonadotropin administration on testicular steroid production in vitro was studied, rats were anaesthetized with sodium pentobarbital. After opening the scrotum and underlying tissue, one testis was removed and was immediately chilled in 0.25 M sucrose, containing 1 mM EDTA at 0°C (zero control testis). Subsequently, the animals were injected into the Vena iliaca either with 25 μg luteinizing hormone or with NaCl/albumin solution only. The second testis was then removed 5—30 min after injection with the luteinizing hormone (LH-treated testis) or the vehicle (control testis). The homogenation procedure and isolation of subcellular fractions from testis tissue were performed in ice-cold 0.25 M sucrose, containing 1 mM EDTA as described previously [9]. When used, 1 mg cycloheximide dissolved in 0.2 ml NaCl/albumin solution was administered intravenously 10 min prior to injection of luteinizing hormone. The activity of 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) was assayed as described previously [9].

Testosterone production from endogenous precursors was estimated by incubating 4—8 mg protein of whole testis tissue homogenates at 33°C in a O_2/CO_2 (95 : 5, v/v) atmosphere in 2-ml medium at pH 7.35 containing sucrose (0.125 M), EDTA (0.5 mM), sodium succinate (20 mM), NAD^+ (1.0 mM), NADPH (1.0 mM), glucose 6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (0.3 unit), $CaCl_2$ (0.7 mM), $MgCl_2$ (5.0 mM), Tris/HCl (25 mM). Pregnenolone production from endogenous precursors in mitochondrial fractions was estimated by incubating 0.8—1.8 mg mitochondrial protein in 2 ml incubation medium as described for testosterone production (see above). NAD^+ was omitted and 0.1 μM cyanoketone (2α-cyano-4,4', 17α-trimethyl-17β-hydroxy-5-androsten-3-one) was added to inhibit further conversion of pregnenolone [9]. The production was calculated from the difference in the amount of testosterone or pregnenolone in the incubation mixtures after incubation and at zero time.

Testosterone in plasma and in tissue samples was estimated with a radioimmunoassay technique described by Verjans et al. [15]. Pregnenolone was determined by a radioimmunoassay technique described by Van der Vusse et al. [10].

The significance of differences between results for different groups was calculated using a two-tailed Student's t-test [16]. Protein was measured by the method of Lowry et al. [17] using bovine serum albumin as a standard.

TABLE I

CONCENTRATIONS OF TESTOSTERONE IN TESTICULAR VENOUS PLASMA OF INTACT MALE RATS AFTER INTRAVENOUS ADMINISTRATION OF LUTEINIZING HORMONE AND CYCLOHEXIMIDE

The treatment consisted of 25 μg luteinizing hormone administered intravenously immediately before collection of testicular venous blood for 20 min. 1 mg cycloheximide was injected intravenously 10 min prior to the administration of 0.2 ml NaCl/albumin solution or 25 μg luteinizing hormone. The number of experiments is given in parentheses. Figures are mean values ± S.D.

Treatment		Testosterone (pmol/ml)
NaCl/albumin solution	(4)	350 ± 100
1 mg cycloheximide + NaCl/albumin solution	(3)	300 ± 90
25 μg luteinizing hormone	(4)	830 ± 190
1 mg cycloheximide + 25 μg luteinizing hormone	(2)	320

Results

Testosterone concentration in testicular venous plasma

Table I shows that the testosterone concentration in plasma obtained from testicular venous blood collected for 20 min after administration of 25 μg luteinizing hormone was enhanced 2.4 times ($P < 0.01$). Cycloheximide injected prior to luteinizing hormone abolished the increase of testosterone concentration in testicular venous plasma. Cycloheximide had no effect on the basal levels of testosterone in testicular venous plasma.

Concentration of testosterone in whole testis homogenates and pregnenolone concentration in isolated mitochondrial fractions

Fig. 1A shows that within 20 min after the administration of 25 μg

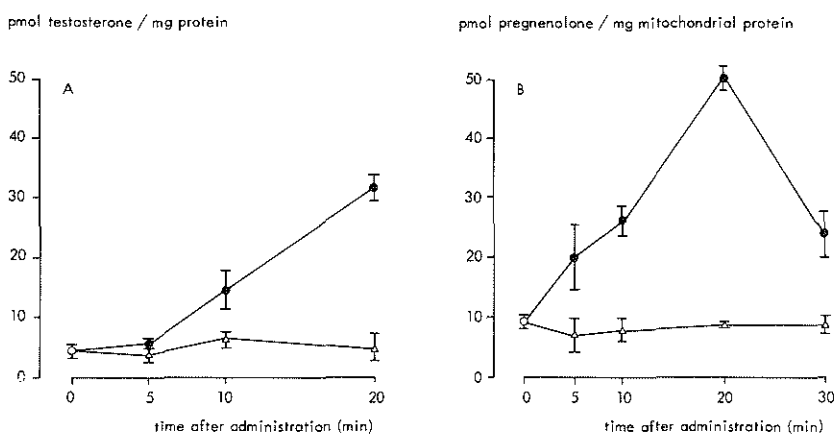

Fig. 1. (A) Concentration of testosterone in whole tissue homogenates of testes removed at various times after intravenous administration of luteinizing hormone or of NaCl/albumin solution (means of at least four experiments ±S.E.M.). (B) Concentration of pregnenolone in isolated mitochondrial fractions obtained from testes removed at various times after intravenous administration of luteinizing hormone or NaCl/albumin solution (means ±S.E.M.). △———△, after injection of 0.2 ml NaCl/albumin solution; ●———●, after injection of 25 μg luteinizing hormone.

luteinizing hormone, testosterone concentrations in whole testis tissue homogenates increased from 4 to 32 pmol per mg protein ($P < 0.001$). Pregnenolone concentrations in mitochondrial fractions obtained from zero control and control testes were about 10 pmol per mg mitochondrial protein (Fig. 1B). Within 10 and 20 min after intravenous administration of luteinizing hormone mitochondrial pregnenolone concentrations were significantly increased to 25 and 50 pmol per mg mitochondrial protein respectively ($P < 0.001$).

Production of testosterone and pregnenolone in vitro

Testosterone production by homogenates obtained from zero control testes and testes removed 5, 10 and 20 min after intravenous administration of NaCl/albumin solution were about 20 pmol testosterone per mg protein per 30 min, whereas 5, 10 and 20 min after administration of luteinizing hormone, testosterone production increased to 39, 66 and 78 pmol testosterone per mg protein per 30 min respectively ($P < 0.05$, $P < 0.025$ and $P < 0.001$ respectively) (Fig. 2A).

Fig. 2B shows the stimulatory effect of intravenous administration of luteinizing hormone on pregnenolone production by mitochondrial fractions isolated from testes removed 5, 10, 20 and 30 min after luteinizing hormone administration. Pregnenolone productions by mitochondrial fractions isolated from zero control and control testes were about 150 pmol pregnenolone per mg mitochondrial protein per 30 min. When testes were removed 20 min after luteinizing hormone injection, mitochondrial pregnenolone production was significantly enhanced to 400 pmol per mg mitochondrial protein per 30 min ($P < 0.001$). When testes were removed 30 min after luteinizing hormone injection, the increase of mitochondrial pregnenolone production was smaller.

In order to obtain an impression about the amount of exogenous luteinizing hormone that is needed in vivo to cause a stimulation of in vitro testosterone production by whole tissue homogenates 25 ng, 250 ng, 2.5 μg, 25 μg and

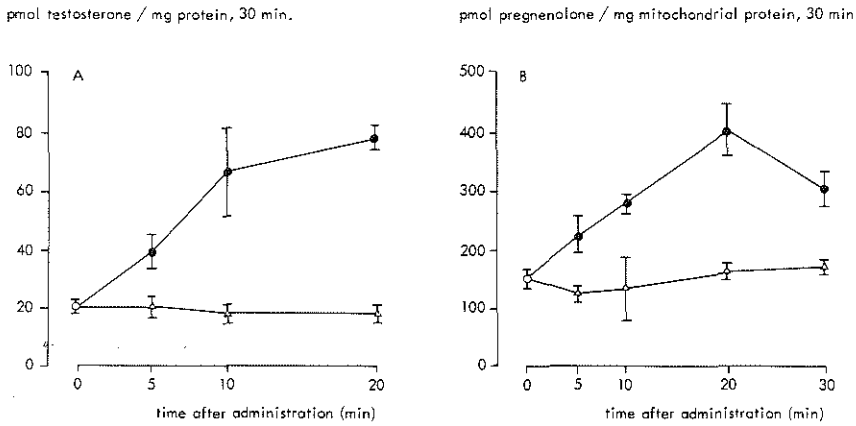

Fig. 2. (A) Production of testosterone by whole tissue homogenates of testes removed various times after intravenous administration of luteinizing hormone or of NaCl/albumin solution (means of at least four experiments ±S.E.M.). (B) Production of pregnenolone by isolated mitochondrial fractions obtained from testes removed various times after intravenous administration of luteinizing hormone or of NaCl/albumin solution (means ±S.E.M.). △———△, after injection of 0.2 ml NaCl/albumin solution; ●———●, after injection of 25 μg luteinizing hormone.

250 μg luteinizing hormone were administered and testes were removed after 20 min. It appeared that under these conditions at least more than 250 ng luteinizing hormone in vivo was required to stimulate testosterone production in vitro (data not shown).

Testosterone and pregnenolone production in vitro as a function of time of incubation

Fig. 3A reflects that testosterone formation from endogenous precursors by whole testis homogenates versus incubation time showed a biphasic pattern. An initial phase of high testosterone production lasting 10 min was followed by a slower rate of production. The production of pregnenolone from endogenous precursors by mitochondrial fractions showed a similar time curve (Fig. 3B). This biphasic pattern was more pronounced in incubation experiments with preparations from luteinizing hormone-treated testes than in experiments with control testes.

Activity of 3β-hydroxysteroid dehydrogenase in whole tissue homogenates

The specific activities of 3β-hydroxysteroid dehydrogenase in homogenates of zero control and control testes were 0.13 ± 0.03 and 0.12 ± 0.01 mU per mg protein (mean value ±S.D., $n = 4$). In homogenates of luteinizing hormone-treated testes, the same specific activity was found: 0.12 ± 0.02 mU per mg protein (testes were removed 20 min after injection of luteinizing hormone or NaCl/albumin solution).

Effect of cycloheximide administration in vivo on steroid concentrations and on steroid production in vitro

Cycloheximide injection prior to luteinizing hormone prevented the luteinizing hormone stimulation of testicular testosterone concentration and

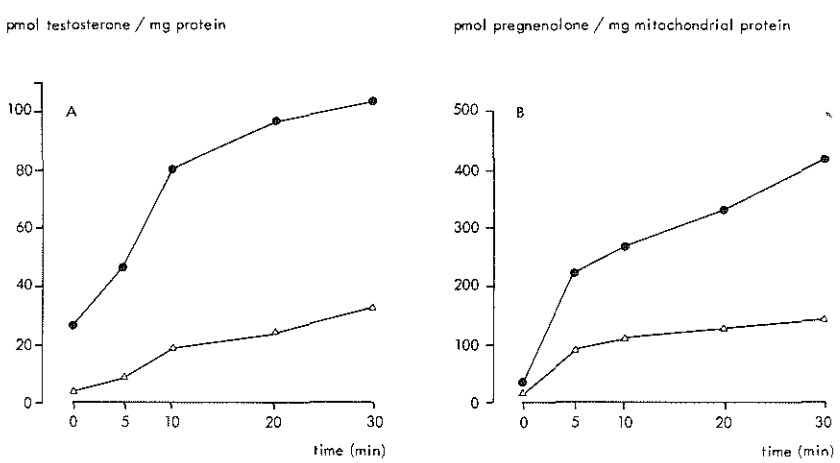

Fig. 3. (A) Production of testosterone by whole testis tissue homogenates as a function of the time of incubation. △———△, homogenates of control testes; ●———●, homogenates of testes removed 20 min after intravenous administration of 25 μg luteinizing hormone. (B) Production of pregnenolone by isolated mitochondrial fractions as a function of the time of incubation. △———△, mitochondrial fractions obtained from control testes; ●———●, mitochondrial fractions obtained from testes, removed 20 min after intravenous administration of 25 μg luteinizing hormone.

TABLE II

STEROID CONCENTRATION AND IN VITRO STEROID PRODUCTION IN RAT TESTIS PREPARATIONS AFTER IN VIVO ADMINISTRATION OF CYCLOHEXIMIDE AND LUTEINIZING HORMONE

Number of experiments (n) is given in parentheses. Figures are mean values ± S.D.

Treatment	Steroid concentration		Steroid production	
	Whole tissue homogenate (pmol testosterone/mg protein)	Mitochondrial fraction (pmol pregnenolone/mg mitochondrial protein)	Whole tissue homogenate (pmol testosterone/mg protein, 30 min)	Mitochondrial fraction (pmol pregnenolone/mg mitochondrial protein, 30 min)
NaCl/albumin* solution	(4) 3.2 ± 1.1	(7) 8.3 ± 1.4	(5) 18 ± 4	(6) 167 ± 27
Cycloheximide** + NaCl/albumin solution	(4) 1.7 ± 0.7	(5) 2.8 ± 2.0	(4) 16 ± 6	(5) 126 ± 26
Luteinizing hormone***	(4) 32 ± 4	(5) 50 ± 4	(4) 78 ± 8	(4) 397 ± 84
Cycloheximide + luteinizing hormone†	(4) 3.3 ± 1.8	(3) 3.4 ± 1.2	(4) 23 ± 9	(3) 142 ± 10

* 0.2 ml 0.9% NaCl, containing 0.2% bovine serum albumin was injected intravenously 30 min before removing the testis.

** 1 mg cycloheximide in NaCl-albumin solution was injected intravenously. Testes were removed 30 min after injection.

*** 25 μg luteinizing hormone was injected i.v. 20 min before removing the testes.

† 1 mg cycloheximide was injected i.v. 10 min prior to administration of 25 μg luteinizing hormone 20 min after luteinizing hormone administration testes were removed.

testosterone production by whole testis homogenates (Table II). Moreover, cycloheximide administration prior to luteinizing hormone injection inhibited the increases of mitochondrial pregnenolone concentration and pregnenolone production which were observed after administration of luteinizing hormone alone (Table II). Administration of cycloheximide to control animals did not significantly alter testosterone concentration and production in vivro. However, cycloheximide injection of control animals resulted in a decreased pregnenolone concentration in mitochondrial fractions ($P < 0.001$) and pregnenolone production by mitochondrial fractions decreased from 167 to 126 pmol pregnenolone per mg protein per 30 min ($P < 0.05$). Addition of 1 mg cycloheximide to the incubation mixture did not inhibit the production of testosterone by whole testis homogenates and of pregnenolone by mitochondrial fractions from control and luteinizing hormone treated testes (data not shown).

Effect of Ca^{2+} concentration on testosterone and pregnenolone synthesis in vitro

The incubation studies mentioned above were performed in a medium with 0.7 mM Ca^{2+}. Fig. 4 shows the effect of variation of Ca^{2+} concentration in the incubation medium on testosterone production by whole testis homogenates. An increase of Ca^{2+} concentration in the range from 0 to 1 mM resulted in a striking activation of testosterone production. The stimulation was decreased at Ca^{2+} concentrations of 50 mM and higher.

Fig. 5 shows the influence of Ca^{2+} concentration in mitochondrial preg-

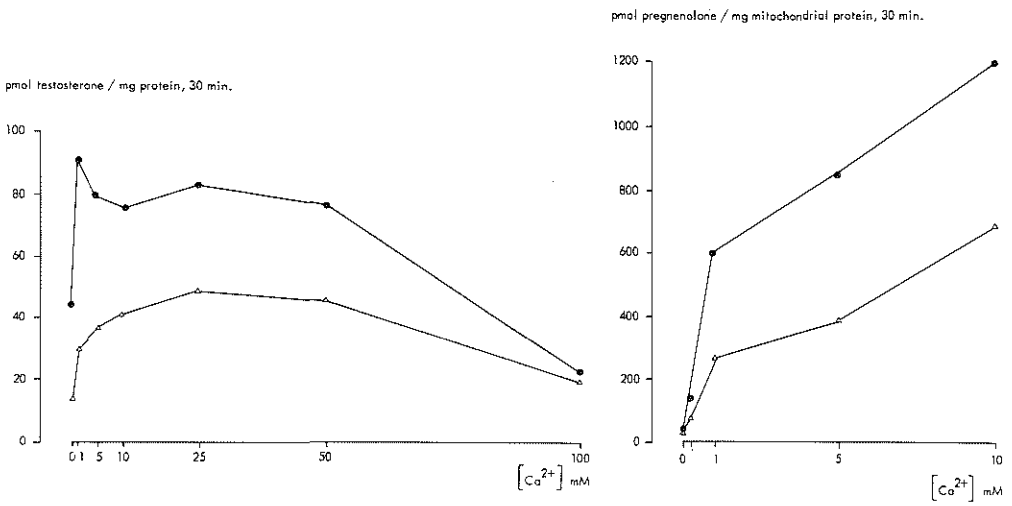

Fig. 4. The effect of Ca^{2+} on the production of testosterone by whole testis tissue homogenates. △———△, homogenates of control testes; ●———●, homogenates of testes, removed 20 min after intravenous administration of 25 μg luteinizing hormone.

Fig. 5. The effect of Ca^{2+} on the production of pregnenolone by isolated mitochondrial fractions. △———△, mitochondrial fractions obtained from control testes; ●———●, mitochondrial fractions obtained from testes, removed 20 min after intravenous administration of 25 μg luteinizing hormone.

nenolone production. The increase of Ca^{2+} concentrations from 0 to 1 mM also caused an increase of mitochondrial pregnenolone synthesis. At 25 mM Ca^{2+} a maximal production rate was achieved; at higher Ca^{2+} concentrations the production rate decreased (not shown). In the absence of Ca^{2+}, mitochondrial fractions of control testes produced 36 ± 10 whereas mitochondrial fractions of luteinizing hormone-treated testes produced 76 ± 36 pmol pregnenolone per mg mitochondrial protein, 30 min (mean values ±S.D., n = 3). Under the experimental conditions used in the present study, changes in low Ca^{2+} concentrations had a more pronounced effect on steroid production in preparations from luteinizing hormone treated testes as compared to the production by control testes (Figs 4 and 5).

Influence of luteinizing hormone and cyclic AMP in vitro on mitochondrial pregnenolone synthesis

The presence of 12.5 and 25 μg luteinizing hormone in the incubation medium had no effect on the production of pregnenolone from endogenous precursors by mitochondrial fractions (data not shown). With our incubation conditions, 10^{-8}—10^{-5} M cyclic AMP in the presence of 0.2 mM 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase activity (EC 3.1.4.1), had no influence on pregnenolone production by isolated mitochondrial fractions of rat restis (data not shown).

Discussion

The present results show a good correlation between the effects of luteinizing hormone injected intravenously on steroid production in vivo and in vitro.

The results (Table I) also confirm that the testosterone concentration in rat testicular venous plasma is a good parameter for steroid secretion by the rat testis. A rapid increase of the testosterone concentration in venous plasma was observed after injection of luteinizing hormone, which is compatible with previous observations of Eik-Nes [1] and of De Jong et al. [8]. The effect of luteinizing hormone administered in vivo is reflected already after 5 min by an increase in the in vitro testosterone production by whole testis homogenates (Fig. 2A). The increased pregnenolone production by isolated mitochondrial fractions after in vivo luteinizing hormone treatment (Fig. 2B) appears a logical cause for this increase in testosterone production. This is in accordance with the theory that gonadotropins stimulate steroid biosynthesis at a step prior to the conversion of pregnenolone [2].

The biphasic time curve of steroid production by whole testis tissue homogenates (Fig. 3A) as well as by mitochondrial fractions (Fig. 3B) may reflect the involvement of a mitochondrial substance which is required for steroid production and which is rapidly depleted (within 5—10 min) during incubation in vitro. Luteinizing hormone treatment may enhance the in vivo accumulation of this factor. A similar time-curve of steroid production has been observed for pregnenolone production by isolated mitochondrial fractions from the rat adrenal gland [13,14] and ovary [6].

After luteinizing hormone treatment, the concentrations as well as the production rates of mitochondrial pregnenolone were increased (Figs 1B and 2B). This observation makes it unlikely that activation of testicular steroid production by trophic hormones is only caused by an increased efflux of pregnenolone from the mitochondrion, thus relieving feedback inhibition of the cholesterol side chain-cleaving enzyme complex by pregnenolone as was proposed by Koritz and Kumar for the adrenal gland [18].

In the present experiments, after short-term luteinizing hormone administration, no effect was observed on 3β-hydroxysteroid dehydrogenase activity. This is in contrast to the effect of long-term human chorionic gonadotropin administration [10] which caused a 2-fold increase of 3β-hydroxysteroid dehydrogenase activity. This may reflect that the rapid effect of luteinizing hormone on testis steroidogenesis does not involve an increase of the synthesis of enzymes involved in testosterone formation. It is generally accepted that cyclic AMP is required as an intracellular messenger for gonadotropin stimulation of testicular steroid biosynthesis [4]. Recently, Cooke et al. [19] have reported that the cyclic AMP concentration in rat testis tissue is about 10^{-7} M. Luteinizing hormone treatment increases the cyclic AMP concentration in isolated testicular interstitial tissue [19]. From the present results it appears that physiological concentrations of cyclic AMP had no direct effect on mitochondrial pregnenolone production. Also, luteinizing hormone, when added directly to mitochondrial incubation mixtures, did not affect mitochondrial pregnenolone production. Although Sulimovici and Lunenfeld [20] have reported that luteinizing hormone in vitro can stimulate rat testis mitochondrial adenylcyclase, the present results do not support the suggestion that mitochondrial cyclic AMP formation plays a role in the regulation of mitochondrial steroid production.

Pretreatment of the animals with cycloheximide in vivo prevented the

effect of luteinizing hormone on activation of steroid production in vivo and in vitro (Tables I and II). This may indicate that extra-mitochondrial protein synthesis is involved in the molecular action of trophic hormones on the mitochondrial pregnenolone production.

Effects of protein synthesis inhibitors have been reported on steroid production by superfused adrenal cells [21] and testicular interstitial tissue [7] and in adrenal mitochondria [22] and on the mitochondrial cholesterol side-chain cleavage activity from luteinized rat ovaries [6]. These results have been interpreted in terms of a cytoplasmic protein which is rapidly turning over and which may be involved in the interaction between cholesterol and mitochondrial cytochrome P-450.

Testosterone production by whole testis tissue homogenates and pregnenolone production by mitochondrial fractions are stimulated by Ca^{2+} in vitro (Figs 4 and 5). After luteinizing hormone treatment in vivo the testosterone and pregnenolone production rates in the absence of Ca^{2+} were increased when compared with steroid production by relevant control samples. In the presence of low Ca^{2+} concentrations the activating effect of this cation is more pronounced in preparations of luteinizing hormone treated testes, than in preparations of control testes. These results could indicate that luteinizing hormone stimulates mitochondrial pregnenolone production via a compound that enhances the mitochondrial sensitivity to Ca^{2+} or increases cellular Ca^{2+} concentrations, which may facilitate the uptake of Ca^{2+} by the mitochondria. On the other hand it cannot be excluded that cellular Ca^{2+} plays a permissive role in the regulation of testicular steroidogenesis. The mechanism of the Ca^{2+} in stimulating pregnenolone production is not known. Recently, Simpson et al. [13] have reported that pregnenolone synthesis in rat adrenal mitochondria can be activated by Ca^{2+}, which also alters the spin state of mitochondrial cytochrome P450, probably caused by a change in conformation of mitochondria. The possible relationship between a cytoplasmic protein factor that may be involved in the trophic stimulation of steroid production and Ca^{2+}-induced mitochondrial pregnenolone production also remains to be elucidated.

Acknowledgements

This work was supported (in part) by the Foundation for Medical Research FUNGO which is subsidized by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). We are grateful to the National Institute of Health, Endocrinology Study Section, Bethesda, Md., U.S.A. for gifts of ovine-luteinizing hormone.

References

1 Eik-Nes, K.B. (1971) Recent Progr. Horm. Res. 27, 517—535
2 Hall, P.F. (1970) in The Androgens of the Testis (Eik-Nes, K.B., ed.), pp. 73—115, Marcel Dekker Inc., New York
3 Cooke, B.A., Rommerts, F.F.G., Van der Kemp, J.W.C.M. and Van der Molen, H.J. (1974) Mol. Cell. Endocrinol. 1, 99—111
4 Rommerts, F.F.G., Cooke, B.A. and Van der Molen, H.J. (1974) J. Steroid Biochem. 5, 279—285
5 Garren, L.D., Gill, G.N., Masui, H. and Walton, G.M. (1971) Recent Progr. Horm. Res. 27, 433—478
6 Arthur, J.R. and Boyd, G.S. (1974) Eur. J. Biochem. 49, 117—127

7 Cooke, B.A., Clotscher, W.F., De Jong, C.M.M., Renniers, A.C.H.M. and Van der Molen, H.J. (1974) J. Endocrinol. 63, 17—18
8 De Jong, F.H., Hey, A.H. and Van der Molen, H.J. (1973) J. Endocrinol. 57, 277—284
9 Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1974) Biochim. Biophys. Acta 348, 404—414
10 Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1975) Biochim. Biophys. Acta in press
11 Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1973) Biochim. Biophys. Acta 297, 179—185
12 Drosdowsky, M., Menon, K.M.J., Forchielli, E. and Dorfman, R.I. (1965) Biochim. Biophys. Acta 104, 229—236
13 Simpson, E.R., Jefcoate, C.R., McCarthy, J.L. and Boyd, G.S. (1974) Eur. J. Biochem. 45, 181—188
14 Bell, J.J. and Harding, B.W. (1974) Biochim. Biophys Acta 348, 285—298
15 Verjans, H.L., Cooke, B.A., De Jong, F.H., De Jong, C.C.M. and Van der Molen, H.J. (1973) J. Steroid Biochem. 4, 665—676
16 Snedecor, G.W. and Cochran, W.G. (1967) in Statistical Methods, 6th edn, Ames: Iowa State University Press
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Koritz, S.B. and Kumar, A.M. (1970) J. Biol. Chem. 245, 152—159
19 Cooke, B.A., Van Beurden, W.M.O., Rommerts, F.F.G. and Van der Molen, H.J. (1972) FEBS Lett. 25, 83—86
20 Sulimovici, S. and Lunenfeld, B. (1973) Biochem. Biophys. Res. Commun. 55, 673—679
21 Schulster, D., Richardson, M.C. and Mackie, C. (1972) Biochem. J. 129, 8p—9p
22 Jefcoate, C.R., Simpson, E.R. and Boyd, G.S. (1974) Eur. J. Biochem. 42, 539—551